Ian Michelow, *et al.*
Griffithsin vs Ebola Virus

Case Adams : Red Algae Extract Fights Ebola ... and HIV, SARS and HCV
High-Dose Mannose-Binding Lectin Therapy for Ebola Virus Infection
Isolation and characterization of griffithsin
US2010331240 : METHODS FOR PREVENTION AND TREATMENT OF INFECTIONS WITH SUPRAPHYSIOLOGICAL DOSES OF MANNAN-BINDING LECTIN
US8394764 : GRIFFITHSIN, GLYCOSYLATION-RESISTANT GRIFFITHSIN, AND RELATED CONJUGATES,
US8088729 : Anti-viral griffithsin compounds, compositions, and methods of use
Scytonema varium red algae / Scytovirin Patents
Nostoc ellipsosporum / Cyanovirin-N Patents

http://www.greenmedinfo.com/blog/red-algae-extract-fights-ebola-and-hiv-sars-and-hcv

Red Algae Extract Fights Ebola ... and HIV, SARS and HCV

by Case Adams

Ebola Antibodies

The research found that nearly half of those who were asymptomatic and seemingly immune developed antibodies (IgM and IgG) to the Ebola virus.

This means these individuals certainly were intimately exposed to the virus, but simply naturally developed the immunity tools - including those discussed below - that prevented the infection from replicating out of control.

Furthermore, the asymptomatic group exhibited greater anti-inflammatory responses in general. They were found to have higher levels of circulating cytokines and chemokines – which speed up the body's natural ability to break down the viral cells and stop their activity within the body.

They concluded: "Asymptomatic individuals had a strong inflammatory response by high circulating concentrations of cytokines and chemokines."

Mannose-Binding Lectins Attack Ebola Virus

The particular mechanism with which the body naturally breaks down and prevents infection from lethal infections including Ebola, HIV, HCV and SARS has gradually emerged.

The mechanism is called mannose-binding lectins. Mannose-binding lectins are apparently produced in the human body via a DNA sequence, called the MBL2.

When this part of our genes is in order, the body will produce and release these mannose-binding lectins into the bloodstream. Mannose-binding lectins will then recognize and glom onto certain carbohydrate molecules that cover and make up various microorganisms.

These include fungi, bacteria and even parasites, which utilize glycoprotein shells to protect themselves. But they also include viruses. Once the lectins attach to these shells, they will break apart the surface of

the microbe and basically break them down, allowing the body's other immune cells to kill off the microbe and prevent it from replicating.

In fact, a healthy body that produces good levels of these mannose-binding lectins will be able to easily fight off colds and flus, as well as other microbial infections. Several animal studies have shown mannose-binding lectins heartily beat down coronaviruses and infectious bronchitis.

Research over the past five years has found that low levels of mannose-binding lectins increases the risk of respiratory infections, including syncytial virus infections, pneumonia and others.

For example, in a study of 121 children, RSV-infections were associated with low levels of mannose-binding lectins. Nearly 70 percent of RSV-infected children had low levels of mannose-binding lectins. But other infections – especially those related to bacterial infections – are not necessarily connected with mannose-binding lectin levels.

When it comes to virulent infections such as Ebola, Hepatitis C and HIV, however, these are different. These viruses come with glycoprotein shells that protect the virus from being broken down.

Furthermore, the glycoprotein shell of the Ebola virus produces glycoproteins that damage cells, allowing the virus to penetrate and replicate within the cell.

Mannose-binding lectins actually break down this shell and the glycoprotein matrix through a mechanism called the lectin pathway.

Humans that don't produce enough of these mannose-binding lectins are not only more susceptible because they don't have enough lectins, but they are typically also immunosuppressed with regard to the rest of their immune system.

One of the reason some humans don't produce enough mannose-binding lectins is because of a slight genetic mutation, where the MBL2 gene is switched off. The reason for this mutation/switch-off has yet to be fully understood. (Guess - something to do with our toxic environment and/or nutritional deficiency.)

Mannose-Binding Lectins From Red Algae

This brings us to the fun part. Yes, humans aren't the only critters that produce mannose-binding lectins. Red algae also produce these profusely, which allow the algae to protect themselves from invasion by viruses.

The most promising form of mannose-binding lectins is a component of the Scytonema varium red algae called Scytovirin. The protein extract was isolated by researchers from the National Cancer Institute at Frederick, Maryland in 2003. The protein contains 95 amino acids, and was found to bind to HIV-1 viral shells.

A similar antiviral protein was found in Nostoc ellipsosporum – called Cyanovirin-N. Both of these antiviral proteins did similar things – they broke down the glycoprotein shells of HIV and HCV.

Yet another anti-viral extract was found from the New Zealand red alga species, Griffithsia sp. This protein is called Griffithsin, abbreviated with GRFT.

Over the next few years, Griffithsin was tested against HIV-1 with great success in laboratory studies, which included studies with mice. The epidemic-potential virus SARS was also tested against Griffithsin, also with great success.

Multiple studies illustrated these effects. Research from the Center for Cancer Research in Frederick, Maryland found that Griffithsin not only stopped HIV-1 virus replication, but stopped cellular intrusion of the virus.

In 2010 Harvard researchers tested a recombinant version of Griffithsin – called rhMBL – against Ebola.

Once again, they found the mannose-binding lectins were able to not only breakdown the viral shells of the Ebola, but when given to mice infected with Ebola, the mice became immune to the virus.

Yes, when the mice given the recombinant mannose-binding lectins were rechallenged with the Ebola virus, they were found to be immune to the Ebola virus.

Since that study other research has tested other animals with Griffithsin, with similar results.

Recombinant Griffithsin Produced in Nicotiana Benthamiana Plants

As modern medical researchers continually strive for isolated and synthesized versions of nature able to be patented, recombinant versions of Griffithsin were eventually produced using Nicotiana benthamiana plants (a relative of the tobacco plant). These plants were genetically modified so they would produce the same mannose-binding lectins.

This form of Griffithsin was tested on mice and guinea pigs infected with HIV-1, with successful antiviral results.

This was also found when testing the recombinant Griffithsin on Ebola-infected mice.

In all the studies, the Griffithsin was found to be safe and tolerated.

As to whether red algae can be taken in natural form to increase immunity, there is no doubt this is the case. Prior to this antiviral research that has spiraled into biopharm research, red algae had been shown to have antiviral and anticancer effects.

So the most logical answer is "yes" – certainly consuming red algae in supplement form has been found to boost antiviral immunity, and from the available research, blood levels of mannose-binding lectins. This should in turn boost immunity and create a natural method of preventing and even treating viral infections such as Ebola, SARS, HIV and Hepatitis-C.

Of course, this strategy should be used with other natural immunity-boosting strategies.

Other plants also produce these mannose-binding lectins, some of which have been used in traditional medicines. A study from Belgium's University of Leuven studied 33 different plant lectins, and found 10 different mannose-binding lectins among the plants that inhibited coronovirus, and intervened upon the replication cycle of SARS-CoV.

Consult with your health professional if you are sick.

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While researchers scramble to develop a vaccine or monoclonal antibody against the Ebola virus – and continue to develop chemo treatments to stem HIV and Hepatitis-C while fearing SARS – nature has already provided a natural treatment.

Research has shown that a healthy strong immune system can allow a person to not only avoid contracting the disease – but become resistant to it as well.

For those of us who need help or extract assurance, red algae proves to provide a key antiviral.

Hunting Natural Immunity For Ebola

After the two 1996 Ebola outbreaks in Gabon Africa, medical scientists determined that about Ebola causes death among about 70 percent of those who contracted the virus.

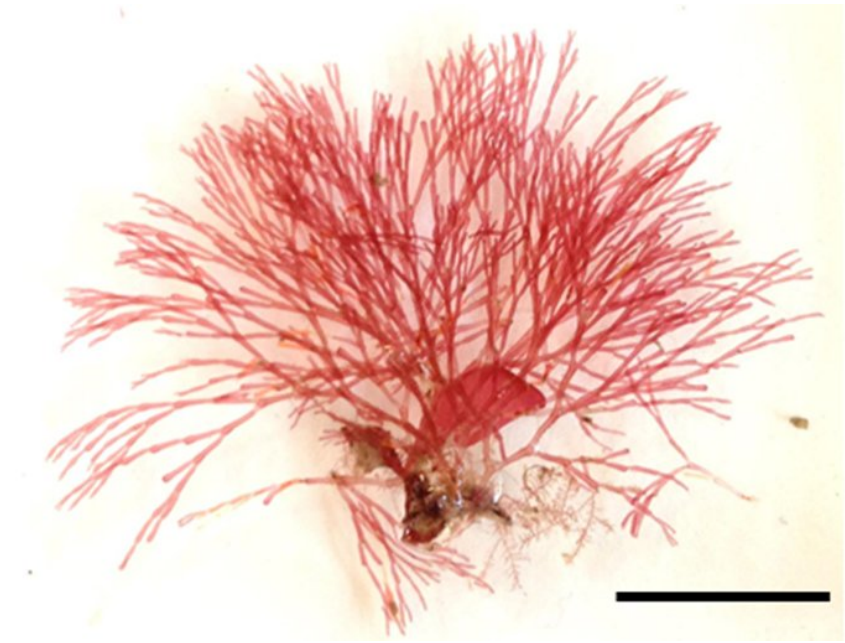
This question led researchers from Gabon's Franceville International Center of Medical Research to investigate. The questions ensued: Why don't the other 30 percent die? How do 30 percent of those infected recover?

Furthermore, medical researchers found many instances where there were close contacts of those who became infected who never were infected at all. Even though they were in contact with the infected patient while the patient was symptomatic.

Note: An infected patient with Ebola must be symptomatic in order to be contagious – with fever and other flu-like symptoms. A person must also have direct mucosal or blood contact in order to become infected with the virus. This means a transfer of saliva, urine, semen or blood from one person to another.

Thus, when the researchers investigated "close contact" individuals, they focused upon those who had this sort of exposure.

Griffithsia species



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High-Dose Mannose-Binding Lectin Therapy for Ebola Virus Infection

Ian C. Michelow,1 Calli Lear,2 Corinne Scully,2 Laura I. Prugar,2 Clifford B. Longley,3 L. Michael Yantosca,1 Xin Ji,4 Marshall Karpel,1 Matthew Brudner,1 Kazue Takahashi,1 Gregory T. Spear,4 R. Alan B. Ezekowitz,1 Emmett V. Schmidt,corresponding author5 and Gene G. Olinger2

Abstract

Mannose-binding lectin (MBL) targets diverse microorganisms for phagocytosis and complement-mediated lysis by binding specific surface glycans. Although recombinant human MBL (rhMBL) trials have focused on reconstitution therapy, safety studies have identified no barriers to its use at higher levels. Ebola viruses cause fatal hemorrhagic fevers for which no treatment exists and that are feared as potential biothreat agents. We found that mice whose rhMBL serum concentrations were increased =7-fold above average human levels survived otherwise fatal Ebola virus infections and became immune to virus rechallenge. Because Ebola glycoproteins potentially model other glycosylated viruses, rhMBL may offer a novel broad-spectrum antiviral approach.

Circulating mannose-binding lectin (MBL) is a first-line host defense against a wide range of viral

and other pathogens. MBL is a C-type lectin that recognizes hexose sugars including mannose, glucose, fucose, and N-acetylglucosamine on the surface of many pathogens. It does not recognize the terminal carbohydrates galactose and sialic acid on normal host cells. Therefore, MBL preferentially recognizes glycosylated viruses including influenza virus, human immunodeficiency virus, severe acute respiratory syndrome coronovirus (SARS-CoV), Ebola virus, and Marburg virus. It also recognizes many glycosylated gram-positive and gram-negative bacteria [1, 2]. As a result of common genetic variants, MBL serum levels in humans range from 0 to 10,000 ng/mL. Thirty percent of the human population has levels <500 ng/mL, which are associated with increased susceptibility to infections in children and immunocompromised individuals [3].

We previously reported preclinical studies that addressed the potential utility of recombinant human MBL (rhMBL) reconstitution therapy. MBL-knockout mice are highly susceptible to several bacteria including Staphylococcus aureus [1]. RhMBL improved survival in MBL-null mice to approximate survival among infected wild-type mice at doses that reconstituted the complement-activating capacity of MBL-knockout serum to a level comparable to that of wild-type mouse serum [1]. Doses of plasma-derived MBL and rhMBL designed to increase MBL concentrations to physiologic levels (>1000 ng/mL) in MBL-deficient humans were safe in early trials and did not elicit antibodies [3–5]. In contrast, although MBL replacement therapy enhanced opsonophagocytic potential, higher levels of plasma-derived MBL were needed to achieve MBL-mediated complement activation comparable to healthy controls [6], suggesting that above-replacement dosing will need attention.

Ebola and Marburg viruses of the filovirus family are among the most virulent causes of the human viral hemorrhagic fevers and cause devastating epidemics of fulminant and rapidly fatal disease. They constitute important biological threat agents because of their high mortality rates, capacity for large-scale dissemination, and potential for causing social disruption. Currently, there are no US Food and Drug Administration–approved therapeutic agents available to prevent or treat these lethal viral infections. Filovirus surface glycoproteins (GPs) are heavily glycosylated and contain high-mannose. As a result, MBL binds to Ebola and Marburg viruses and mediates complement-dependent virus neutralization [2]. Importantly, their surface glycoprotein structures are characteristic of a broad group of viruses in which N-linked glycosylation contributes to viral virulence [7]. Reasoning that MBL treatment is likely to be safe at supraphysiological levels, we evaluated an in vivo Ebola virus model to explore the possibility of using MBL as an immunotherapeutic agent. Our results showed that supraphysiological doses of MBL rescued ~40% of mice from lethal challenges when administered pre– or post–Ebola virus exposure. This novel paradigm suggests that high-dose MBL should be evaluated more broadly as an immunotherapeutic agent for a wide spectrum of glycosylated pathogens.

MATERIALS AND METHODS

Production and pharmacokinetics of rhMBL

Commercial-grade rhMBL was provided by Enzon Pharmaceuticals [8]. Human MBL concentrations and complement cleavage activity were measured as described elsewhere [9]. Pharmacokinetics of rhMBL concentration–time data were evaluated using noncompartmental modeling with WinNonlin Professional Edition (version 5.2; Pharsight). The area under the curve from zero to infinity (AUC0–8) values were calculated using the linear trapezoidal method.

Murine Ebola model

We used a validated lethal Ebola Zaire mouse model developed at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) [10], with a double plaque-purified, mouse-adapted, Ebola isolate, EZ'76 Mp3 Vp2 Mp9 GH. The virus was inoculated intraperitoneally (i.p.) at 100 pfu (3000 × LD50) producing uniformly lethal disease in C57B6 mice using biosafety level-4 facilities. Research was conducted in compliance with the Animal Welfare Act and federal regulations in a fully accredited facility. To assess the effect of rhMBL on virus lethality, we treated Ebola virus–infected C57B6 mice i.p. with either 4.3 mg/kg or 20 mg/kg of rhMBL twice daily ~12 hours apart for 10 days. On the day of virus exposure, mice were treated and exposed to 100 pfu of mouse-adapted Ebola Zaire either 12 hours before or 1 hour after the first dose of rhMBL as indicated in

Figure 1.

Figure 1.
Survival and laboratory indices of filovirus-infected mice treated with recombinant human mannose-binding lectin (rhMBL). (A) Mouse survival when treated with rhMBL before Ebola virus inoculation. Sham-treated wild-type mice were compared with wild-type ...

Mice were assessed daily for changes in physical appearance and weight. Viremia was assessed by reverse transcription-polymerase chain reaction (RT-PCR) and plaque assays as described elsewhere [11], and anti–Ebola virus antibodies were measured using standard enzyme-linked immunosorbent assays (ELISAs) [12]. Standard blood counts were evaluated with a Coulter AC·T diff (Beckman Coulter). For analysis with flow cytometry, spleens were ground into single cell suspensions with the BD Medimachine tissue grinder. After incubation with Fc Block (BD), cells were washed and incubated with antibody (CD3 FITC BD no. 555274, CD8 V450 BD no. 560469, CD14 PerCP eBio no. 45-0141, CD4 PE eBio no. 12-0041-82, CD11b APC BD no. 553312, and CD19 PE-Cy7 BD no. 557655). Cells were washed with PBS and fixed in BD cytofix. Data were immediately acquired with a BD FACSCantoII and analyzed with FlowJo (version 7). The Bio-Plex Mouse Cytokine 23-Plex Panel assay (Bio-Rad 171-F11241) was used to measure multiple cytokines, chemokines, and growth factors in serum and tissue supernatants according to the manufacturer's instructions. Mice that survived the initial infection were tested for Ebola-specific serological response on day 21 and rechallenged with the same virus dose without further treatment, and antibody titers were retested 28 days later.

RESULTS

We previously found that rhMBL bound Ebola (Zaire) and Marburg (Musoke) envelope GPs [2]. RhMBL effectively blocked Ebola GP interactions with DC-SIGN, and HIV particles lacking gp120/gp41 pseudotyped with Ebola or Marburg GPs were neutralized by the lectin complement pathway [2]. To develop an in vivo test of rhMBL effectiveness, we determined that 100 ng/mL of rhMBL was the minimum concentration needed to inhibit =90% infectivity of HepG2 cells using Ebola GP pseudotyped lentiviral particles and to inhibit =90% infectivity of Vero E6 cells using recombinant Ebola Zaire virus (Mayinga strain)-eGFP (data not shown). We had previously found that a single intraperitoneal dose of 75 μg of rhMBL reconstituted the lectin complement pathway in MBL-knockout mice [1]. We compared the pharmacokinetic parameters (Table 1) of that single reconstitution dose (4.3 mg/kg) with a higher single intraperitoneal dose of 350 μg (20 mg/kg) to identify a potentially supraphysiological dose to test in model infections. The average maximum serum concentration (Cmax) of both doses exceeded the minimum concentration of MBL that inhibited infection in vitro by at least 55-fold. The average ratio of maximum to baseline complement component 4 cleavage activity was 1.7 for the 75-μg rhMBL dose and 5.4 for the 350-μg dose.

Table 1.
Pharmacokinetic Parameters of Low- vs High-Dose Recombinant Human Mannose-Binding Lectin (rhMBL) Therapy in Uninfected Mice

Intraperitoneal administration of 100 pfu of native Ebola Zaire virus (3000 × LD50) is uniformly fatal in mice. Treatment with 75 μg of rhMBL per dose every 12 hours failed to protect mice from that virus inoculum. Therefore, we increased rhMBL to 350 μg administered every 12 hours for 10 days starting either 1 hour before or 12 hours after Ebola virus challenge (Figure 1A and 1B). When treatment was started 1 hour before virus infection, the supraphysiological dose increased survival to > 40% of mice in several trials (Figure 1A). We then started treatment 12 hours after viral infection. We compared survival in wild-type and complement component 3 (C3)–deficient mice as the inhibitory effects of MBL on Ebola virus are mediated by complement in cell culture [2]. Once again we saw an increase in survival from 0% to >40% in rhMBL-treated mice, and survival was dependent on an intact complement pathway, since C3-deficient mice did not survive (Figure 1B). All inoculated mice showed signs of infection according to standardized observation scores and weight loss, and surviving mice had detectable Ebola virus–specific antibodies 28 days after infection (data not shown).

We monitored the effect of treatment started 12 hours after infection on a variety of laboratory indices. Mean white blood cell counts were 9100 cells/mL in MBL-treated mice (n = 5) compared with 4525 cells/mL on day 7 after infection in the surviving sham-treated mice (n = 4). Average lymphocyte counts were also higher in MBL-treated mice compared with controls (5500 cells/mL vs 2800 cells/mL, respectively). A similar trend was seen for platelet counts, which averaged 726,000 cells/mL in the treatment group and 239,000 cells/mL in the controls. These differences were statistically significant for platelet counts on day 5 (672,000 cells/mL vs 322,000 cells/mL, P = .014; Figure 1C).

In a separate experiment, spleens were harvested on day 5 after infection (4 sham-treated and 4 MBL-treated mice). Constituent cell populations were assayed by flow cytometry. Numbers of splenic CD3-CD19+ cells (B lymphocytes) and CD11b+ granulocytes were higher in MBL-treated mice (89.2% vs 85.1%, P = .019; 17.6% vs 12.8%, P = .04, respectively). The RNA viral loads as determined by RT-PCR in blood, liver, and spleen 5 days after infection were similar in sham- and rhMBL-treated mice (P > .05). Virus titers in blood were generally lower on days 1 and 3 in rhMBL-treated mice as determined by plaque assays (P > .05; Figure 1D). Of 23 cytokines and chemokines tested in serum, liver, and spleen on day 5 after inoculation, lower values (fluorescence intensity units) for interleukin (IL)-1b (170 vs 253, P = .07), IL-5 (89 vs 112, P = .03), IL-10 (379 vs 518, P = .004), IL-13 (264 vs 384, P = .008), and IL-17 (120 vs 174, P = .028) were found in liver homogenates from rhMBL-treated mice (Figure 1E). We tested protective immunity in 5 seropositive mice that survived initial infection by rechallenging them with native Ebola virus 28 days after initial infection. It is noteworthy that all MBL-treated survivors also survived the second viral challenge. Similar or higher immunoglobulin G, A, and M antibody titers were seen 28 days after the second challenge with the virus (Figure 1F).

DISCUSSION

In the past 3 decades, approved antivirals have increased from a few nucleoside analogues to well over 40 drugs [13]. The human immunodeficiency virus (HIV) and hepatitis C virus (HCV) epidemics particularly drove antiviral discovery toward rationally designed drugs targeting specific viral enzymes. Although this approach was remarkably effective, the advent of newly emerging or drug-resistant viruses that threaten humans calls for the development of more broadly active agents targeting viral components shared among viruses. N-glycosylation of viral envelopes is an important such target shared between influenza, HIV, HCV, West Nile virus, SARS-CoV, Hendra virus, Nipah virus, and filoviruses (Ebola and Marburg viruses) [7]. To assess one possible strategy against N-glycosylated viruses, we tested a stringent Ebola virus infection model (3000 × LD50) in mice.

Filovirus infections are characterized by marked lymphopenia, severe degeneration of lymphoid tissues, dysregulated dendritic cell function, and cytokine storms—all hallmarks of pathogens that subvert both innate and adaptive immune responses [14]. Nevertheless, survivors exhibit detectable virus-specific antibody responses [15]. Therefore, we hypothesized that administration of a recombinant innate immune molecule that targets glycosylated viruses might bridge an infected individual to recovery. Here we show for the first time that rhMBL can be used as a therapeutic agent to achieve serum concentrations in mice that correspond to levels in humans that are 7–24-fold higher than average human concentrations and complement cleaving activity that is >5-fold higher than baseline values in mice. This result confirms our previous in vitro data showing that MBL possesses complement-dependent intrinsic antimicrobial activity [2].

Biological responses of the infected mice to rhMBL treatment further indicated that our strategy targeted the main pathogenic effects of Ebola viruses. MBL-treated mice had higher B lymphocyte and CD11b+ granulocyte counts and demonstrated down-regulation of intrahepatic proinflammatory (IL-1b and IL-17) and Th2 cytokines (IL-5, IL-10, and IL-13) early in the course of infection (Figure 1E), suggesting that rhMBL may mitigate the detrimental effects of the characteristic cytokine storm. MBL-treated mice tended to have greater inhibition of viral replication on days 1 and 3 after infection (P > .05; Figure 1D). Most important, rhMBL treatment bridged surviving mice to development of an effective adaptive immune response (Figure 1F). Future experiments will be needed to scale high-dose rhMBL therapy for use in larger animal models and to test rhMBL in combination with other promising experimental therapies such as

small molecule inhibitors, coagulation modulators, antisense technologies, therapeutic antibodies and cytokines, and postexposure vaccination. In summary, we report that supraphysiologic rhMBL therapy may be an effective immunotherapeutic strategy against Ebola virus, and since Ebola glycoproteins potentially model other glycosylated viruses, rhMBL therapy may offer a novel broad-spectrum antiviral approach.

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Mannose-binding lectin binds to Ebola and Marburg envelope glycoproteins, resulting in blocking of virus interaction with DC-SIGN and complement-mediated virus neutralization.[J Gen Virol. 2005]

A novel L-ficolin/mannose-binding lectin chimeric molecule with enhanced activity against Ebola virus.[J Biol Chem. 2010]

Cyanovirin-N binds to the viral surface glycoprotein, GP1,2 and inhibits infectivity of Ebola virus. [Antiviral Res. 2003]

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Imino sugar glucosidase inhibitors as broadly active anti-filovirus agents[Emerging Microbes & Infections. 2013]

Activity of and Effect of Subcutaneous Treatment with the Broad-Spectrum Antiviral Lectin Griffithsin in Two Laboratory Rodent Models[Antimicrobial Agents and Chemotherapy. 2014...]

Animal models for Ebola and Marburg virus infections[Frontiers in Microbiology.]

Lectin-Dependent Enhancement of Ebola Virus Infection via Soluble and Transmembrane C-type Lectin Receptors[PLoS ONE.]

A Syrian Golden Hamster Model Recapitulating Ebola Hemorrhagic Fever[The Journal of Infectious Diseases. 2013]

Recombinant expression of human mannan-binding lectin.[Int Immunopharmacol. 2001]

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A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever.[J Infect Dis. 1998]

Rapid detection protocol for filoviruses.[J Clin Virol. 2004]

Filovirus-like particles produced in insect cells: immunogenicity and protection in rodents.[J Infect Dis. 2007]

Mannose-binding lectin binds to Ebola and Marburg envelope glycoproteins, resulting in blocking of virus interaction with DC-SIGN and complement-mediated virus neutralization.[J Gen Virol. 2005]

Mannose-binding lectin-deficient mice are susceptible to infection with Staphylococcus aureus.[J Exp Med. 2004]

Mannose-binding lectin binds to Ebola and Marburg envelope glycoproteins, resulting in blocking of virus interaction with DC-SIGN and complement-mediated virus neutralization.[J Gen Virol. 2005]

Review Virus glycosylation: role in virulence and immune interactions.[Trends Microbiol. 2007]

Review Immunopathology of highly virulent pathogens: insights from Ebola virus.[Nat Immunol. 2007]

Defective humoral responses and extensive intravascular apoptosis are associated with fatal

outcome in Ebola virus-infected patients.[Nat Med. 1999]

Mannose-binding lectin binds to Ebola and Marburg envelope glycoproteins, resulting in blocking of virus interaction with DC-SIGN and complement-mediated virus neutralization.[J Gen Virol. 2005]

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J Biol Chem. 2005 Mar 11;280(10):9345-53. Epub 2004 Dec 21.

Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp.

Mori T, O'Keefe BR, Sowder RC, Bringans S, Gardella R, Berg S, Cochran P, Turpin JA, Buckheit RW Jr, McMahon JB, Boyd MR.

Abstract

Griffithsin (GRFT), a novel anti-HIV protein, was isolated from an aqueous extract of the red alga Griffithsia sp. The 121-amino acid sequence of GRFT has been determined, and biologically active GRFT was subsequently produced by expression of a corresponding DNA sequence in Escherichia coli. Both native and recombinant GRFT displayed potent antiviral activity against laboratory strains and primary isolates of T- and M- tropic HIV-1 with EC50 values ranging from 0.043 to 0.63 nM. GRFT also aborted cell-to-cell fusion and transmission of HIV-1 infection at similar concentrations. High concentrations (e.g. 783 nM) of GRFT were not lethal to any tested host cell types. GRFT blocked CD4-dependent glycoprotein (gp) 120 binding to receptor-expressing cells and bound to viral coat glycoproteins (gp120, gp41, and gp160) in a glycosylation-dependent manner. GRFT preferentially inhibited gp120 binding of the monoclonal antibody (mAb) 2G12, which recognizes a carbohydrate-dependent motif, and the (mAb) 48d, which binds to CD4-induced epitope. In addition, GRFT moderately interfered with the binding of gp120 to sCD4. Further data showed that the binding of GRFT to soluble gp120 was inhibited by the monosaccharides glucose, mannose, and N-acetylglucosamine but not by galactose, xylose, fucose, N-acetylgalactosamine, or sialic acid-containing glycoproteins. Taken together these data suggest that GRFT is a new type of lectin that binds to various viral glycoproteins in a monosaccharide-dependent manner. GRFT could be a potential candidate microbicide to prevent the sexual transmission of HIV and AIDS.

US2010331240
METHODS FOR PREVENTION AND TREATMENT OF INFECTIONS WITH SUPRAPHYSIOLOGICAL DOSES OF MANNAN-BINDING LECTIN (MBL) AND FICOLIN-MBL FUSION PROTEINS

Inventor: MICHELOW IAN // SCHMIDT EMMETT

The present invention provides methods of treatment and/or prevention of infections, for example, viral and bacterial infections, in individuals, wherein the method comprises administering a supraphysiological amount of mannose-binding lectin (MLB) and/or ficolin-MBL fusion protein to an individual afflicted with an infection or at risk of an infection, such as a bacterial or a viral infection. For example, methods for treatment and/or prevention of Ebola virus infection are provided.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The present invention pertains to the use of subunits and oligomers of mannan-binding lectin (MBL) and ficolin-MBL fusion proteins for prevention and/or treatment of infections, particularly in subjects who have normal and functional MBL serum levels.

[0004] 2. Background of the Invention

[0005] Infections count for a large part of morbidity and mortality in the world. While bacterial infections have been tackled by antibiotics and bacteriophages, new treatment methods are sorely needed for the growing amount of bacteria that have become resistant to these treatments. Viruses are a difficult target for treatment in humans and other animals because they use animal cells to replicate and spread. While some viral infections can be prevented using vaccination or antibody-based therapies, several serious and lethal viruses remain currently without effective treatment.

[0006] One of such lethal virus family is filoviruses. The two most known lethal filoviruses are Ebola and Marburg viruses. Ebola and Marburg virus can cause acute, lethal hemorrhagic fevers for which no vaccines or effective treatments currently exist. Marburg and Ebola envelope glycoproteins consist of glycoprotein 1 (GP1) and membrane-bound glycoprotein 2 (GP2) protein that are covalently linked by a disulfide bond (Sanchez et al., Proc Natl Acad Sci USA 93:3602-3607, 1996). Although the causes of filovirus virulence are not well known, there is evidence that glycans on the viral glycoproteins play distinct roles in pathogenesis of these viruses (Takeda and Kawaoke, Trends Microbiol 9:506-511, 2001).

[0007] It would be useful to discover and develop new treatments for infections, such as viral and bacterial infections that could be used in prevention and/or treatment of infections and/or to supplement the currently available treatment methods to combat infections. In addition, it would be useful to discover new treatments for infectious diseases that do not currently have an effective treatment method, such as filovirus infection or infections by bacteria that have developed resistance to the available antibiotics.

SUMMARY OF THE INVENTION

[0008] The present invention is directed to methods of treatment and/or prevention of infections, for example, viral and bacterial infections, in individuals, wherein the method comprises administering a supraphysiological amount of mannose-binding lectin (MLB) or ficolin-MBL fusion protein to an individual afflicted with an infection or at risk of infection, such as a viral or bacterial infection.

[0009] The invention is based upon a surprising discovery, that an infection in an individual with normal MBL serum concentration and function, i.e., who has no defect in MBL, can be successfully treated or prevented by using supraphysiological amounts of MBL or by using ficolin-MBL fusion protein.

[0010] The terms "supraphysiological" or "supraphysiologic" are intended to encompass amounts of MBL or ficolin-MBL fusion protein that exceed the normal serum concentration of MBL in an individual, preferably a human individual. The normal serum concentration of MBL can be either measured individually, or estimated based upon a normal range or average normal serum concentration in humans or particular human populations. Typically, the "normal" human serum concentration of MBL is considered a concentration in individuals who do not carry genetic alterations or mutations that are known to reduce the amount or function of MBL in said individual.

[0011] In one embodiment, and all other embodiments described herein, one uses amounts of MBL that result in blood concentration of >2* to 10* the average human serum concentration, which is considered a normal serum concentration. In one embodiment, the human average MBL serum concentration is estimated to be about 2 [mu]g/mL. Accordingly, one can use any amount that results in serum concentration of between 4-20 [mu]g/mL. For example, an amount that results in serum concentration of about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 [mu]g/mL In one embodiment, similar amounts of ficolin-MBL are used.

[0012] Viral infections that can be prevented, ameliorated/treated or cured using the methods of the present invention include, but are not limited to, filoviruses, including Ebola and Marburg viruses, HIV, influenza, severe acute respiratory syndrome coronavirus (SARS-CoV), hepatitis B virus, hepatitis C virus, respiratory syncytial virus, and herpes simplex virus.

[0013] Bacterial infections that can be prevented ameliorated/treated or cured using the methods of the present invention include, but are not limited to Staphylococcus aureus; Neisseria meningitidis; Burkholderia multivorans, group B. streptococcus, Escherichia coli, Pseudomonas aeruginosa, Mycoplasma pneumoniae, and Chlamydia pneumoniae.

[0014] While the methods of the invention can be used for treatment and/or prevention of infections in any animal or bird, a preferred target individual is human.

[0015] In one embodiment, and all other embodiments described herein, the target individual is affected with a bacterium that has become resistant to currently available antibiotics. In one embodiment, one uses the method of the present invention in combination with antibiotics or bacteriophages, or anti-viral agents.

[0016] In one embodiment, and all other embodiments described herein, the individual is affected with a filovirus, such as Ebola or Marburg virus.

[0017] In one embodiment, and all other embodiments described herein, the method comprises first selecting a patient who is infected with a virus or bacterium, and then administering to the selected individual a supraphysiological amount of MLB or ficolin-MBL fusion protein.

[0018] In one embodiment, and all other embodiments described herein, the individual affected with, exposed to or susceptible to be exposed to an infection, such as bacterial or viral infection does not have a congenital or acquired MBL deficiency. In one embodiment, and all other embodiments described herein, one first determines if the individual has a congenital or acquired MBL deficiency. If the individual does not have such a deficiency, the individual can be administered a supraphysiological amount of MBL or ficolin-MBL fusion protein as a treatment or preventive measure to fight a viral infection or a suspected viral infection or exposure to an environment likely to carry viruses, such as an Ebola virus.

[0019] MBL can be purified from natural sources or from material produced by recombinant technologies, or by any other suitable MBL-producing cell line, for the prophylaxis and/or treatment of infections. Preparations and pharmaceutical compositions of MBL are known. In one embodiment, one uses the MBL as described in U.S. Pat. No. 5,270,199, which is herein incorporated by reference in its entirety. Also preparations and pharmaceutical compositions of ficolin-MBL fusion proteins are known. In one embodiment, one uses ficolin-MBL chimeric proteins described in, e.g., U.S. Patent Application Publication No. 20060188963. In one embodiment, one uses SEQ ID NO: 1 to produce MBL. Ficolin sequences, for example SEQ ID NO: 3, and SEQ ID NO: 8 can be used to make constructs for recombinantly producing various ficolin-MBL fusion proteins.

[0020] One aspect of the invention relates to treatment and/or prophylaxis of infections in individuals affected with a viral or bacterial infection using supraphysiological amount of MBL or ficolin-MBL fusion protein. In one embodiment, the individuals are not immunocompromised.

[0021] Without wishing to be bound by a theory, we believe that MBL exerts its antimicrobial activity mainly through its opsonizing activity (preparation of microorganisms for phagocytosis). This activity is dependent on activation of complement after binding of MBL to the microbial surface and deposition of C4b and C3b on the microorganism. MBL can also promote direct complement-mediated killing of the microorganism through an activation of the terminal lytic pathway of complement and insertion of the membrane attack complex (MAC) in the membrane. Without wishing to be bound by a theory, this mechanism is considered of minor importance. Many microorganisms, such as Gram-positive bacteria, e.g., Streptococcus pneumonia, are resistant to MAC, but can be eliminated by opsonophagocytosis. The inhibition of infection may be mediated by MBL directly neutralizing the pathogen, enhancing uptake by phagocytic cells that eliminate the

infection, or by killing the pathogens by activation of the complement protein pathway.

[0022] Because the MBL is normally present at physiological amounts in individuals who do not have congenital defects in it or who are not immunocompromised, it was surprising that one can exert a virus dose reducing effect by administering additional, supraphysiological amount of MBL into such an individual.

[0023] In another aspect, the present invention relates to the use of a composition comprising at least one mannan-binding lectin (MBL) subunit, or at least one oligomer comprising the at least one mannan-binding lectin (MBL) subunit, in the manufacture of a medicament for prophylactic, ameliorating or curative treatment of an infection, including a viral or bacterial infection, in an individual initially having plasma levels of MBL of about 5 [mu]g/mL. In one embodiment, the individual is not genetically disposed to an MBL deficiency or does not have acquired MBL deficiency.

[0024] Accordingly, in one embodiment, the methods are used as prophylaxis for individuals who are likely to be exposed, or who have already been exposed to viruses and/or bacteria, but do not yet have symptoms of infection, wherein the presence of supraphysiological amount of MBL or ficolin-MBL will prevent infection or ameliorate symptoms of an infection.

[0025] In one embodiment, the invention provides a method of preventing a filovirus infection by administering a supraphysiological dose of MBL or ficolin-MBL fusion protein to an individual who is likely to be exposed to a filovirus. In one embodiment, the filovirus is Ebola virus. In one embodiment, the filovirus is Marburg virus.

[0026] In one embodiment, the invention provides use of MBL or ficolin-MBL fusion protein as a medicament for treatment of infections, particularly viral and bacterial infections, in amounts that are supraphysiological.

BRIEF DESCRIPTION OF DRAWINGS

[0027] FIG. 1 shows a schematic drawing of the mannose-binding lectin (MBL) protein and L-ficolin.

[0028] FIG. 2 shows a schematic drawing showing the functional and structural domains of MBL and L-ficolin.

[0029] FIG. 3 shows a schematic drawing showing the construction of the three chimeric FCN-MBL fusion proteins.

[0030] FIG. 4 shows an SDS-PAGE protein gel showing the purified recombinant chimeric FCN-MBL fusion proteins and the denatured purified recombinant MBL under reducing conditions.

[0031] FIG. 5 shows a protein gel of the purified recombinant chimeric FCN-MBL fusion proteins and recombinant MBL under non-reducing conditions.

[0032] FIG. 6 shows a competitive ELISA comparing avidity of rhMBL and chimeric proteins (100 ng each) binding to mannan.

[0033] FIG. 7 shows a C4 deposition assay. The C4 deposition assay is an ELISA-based functional assay that measures the relative capacities of MBL or the chimeric protein to bind human C4. Mannan (10 ug/mL) is coated on a 96-well ELISA plate, blocked with BSA, and incubated with varying concentrations of rhMBL or chimeric proteins. Human C4 (10 ug/mL) is then added and detected with biotin-streptavidin conjugated antibodies. FCN-MBL76 had significantly greater C4 binding activity compared with rhMBL and the other chimerics. This result suggests that FCN-MBL76 has greater complement pathway activating capacity which may result in enhanced pathogen lysis or neutralization.

[0034] FIG. 8 shows calreticulin binding assay. The 96-well ELISA plate was coated with rhMBL or

chimeric proteins (10 ug/mL), blocked with BSA and incubated with 5 ug/mL biotinylated human placental calreticulin that was measured at absorbance O.D. 405. FCN-MBL76 bound to human placental calreticulin significantly better than rhMBL or the other chimeric proteins. This may have important implications for the relative functions of the proteins because calreticulin is the putative cellular receptor on phagocytes for native MBL and therefore, enhanced binding of the chimeric molecule may result in improved pathogen clearance by opsonophagocytosis.

[0035] FIG. 9 shows an inhibition assay using Hep G2 cells infected with lentivirus (HIV) pseudotyped with Ebola glycoprotein. Hep G2 cells at approximately 80% confluence in 96-well tissue culture plates were infected with HIV particles without an envelope (HIV-env neg; solid square) or with an envelope consisting of Ebola glycoprotein (other symbols). The virions encoded luciferase that was expressed only in infected cells and detected with a commercial luciferase assay. Before addition of viral particles to the cells, the viruses were preincubated with 0, 0.1 or 1 ug/mL of rhMBL or chimeric proteins in veronal-buffered saline with 5 mM CaCl2 for 1 hour at 37 C. Infection was achieved by spinoculation of cells at 1000 g*2 hrs. The viral protein mixture was replaced with EMEM culture media and incubated at 37 C for 40 hrs after which, the cells were lyzed and luciferase expression was quantified. rhMBL and the chimeric proteins inhibited viral infection to similar significant extents (1 ug/mL vs no protein, $p<0.001$)

[0036] FIG. 10 shows an inhibition assay using Hep G2 cells infected with native Ebola-Zaire virus. 30,000 Hep G2 cells/well in 96-well tissue culture plates were infected with native Ebola virus (Zaire strain) that was genetically engineered to express GFP. The viral particles were preincubated with 0, 0.1 or 1 ug/mL of rhMBL or chimeric proteins in veronal-buffered saline with 10 mM CaCl2 for 1 hour at 37 C. The viral protein mixture was added to the cells and incubated for 48 hrs after which time the cells were washed. Viral infection of cells was quantified by measuring GFP expression. rhMBL and the chimeric proteins inhibited viral infection but FCN-MBL76 was the most effective.

[0037] FIG. 11 shows that a pharmacokinetic modeling of rhMBL (recombinant human MBL) in immunocompetent C57B/6J mice revealed that doses of 75 mcg and 350 mcg doses produced Cmax of ~5 [mu]g/mL and ~15 [mu]g/mL, respectively and half-life of ~11 hours at both doses. A previous study showed that 75 [mu]g is the minimum dose of rhMBL required to activate complement in an MBL-deficient mouse model.

[0038] FIG. 12 shows a Kaplan Meier survival analyses: 350 [mu]g rhMBL was given immediately pre-challenge with EBOV Zaire and continued every 12 hrs*10 days resulting in 42% survival rate (log rank, $p<0.008$).

[0039] FIG. 13 shows a Kaplan Meier survival curve with a post-challenge analysis which demonstrated that recombinant human MBL-treated wild-type mice had a significant survival advantage: 40% survived compared to 100% mortality among wild-type and C3 knock-out mice treated with saline or rhMBL indicating that rhMBL provides protection but that the protection is dependent on C3. MBL treated mice survived significantly longer than mice not treated with MBL. EBOV was administered IV 100pfu (plaque forming units) 3000xLD50. WT (wildtype, C57B/6J mice) versus C3 knock out (KO). Recombinant MBL (rhMBL) was administered at 350 mcg IL 12 hors post challenge, then q12hx10 days vs. sham Rx.*log rank, $p<0.0004$.

[0040] FIGS. 14A-14D show that sham treated wild-type mice all died before the 10 day time point. rhMBL-treated wild-type mice had significantly higher total white blood cell and lymphocyte counts after day 5 suggesting that lymphocyte responses in these mice may be protective.

[0041] FIG. 15 shows A Rush HepG2 Infection Assay for HIV-EBOZ vs. HIV-env as a negative control with 400 pg/well, 96 well format. MDS (M.R. 1:2, non-HI). The results demonstrate that MBL significantly inhibited infection of HepG2 cells by HIV particles pseudotyped with Ebola glycoprotein. The control virus is an HIV particle without viral surface glycoproteins.

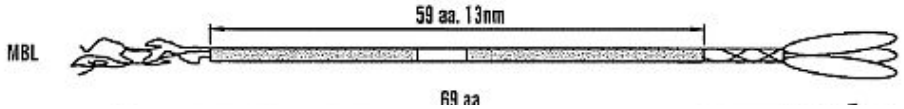


FIG. 1

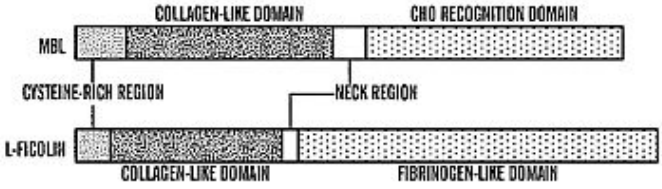


FIG. 2

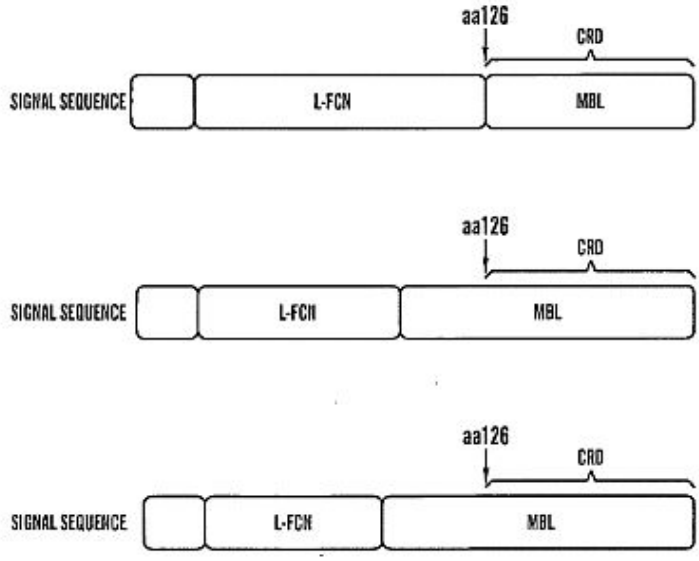


FIG. 3

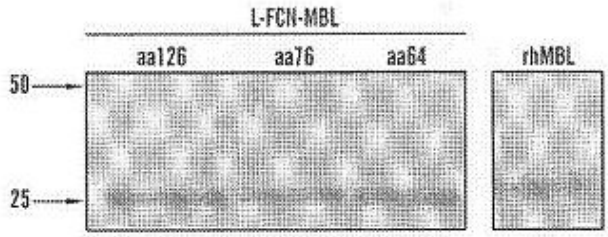
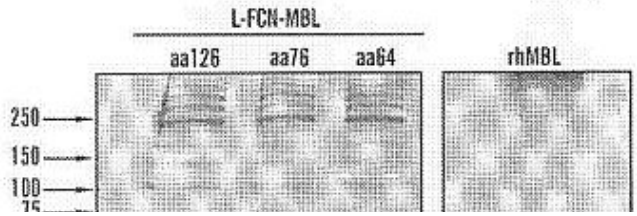


FIG. 4



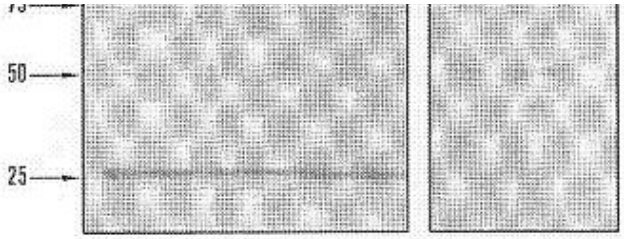


FIG. 5

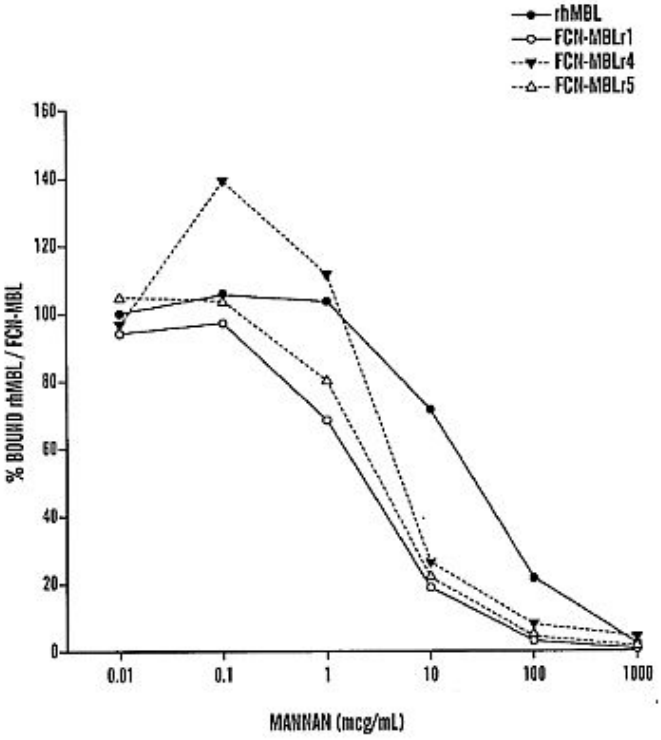


FIG. 6

* Chimerics vs rhMBL, p<0.001
** FCN-MBL76 vs rhMBL or other chimerics, p<0.001
*** Chimerics vs rhMBL, p<0.001

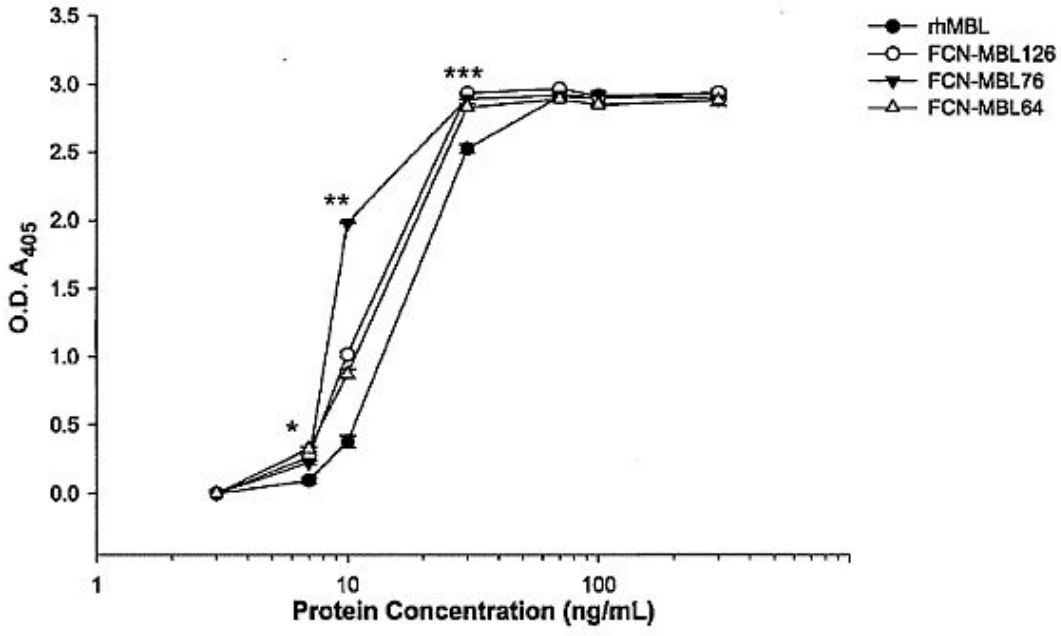


FIG. 7

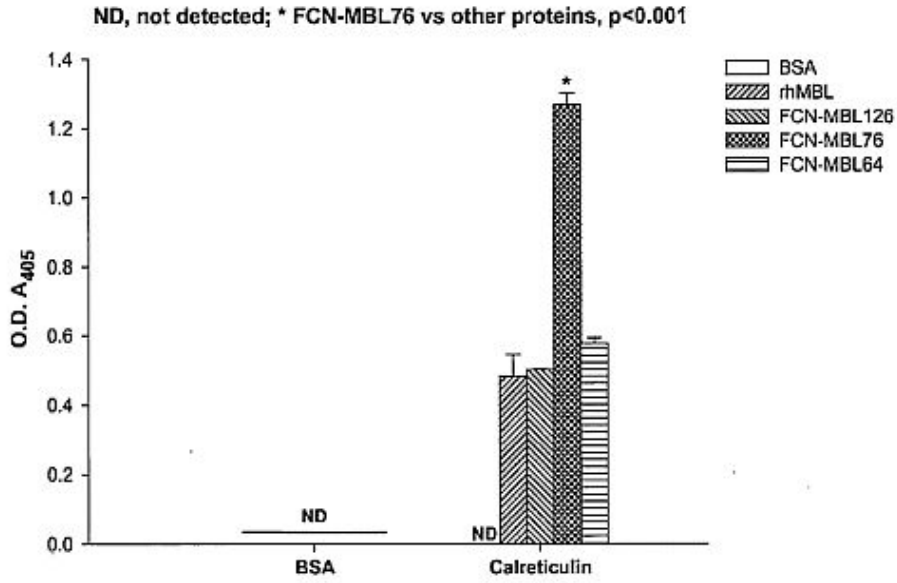

ND, not detected; * FCN-MBL76 vs other proteins, p<0.001
O.D. A405
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BSA
rhMBL
FCN-MBL126
FCN-MBL76
FCN-MBL64
*
ND
ND
BSA
Calreticulin

FIG. 8

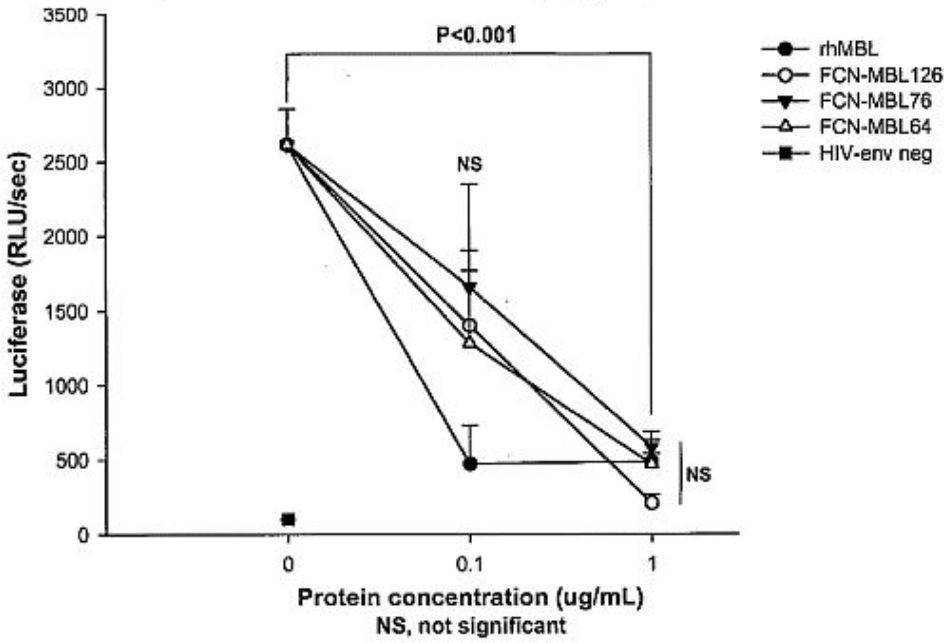
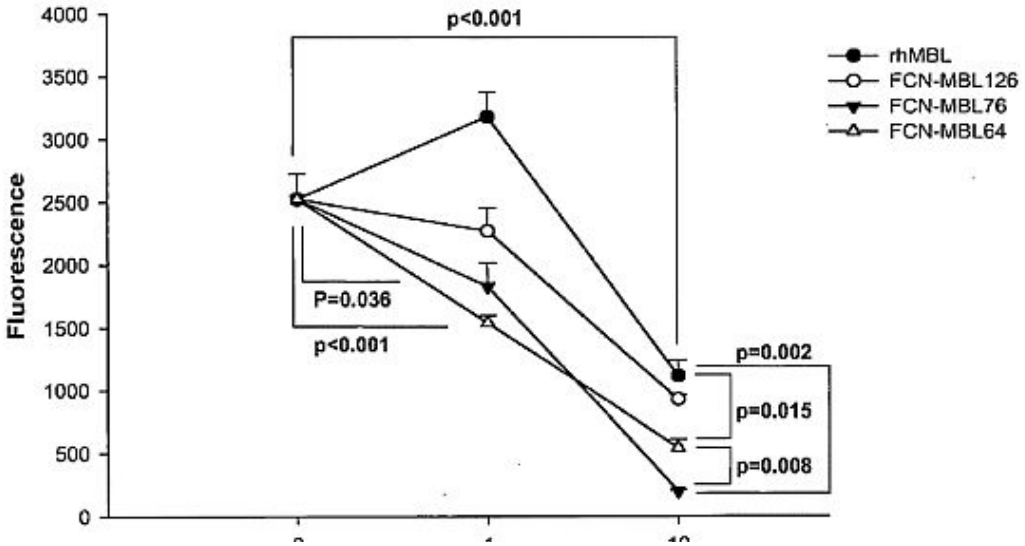

Luciferase (RLU/sec)
3500
3000
2500
2000
1500
1000
500
0
P<0.001
NS
NS
0
0.1
1
Protein concentration (ug/mL)
NS, not significant
rhMBL
FCN-MBL126
FCN-MBL76
FCN-MBL64
HIV-env neg

FIG. 9


Fluorescence
4000
3500
3000
2500
2000
1500
1000
500
0
p<0.001
P=0.036
p<0.001
p=0.002
p=0.015
p=0.008
rhMBL
FCN-MBL126
FCN-MBL76
FCN-MBL64

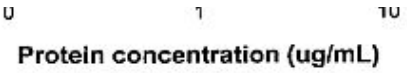


FIG. 10

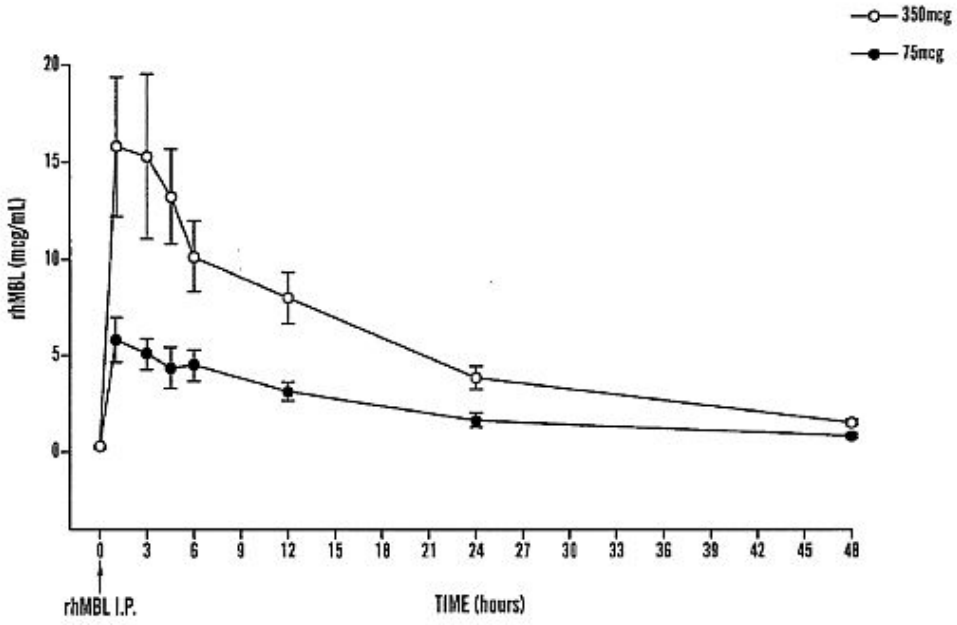


FIG. 11

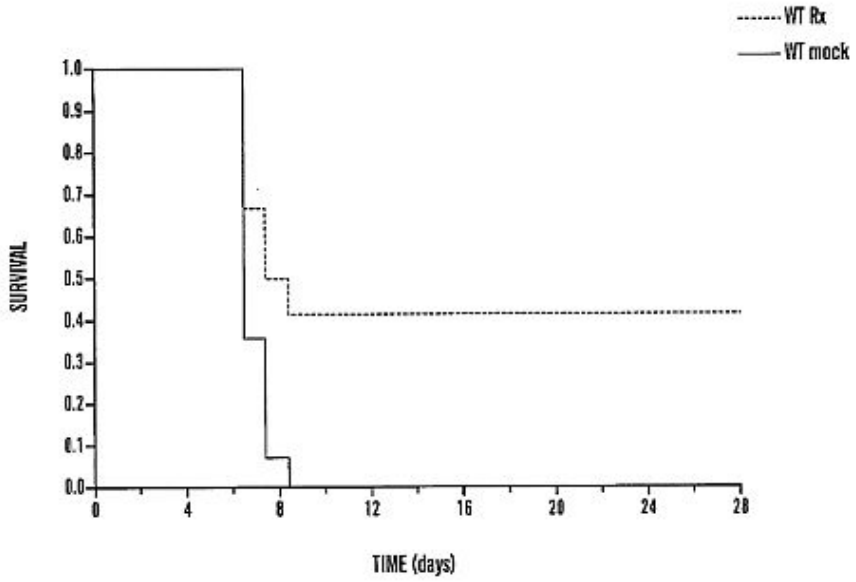


FIG. 12

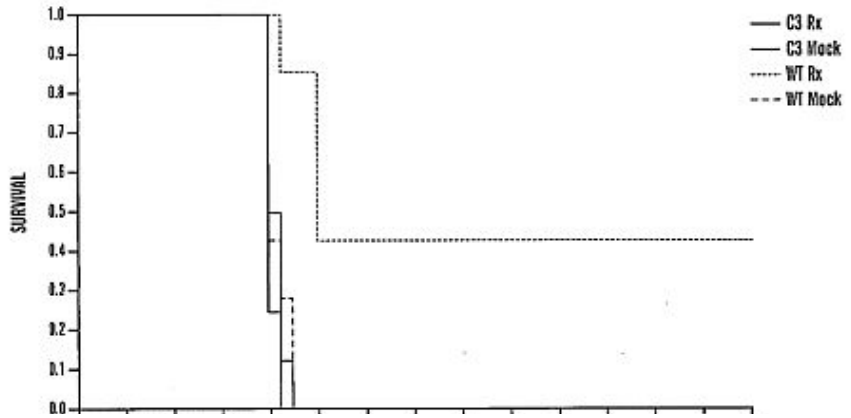


FIG. 13

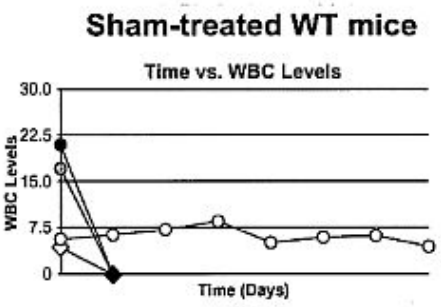


FIG. 14A

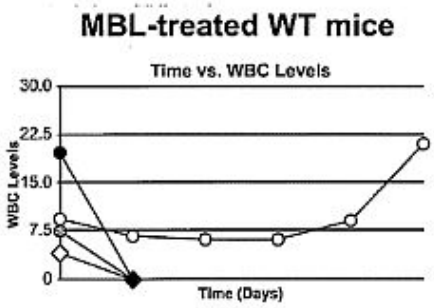


FIG. 14B

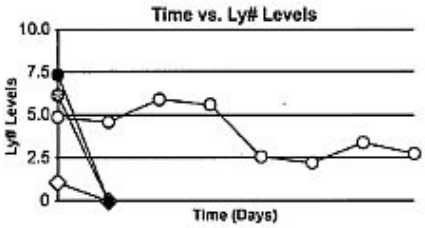


FIG. 14C

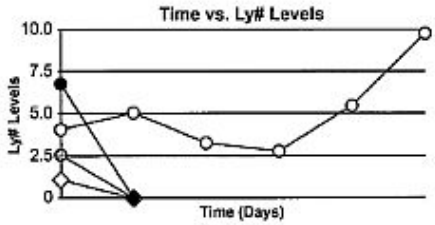


FIG. 14D

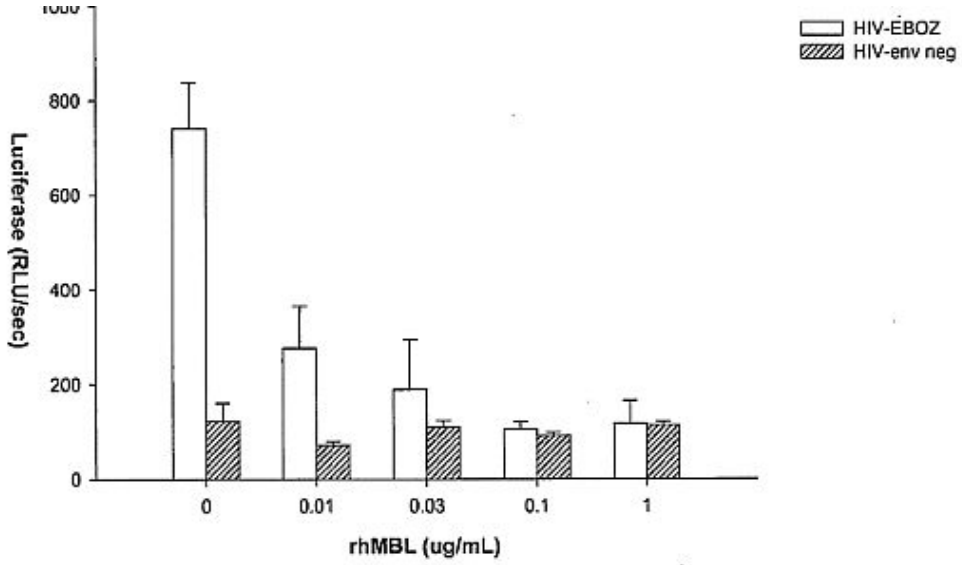


FIG. 15

DETAILED DESCRIPTION OF THE INVENTION

[0042] The present invention is directed to methods and uses of MBL and ficolin-MBL fusion proteins for the treatment of infections.

[0043] The innate immune system that defends humans from infections is comprised of a network of recognition and effector molecules that act together to protect the host in the first minutes or hours of exposure to an infectious challenge.

[0044] The mannan-binding lectin (MBL), synonymous to mannose-binding lectin, mannan-binding protein or mannose-binding protein (MBP), is an evolutionarily conserved circulating host defense protein that acts as a broad spectrum recognition molecule against a wide variety of infectious agents (see, e.g., review by Takahashi et al. Current Opinion in Immunology 18:16-23, 2006).

[0045] Several groups of lectins, i.e., carbohydrate-binding proteins, are known in humans. One group is the C-type lectins. The C-type lectins contain a calcium-dependent carbohydrate recognition domain (a C-type CRD)(Weis W I, et al. Immunological Reviews 163: 19-34, 1998). MBL belongs to the subgroup of C-type lectins, termed collectins, since these soluble proteins are composed of subunits presenting three CRDs attached to a collagenous stalk (Holmskov, U., et al., Immunol. Today 15:67-74, 1994). MBL interacts with carbohydrates presented by a wide range of micro-organisms playing an important role in the innate immune defense (Turner, M. W. Immunol. Today 17:532-540, 1996 and Takahashi et al., Current Opinion in Immunology, 18:16-23, 2006). When bound to carbohydrate MBL is able to activate the complement system.

[0046] The complement system may be activated via three different pathways: the classical pathway, the alternative pathway, and the third pathway, the mannan-binding lectin (MBL) pathway, which is initiated by the binding of MBL to carbohydrates presented by micro-organisms. The components of the alternative pathway and of the MBL pathway are parts of the innate immune defense, also termed the natural or the non-clonal, immune defense, while the classical pathway involves cooperation with antibodies of the specific immune defense (Janeway C A, Travers P, Walport M and Capra J D, 1999, Immunobiology, the immune system in health and disease, Fourth Edition, Churchill Livingstone).

[0047] The human MBL protein is composed of up to 18 identical 32 kDa polypeptide chains (Lu, J., et al., (1990) J. Immunol. 144:2287-2294), each comprising a short N-terminal segment of 21 amino acids including three cysteine residues, followed by 7 repeats of the collagenous motif Gly-X-Y interrupted by a Gln residues followed by another 12 Gly-X-Y repeats. A small 34 residue 'neck-region' joins the C-terminal Ca<2+>-dependent lectin domain of 93 amino acids with the collagenous part of the molecule (Sastry, K., et al., (1989) J. Exp. Med. 170:1175-1189).

[0048] The collagenous regions of the three polypeptide chains combine to form a subunit which is stabilized covalently by disulphide bridges. Individual subunits are joined by disulphide bridges as well as by non-covalent interactions (Lu, J., et al., J. Immunol. 144:2287-2294, 1990).

[0049] The position of these disulphide bridges has, however, not been fully resolved. SDS-PAGE analysis under non-reducing conditions of MBL shows bands with an apparent molecular weight (m.w.) larger than 200 kDa presumably representing blocks of 3, 4, 5 and even 6 assembled subunits (Lu, J., et al., J. Immunol. 144:2287-2294, 1990).

[0050] The actual number of subunits in the natural human MBL protein has been controversial. Lipscombe et al. (1995) obtained data by use of ultracentrifugation suggesting 25% of human serum MBL to be made of 2-3 subunits and only a minor fraction reaching the size of 6 subunits (Lipscombe, R. J., et al., Immunology 85:660-667, 1995). The relative quantification was carried out by densitometry of Western blots developed by chemiluminescence (Lu, J., et al., J. Immunol. 144:2287-2294, 1990) found by SDS-PAGE analysis of fractions from ion exchange chromatography that the predominant species of covalently linked MBL subunit chains consisted of tetramers while only pentameric or hexameric complexes activated complement. Gel permeation chromatography (GPC) analysis, in contrast, suggests that MBL is comparable in size with the C1 complex. GPC can be carried out under conditions which allow for a study of the importance of weak protein-protein interactions in the formation of MBL molecules. MBL content in the GPC fractions can be determined by standard MBL assay techniques.

[0051] MBL is synthesized in the liver by hepatocytes and secreted into the blood. It binds to carbohydrate structures on bacteria, yeast, parasitic protozoa and viruses, and exhibits antibacterial activity through killing of the microorganisms by activation of the terminal, lytic complement components or through promotion of phagocytosis (opsonization). The sertiform structure of MBL is quite similar to the bouquet-like structure of C1q, the immunoglobulin-binding subcomponent of the first component in the classical pathway (Turner, M. W. Mannose-binding lectin: the pluripotent molecule of the innate immune system. Immunol. Today 17:532-540, 1996). C1q is associated with two serine proteases, C1r and C1s, to form the C1 complex. Similarly, MBL is associated with two serine proteases MASP-1 (Matsushita, M. and Fujita, T, J. Exp. Med. 176:1497-1502, 1992) and MASP-2 (Thiel S, et al., Nature, 386(6624): 506-510, 1997), and an additional protein called Map19 (Stover C M, et al., J Immunol 162: 3481-3490, 1999). MASP-1

and MASP-2 have modular structures identical to those of C1r and C1s (Thiel S, et al., Nature, 386(6624): 506-510, 1997). The binding of MBL to carbohydrates induces the activation of MASP-1 and MASP-2. MASP-2 then generates the C3 convertase, C4b2a, through cleavage of C4 and C2. Reports suggest that MASP-1 may activate C3 directly. Nothing is known about the stoichiometry and activation sequence of the MBL/MASP complexes. MBL has also been characterized in other animals such as rodents, cattle, chicken and monkeys.

[0052] Based on presence and function of MBL in at least rodents, cattle, chicken and monkeys, in addition to humans, makes the methods of the present invention applicable to at least these animals as well.

[0053] Human mannose-binding protein has been disclosed in U.S. Pat. No. 5,270,199. Moreover, use of MBL in treatment of immunocompromised individuals has been described (U.S. Pat. Nos. 6,562,784 and 7,202,207, and U.S. Patent Application Publication No. 2007-0197428). However, because MBL is a naturally occurring molecule present in the serum, no one has suggested its use in treatment or prevention of infections in individuals with normal serum concentration of MBL. Our discovery that supraphysiological amounts of MBL can increase the infection fighting capacity of an individual with normal MBL concentrations and function was thus surprising.

[0054] Accordingly, one aspect of the invention provides a method for prevention and treatment of infections in individuals, such as human individuals, comprising administering to said individual a supraphysiological amount of MBL.

[0055] The term "supraphysiological" as used in the present application means amounts greater than the physiological amount normally present in an individual or greater than minimal concentration of MBL required to activate a complement, i.e. to bind to C4. Similar concentrations of ficolin-MBL fusion proteins can also be used.

[0056] In one embodiment, one uses MBL and/or ficolin-MBL fusion proteins or combinations thereof in the amount that results in the amount of about 2-10 times greater than the physiological amount of MBL in an individual. In one embodiment, one uses, for example, 2, 3, 4, 5, 6, 7, 8, 9 or 10 times greater than the physiological amount in the individual. In one embodiment, the average physiological amount is considered about 2 [mu]g/mL. In one embodiment, one first determines the physiological amount of MBL in an individual prior to administering MBL or ficolin-MBL fusion protein composition to said individual. This is particularly useful when using MBL or ficolin-MBL as a prophylaxis for individuals who will be at risk of encountering infective agents, such as medical personnel or armed forces who may be a target for a biological attack.

[0057] The concentration of MBL in human serum is largely genetically determined, but reportedly increases up to threefold during acute phase infection reactions (Thiel S, et al., Clin Exp Immunol 90: 31-35, 1992). Three mutations causing structural alterations and two mutations in the promotor region are associated with MBL deficiency (Madsen, H. O., et al., Immunogenetics 40:37-44, 1994). MBL deficiency is associated with susceptibility to a variety of infections (Summerfield J A, et al., Lancet 345: 886-889, 1995; Garred P, et al., Lancet 346: 941-943, 1995).

[0058] It has been estimated that the average physiological amount of MBL in human serum is about 2 [mu]g/mL. Accordingly, in one embodiment, one uses the average physiological amount as the physiological amount, and consequently, the supraphysiological amount of MBL or ficolin-MBL according to the present invention is about 2-10 times above the average physiological MBL level.

[0059] In one embodiment, one takes into account the impact of MBL haplotypes when considering the physiological serum MBL concentrations. At least ten distinct MBL haplotypes have been described in human, four of which (LYPB, LYQC, HYPD and LXPA) dictate low serum MBL concentrations (Madsen et al. J Immunol 161:3169-3175, 1998; Takahashi et al., Current Opinion in Immunology 18:16-23, 2006). Human populations from diverse geographic locations and ethnic and genetic backgrounds have higher rate of haplotype variation, with a rate of heterozygosity from 15% in white populations to 30% in certain African populations. Accordingly, in one embodiment, to establish the physiological serum MBL level to adjust the amount of MBL used in

the methods of the present invention, one correlates the level of MBL with a functional measurement of the MBL:MASP pathway (Takahashi et al., Current Opinion in Immunology 18:16-23, 2006; Petersen et al., J Immunol Methods 257:107-116, 2001). Accordingly, if the average serum concentration of MBL in a particular individual or population is higher, the supraphysiologic dosage is adjusted accordingly. Similarly, if the average serum concentration of MBL is lower, a lower amount is needed for the treatment of prevention of infections. A skilled artisan is easily able to make these determinations based on the description herein.

[0060] A wide range of oligosaccharides can bind to MBL. As the target sugars are not normally exposed on mammalian cell surfaces at high densities, MBL does not usually recognize self-determinants, but is well suited to interactions with microbial cell surfaces presenting repetitive carbohydrate determinants. In vitro, yeast (Candida albicans and Cryptococcus neoformans), viruses (HIV-1, HIV-2, HSV-2, and various types of influenza A) and a number of bacteria have been shown to be recognized by MBL. In the case of some bacteria, the binding with MBL is impaired by the presence of a capsule (van Emmerik, L C, et al., Clin.Exp.Immunol. 97:411-416, 1994). However, even encapsulated bacteria (Neisseria meningitidis) can show strong binding of MBL (Jack D L, et al., J Immunol 160: 1346-1353, 1998), and is thus one target infection according to the present invention.

[0061] The microorganisms, which infect MBL deficient individuals, represent many different species of bacterial, viral and fungal origin (Summerfield J A, et al., BioMed J 314: 1229-1232, 1997; Miller, M. E., et al., Lancet: 60-63, 1968; Super, M., et al., Lancet 2:1236-1239, 1989; and Nielsen, S. L., et al., Clin. Exp. Immunol. 100:219-222, 1995). Deficiency is also associated with habitual abortions (Christiansen, O. B., et al., Scand. J. Immunol., 49, 193-196, 1999). Indeed, MBL appears to be a general defense molecule against most bacteria, and thus be considered as one reason why so many bacteria are non-pathogenic.

[0062] Accordingly, in one embodiment, the methods of the invention pertain to prevention and/or treatment of infections caused by any of the foregoing infective agents, including viruses, yeast, fungus, and bacteria.

[0063] While accumulating data support the notion of a protective effect of MBL there are also observations suggesting that infections with some microorganisms, notably intracellular pathogens, attain a higher frequency in MBL sufficient than in MBL deficient individuals (Garred, P, et al., Eur. J. Immunogen. 21:125-131, 1994; Hoal-Van Helden E G, et al., Pediatr Res 45:459-64, 1999). This is in concordance with the results of an animal experiment, where an increased number of HSV-2 were found in the liver of mice pre-injected with human MBL (Fischer, P B, et al., Scand J Immunol 39:439-445, 1994). Our results contradict these findings by showing a strong protective and treatment effect of administering to a subject a supraphysiological amount of MBL and/or ficolin-MBL fusion proteins or combinations thereof.

[0064] Clinical grade MBL has been obtained from blood donor plasma and shown to be safe upon infusion (Valdimarsson, H., M. et al., Scand. J. Immunol. 48:116-123, 1998). Accordingly, one can use such preparations in the methods of the present invention. Similarly, one can make recombinant MBL using any well known gene expression system.

[0065] Ficolins, like MBL, are lectins that contain a collagen-like domain. However, unlike MBL, they have a fibrinogen-like domain, which is similar to fibrinogen beta- and gamma-chains. Ficolin also forms oligomers of structural subunits, each of which is composed of three identical 35 kDa polypeptides. Each subunit is composed of an amino-terminal, cysteine-rich region; a collagen-like domain that consists of tandem repeats of Gly-Xaa-Yaa triplet sequences (where Xaa and Yaa represent any amino acid); a neck region; and a fibrinogen-like domain. The oligomers of ficolins comprise two or more subunits, especially a tetrameric form of ficolin has been observed.

[0066] Some of the ficolins trigger an activation of the complement system substantially in similar way as done by MBL. This triggering of the complement system results in the activation of novel serine proteases (MASPs).

[0067] The fibrinogen-like domain of several lectins has a similar function to the CRD of C-type

lectins including MBL, and function as pattern-recognition receptors to discriminate pathogens from self.

[0068] Serum ficolins have a common binding specificity for GlcNAc (N-acetyl-glucosamine), elastin or GalNAc (N-acetyl-galactosamine). The fibrinogen-like domain is responsible for the carbohydrate binding. In human serum, two types of ficolin, known as L-ficolin (also called P35, ficolin L, ficolin 2 or hucolin) and H-ficolin (also called Hakata antigen, ficolin 3 or thermolabile b2-macroglycoprotein), have been identified, and both of them have lectin activity. L-ficolin recognises GlcNAc and H-ficolin recognises GalNAc. Another ficolin known as M-ficolin (also called P35-related protein, ficolin 1 or ficolin A) is not considered to be a serum protein and is found in leucocytes and in the lungs. L-ficolin and H-ficolin activate the lectin-complement pathway in association with MASPs. M-Ficolin, L-ficolin and H-ficolin has calcium-independent lectin activity.

[0069] Accordingly, in one embodiment, the invention provides methods of prevention and/or treatment of infectious diseases using MBL-L-ficolin or MBL-H-ficolin fusion proteins, or a combination thereof.

[0070] Naturally, one can also use a combination of MBL and MBL-ficolin, such as MBL-L-ficolin and/or MBL-H-ficolin.

[0071] Chimeric molecules of MBL and ficolin have been described, for example, in U.S. Patent Application Publication No. 2006-0188963. Although it has been suggested that the chimeric molecules could be used to prevent and/or treat infections in patients having clinical symptoms associated with congenital or acquired MBL deficiency or being at risk of developing such symptoms (Id.), no one has proposed or shown that individuals with normal MBL activity would benefit from additional, supraphysiological amounts of MBL or MBL-ficolin in combating infectious diseases.

[0072] Based on our findings, the present invention provides a novel method for treatment or prevention of infections in an individual having normal expression and normal function of MBL.

[0073] In addition, fusion proteins useful according to the methods of the invention can designed in such a way as to test whether the source of the MASP-binding site and flanking sequences, and presence of the "kink" from MBL affect ligand binding activity and/or complement activation. Without wishing to be bound by a theory, we designed the proteins in the examples based on the assumption that differences at these sites alter the protein conformational structure which in turn alters protein-protein interactions. Therefore any differences in protein activity can assist in understanding the functional parts of the molecules. We discovered that FCN-MBL76 has a greater activity in various assays. Without wishing to be bound by a theory, we concluded that is because of differences in spatial orientation of the CRDs. We have shown that FCN-MBL76 binds the best to a sugar, mannan.

[0074] Examples of useful fusion proteins are presented in FIG. 3. In our test molecules, FCN-MBL126 has only the carbohydrate recognition domain (CRD) from MBL and the rest includes the MASP-binding site. The amino-terminus is from L-FCN.

[0075] FCN-MBL76 has the CRD, neck and part of the flanking sequences of the MASP-binding site from MBL; the lysine and other flanking sequences of the MASP-binding site, and the amino-terminal is from L-FCN.

[0076] FCN-MBL64 has the CRD, neck, MASP-binding site and flanking sequences and the "kink" from MBL; the amino-terminal is from L-FCN.

[0077] In one embodiment, one uses a fusion protein which includes the signal peptide from L-FCN because this component is important to signal the protein to be transported from the cytosol to the endoplasmic reticulum for packaging and secretion.

[0078] Accordingly, based on the description herein and throughout this specification and

examples, a skilled artisan can design various fusion proteins, including proteins with stability-increasing modifications using routine methods.

[0079] In certain embodiments, the methods of the present invention include treatment and/or prevention of infections including bacterial, viral and fungal infections. The viral infections according to the present invention can be caused by any virus, such as viruses including but not limited to the viruses of the herpes family, such as Herpes Simpex I, Herpes Simplex II, Human Herpesvirus 6 (HHV-6), herpes zoster; poxviruses; corona viruses; paramyxoviruses; and togaviruses, HIV, Ebola, and the like.

[0080] In certain embodiments, the methods of the present invention provide for treatment of bacterial infections and/or preventing bacterial infection for bacteria such as Staphylococcus spp., Streptococcus spp., Escherichia spp., Enterococcus spp., Pseudomonas spp. bacteria and combinations thereof, and more particularly Staphylococcus aureus, including antibiotic resistant strains such as methicillin resistant Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli (E. coli), Pseudomonas aeruginosa (Pseudomonasae), Streptococcus pyogenes, and combinations thereof.

[0081] In certain embodiments, the method of the present invention provide treatment and/or prevention for infections caused by Staphylococcus aureus; Neisseria meningitidis; Burkholderia multivorans, group B streptococcus, Escherichia coli, Pseudomonas aeruginosa, Mycoplasma pneumoniae, and Chlamydia pneumoniae.

[0082] In one embodiment, the method of the present invention provide treatment and/or prevention for infections caused by HIV, influenza, severe acute respiratory syndrome coronavirus SARS-CoV), hepatitis B virus, hepatitis C virus, respiratory syncytial virus, herpes simplex virus, or filovirus, for example Ebola or Marburg virus.

[0083] A medicament comprising MBL and/or MBL-ficolin fusion protein, may be produced by using the eluant obtained from the affinity chromatography as such. It is however preferred that the eluant is subjected to further purification steps before being used in a pharmaceutically acceptable carrier.

[0084] In one embodiment, the composition or medicament consists essentially of MBL and/or MBL-ficolin fusion protein or functional, i.e. infectious agents binding derivatives thereof in a pharmaceutically acceptable carrier.

[0085] In addition to the MBL oligomers or ficolin-MBL fusion proteins, the medicament may comprise a pharmaceutically acceptable carrier substance and/or vehicles. In particular, a stabilizing agent may be added to stabilize the MBL proteins or the ficolin-MBL fusion proteins. The stabilizing agent may be a sugar alcohol, saccharides, proteins and/or amino acids. Examples of stabilizing agents are maltose or albumin.

[0086] The term "derivative" as used herein refers to MBL or ficolin-MBL fusion proteins which are functional in the sense that they can bind infectious agents but have also have been chemically modified, for example but not limited to by techniques such as ubiquitination, labeling, pegylation (derivatization with polyethylene glycol, PEG) or addition of other molecules. A molecule also a "derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties can improve the molecule's solubility, absorption, biological half life, etc. The moieties can alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., MackPubl., Easton, Pa. (1990), and PROTEINS-STRUCTURE AND MOLECULAR PROPERTIES, 2nd Ed., T. E. Creighton, W.H. Freeman and Company, New York (1993); POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS, B. C. Johnson, Ed., Academic Press, New York, pgs. 1-12 (1983); Seifter et al., Meth Enzymol 182:626-646 (1990); Rattan et al., Ann NY Acad Sci 663:48-62 (1992).).

[0087] In one embodiment, the MBL and/or ficolin-MBL fusion protein is fused to a second fusion

partner, such as a carrier molecule to enhance its bioavailability. Such carriers are known in the art and include poly (alkyl) glycol such as poly ethylene glycol (PEG). Fusion to serum albumin can also increase the serum half-life of therapeutic polypeptides.

[0088] The MBL and/or ficolin MBL fusion polypeptide can also be fused to a second fusion partner, for example, to a polypeptide that targets the product to a desired location, or, for example, a tag that facilitates its purification, if so desired. Tags and fusion partners can be designed to be cleavable, if so desired. Another modification specifically contemplated is attachment, e.g., covalent attachment, to a polymer. In one aspect, polymers such as polyethylene glycol (PEG) or methoxypolyethylene glycol (mPEG) can increase the in vivo half-life of proteins to which they are conjugated. Methods of PEGylation of polypeptide agents are well known to those skilled in the art, as are considerations of, for example, how large a PEG polymer to use.

[0089] As used herein, the term "conjugate" or "conjugation" refers to the attachment of two or more entities to form one entity. For example, the methods of the present invention provide conjugation of a MBL or ficolin-MBL fusion polypeptide or fragments, derivatives or variants thereof, joined with another entity, for example a moiety such as a first fusion partner that makes the MBL or ficolin-MBL fusion protein stable, such as Ig carrier particle, for example IgG1 Fc. The attachment can be by means of linkers, chemical modification, peptide linkers, chemical linkers, covalent or non-covalent bonds, or protein fusion or by any means known to one skilled in the art. The joining can be permanent or reversible. In some embodiments, several linkers can be included in order to take advantage of desired properties of each linker and each protein in the conjugate. Flexible linkers and linkers that increase the solubility of the conjugates are contemplated for use alone or with other linkers as disclosed herein. Peptide linkers can be linked by expressing DNA encoding the linker to one or more proteins in the conjugate. Linkers can be acid cleavable, photocleavable and heat sensitive linkers. Methods for conjugation are well known by persons skilled in the art and are encompassed for use in the present invention.

[0090] According to the present invention, the MBL or ficolin-MBL fusion polypeptide or fragments, derivatives or variants thereof, can be linked to the first fusion partner via any suitable means, as known in the art, see for example U.S. Pat. Nos. 4,625,014, 5,057,301 and 5, 514,363, which are incorporated herein in their entirety by reference. For example, the MBL or ficolin-MBL fusion polypeptide can be covalently conjugated to the IgG1 Fc, either directly or through one or more linkers. In one embodiment, a MBL or ficolin-MBL fusion polypeptide as disclosed herein is conjugated directly to the first fusion partner (e.g. Fc), and in an alternative embodiment, a MBL or ficolin-MBL fusion polypeptide as disclosed herein can be conjugated to a first fusion partner (such as IgG1 Fc) via a linker, e.g. a transport enhancing linker.

[0091] As used herein, the term "treating" includes reducing or alleviating at least one adverse effect or symptom of an infection. Accordingly, the anti-viral medicament according to the present invention may be a medicament capable of virus attenuation and/or elimination. Similarly, antibacterial medicament according to the present invention may be a medicament capable of stabilizing the bacterial infection and/or eliminating such an infection.

[0092] The MBL or ficolin-MBL fusion protein can be administered by any appropriate route which results in an effective treatment of an infection in the subject. In one embodiment, the administration is performed systemically.

[0093] In one embodiment, one administers the MBL or ficolin-MBL fusion proteins enterally, topically or parenterally. The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and infrasternal injection and infusion. The phrases "systemic administration," "administered systemically", "peripheral administration" and "administered peripherally" as used herein mean the administration of MBL and/or ficolin-MBL fusion protein other than directly into the central nervous system, such that it enters the animal's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous

administration.

[0094] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0095] The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

[0096] Other conventional additives may be added to the medicament depending on administration form for example. In one embodiment the medicament is in a form suitable for injections. Conventional carrier substances, such as isotonic saline, may be used.

[0097] In another embodiment the pharmaceutical composition or medicament is in a form suitable for pulmonal administration, such as in the form of a powder for inhalation or cream or fluid for topical application.

[0098] The route of administration may be any suitable route, such as intravenously, intramusculary, intraperitoneally, subcutanously or intradermally. Also, pulmonal or topical administration is envisaged by the present invention.

[0099] The MBL composition may also be administered simultaneously, sequentially or separately with another anti-bacterial, anti-viral or viral or bacterial infection symptom alleviating treatment.

[0100] The MBL and/or ficolin-MBL composition is administered in suitable dosage regimes, in particular, it is administered repeatedly at suitable intervals, such as once or twice a week. For example, one can start before exposure to the virus and maintain the periodic administration at intervals, for example 1, 2, 3, 4, 5, 6, or 7 times a week, or, for example, 1, 2, 3, or 4 times a day, at least during a part of the exposure or suspected exposure of the individual to the virus. One can also begin administering the MBL composition at the time of suspected exposure and continue with periodic additional dosages for at least 2, 3, 4, 5, 6, or 7 days or even longer. One can also begin the treatment at the onset of the symptoms of the infection, such a viral infection and continue the periodic administration of at least one additional dosages until the symptoms begin to diminish or until there are no symptoms, or until at least 1, 2, 3, 4, 5, 6, 7 days after the symptoms have disappeared.

[0101] In one embodiment, the invention provides a method wherein recombinant human MBL is administered every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 hours. In one embodiment, one administers recombinant human MBL every 12 hours.

[0102] The use of an MBL or ficolin-MBL fusion protein composition may also be in a kit-of-parts further comprising another medicament, such as an anti-fungal, anti-yeast, anti-bacterial and/or anti-viral medicament.

[0103] Accordingly, in one embodiment, the invention provides a method for treatment and/or prevention of an infection in an individual comprising administering to said individual a supraphysiological amount of mannose-binding lectin (MBL) or ficolin-MBL fusion protein or a combination thereof and a pharmaceutically acceptable carrier. In one embodiment, the infection is a viral infection. In one embodiment, the viral infection is an Ebola virus infection. In another embodiment, the infection is a bacterial infection.

[0104] In one embodiment the supraphysiological amount is an amount that results in blood concentrations of the MBL or the ficolin-MBL fusion protein at about 2-10 times the average physiological MBL serum concentration. In one embodiment, the average physiological MBL

serum concentration is about 2 [mu]g/mL.

[0105] In one aspect of the invention and all other aspects described herein, the invention provides use of supraphysiological amount of mannose-binding lectin (MBL) or ficolin-MBL fusion protein for treatment and/or prevention of an infection. In one embodiment, the infection is a viral infection. In one embodiment, the viral infection is an Ebola virus infection. In another embodiment, the infection is a bacterial infection.

[0106] In one aspect of the invention and all other aspects described herein, the invention provides, one uses MBL2, SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 6 and/or SEQ ID NO: 7, or combinations thereof in the methods of the invention. A skilled artisan can create alternative protein variants and fusion proteins for the use in the methods of the present invention using routine gene manipulation techniques and sequences available in public and proprietary databases alike.

EXAMPLES

[0107] We have developed novel immunotherapeutic agents that effectively prevent and treat infections, such as life-threatening infections, like infections caused by Ebola (EBOV) or Marburg viruses. In our experiments we used Ebola viruses as examples. However, based on our discovery, any other viral or bacterial infection can be treated using similar methods.

[0108] Ebola viruses are filamentous, enveloped, non-segmented, negative-strand RNA viruses. EBOV subspecies Zaire and Sudan are highly pathogenic in humans and cause as high as 90% mortality in outbreaks in equatorial Africa. There are no FDA-approved vaccines or therapeutic agents available to prevent or treat EBOV.

[0109] MBL is a broad-spectrum ligand-specific C-type lectin that plays an important role in innate immunity by acting as an opsonin and by activating the lectin complement pathway. Preliminary data indicated that recombinant human MBL (rhMBL) 1) binds high-mannose residues in EBOV envelope glycoproteins (GP) which are the principal virulence and immunogenic determinants of EBOV, and Holmskov, U., et al., Immunol. Today 15:67-74, 1994) inhibits experimental EBOV infection in a complement-dependent manner manner (Ji X et al. J Gen Virol 2005; 86:2535-42). These observations strongly support rhMBL's role as a novel immunotherapeutic agent for EBOV.

[0110] We also tested three chimeric proteins (FCN-MBL) comprising varying lengths of the carboxy terminal of MBL and the collagen stalk of L-ficolin, another lectin-like protein that activates the lectin complement pathway (see FIGS. 3 and 3).

[0111] RhMBL and FCN-MBL chimeras have comparable ligand-binding specificity but FCN-MBL has a simpler multimeric structure and different functional characteristics, and rhMBL reduces mortality in a mouse model of native EBOV Zaire infection.

[0112] Clinical grade rhMBL was provided by ENZON Pharmaceuticals, NJ.

[0113] Chimeric FCN-MBL proteins were expressed in stably transfected HEK293F cells cultured in an artificial capillary cell culture system (CELLMAX, Spectrum Laboratories, CA). Plasmids were provided by ENZON Pharmaceuticals, NJ. Proteins were batch purified with mannose-agarose beads, eluted with EDTA-containing buffer, and then dialyzed with the same buffer as for rhMBL.

[0114] The designs of the three chimeric FCN-MBL fusion proteins are as follows:

[0115] L-FCN-MBL126: L-FCN (signal sequence+collagen+"hinge" to ficolin domain amino acid [aa]128)+MBL (from aa126 carbohydrate binding domain [CRD]) (SEQ ID NO: 5).

[0116] L-FCN-MBL76: L-FCN (signal sequence+part of collagen to aa82)+MBL (from aa76 including rest of collagen+coil-coil+carbohydrate binding domain) (SEQ ID NO: 6).

[0117] L-FCN-MBL64: L-FCN (signal sequence+part of collagen to aa69)+MBL (from aa64 rest of collagen [containing "kink"]+coil-coil+carbohydrate binding domain) (SEQ ID NO: 7).

[0118] Endotoxin assay: endotoxin was <5 EU/mL (FDA standard) in all protein preparations as determined by the kinetic Limulus amebocyte lysate test.

[0119] Ebola viruses: For in vitro experiments, HIV particles (env-negative pNL 4-3) that lacked gp120/gp41 and that expressed luciferase were pseudo-typed with Ebola glycoprotein. Viral concentrations were determined by ELISA for p24 core protein. Native EBOV subspecies Zaire was used for mouse experiments.

[0120] Structure and function of rhMBL and chimeric proteins: the relative oligomerization was demonstrated with SDS-PAGE; protein composition was determined by amino acid analysis. Relative capacity to activate complement was determined by C4 deposition in an ELISA format with mannan as the capture antigen; and relative avidity was studied with a range of acetylated and non-acetylated carbohydrates in a competitive ELISA format.

[0121] HEK293F infection-inhibition assay: 400 pg p24 HIV-Ebola GP particles were preincubated with MBL-deficient serum (1:2 dilution) that was supplemented with varying amounts of rhMBL to test the relative capacity of rhMBL to inhibit infection. HEK293F cells (5*10<3>/well in a 96-well format) were then infected with the viral particles by means of spinoculation*2 hrs and then incubated with virus-free fresh media*40 hrs. Cell infection was determined by expression of luciferase.

[0122] Murine model of EBOV infection: relevant PK parameters (t1/2, Cmax) of rhMBL were calculated in 8-week old C57B/6J mice (n=5 per group) after I.P. injection of 75 mcg (3 mg/kg) or 350 mcg (14 mg/kg). C57B/6J mice were challenged with 100pfu EBOV Zaire (3000*LD50) I.P. immediately after or 12 hours before treatment with rhMBL 350 mcg I.P. that was continued every 12 hours*10 days.

Results

[0123] Three chimeric proteins (FCN-MBL) were designed (FIG. 3). The chimeric FCN-MBL proteins comprise varying lengths of the carboxyl terminal of MBL (FIGS. 1 and 2) and the collagen stalk of L-ficolin, another lectin-like protein that activates the lectin complement pathway (FIG. 2). The carboxyl terminal of MBL has been shown to be the region responsible for binding carbohydrates, the carbohydrate recognition domain (CRD) (FIG. 2). Human MBL nucleotide reference sequence, MBL2 CDS was derived from a consensus CDS 7247.1/NCBI NM-000242.2, the sequence of the 747 by nucleic acid is as follows: atgtccctgt ttccatcact ccctctcctt ctcctgagta tggtggcagc gtcttactca gaaactgtga cctgtgagga tgcccaaaag acctgccctg cagtgattgc ctgtagctct ccaggcatca acggcttccc aggcaaagat gggcgtgatg gcaccaaggg agaaaagggg gaaccaggcc aagggctcag aggcttacag ggccccccctg gaaagttggg gcctccagga aatccagggc cttctgggtc accaggacca aagggccaaa aaggagaccc tggaaaaagt ccggatggtg atagtagcct ggctgcctca gaaagaaaag ctctgcaaac agaaatggca cgtatcaaaa agtggctcac cttctctctg ggcaaacaag ttgggaacaa gttcttcctg accaatggtg aaataatgac ctttgaaaaa gtgaaggcct tgtgtgtcaa gttccaggcc tctgtggcca cccccaggaa tgctgcagag aatggagcca ttcagaatct catcaaggag gaagccttcc tgggcatcac tgatgagaag acagaagggc agtttgtgga tctgacagga aatagactga cctacacaaa ctggaacgag ggtgaaccca acaatgctgg ttctgatgaa gattgtgtat tgctactgaa aaatggccag tggaatgacg tcccctgctc cacctcccat ctggccgtct gtgagttccc tatctga (SEQ ID NO: 1).

[0124] Human MBL protein reference sequence, translation of MBL2 results in a 248 amino acid sequence as follows: mslfpslpll llsmvaasys etvtcedaqk tcpaviacss pgingfpgkd grdg/kgekg epgqglrglq gppgklgppg npgpsgspgp kgqkgdpgks pdgdsslaas erkalqtema rikkwltfsl gkqvgnkffl tngeimtfek vkalcvkfqa svatprnaae ngaiqnlike eaflgitdek tegqfvdltg nrltytnwne gepnnagsde dCvlllkngq wndvpcstsh lavcefpi (SEQ ID NO: 2).

[0125] Human L-ficolin nucleotide reference sequence FCN2 CDS was derived from consensus 6983.1/NCBI NM-004108.2. The sequence of this 942 nucleic acid sequence is as follows:

[0000]

(SEQ ID NO: 3)

atggagctgg acagagctgt gggggtcctg ggcgctgcca
ccctgctgct ctctttcctg ggcatggcct gggctctcca
ggcggcagac acctgtccag aggtgaagat ggtgggcctg
gagggctctg acaagctcac cattctccga ggctgtccgg
ggctgcctgg ggcccctggg cccaagggag aggcaggca
caatggaaag agaggagaac gtggcccccc tggacctcct
gggaaggcag gaccacctgg gcccaacgga gcacctgggg
agccccagcc gtgcctgaca ggcccgcgta cctgcaagga
cctgctagac cgagggcact tcctgagcgg ctggcacacc
atctacctgc ccgactgccg gcccctgact gtgctctgtg
acatggacac ggacggaggg ggctggaccg ttttccagcg
gagggtggat ggctctgtgg acttctaccg ggactgggcc
acgtacaagc agggcttcgg cagtcggctg ggggagttct
ggctggggaa tgacaacatc cacgccctga ccgcccaggg
aaccagcgag ctccgtgtag acctggtgga ctttgaggac
aactaccagt ttgctaagta cagatcattc aaggtggccg
acgaggcgga gaagtacaat ctggtcctgg gggccttcgt
ggagggcagt gcgggagatt ccctgacgtt ccacaacaac
cagtccttct ccaccaaaga ccaggacaat gatcttaaca
ccggaaattg tgctgtgatg tttcagggag cttggtggta
caaaaactgc catgtgtcaa acctgaatgg tcgctacctc
agggggactc atggcagctt tgcaaatggc atcaactgga
agtcggggaa aggatacaat tatagctaca aggtgtcaga
gatgaaggtg cgacctgcct ag.

[0126] Human L-ficolin protein reference sequence translation of FCN2, isoform a, results in a 313 amino acid protein: meldravgvl gaatlllsfl gmawalqaad tcpevkmvgl egsdkltilr gcpglpgapg pkgeagtngk rgergpppgpp gkagppgpng apgepqpclt gprtckdlld rghflsgwht iylpdcrplt vlcdmdtdgg gwtvfqrrvd gsvdfyrdwa tykqgfgsrl gefwlgndni haltaqgtse lrvdlvdfed nyqfakyrsf kvadeaekyn lylgafvegs agdsltfhnn qsfstkdqdn dlntgncavm fqgawwyknc hvsnlngryl rgthgsfang inwksgkgyn ysykvsemkv rpa (SEQ ID NO: 4).

[0127] Human H-ficolin nucleotide reference sequence, FCN3 CDS transcript variant 1 derived from consensus CDS 300.1/NCBI NM-003665.2, comprises a 990 by sequence as follows:

[0000]

(SEQ ID NO: 8)

atggatctac tgtggatcct gccctccctg tggcttctcc
tgcttggggg gcctgcctgcctgaagaccc aggaacaccc
cagctgccca ggacccaggg aactggaagc
cagcaaagttgtcctcctgc ccagttgtcc cggagctcca
ggaagtcctg gggagaaggg agccccaggtcctcaagggc
cacctggacc accaggcaag atgggcccca agggtgagcc
aggagatccagtgaacctgc tccggtgcca ggaaggccc
agaaactgcc gggagctgtt gagccagggcgccaccttga
gcggctggta ccatctgtgc ctacctgagg gcagggccct
cccagtcttttgtgacatgg acaccgaggg gggcggctgg
ctggtgtttc agaggcgcca ggatggttctgtggatttct
tccgctcttg gtcctcctac agagcaggtt ttgggaacca
agagtctgaattctggctgg gaaatgagaa tttgcaccag
cttactctcc agggtaactg ggagctgcgggtagagctgg
aagactttaa tggtaaccgt actttcgccc actatgcgac
cttccgcctcctcggtgagg tagaccacta ccagctggca
ctgggcaagt tctcagaggg cactgcaggggattccctga
gcctccacag tgggaggccc tttaccacct atgacgctga

ccacgattcaagcaacagca actgtgcagt gattgtccac
ggtgcctggt ggtatgcatc ctgttaccga tcaaatctca
atggtcgcta tgcagtgtct gaggctgccg cccacaaata
tggcattgactgggcctcag gccgtggtgt gggccacccc
taccgcaggg ttcggatgat gcttcgatag.

[0128] Human H-ficolin protein reference sequence of 299 amino acids (translation of FCN3 transcript variant 1) is as follows: mdllwilpsl wllllggpac lktqehpscp gpreleaskv vllpscpgap gspgekgapgpqgppgppgk mgpkgepgdp vnllrcqegp rncrellsqg atlsgwyhlc 1pegralpvf cdmdtegggw lvfqrrqdgs vdffrswssy ragfgnqese fwlgnenlhq ltlqgnwelrveledfngnr tfahyatfrl lgevdhyqla lgkfsegtag dslslhsgrp fttydadhds snsncavivh gawwyascyr snlngryays eaaahkygid wasgrgvghp yrrvrmmlr (SEQ ID NO: 9).

[0129] The sequence of the L-ficolin MBL fusion proteins used in the experiments is set forth as follows:

[0130] L-FCN-MBL126, a 251 amino acid protein:

[0000]

(SEQ ID NO: 5)

MELDRAVGVLGAATLLLSFLGMAWALQAADTCPEVKMVGLEGSDKLTILR
GCPGLPGAPGPKGEAGTNGKRGERGPPGPPGKAGPPGPNGAPGEPQPCLT
GPRTCKDLLDRGHFLSGWHTIYLPDCRPLTFSLGKQVGNKFFLTNGEIMT
FEKVKALCVKFQASVATPRNAAENGAIQNLIKEEAFLGITDEKTEGQFVD
LTGNRLTYTNWNEGEPNNAGSDEDCVLLLKNGQWNDVPCSTSHLAVCEFP

I.
[0131] L-FCN-MBL76, a 255 amino acid protein:

[0000]

(SEQ ID NO: 6)
MELDRAVGVLGAATLLLSFLGMAWALQAADTCPEVKMVGLEGSDKLTILR
GCPGLPGAPGPKGEAGTNGKRGERGPPGPPGKLGPPGNPGPSGSPGPKGQ
KGDPGKSPDGDSSLAASERKALQTEMARIKKWLTFSLGKQVGNKFFLTNG
EIMTFEKVKALCVKFQASVATPRNAAENGAIQNLIKEEAFLGITDEKTEG
QFVDLTGNRLTYTNWNEGEPNNAGSDEDCVLLLKNGQWNDVPCSTSHLAV
CEFPI.

[0132] L-FCN-MBL64, a 254 amino acid protein:

[0000]

(SEQ ID NO: 7)
MELDRAVGVLGAATLLLSFLGMAWALQAADTCPEVKMVGLEGSDKLTILR
GCPGLPGAPGPKGEAGTNGQGLRGLQGPPGKLGPPGNPGPSGSPGPKGQK
GDPGKSPDGDSSLAASERKALQTEMARIKKWLTFSLGKQVGNKFFLTNGE
IMTFEKVKALCVKFQASVATPRNAAENGAIQNLIKEEAFLGITDEKTEGQ
FVDLTGNRLTYTNWNEGEPNNAGSDEDCVLLLKNGQWNDVPCSTSHLAVC
EFPI.

[0133] In the above-identified sequences, the protein part indicated in bold indicates L-ficolin signal sequence; italics indicates L-ficolin component of fusion protein; and the remaining part is MBL component of fusion protein. The double-underlined K indicates a Lysine=MBL-associated serine protease (MASP) binding site.

[0134] The fusion protein numbers used herein refer to the corresponding amino acid from the

MBL protein sequence (1-248). Accordingly, in the L-FCN-MBL126, the number 126 corresponds to the first amino acid (L) of the MBL component of this fusion protein (aa126-248 is the Carbohydrate Recognition Domain) L-ficolin component=aa1-128; in the L-FCN-MBL76, the number 76 corresponds to the first amino acid (L) of the MBL component of this fusion protein (aa126-248 is the Carbohydrate Recognition Domain) L-ficolin component=aa1-82; and in the L-FCN-MBL64, the number 64 corresponds to the first amino acid (Q) of the MBL component of this fusion protein (aa126-248 is the Carbohydrate Recognition Domain) L-ficolin component=aa1-64.

[0135] The chimeric FCN-MBL fusion proteins were expressed, purified and analyzed on a 4-20% gradient SDS-PAGE gel, which was stained with Imperial Blue after electrophoresis. The recombinant chimeric proteins were compared to the full length rhMBL. An aliquot of 450 ng of each of the recombinant proteins was separated under reducing conditions. On the gel, the chimeric proteins exhibited an apparent molecular weight of ~30 kDa, slightly smaller than the full length rhMBL (FIG. 4). Only a single polypeptide was expressed for each construct.

[0136] Under non-reducing conditions (1200 ng purified protein), the recombinant chimeric proteins primarily form trimers and tetramers whereas rhMBL forms larger multimers (FIG. 5). The full-length rhMBL forms larger multimers than 3 chimeric proteins that comprise varying lengths of the carboxy-terminal of MBL and the amino-terminal of L-ficolin.

[0137] Binding of the rhMBL or any of the three chimeric FCN-MBL fusion proteins (10 mcg/mL in a 96-well ELISA format) to mannan was competed with mannan and then detected with anti-hMBL mAb (131-01). Binding to mannan was similar for all proteins except in a narrow range of concentrations (FIG. 6). The chimeric proteins bind a similar spectrum of carbohydrate ligands as demonstrated in the competitive ELISA assay.

[0138] FIG. 13 shows a Kaplan Meier survival curve with a post-challenge analysis which demonstrated that recombinant human MBL-treated wild-type mice had a significant survival advantage: 40% survived compared to 100% mortality among wild-type and C3 knock-out mice treated with saline or rhMBL indicating that rhMBL provides protection but that the protection is dependent on C3. MBL treated mice survived significantly longer than mice not treated with MBL. EBOV was administered N 100pfu (plaque forming units) 3000xLD50. WT (wildtype, C57B/6J mice) versus C3 knock out (KO). Recombinant MBL (rhMBL) was administered at 350 mcg IL 12 hors post challenge, then q12 h*10 days vs. sham Rx. *log rank, $p<0.0004$.

[0139] The rhMBL and the three chimeric FCN-MBL fusion proteins exhibited similar functional capacity to activate complement as determined by C4 deposition. FIG. 7 shows the C4 deposition assay that compared the capacity of rhMBL and the chimeric proteins to bind C4. This test indicates the complement activating activity of lectins. We showed that FCN-MBL76 has significantly greater C4 binding activity compared with rhMBL and the other chimerics.

[0140] Calreticulin binding assay. The 96-well ELISA plate was coated with rhMBL or chimeric proteins (10 ug/mL), blocked with BSA and incubated with 5 ug/mL biotinylated human placental calreticulin that was measured at absorbance O.D. 405. FCN-MBL76 bound to human placental calreticulin significantly better than rhMBL or the other chimeric proteins. This likely has important implications for the relative functions of the proteins because calreticulin is the putative cellular receptor on phagocytes for native MBL and therefore, enhanced binding of the chimeric molecule results in improved pathogen clearance by opsonophagocytosis (FIG. 8).

[0141] Inhibition assay using Hep G2 cells infected with lentivirus (HIV) pseudotyped with Ebola glycoprotein. Hep G2 cells at approximately 80% confluence in 96-well tissue culture plates were infected with HIV particles without an envelope (HIV-env neg; solid square) or with an envelope consisting of Ebola glycoprotein (other symbols). The virions encoded luciferase that was expressed only in infected cells and detected with a commercial luciferase assay. Before addition of viral particles to the cells, the viruses were preincubated with 0, 0.1 or 1 ug/mL of rhMBL or chimeric proteins in veronal-buffered saline with 5 mM CaCl2 for 1 hour at 37 C. Infection was achieved by spinoculation of cells at 1000 g*2 hrs. The viral protein mixture was replaced with EMEM culture media and incubated at 37 C for 40 hrs after which, the cells were lyzed and luciferase expression was quantified. rhMBL and the chimeric proteins inhibited viral infection to similar significant

extents (1 ug/mL vs no protein, p<0.001) (FIG. 9).

[0142] The pharmacokinetic modeling of rhMBL in immunocompetent C57B/6J mice revealed that doses of 75 mcg and 350 mcg doses produced Cmax of ~5 mcg/mL and ~15 mcg/mL, respectively and half-life of ~11 hours at both doses (FIG. 10). A previous study showed that 75 mcg is the minimum dose of rhMBL required to activate complement in an MBL-deficient mouse model.

[0143] Using the higher dosage of 350 mcg/ml, the survival rate of mice infected with the EBOV Zaire virus was analyzed. Data is presented as the Kaplan Meier survival analyses. 350 mcg rhMBL was given immediately pre-challenge with EBOV Zaire and continued every 12 hrs*10 days. These group of mice had a 42% survival rate (log rank, p<0.008). When 350 mcg rhMBL was given 12 hours post-challenge with EBOV Zaire and continued every 12 hrs*10 days, the mice also had a 42% survival rate (log rank, p<0.002). Therefore, the MBL proteins have preventive/prophylactic function as well as treatment function against viral infections.

[0144] Accordingly, our data clearly demonstrate that rhMBL and chimeric FCN-MBL proteins activate complement (bind C4) to a similar extent. Since rhMBL has a half-life in mice of -11 hours, supraphysiologic doses of rhMBL administered in prophylactic and therapeutic regimens every 12 hours significantly reduced mortality by >40%.

[0145] The references cited herein and throughout the specification and examples are herein incorporated by reference in their entirety.

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GRIFFITHSIN, GLYCOSYLATION-RESISTANT GRIFFITHSIN, AND RELATED CONJUGATES, COMPOSITIONS, NUCLEIC ACIDS, VECTORS, HOST CELLS, METHODS OF PRODUCTION AND METHODS OF USE

An isolated and purified nucleic acid molecule that encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, as well as an isolated and purified nucleic acid molecule that encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, and, when the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO:; 3, the at least eight contiguous amino acids have been rendered glycosylation-resistant, a vector comprising such an isolated and purified nucleic acid molecule, a host cell comprising the nucleic acid molecule, optionally in the form of a vector, a method of producing an anti-viral polypeptide or conjugate thereof, the anti-viral polypeptide itself, a conjugate or fusion protein comprising the anti-viral polypeptide, and compositions comprising an effective amount of the anti-viral polypeptide or conjugate or fusion protein thereof. Further provided are methods of inhibiting prophylactically or therapeutically a viral infection of a host.

TECHNICAL FIELD OF THE INVENTION

[0004] The invention relates to an anti-viral polypeptide, a glycosylation-resistant anti-viral polypeptide, and related conjugates, compositions, nucleic acids, vectors, host cells, antibodies and methods of production and use.

BACKGROUND OF THE INVENTION

[0005] The field of viral therapeutics has developed in response to the need for agents effective against retroviruses, especially HIV. There are many ways in which an agent can exhibit anti-retroviral activity (e.g., see DeClercq, Adv. Virus Res., 42: 1-55 (1993); DeClercq, J. Acquir. Immun. Def. Synd., 4: 207-218 (1991); and Mitsuya et al., Science, 249: 1533-1544 (1990). Nucleoside derivatives, such as AZT, which inhibit the viral reverse transcriptase, were among the first clinically active agents available commercially for anti-HIV therapy. Although very useful in

some patients, the utility of AZT and related compounds is limited by toxicity and insufficient therapeutic indices for fully adequate therapy. Also, given the subsequent revelations about the true dynamics of HIV infection (Coffin, Science, 267: 483-489 (1995); and Cohen, Science, 267: 179 (1995)), it has become increasingly apparent that agents acting as early as possible in the viral replicative cycle are needed to inhibit infection of newly produced, uninfected immune cells generated in the body in response to the virus-induced killing of infected cells. Also, it is essential to neutralize or inhibit new infectious virus produced by infected cells.

[0006] Effective means for preventing HIV infection also are needed as a global priority. Heterosexual transmission accounts for the majority of new cases of HIV infection each year. Current reports from the World Health Organization estimate that a total of more than 40 million people are now infected with HIV. HIV prevention research has to date focused predominantly on vaccine development. However, no effective preventative or therapeutic vaccine has been identified thus far. New approaches to vaccine development, as well as entirely different strategies and agents for preventing person-to-person transmission of HIV infection, are needed. One approach showing great promise is the development and use of topical microbicides. In this approach, a suitable antiviral agent is applied directly at the potential site of virus exposure, e.g., the genital mucosa in the case of HIV. A suitable antiviral agent is one which inactivates or inhibits infectivity of a virus upon contact of the antiviral agent with the virus. Suitable animal models are available for demonstrating in vivo efficacy of such approaches for preventing transmission of immunodeficiency viruses, such as HIV. For instance, the HIV-inactivating protein, cyanovirin-N, has been shown to inhibit the sexual transmission of a chimeric simian/human immunodeficiency virus (SHIV) infection in a primate model employing macaques exposed to the virus vaginally or rectally (C-C Tsai et al., AIDS Res. Hum. Retroviruses, 19, 535-541 (2003) and C-C Tsai et al., AIDS Res. Hum. Retroviruses, 20, 11-18 (2004)).

[0007] Infection of people by influenza viruses is also a major cause of pandemic illness, morbidity and mortality worldwide. The adverse economic consequences, as well as human suffering, are enormous. Available treatments for established infection by this virus are either minimally effective or ineffective; these treatments employ amantatadine, rimantadine and neuraminidase inhibitors. Of these drugs, only the neuraminidase inhibitors are substantially active against multiple strains of influenza virus that commonly infect humans, yet these drugs still have limited utility or efficacy against pandemic disease.

[0008] Currently, the only effective preventative treatment against influenza viral infection is vaccination. However, this, like the drug treatments, is severely limited by the propensity of influenza viruses to mutate rapidly by genetic exchange, resulting in the emergence of highly resistant viral strains that rapidly infect and spread throughout susceptible populations. In fact, a vaccination strategy is only effective from year-to-year if the potential pandemic strains can be identified or predicted, and corresponding vaccines prepared and administered early enough that the year's potential pandemic can be aborted or attenuated. Thus, new preventative and therapeutic interventions and agents are urgently needed to combat influenza viruses.

[0009] New agents with broad anti-influenza virus activity against diverse strains, clinical isolates and subtypes of influenza virus would be highly useful, since such agents would most likely remain active against the mutating virus. The two major types of influenza virus that infect humans are influenza A and B, both of which cause severe acute illness that may include both respiratory and gastrointestinal distress, as well as other serious pathological sequellae. An agent that has anti-influenza virus activity against diverse strains and isolates of both influenza A and B, including recent clinical isolates thereof, would be particularly advantageous for use in prevention or treatment of hosts susceptible to influenza virus infection.

[0010] The predominant mode of transmission of influenza viral infection is respiratory, i.e., transmission via inhalation of virus-laden aerosolized particles generated through coughing, sneezing, breathing, etc., of an influenza-infected individual. Transmission of infectious influenza virions may also occur through contact (e.g., through inadvertent hand-to-mouth contact, kissing, touching, etc.) with saliva or other bodily secretions of an infected individual. Thus, the primary first points of contact of infectious influenza virions within a susceptible individual are the mucosal surfaces within the oropharyngeal mucosa, and the mucosal surfaces within the upper and lower

respiratory tracts. Not only do these sites comprise first points of virus contact for initial infection of an individual, they are also the primary sites for production and exit (e.g., by coughing, sneezing, salivary transmission, etc.) of bodily fluids containing infectious influenza viral particles. Therefore, availability of a highly potent anti-influenza virus agent, having broad-spectrum activity against diverse strains and isolates of influenza viruses A and B, which could be applied or delivered topically to the aforementioned mucosal sites of contact and infection and transmission of infectious influenza viruses, would be highly advantageous for therapeutic and preventative inhibition of influenza viral infection, either in susceptible uninfected or infected hosts.

[0011] In this regard, new classes of anti-viral agents, to be used alone or in combination existing anti-viral agents, are needed for effective anti-viral therapy. New agents are also important for the prophylactic inhibition of viral infection. In both areas of need, the ideal new agent(s) would act as early as possible in the viral life cycle; be as virus-specific as possible (i.e., attack a molecular target specific to the virus but not the host); render the intact virus noninfectious; prevent the death or dysfunction of virus-infected cells; prevent further production of virus from infected cells; prevent spread of virus infection to uninfected cells; be highly potent and active against the broadest possible range of strains and isolates of a given virus; be resistant to degradation under physiological and rigorous environmental conditions; and be readily and inexpensively produced.

[0012] Accordingly, the invention provides a novel anti-viral polypeptide and related conjugates, nucleic acids, vectors, host cells and methods of production and use. This and other advantages of the invention, as well as additional inventive features, will become apparent from the description provided herein.

BRIEF SUMMARY OF THE INVENTION

[0013] The invention provides, among other things, an isolated and purified nucleic acid molecule that encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, optionally as part of an encoded fusion protein. In this regard, the invention also provides an isolated and purified nucleic acid molecule that encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO: 3 which have been rendered glycosylation-resistant and wherein the at least eight contiguous amino acids have antiviral activity, optionally as part of an encoded fusion protein. Further provided are vectors comprising an aforementioned isolated and purified nucleic acid molecule and a host cell or organism comprising such a vector.

[0014] Accordingly, the invention also provides a method of producing an anti-viral polypeptide, which method comprises expressing the nucleic acid molecule, optionally in the form of a vector, in a host cell or organism. Thus, an anti-viral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, and an antiviral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO: 3, which have been rendered glycosylation-resistant and wherein the at least eight contiguous amino acids have antiviral activity, are also provided, as are conjugates comprising an aforementioned anti-viral polypeptide and at least one effector component. Compositions comprising an effective amount of an aforementioned anti-viral polypeptide or anti-viral polypeptide conjugate are also provided.

[0015] The invention further provides a method of inhibiting prophylactically or therapeutically a viral infection of a host, specifically a retroviral infection of a host, such as an infection of a host with a human immunodeficiency virus (HIV), e.g., HIV-1 or HIV-2, or influenza virus. The method comprises administering to the host an effective amount of an anti-viral polypeptide or anti-viral polypeptide conjugate comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, whereupon the viral infection is inhibited.

[0016] Still further provided is a method of inhibiting a viral infection of an animal comprising transforming host cells in vivo with a nucleic acid molecule encoding an above-described

polypeptide. Even still further provided is a method of inhibiting a viral infection of an animal comprising transforming host cells with a nucleic acid molecule encoding an above-described polypeptide and placing the transformed host cells into or onto the animal.

[0017] An antibody that binds griffithsin is provided as is a composition comprising same. Similarly, an anti-griffithsin antibody is provided as is a composition comprising same. A method of administering an anti-griffithsin antibody or a composition comprising same to a mammal so as to inhibit infection of the mammal with a virus is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 is a flow diagram illustrating an anti-HIV bioassay-guided method of isolating, purifying, and elucidating the amino acid sequence of griffithsin.

[0019] FIG. 2 is a flow diagram illustrating a method of synthesizing a recombinant griffithsin gene.

[0020] FIG. 3 is a flow diagram illustrating a method of expressing a synthetic griffithsin gene encoding a His-tagged griffithsin polypeptide protein and purification of the recombinant His-tagged griffithsin.

[0021] FIG. 4a is a line graph illustrating the anti-HIV activity of native griffithsin, in terms of concentration of griffithsin (nM) (X-axis) versus % control (Y-axis). FIG. 4b is a line graph illustrating the anti-HIV activity of recombinant, His-tagged griffithsin in terms of concentration of griffithsin (nM) (X-axis) versus % control (Y-axis).

[0022] FIG. 5a is a bar graph comparing test proteins bound by griffithsin (Y-axis) and absorbance of the griffithsin-test protein complex at 405 nm (X-axis). FIG. 5b illustrates the concentration-dependent binding of griffithsin to glycosylated (-) or nonglycosylated () gp120 by comparing griffithsin (GRFT) concentration (pmol) and absorbance of griffithsin-gp120 complexes at 405 nm.

[0023] FIG. 6 is a flow diagram illustrating a method of producing anti-griffithsin antibodies.

[0024] FIG. 7 is the amino acid sequence of griffithsin polypeptide (SEQ ID NO: 3) isolated and purified from Griffithsin sp.

[0025] FIG. 8 shows the nucleic acid (SEQ ID NO: 1) sequence of recombinant griffithsin.

[0026] FIG. 9 is the amino acid sequence of a recombinant griffithsin polypeptide (SEQ ID NO: 2).

[0027] FIG. 10 shows the nucleic acid sequence of a recombinant griffithsin polypeptide comprising a His tag (SEQ ID NO: 4).

[0028] FIG. 11 is the amino acid sequence of a recombinant griffithsin polypeptide comprising a His tag (SEQ ID NO: 5).

Figure 1

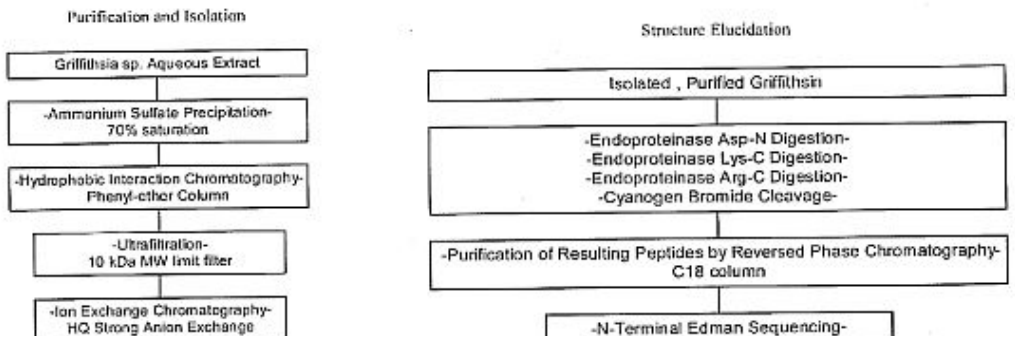


Figure 2

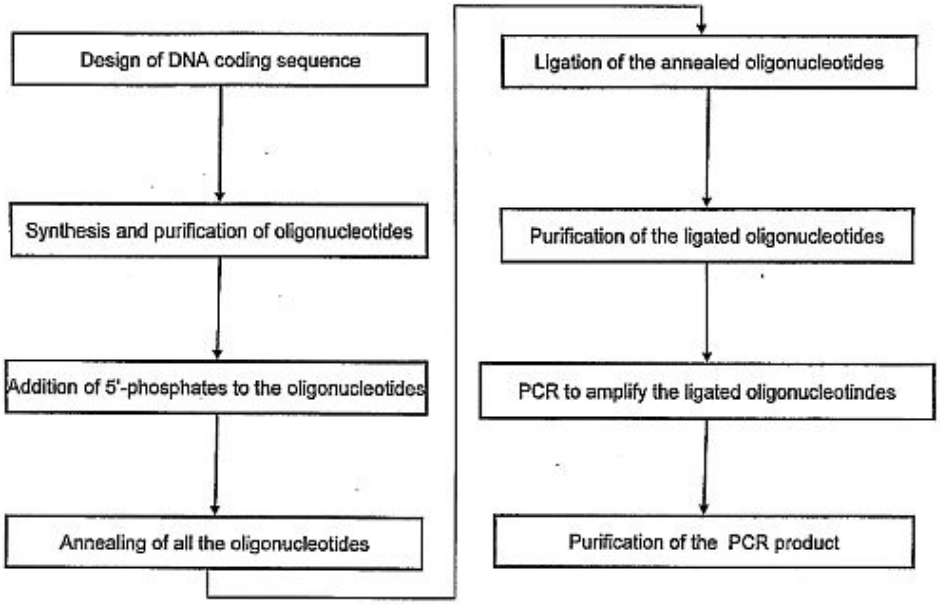
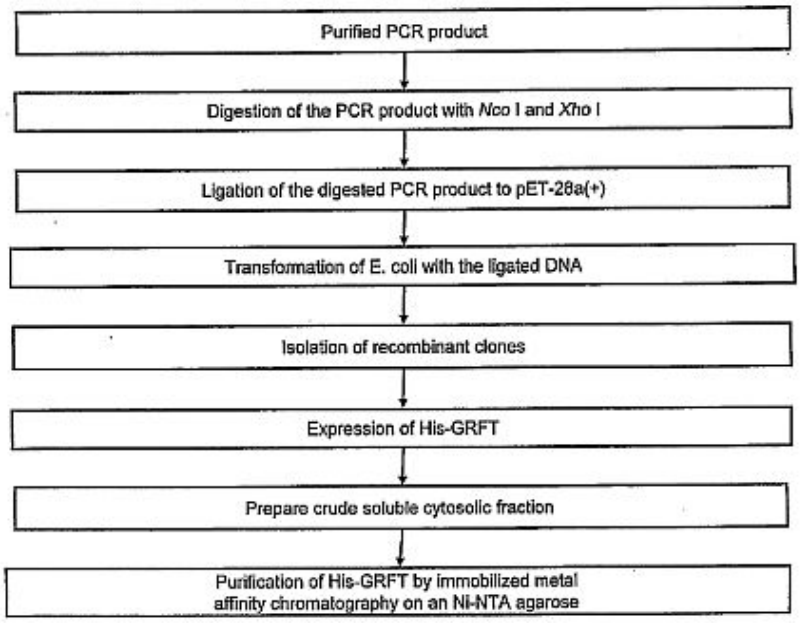
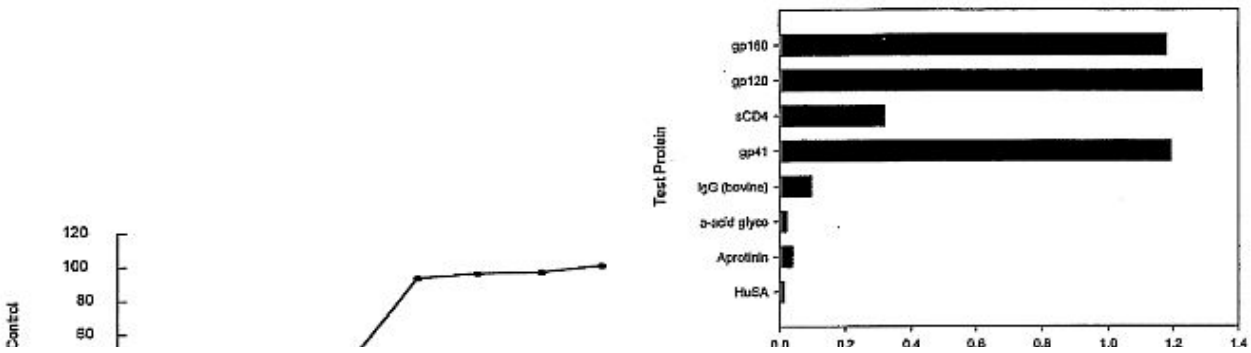


Figure 3





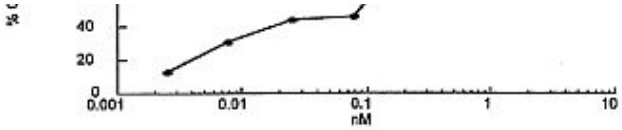


Figure 4a

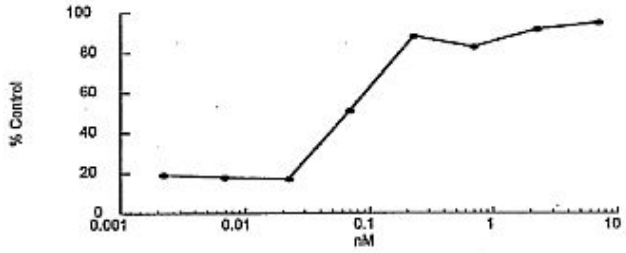


Figure 4b

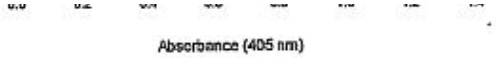


Figure 5a

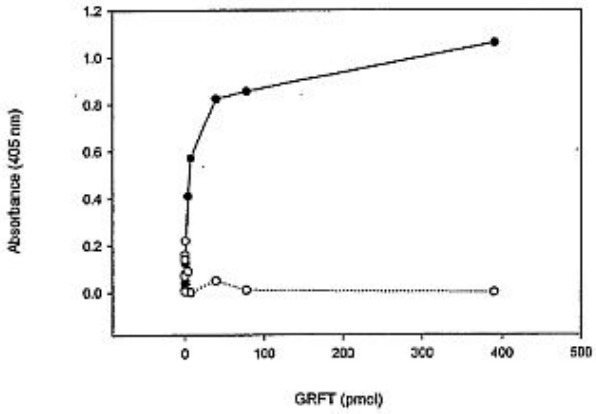
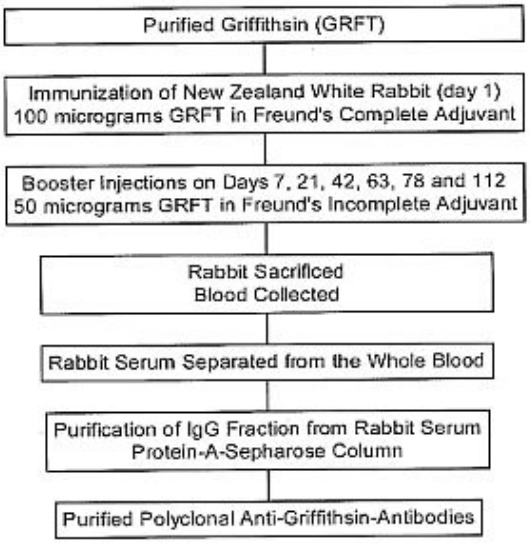


Figure 5b

Figure 6



SEQ ID NO: 3,Native Griffithsin Amino Acid Sequence

```
Ser Leu Thr His Arg Lys Phe Gly Gly Ser Gly Gly Ser Pro Phe Ser 16
Gly Leu Ser Ser Ile Ala Val Arg Ser Gly Ser Tyr Leu Asp Xxx Ile  32
Ile Ile Asp Gly Val His His Gly Gly Ser Gly Gly Asn Leu Ser Pro  48
Thr Phe Thr Phe Gly Ser Gly Glu Tyr Ile Ser Asn Met Thr Ile Arg  64
Ser Gly Asp Tyr Ile Asp Asn Ile Ser Phe Glu Thr Asn Met Gly Arg  80
Arg Phe Gly Pro Tyr Gly Gly Ser Gly Gly Ser Ala Asn Thr Leu Ser  96
Asn Val Lys Val Ile Gln Ile Asn Gly Ser Ala Gly Asp Tyr Leu Asp 112
Ser Leu Asp Ile Tyr Tyr Glu Gln Tyr 121
```

Figure 7

SEQ ID NO: 1, DNA Sequence Encoding Native Griffithsin Polypeptide

```
AGC CTG ACC CAT CGC AAG TTC GGT GGT AGT GGT GGA AGT CCG TTC AGC  48
Ser Leu Thr His Arg Lys Phe Gly Gly Ser Gly Gly Ser Pro Phe Ser  16

GGT CTG AGC AGC ATT GCA GTT CGT AGT GGC AGC TAT CTG GAT GCG ATC  96
Gly Leu Ser Ser Ile Ala Val Arg Ser Gly Ser Tyr Leu Asp Ala Ile  32

ATC ATT GAT GGT GTA CAT CAC GGT GGC TCT GGT GGT AAC CTG AGT CCG 144
Ile Ile Asp Gly Val His His Gly Gly Ser Gly Gly Asn Leu Ser Pro  48

ACC TTC ACC TTT GGA TCC GGT GAG TAC ATC AGC AAC ATG ACC ATT CGT 192
Thr Phe Thr Phe Gly Ser Gly Glu Tyr Ile Ser Asn Met Thr Ile Arg  64

AGT GGA GAC TAC ATT GAC AAC ATC AGC TTT GAA ACC AAC ATG GGT CGT 240
Ser Gly Asp Tyr Ile Asp Asn Ile Ser Phe Glu Thr Asn Met Gly Arg  80

CGC TTT GGT CCG TAT GGT GGA TCT GGT GGC AGT GCA AAC ACC CTG AGC 288
Arg Phe Gly Pro Tyr Gly Gly Ser Gly Gly Ser Ala Asn Thr Leu Ser  96

AAC GTG AAA GTC ATC CAG ATC AAC GGT AGT GCA GGT GAC TAT CTG GAT 336
Asn Val Lys Val Ile Gln Ile Asn Gly Ser Ala Gly Asp Tyr Leu Asp 112

AGC CTG GAC ATC TAC TAT GAA CAG TAC 363
Ser Leu Asp Ile Tyr Tyr Glu Gln Tyr 121
```

Figure 8

SEQ ID NO: 2, Recombinant Griffithsin Amino Acid Sequence

```
Ser Leu Thr His Arg Lys Phe Gly Gly Ser Gly Gly Ser Pro Phe Ser  16

Gly Leu Ser Ser Ile Ala Val Arg Ser Gly Ser Tyr Leu Asp Ala Ile  32

Ile Ile Asp Gly Val His His Gly Gly Ser Gly Gly Asn Leu Ser Pro  48

Thr Phe Thr Phe Gly Ser Gly Glu Tyr Ile Ser Asn Met Thr Ile Arg  64

Ser Gly Asp Tyr Ile Asp Asn Ile Ser Phe Glu Thr Asn Met Gly Arg  80

Arg Phe Gly Pro Tyr Gly Gly Ser Gly Gly Ser Ala Asn Thr Leu Ser  96

Asn Val Lys Val Ile Gln Ile Asn Gly Ser Ala Gly Asp Tyr Leu Asp 112

Ser Leu Asp Ile Tyr Tyr Glu Gln Tyr 121
```

Figure 9

SEQ ID NO: 4, DNA Sequence Encoding Recombinant His-tagged Griffithsin

```
ATG GGC AGC AGC CAT CAT CAT CAT CAT CAC AGC AGC GGC CTG GTG CCG  48
Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro  16

CGC GGC AGC CTG ACC CAT CGC AAG TTC GGT GGT AGT GGT GGA AGT CCG  96
Arg Gly Ser Leu Thr His Arg Lys Phe Gly Gly Ser Gly Gly Ser Pro  32

TTC AGC GGT CTG AGC AGC ATT GCA GTT CGT AGT GGC AGC TAT CTG GAT 144
Phe Ser Gly Leu Ser Ser Ile Ala Val Arg Ser Gly Ser Tyr Leu Asp  48

GCG ATC ATC ATT GAT GGT GTA CAT CAC GGT GGC TCT GGT GGT AAC CTG 192
Ala Ile Ile Ile Asp Gly Val His His Gly Gly Ser Gly Gly Asn Leu  64

AGT CCG ACC TTC ACC TTT GGA TCC GGT GAG TAC ATC AGC AAC ATG ACC 240
Ser Pro Thr Phe Thr Phe Gly Ser Gly Glu Tyr Ile Ser Asn Met Thr  80

ATT CGT AGT GGA GAC TAC ATT GAC AAC ATC AGC TTT GAA ACC AAC ATG 288
Ile Arg Ser Gly Asp Tyr Ile Asp Asn Ile Ser Phe Glu Thr Asn Met  96

GGT CGT CGC TTT GGT CCG TAT GGT GGA TCT GGT GGC AGT GCA AAC ACC 336
```

```
Gly Arg Arg Phe Gly Pro Tyr Gly Gly Ser Gly Gly Ser Ala Asn Thr 112

CTG AGC AAC GTG AAA GTC ATC CAG ATC AAC GGT AGT GCA GGT GAC TAT 384
Leu Ser Asn Val Lys Val Ile Gln Ile Asn Gly Ser Ala Gly Asp Tyr 128

CTG GAT AGC CTG GAC ATC TAC TAT GAA CAG TAC 417
Leu Asp Ser Leu Asp Ile Tyr Tyr Glu Gln Tyr 139
```

Figure 10

SEQ ID NO: 5, Recombinant His-Tagged Griffithsin Amino Acid Sequence

```
Met Gly Ser Ser His His His His His His Ser Ser Gly Leu Val Pro  16

Arg Gly Ser Leu Thr His Arg Lys Phe Gly Gly Ser Gly Gly Ser Pro  32

Phe Ser Gly Leu Ser Ser Ile Ala Val Arg Ser Gly Ser Tyr Leu Asp  48

Ala Ile Ile Ile Asp Gly Val His His Gly Gly Ser Gly Gly Asn Leu  64

Ser Pro Thr Phe Thr Phe Gly Ser Gly Glu Tyr Ile Ser Asn Met Thr  80

Ile Arg Ser Gly Asp Tyr Ile Asp Asn Ile Ser Phe Glu Thr Asn Met  96

Gly Arg Arg Phe Gly Pro Tyr Gly Gly Ser Gly Gly Ser Ala Asn Thr 112

Leu Ser Asn Val Lys Val Ile Gln Ile Asn Gly Ser Ala Gly Asp Tyr 128

Leu Asp Ser Leu Asp Ile Tyr Tyr Glu Gln Tyr 139
```

Figure 11

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0029] The principal overall objective of the invention is to provide an anti-viral polypeptide and derivatives thereof, and broad uses thereof (e.g., medical and research uses), including prophylactic and/or therapeutic applications against viruses. An initial observation, which led to the invention, was anti-viral activity of certain extracts from a marine organism, namely Rhodophyte (Griffithsia sp.), originally collected in the territorial waters of New Zealand. Low picomolar concentrations of a protein isolated from the extracts, referred to herein as griffithsin, irreversibly inactivated human clinical isolates of HIV. Its HIV molecular target is high mannose-comprised oligosaccharide constituents of Env glycoproteins. Upon binding, griffithsin inhibits viral binding, fusion, and entry. Griffithsin also targets other viruses having oligosaccharide constituents similar to HIV, such as other retroviruses, e.g., FIV, SIV and HTLV, and non-retroviruses, e.g., influenza, ebola, and measles.

[0030] Accordingly, the invention provides an isolated and purified anti-viral polypeptide of SEQ ID NO: 3 from Griffithsia sp. and functional homologs thereof, referred to collectively as "griffithsin." Herein the term "griffithsin" is used generically to refer to a natural griffithsin or any related, functionally equivalent (i.e., anti-viral) polypeptide or derivative thereof. By definition, in this context, a related, functionally equivalent polypeptide or derivative thereof (a) contains a sequence of at least eight contiguous amino acids directly identical to a sub-sequence of eight contiguous amino acids contained within a natural griffithsin, and (b) can specifically bind to a virus, in particular an influenza virus or a retrovirus, more specifically a primate immunodeficiency virus, more specifically HIV-1, HIV-2 or SIV, or to an infected host cell expressing one or more viral antigen(s), more specifically an envelope glycoprotein, such as gp120, of the respective virus. In addition, such a functionally equivalent polypeptide or derivative thereof can comprise the amino acid sequence of a natural griffithsin (see SEQ ID NO: 3), in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been removed from one or both ends, preferably from only one end, e.g., removed from the amino-terminal end, of natural griffithsin. Alternatively, a functionally equivalent polypeptide or derivative thereof can comprise the amino acid sequence of a native griffithsin (see SEQ ID NO: 3), in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been added to one or both ends, preferably from only one end, e.g., the amino-terminal

end, of the native griffithsin.

[0031] The invention further provides an isolated and purified polypeptide encoded by a nucleic acid molecule comprising a sequence of SEQ ID NO: 1 or a nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3. Upon examination of the antiviral griffithsin polypeptide, the amino acid at position 31 of SEQ ID NO: 3 (represented as Xaa) was found not to be a familiar amino acid residue. Placement of an alanine at position 31, such as achieved in the recombinant griffithsin polypeptide described herein (SEQ ID NO: 2), results in a polypeptide exhibiting equivalent activity as the natural griffithsin polypeptide. If desired, the amino acid at position 31 can be substituted with any other amino acid to facilitate protein production. Ideally, the substitution at position 31 of SEQ ID NO: 3 does not diminish the anti-viral activity of the protein (e.g., does not diminish the anti-viral activity more than 50%, more than 30% or more than 10%) as compared to the anti-viral activity of the native protein. Preferably, the aforementioned nucleic acid molecules encode at least eight (e.g., at least 10, at least 20, at least 30, at least 50, at least 70, at least 80, at least 90, or at least 100) contiguous amino acids of the amino acid sequence of SEQ ID NO: 3, which desirably have anti-viral activity. If the at least eight contiguous amino acids of SEQ ID NO: 3 comprise amino acids 1-121, desirably amino acid residue 45, 60, 71, and/or 104 has been rendered glycosylation resistant, while maintaining antiviral activity of the polypeptide.

[0032] The term "isolated" as used herein means having been removed from its natural environment. The term "purified" as used herein means having been increased in purity, wherein "purity" is a relative term and not to be construed as absolute purity. By "antiviral" is meant that the polypeptide or fragment thereof can inhibit a virus (e.g., inhibit entry of a virus into a host cell, limit the spread of viral infection by inhibiting cell to cell fusion, and the like), in particular an influenza virus, such as influenza virus or a strain A or strain B, or a retrovirus, specifically a primate immunodeficiency virus, more specifically a human immunodeficiency virus (HIV), such as HIV-1, HIV-2 or SIV.

[0033] Preferably, the polypeptide or derivative thereof comprises an amino acid sequence that is substantially homologous to that of an anti-viral protein from Griffithsia sp. By "substantially homologous" is meant sufficient homology to render the polypeptide or derivative thereof anti-viral, with anti-viral activity characteristic of an anti-viral protein isolated from Griffithsia sp. At least about 50% homology (e.g., at least about 60% homology, at least about 65% homology, or at least about 70% homology), preferably at least about 75% homology (e.g., at least about 80% homology or at least about 85% homology), and most preferably at least about 90% homology (e.g., at least about 95% homology) should exist.

[0034] Alterations of the natural amino acid sequence to produce variant polypeptides can be done by a variety of means known to those skilled in the art. For instance, amino acid substitutions can be conveniently introduced into the polypeptides at the time of synthesis. Alternatively, site-specific mutations can be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the modified site. Alternately, oligonucleotide-directed, site-specific mutagenesis procedures can be used, such as disclosed in Walder et al., Gene, 42: 133 (1986); Bauer et al., Gene, 37: 73 (1985); Craik, Biotechniques, 12-19 (January 1995); and U.S. Pat. Nos. 4,518,584 and 4,737,462.

[0035] It is within the skill of the ordinary artisan to select synthetic and naturally-occurring amino acids that effect conservative or neutral substitutions for any particular naturally-occurring amino acids. The ordinarily skilled artisan desirably will consider the context in which any particular amino acid substitution is made, in addition to considering the hydrophobicity or polarity of the side-chain, the general size of the side chain and the pK value of side-chains with acidic or basic character under physiological conditions. For example, lysine, arginine, and histidine are often suitably substituted for each other, and more often arginine and histidine. As is known in the art, this is because all three amino acids have basic side chains, whereas the pK value for the side-chains of lysine and arginine are much closer to each other (about 10 and 12) than to histidine (about 6). Similarly, glycine, alanine, valine, leucine, and isoleucine are often suitably substituted for each other, with the proviso that glycine is frequently not suitably substituted for the other members of the group. This is because each of these amino acids are relatively hydrophobic when

incorporated into a polypeptide, but glycine's lack of an [alpha]-carbon allows the phi and psi angles of rotation (around the [alpha]-carbon) so much conformational freedom that glycinyl residues can trigger changes in conformation or secondary structure that do not often occur when the other amino acids are substituted for each other. Other groups of amino acids frequently suitably substituted for each other include, but are not limited to, the group consisting of glutamic and aspartic acids; the group consisting of phenylalanine, tyrosine and tryptophan; and the group consisting of serine, threonine and, optionally, tyrosine. Additionally, the ordinarily skilled artisan can readily group synthetic amino acids with naturally-occurring amino acids.

[0036] The ordinarily skilled artisan can generate griffithsin mutants or variants by, for example, substituting or mutating amino acids which are not critical for the anti-viral function of the polypeptide. Ideally, mutations that do not modify the electronic or structural environment of the peptide are generated to retain optimal antiviral activity. For example, natural griffithsin forms dimers, which can be advantageous in some embodiments. Therefore, alterations which do not disrupt dimer formation can be preferred. Amino acid residues which are not responsible for folding or stability of the three-dimensional conformation of the griffithsin polypeptide are candidate residues for mutation. Alternatively or in addition, amino acids which are not involved in glycoprotein binding can be mutated or replaced. It is understood that surface hydrophobicity plays a key role in protein-protein interactions and surface electrophilicity is important to protein-sugar interactions, such as the interaction between griffithsin and viral proteins. Hydrophobic surface clusters and electrophilic surface clusters on the griffithsin peptide or homologs which suggest regions critical for interaction with the viral envelope can be mapped using routine methods such as those disclosed in Bewley et al., Nature Structural Biology, 5(7): 571-578 (1998). Amino acid residues not found either in electrophilic or hydrophobic surface clusters are likely not critical for hydrophobicity or electrophilicity of these clusters and, thus, are appropriate targets for mutation to create griffithsin fragments (e.g., anti-viral polypeptides comprising at least about eight contiguous amino acids of SEQ ID NO: 2 or SEQ ID NO: 3), variants, mutants, or homologs (e.g., griffithsin variants having 80%, 85%, or 90% homology to SEQ ID NO: 2 or SEQ ID NO: 3) which retain antiviral activity. If desired, amino acid residues which are responsible for binding to high-mannose oligosaccharide-containing glycoproteins on the viral surface can be mutated to increase the specificity or affinity of glycoprotein binding.

[0037] If desired, the proteins and peptides of the invention (including antiviral fragments, variant polypeptides, fusion proteins, and conjugates) can be modified, for instance, by glycosylation, amidation, carboxylation, or phosphorylation, or by the creation of acid addition salts, amides, esters, in particular C-terminal esters, and N-acyl derivatives of the proteins of the invention. The polypeptides also can be modified to create protein derivatives by forming covalent or noncovalent complexes with other moieties in accordance with methods known in the art. Covalently-bound complexes can be prepared by linking the chemical moieties to functional groups on the side chains of amino acids comprising the proteins, or at the N- or C-terminus. Desirably, such modifications and conjugations do not adversely affect the activity of the polypeptides (and variants thereof). While such modifications and conjugations can have greater or lesser activity, the activity desirably is not negated and is characteristic of the unaltered polypeptide.

[0038] The polypeptides (and fragments, homologs, variants, and fusion proteins) can be prepared by any of a number of conventional techniques. The polypeptide can be isolated or purified from a naturally occurring source or from a recombinant source. For instance, in the case of recombinant proteins, a DNA fragment encoding a desired polypeptide can be subcloned into an appropriate vector using well-known molecular genetic techniques (see, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory (1989)) and other references cited herein under "EXAMPLES"). The fragment can be transcribed and the polypeptide subsequently translated in vitro. Commercially available kits also can be employed (e.g., such as manufactured by Clontech, Palo Alto, Calif.; Amersham Life Sciences, Inc., Arlington Heights, Ill.; InVitrogen, San Diego, Calif.; and the like). The polymerase chain reaction optionally can be employed in the manipulation of nucleic acids.

[0039] Such polypeptides also can be synthesized using an automated peptide synthesizer in accordance with methods known in the art. Alternately, the polypeptide (and fragments, homologs, variants, and fusion proteins) can be synthesized using standard peptide synthesizing techniques

well-known to those of skill in the art (e.g., as summarized in Bodanszky, Principles of Peptide Synthesis, (Springer-Verlag, Heidelberg: 1984)). In particular, the polypeptide can be synthesized using the procedure of solid-phase synthesis (see, e.g., Merrifield, J. Am. Chem. Soc., 85: 2149-54 (1963); Barany et al., Int. J. Peptide Protein Res., 30: 705-739 (1987); and U.S. Pat. No. 5,424,398). If desired, this can be done using an automated peptide synthesizer. Removal of the t-butyloxycarbonyl (t-BOC) or 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid blocking groups and separation of the polypeptide from the resin can be accomplished by, for example, acid treatment at reduced temperature. The protein-containing mixture then can be extracted, for instance, with diethyl ether, to remove non-peptidic organic compounds, and the synthesized polypeptide can be extracted from the resin powder (e.g., with about 25% w/v acetic acid). Following the synthesis of the polypeptide, further purification (e.g., using HPLC) optionally can be preformed in order to eliminate any incomplete proteins, polypeptides, peptides or free amino acids. Amino acid and/or HPLC analysis can be performed on the synthesized polypeptide to validate its identity. For other applications according to the invention, it may be preferable to produce the polypeptide as part of a larger fusion protein, either by chemical conjugation or through genetic means, such as are known to those skilled in the art. In this regard, the invention also provides a fusion protein comprising the isolated or purified antiviral polypeptide (or fragment thereof) or variant thereof and one or more other protein(s) having any desired properties or effector functions, such as cytotoxic or immunological properties, or other desired properties, such as to facilitate isolation, purification, analysis, or stability of the fusion protein.

[0040] A griffithsin conjugate comprising a griffithsin coupled to at least one effector component, which can be the same or different, is also provided. The effector component can be polyethylene glycol, dextran, albumin, an immunological reagent, a toxin, an antiviral agent, or a solid support matrix. "Immunological reagent" will be used to refer to an antibody, an antibody fragment (e.g., an F(ab')2, an Fab', an Fab, an Fv, an sFv, a dsFv, or an Fc antibody fragment), an immunoglobulin, and an immunological recognition element. An immunological recognition element is an element, such as a peptide, e.g., the FLAG sequence of a recombinant griffithsin-FLAG fusion protein, which facilitates, through immunological recognition, isolation and/or purification and/or analysis of the protein or peptide to which it is attached. An immunological reagent also can be an immunogenic peptide, which can be fused to griffithsin for enhancing an immune response. In this respect, the invention provides an anti-viral conjugate comprising a griffithsin polypeptide or fragment thereof bound to a virus or viral envelope glycoprotein. A griffithsin fusion protein is a type of griffithsin conjugate, wherein a griffithsin is coupled to one or more other protein(s) having any desired properties or effector functions, such as cytotoxic or immunological properties, or other desired properties, such as to facilitate isolation, purification or analysis of the fusion protein or increase the stability or in vivo half-life of the fusion protein. Griffithsin also can be attached to a chemical moiety which allows recognition, isolation, purification, and/or analysis of the protein or peptide. An example of such a chemical moiety is a His tag of a recombinant griffithsin-His fusion protein.

[0041] A "toxin" can be, for example, Pseudomonas exotoxin. An "antiviral agent" can be AZT, ddI, ddC, 3TC gancyclovir, fluorinated dideoxynucleosides, nevirapine, R82913, Ro 31-8959, BI-RJ-70, acyclovir, [alpha]-interferon, recombinant sCD4, michellamines, calanolides, nonoxynol-9, gossypol and derivatives thereof, gramicidin, amantatadine, rimantadine, and neuraminidase inhibitors, and cyanovirin-N or a functional homolog or derivative thereof (see, for example, U.S. Pat. No. 5,843,882). A "solid support matrix" can be a magnetic bead, a flow-through matrix, a sponge, a stent, a culture plate, or a matrix comprising a contraceptive device, such as a condom, diaphragm, cervical cap, vaginal ring or contraceptive sponge. In an alternative embodiment, a solid support matrix can be an implant for surgical implantation in a host and, if appropriate, later removal.

[0042] In view of the foregoing, the invention further provides a composition comprising (i) the isolated or purified antiviral polypeptide (or fragment thereof), a variant thereof, a fusion protein of the antiviral polypeptide (or fragment thereof) or variant thereof, and a conjugate of the antiviral polypeptide (or fragment thereof) or variant thereof, and/or (ii) a carrier, excipient or adjuvant therefor. Preferably, component (i) of the composition is present in an antiviral effective amount and the carrier is pharmaceutically acceptable. By "antiviral effective amount" is meant an amount sufficient to inhibit the infectivity of the virus.

[0043] The carrier can be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the active agent of the invention, and by the route of administration. It is preferred that the pharmaceutically acceptable carrier be one which is chemically inert to the active agent and one which has no detrimental side effects or toxicity under the conditions of use. The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those ordinarily skilled in the art and are readily available to the public. Typically, the composition, such as a pharmaceutical composition, can comprise a physiological saline solution; dextrose or other saccharide solution; or ethylene, propylene, polyethylene, or other glycol. The pharmaceutical composition preferably does not comprise mannose or N-acetyl-glucosamine, as these molecules may interfere with the functioning of the antiviral agent.

[0044] The invention also provides a method of obtaining a griffithsin from Griffithsia sp. Such a method comprises (a) identifying an extract of Griffithsia sp. containing anti-viral activity, (b) optionally removing high molecular weight biopolymers from the extract, (c) anti-viral bioassay-guided fractionating the extract to obtain a crude extract of griffithsin, and (d) purifying the crude extract by reverse-phase HPLC to obtain griffithsin (see, also, Example 1). More specifically, the method involves the use of ethanol to remove high molecular weight biopolymers from the extract and the use of an anti-HIV bioassay to guide fractionation of the extract.

[0045] Griffithsin (a polypeptide of exactly SEQ ID NO: 3), which was isolated and purified using the aforementioned method, was subjected to conventional procedures typically used to determine the amino acid sequence of a given pure protein. Thus, the griffithsin was initially sequenced by N-terminal Edman degradation of intact protein and numerous overlapping peptide fragments generated by endoproteinase digestion. Amino acid analysis was in agreement with the deduced sequence. ESI mass spectrometry of reduced, HPLC-purified griffithsin showed a molecular ion consistent with the calculated value. These studies indicated that griffithsin from Griffithsia was comprised of a unique sequence of 121 amino acids having little or no significant homology or identity to previously described proteins or transcription products of known nucleotide sequences. No more than eight contiguous amino acids from griffithsin were found in any amino acid sequences from known proteins, nor were there any known proteins from any source having significant sequence identity with griffithsin. Given the chemically deduced amino acid sequence of griffithsin, a corresponding recombinant griffithsin (r-griffithsin) was created and used to establish definitively that the deduced amino acid sequence was, indeed, active against virus, such as HIV and influenza.

[0046] Accordingly, the invention provides isolated and purified nucleic acid molecules and synthetic nucleic acid molecules, which comprise a coding sequence for a griffithsin, such as an isolated and purified nucleic acid molecule comprising a sequence of SEQ ID NO: 1, an isolated and purified nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2, an isolated and purified nucleic acid sequence encoding an amino acid sequence SEQ ID NO: 3, an isolated and purified nucleic acid molecule comprising a sequence of SEQ ID NO: 4, an isolated and purified nucleic acid sequence encoding an amino acid sequence of SEQ ID NO: 5, and a nucleic acid molecule that is substantially homologous or substantially identical to any one of the aforementioned nucleic acid molecules. By "substantially homologous" is meant sufficient homology to render the polypeptide or derivative thereof anti-viral, with anti-viral activity characteristic of an anti-viral protein isolated from Griffithsia. At least about 50% homology or identity (e.g., at least about 60%, at least about 65%, or at least about 70% homology or identity), preferably at least about 75% homology or identity (e.g., at least about 80% or at least about 85% homology or identity), and most preferably at least about 90% homology or identity (e.g., at least about 95% homology or identity) should exist.

[0047] The inventive nucleic acid molecule preferably comprises a nucleic acid sequence encoding at least eight (preferably at least 10, more preferably at least 20, and most preferably at least 30) contiguous amino acids of the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 2. The inventive nucleic acid molecule also comprises a nucleic acid sequence encoding a polypeptide comprising the amino acid sequence of a native griffithsin, in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been removed from one or both ends, preferably from only one end, e.g., removed from the amino-terminal end, of the native

griffithsin. Alternatively, the nucleic acid molecule can comprise a nucleic acid sequence encoding a polypeptide comprising the amino acid sequence of a natural griffithsin (see SEQ ID NO: 3), in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been added to one or both ends, preferably from only one end, e.g., the amino-terminal end, of the native griffithsin. Preferably, the isolated and purified nucleic acid molecule encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, which desirably have anti-viral activity. If the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO: 3, desirably amino acids 46, 60, 71, and/or 104 have been rendered glycosylation resistant, while maintaining antiviral activity of the polypeptide. Deletions and substitutions of SEQ ID NO: 2 or SEQ ID NO: 3 are within the skill in the art.

[0048] Given the present disclosure, it will be apparent to one skilled in the art that a partial griffithsin gene sequence will likely suffice to code for a fully functional, i.e., anti-viral, such as anti-influenza or anti-HIV, griffithsin. A minimum essential DNA coding sequence(s) for a functional griffithsin can readily be determined by one skilled in the art, for example, by synthesis and evaluation of sub-sequences comprising the native griffithsin, and by site-directed mutagenesis studies of the griffithsin DNA coding sequence.

[0049] Using an appropriate DNA coding sequence, a recombinant griffithsin can be made by genetic engineering techniques (for general background see, e.g., Nicholl, in An Introduction to Genetic Engineering, Cambridge University Press: Cambridge (1994), pp. 1-5 & 127-130; Steinberg et al., in Recombinant DNA Technology Concepts and Biomedical Applications, Prentice Hall: Englewood Cliffs, N.J. (1993), pp. 81-124 & 150-162; Sofer in Introduction to Genetic Engineering, Butterworth-Heinemann, Stoneham, Mass. (1991), pp. 1-21 & 103-126; Old et al., in Principles of Gene Manipulation, Blackwell Scientific Publishers: London (1992), pp. 1-13 & 108-221; and Emtage, in Delivery Systems for Peptide Drugs, Davis et al., eds., Plenum Press: New York (1986), pp. 23-33). For example, a Griffithsia gene or cDNA encoding a griffithsin can be identified and subcloned. The gene or cDNA then can be incorporated into an appropriate expression vector and delivered into an appropriate polypeptide-synthesizing organism (e.g., E. coli, S. cerevisiae, P. pastoris, or other bacterial, yeast, insect, plant or mammalian cells), where the gene, under the control of an endogenous or exogenous promoter, can be appropriately transcribed and translated. Alternatively, the expression vector can be administered to a plant or animal, for example, for large-scale production (see, e.g., Fischer et al., Transgenic Res., 9 (4-5): 279-299 (2000); Fischer et al., J. Biol. Regul. Homeost. Agents, 14: 83-92 (2000); deWilde et al., Plant Molec. Biol., 43: 347-359 (2000); Houdebine, Transgenic Research, 9: 305-320 (2000); Brink et al., Theriogenology, 53: 139-148 (2000); Pollock et al., J. Immunol. Methods, 231: 147-157 (1999); Conrad et al., Plant Molec. Biol., 38: 101-109 (1998); Staub et al., Nature Biotech., 18: 333-338 (2000); McCormick et al., PNAS USA, 96: 703-708 (1999); Zeitlin et al., Nature Biotech., 16: 1361-1364 (1998); Tacker et al., Microbes and Infection, 1: 777-783 (1999); Tacket et al., Nature Med., 4(5): 607-609 (1998); and Methods in Biotechnology, Recombinant Proteins from Plants, Production and Isolation of Clinically Useful Compounds, Cunningham and Porter, eds., Humana Press: Totowa, N.J. (1998)). Such expression vectors (including, but not limited to, phage, cosmid, viral, and plasmid vectors) are known to those skilled in the art, as are reagents and techniques appropriate for gene transfer (e.g., transfection, electroporation, transduction, micro-injection, transformation, etc.). If a griffithsin is to be recombinantly produced in isolated eukaryotic cells or in a eukaryotic organism, such as a plant (see above references and also Methods in Biotechnology, Recombinant Proteins from Plants, Production and Isolation of Clinically Useful Compounds, Cunningham and Porter, eds., Humana Press: Totowa, N.J. (1998)), desirably the N-linked glycosylation sites at positions 45, 60, 71, and/or 104 is rendered glycosylation-resistant, such as in accordance with the methods described herein. Subsequently, the recombinantly produced polypeptide can be isolated and purified using standard techniques known in the art (e.g., chromatography, centrifugation, differential solubility, isoelectric focusing, etc.), and assayed for anti-viral activity.

[0050] Alternatively, a natural griffithsin can be obtained from Griffithsia by non-recombinant methods, and sequenced by conventional techniques. The sequence can then be used to synthesize the corresponding DNA, which can be subcloned into an appropriate expression vector and delivered into a polypeptide-producing cell for en mass recombinant production of the desired polypeptide.

[0051] In this regard, the invention also provides a vector comprising a DNA sequence, e.g., a Griffithsia gene sequence for griffithsin, a cDNA encoding a griffithsin, or a synthetic DNA sequence encoding griffithsin. The vector can be targeted to a cell-surface receptor if so desired. A nucleic acid molecule as described above can be cloned into any suitable vector and can be used to transform or transfect any suitable host. The selection of vectors and methods to construct them are commonly known to persons of ordinary skill in the art and are described in general technical references (see, in general, "Recombinant DNA Part D," Methods in Enzymology, Vol. 153, Wu and Grossman, eds., Academic Press (1987) and the references cited herein under "EXAMPLES"). Desirably, the vector comprises regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of host (e.g., bacterium, fungus, plant or animal) into which the vector is to be introduced, as appropriate and taking into consideration whether the vector is DNA or RNA. Preferably, the vector comprises regulatory sequences that are specific to the genus of the host. Most preferably, the vector comprises regulatory sequences that are specific to the species of the host.

[0052] Constructs of vectors, which are circular or linear, can be prepared to contain an entire nucleic acid as described above or a portion thereof ligated to a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived from Co1E1, 2 m[mu] plasmid, [lambda], SV40, bovine papilloma virus, and the like.

[0053] In addition to the replication system and the inserted nucleic acid, the construct can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to antibiotics, heavy metals, etc., complementation in an auxotrophic host to provide prototrophy, and the like.

[0054] One of ordinary skill in the art will appreciate that any of a number of vectors known in the art are suitable for use in the invention. Suitable vectors include those designed for propagation and expansion or for expression or both. Examples of suitable vectors include, for instance, plasmids, plasmid-liposome complexes, and viral vectors, e.g., parvoviral-based vectors (i.e., adeno-associated virus (AAV)-based vectors), retroviral vectors, herpes simplex virus (HSV)-based vectors, and adenovirus-based vectors. Any of these expression constructs can be prepared using standard recombinant DNA techniques described in, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2<nd >edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994); Fischer et al., Transgenic Res., 9 (4-5): 279-299 (2000); Fischer et al., J. Biol. Regul. Homeost. Agents, 14: 83-92 (2000); deWilde et al., Plant Molec. Biol., 43: 347-359 (2000); Houdebine, Transgenic Research, 9: 305-320 (2000); Brink et al., Theriogenology, 53: 139-148 (2000); Pollock et al., J. Immunol. Methods, 231: 147-157 (1999); Conrad et al., Plant Molec. Biol., 38: 101-109 (1998); Staub et al., Nature Biotech., 18: 333-338 (2000); McCormick et al., PNAS USA, 96: 703-708 (1999); Zeitlin et al., Nature Biotech., 16: 1361-1364 (1998); Tacker et al., Microbes and Infection, 1: 777-783 (1999); and Tacket et al., Nature Med., 4(5): 607-609 (1998). Examples of cloning vectors include the pUC series, the pBluescript series (Stratagene, LaJolla, Calif.), the pET series (Novagen, Madison, Wis.), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clonetech, Palo Alto, Calif.). Bacteriophage vectors, such as [lambda]GT10, [lambda]GT11, [lambda]ZapII (Stratagene), [lambda] EMBL4, and [lambda] NM1149, also can be used. Examples of plant expression vectors include pBI101, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clonetech, Palo Alto, Calif.). Examples of animal expression vectors include pEUK-C1, pMAM and pMAMneo (Clonetech).

[0055] An expression vector can comprise a native or normative promoter operably linked to an isolated or purified nucleic acid as described above. The selection of promoters, e.g., strong, weak, inducible, tissue-specific and developmental-specific, is within the skill in the art. Similarly, the combining of a nucleic acid molecule as described above with a promoter is also within the skill in the art.

[0056] The DNA, whether isolated and purified or synthetic, or cDNA encoding a griffithsin can encode for either the entire griffithsin or a portion thereof. Where the DNA or cDNA does not comprise the entire coding sequence of the native griffithsin, the DNA or cDNA can be subcloned as part of a gene fusion. In a transcriptional gene fusion, the DNA or cDNA will contain its own

control sequence directing appropriate production of protein (e.g., ribosome binding site, translation initiation codon, etc.), and the transcriptional control sequences (e.g., promoter elements and/or enhancers) will be provided by the vector. In a translational gene fusion, transcriptional control sequences as well as at least some of the translational control sequences (i.e., the translational initiation codon) will be provided by the vector. In the case of a translational gene fusion, a chimeric protein will be produced.

[0057] Genes also can be constructed for specific fusion proteins containing a functional griffithsin component plus a fusion component conferring additional desired attribute(s) to the composite protein. For example, a fusion sequence for a toxin or immunological reagent can be added to facilitate purification and analysis of the functional protein.

[0058] Genes can be specifically constructed to code for fusion proteins, which contain a griffithsin coupled to an effector protein, such as a toxin or immunological reagent, for specific targeting to a virus or viral-infected cells, e.g., HIV and/or HIV-infected cells or influenza and/or influenza-infected cells. In these instances, the griffithsin moiety serves not only as a neutralizing agent but also as a targeting agent to direct the effector activities of these molecules selectively against a given virus, such as HIV or influenza. Thus, for example, a therapeutic agent can be obtained by combining the HIV-targeting function or influenza-targeting function of a functional griffithsin with a toxin aimed at neutralizing infectious virus and/or by destroying cells producing infectious virus, such as HIV or influenza. Similarly, a therapeutic agent can be obtained, which combines the viral-targeting function of a griffithsin with the multivalency and effector functions of various immunoglobulin subclasses. Example 6 further illustrates the viral-targeting, specifically gp120-targeting, properties of a griffithsin.

[0059] Similar rationales underlie extensive developmental therapeutic efforts exploiting the HIV gp120-targeting properties of sCD4. For example, sCD4-toxin conjugates have been prepared in which sCD4 is coupled to a Pseudomonas exotoxin component (Chaudhary et al., in The Human Retrovirus, Gallo et al., eds., Academic Press: San Diego, Calif. (1991), pp. 379-387; and Chaudhary et al., Nature, 335: 369-372 (1988)), or to a diphtheria toxin component (Aullo et al., EMBO J., 11: 575-583 (1992)) or to a ricin A-chain component (Till et al., Science, 242: 1166-1167 (1988)). Likewise, sCD4-immunoglobulin conjugates have been prepared in attempts to decrease the rate of in vivo clearance of functional sCD4 activity, to enhance placental transfer, and to effect a targeted recruitment of immunological mechanisms of pathogen elimination, such as phagocytic engulfment and killing by antibody-dependent cell-mediated cytotoxicity, to kill and/or remove HIV-infected cells and virus (Capon et al., Nature, 337: 525-531 (1989); Traunecker et al., Nature, 339: 68-70 (1989); and Langner et al. (1993), supra). While such CD4-immunoglobulin conjugates (sometimes called "immunoadhesins") have, indeed, shown advantageous pharmacokinetic and distributional attributes in vivo, and anti-HIV effects in vitro, clinical results have been discouraging (Schooley et al. (1990), supra; Husson et al. (1992), supra; and Langner et al. (1993), supra). This is not surprising since clinical isolates of HIV, as opposed to laboratory strains, are highly resistant to binding and neutralization by sCD4 (Orloff et al. (1995), supra; and Moore et al. (1992), supra). The griffithsin polypeptide binds to a wide range of sugars present on viral glycoproteins and, therefore, can inhibit a wide range of viruses which display those glycoproteins. The extraordinarily broad targeting properties of a functional griffithsin to viruses, e.g., primate retroviruses, in general, and clinical and laboratory strains, in particular, can be especially advantageous for combining with toxins, immunoglobulins and other selected effector proteins.

[0060] Viral-targeted conjugates can be prepared either by genetic engineering techniques (see, for example, Chaudhary et al. (1988), supra) or by chemical coupling of the targeting component with an effector component. The most feasible or appropriate technique to be used to construct a given griffithsin conjugate or fusion protein will be selected based upon consideration of the characteristics of the particular effector molecule selected for coupling to a griffithsin. For example, with a selected non-proteinaceous effector molecule, chemical coupling, rather than genetic engineering techniques, may be the only feasible option for creating the desired griffithsin conjugate.

[0061] Accordingly, the invention also provides nucleic acid molecules encoding griffithsin fusion proteins. In particular, the invention provides a nucleic acid molecule comprising SEQ ID NO: 4

and substantially homologous sequences thereof. Also provided is a vector comprising a nucleic acid sequence encoding a griffithsin fusion protein and a method of obtaining a griffithsin fusion protein by expression of the vector encoding a griffithsin fusion protein in a protein-synthesizing organism as described above. Accordingly, griffithsin fusion proteins are also provided.

[0062] In view of the above, the invention further provides an isolated and purified nucleic acid molecule, which comprises a griffithsin coding sequence, such as one of the aforementioned nucleic acids, namely a nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3 or a nucleic acid molecule comprising a sequence of SEQ ID NO: 1 coupled to a second nucleic acid encoding an effector protein. The first nucleic acid preferably comprises a nucleic acid sequence encoding at least eight contiguous amino acids of the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, which encodes a functional griffithsin, and the second nucleic acid preferably encodes an effector protein, such as a toxin or immunological reagent as described herein.

[0063] Accordingly, the invention also further provides an isolated and purified fusion protein encoded by a nucleic acid molecule comprising a sequence of SEQ ID NO: 1 or a nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, either one of which is coupled to a second nucleic acid encoding an effector protein. Preferably, the aforementioned nucleic acid molecules encode at least eight contiguous amino acids of the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, which desirably have anti-viral activity, coupled to an effector molecule, such as a toxin or immunological reagent as described above. Preferably, the effector molecule targets a virus, more preferably HIV or influenza, and, most preferably glycoprotein gp120 of HIV or hemagluttinin of influenza. If the at least eight contiguous amino acids of SEQ ID NO: 3 (or SEQ ID NO: 2) comprise amino acids 1-121, desirably amino acids 46, 60, 71, and/or 104 have been rendered glycosylation-resistant, yet maintain antiviral activity by substitution of the asparagine at those positions with, for example, an alanine or a glutamine residue.

[0064] The coupling can be effected at the DNA level or by chemical coupling as described above. For example, a griffithsin-effector protein conjugate of the invention can be obtained by (a) selecting a desired effector protein or peptide; (b) synthesizing a composite DNA coding sequence comprising a first DNA coding sequence comprising one of the aforementioned nucleic acid sequences, which codes for a functional griffithsin, coupled to a second DNA coding sequence for an effector protein or peptide, e.g., a toxin or immunological reagent; (c) expressing said composite DNA coding sequence in an appropriate protein-synthesizing organism; and (d) purifying the desired fusion protein to substantially pure form. Alternatively, a griffithsin-effector molecule conjugate of the invention can be obtained by (a) selecting a desired effector molecule and a griffithsin or griffithsin fusion protein; (b) chemically coupling the griffithsin or griffithsin fusion protein to the effector molecule; and (c) purifying the desired griffithsin-effector molecule conjugate to substantially pure form.

[0065] Conjugates comprising a functional griffithsin (e.g., an anti-viral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, such as SEQ ID NO: 3, wherein the at least eight contiguous amino acids bind to a virus, in particular an infectious virus, such as influenza virus or HIV, in which case the griffithsin binds to gp120 or hemagluttinin) coupled to an anti-griffithsin antibody, a virus, a viral glycoprotein, or at least one effector component, which can be the same or different, such as a toxin, an immunological reagent, an antiviral agent, or other functional reagent, can be designed even more specifically to exploit the unique viral targeting, e.g., gp120-targeting properties, of griffithsins.

[0066] Other functional reagents that can be used as effector components in the inventive conjugates can include, for example, polyethylene glycol, dextran, albumin, a solid support matrix, and the like, whose intended effector functions may include one or more of the following: to improve stability of the conjugate; to increase the half-life of the conjugate; to increase resistance of the conjugate to proteolysis; to decrease the immunogenicity of the conjugate; to provide a means to attach or immobilize a functional griffithsin onto a solid support matrix (e.g., see, for example, Harris, in Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Harris, ed., Plenum Press: New York (1992), pp. 1-14). Conjugates furthermore can comprise a functional griffithsin coupled to more than one effector molecule, each of which, optionally, can have different effector functions (e.g., such as a toxin molecule (or an immunological reagent) and a polyethylene

glycol (or dextran or albumin) molecule). Diverse applications and uses of functional proteins and peptides, such as in the present instance a functional griffithsin, attached to or immobilized on a solid support matrix, are exemplified more specifically for poly(ethylene glycol) conjugated proteins or peptides in a review by Holmberg et al. (In Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Harris, ed., Plenum Press: New York (1992), pp. 303-324). Preferred examples of solid support matrices include magnetic beads, a flow-through matrix, and a matrix comprising a contraceptive device, such as a condom, a diaphragm, a cervical cap, a vaginal ring or a sponge.

[0067] Example 4 reveals novel gp120-directed effects of griffithsins. Solid-phase ELISA experiments show that griffithsin is capable of global conformational effects on gp120, as observed as a decrease of immunoreactivity at multiple, distinct, non-overlapping epitopes.

[0068] The range of anti-viral activity of griffithsin against diverse CD4<+>-tropic immunodeficiency virus strains in various target cells is remarkable; virtually all tested strains of HIV-1, HIV-2 and SIV were similarly sensitive to griffithsin; clinical isolates and laboratory strains showed essentially equivalent sensitivity. Cocultivation of chronically infected and uninfected CEM-SS cells with griffithsin did not inhibit viral replication, but did cause a concentration-dependent inhibition of cell-to-cell fusion and virus transmission; similar results from binding and fusion inhibition assays employing HeLa-CD4-LTR-[beta]-galactosidase cells were consistent with griffithsin inhibition of virus-cell and/or cell-cell binding.

[0069] The anti-viral, e.g., anti-HIV, activity of the griffithsins and conjugates thereof of the invention can be further demonstrated in a series of interrelated in vitro anti-viral assays (Gulakowski et al., J. Virol. Methods, 33: 87-100 (1991)), which accurately predict for anti-viral activity in humans. These assays measure the ability of compounds to prevent the replication of HIV and/or the cytopathic effects of HIV on human target cells. These measurements directly correlate with the pathogenesis of HIV-induced disease in vivo. The results of the analysis of the anti-viral activity of griffithsins or conjugates, as set forth in Examples 5-7 and 9, predict accurately the anti-viral activity of these products in vivo in humans and, therefore, establish the utility of the invention. Furthermore, since the invention also provides methods of ex vivo use of griffithsins and conjugates, the utility of griffithsins and conjugates thereof is even more certain.

[0070] The griffithsins and conjugates thereof of the invention can be shown to inhibit a virus, specifically a retrovirus, more specifically an immunodeficiency virus, such as the human immunodeficiency virus, i.e., HIV-1 or HIV-2. The griffithsins and conjugates of the invention can be used to inhibit other retroviruses as well as other viruses (see, e.g., Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint et al., eds., ASM Press: Washington, D.C. (2000), particularly Chapter 19). Examples of viruses that may be treated in accordance with the invention include, but are not limited to, Type C and Type D retroviruses, HTLV-1, HTLV-2, HIV, FIV, FLV, SIV, MLV, BLV, BIV, equine infectious virus, anemia virus, avian sarcoma viruses, such as Rous sarcoma virus (RSV), hepatitis type A, B, non-A and non-B viruses, arboviruses, varicella viruses, human herpes virus (e.g., HHV-6), measles, mumps, filovirus (e.g., Ebola, such as Ebola strains Sudan, Zaire, Cote d'Ivoire, and Reston) and rubella viruses. Griffithsins and conjugate thereof also can be used to inhibit influenza viral infection (see, e.g., Fields Virology, third edition, Fields et al., eds., Lippincott-Raven Publishers: Philadelphia, Pa. (1996), particularly Chapter 45) prophylactically and therapeutically in accordance with the methods set forth herein.

[0071] Thus, the invention further provides a composition comprising (i) one or more of an above-described purified or isolated nucleic acid or variant thereof, optionally as part of an encoded fusion protein, and (ii) a carrier, excipient or adjuvant. Preferably, (i) is present in an antiviral effective amount and the composition is pharmaceutically acceptable. The composition can further comprise at least one additional active agent, such as an antiviral agent other than a griffithsin (or antiviral fragment, fusion protein or conjugate thereof), in an antiviral effective amount. Suitable antiviral agents include AZT, ddA, ddI, ddC, 3TC gancyclovir, fluorinated dideoxynucleosides, acyclovir, [alpha]-interferon, nonnucleoside analog compounds, such as nevirapine (Shih et al., PNAS, 88: 9878-9882, (1991)), TIBO derivatives, such as R82913 (White et al., Antiviral Res., 16: 257-266 (1991)), Ro31-8959, BI-RJ-70 (Merigan, Am. J. Med., 90 (Suppl. 4A): 8S-17S (1991)), michellamines (Boyd et al., J. Med. Chem., 37: 1740-1745 (1994)) and calanolides (Kashman et al.,

J. Med. Chem., 35: 2735-2743 (1992)), nonoxynol-9, gossypol and derivatives, gramicidin, Enfurtide (i.e., T20), cyanovirin-N and functional homologs thereof (Boyd et al. (1997), supra). Other exemplary antiviral compounds include protease inhibitors (see R. C. Ogden and C. W. Flexner, eds., Protease Inhibitors in AIDS Therapy, Marcel Dekker, N Y (2001)), such as saquinavir (see I. B. Duncan and S. Redshaw, in R. C. Ogden and C. W. Flexner, supra, pp. 27-48), ritonavir (see D. J. Kempf, in R. C. Ogden and C. W. Flexner, supra, pp. 49-64), indinavir (see B. D. Dorsey and J. P. Vacca, in R. C. Ogden and C. W. Flexner, supra, pp. 65-84), nelfinavir (see S. H. Reich, in R. C. Ogden and C. W. Flexner, supra, pp. 85-100), amprenavir (see R. D. Tung, in R. C. Ogden and C. W. Flexner, supra, pp. 101-118), and anti-TAT agents. If the composition is to be used to induce an immune response, it comprises an immune response-inducing amount of the inventive agent and can further comprise an immunoadjuvant, such as polyphosphazene polyelectrolyte.

[0072] The pharmaceutical composition can contain other pharmaceuticals, such as virucides, immunomodulators, immunostimulants, antibiotics and absorption enhancers. Exemplary immunomodulators and immunostimulants include various interleukins, sCD4, cytokines, antibody preparations, blood transfusions, and cell transfusions. Exemplary antibiotics include antifungal agents, antibacterial agents, and anti-Pneumocystitis carnii agents. Exemplary absorption enhancers include bile salts and other surfactants, saponins, cyclodextrins, and phospholipids (Davis (1992), supra).

[0073] An isolated cell comprising an above-described purified or isolated nucleic acid or variant thereof, optionally in the form of a vector, which is optionally targeted to a cell-surface receptor, is also provided. Examples of host cells include, but are not limited to, a human cell, a human cell line, E. coli, B. subtilis, P. aerugenosa, S. cerevisiae, and N. crassa. E. coli, in particular E. coli TB-1, TG-2, DH5[alpha], XL-Blue MRF' (Stratagene), SA2821 and Y1090. Preferably, the cell is a mammalian cell, bacterium, or yeast. A preferred bacterium is lactobacillus or other commensal microorganism. The above-described nucleic acid or variant thereof, optionally in the form of a vector, can be introduced into a host cell using such techniques as transfection, electroporation, transduction, micro-injection, transformation, and the like.

[0074] Accordingly, the invention provides a method of inhibiting prophylactically or therapeutically a viral infection, in particular an influenza viral infection or HIV infection, of a host. The method comprises administering to the host an effective amount of an anti-viral polypeptide or anti-viral polypeptide conjugate comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids are nonglycosylated and have anti-viral activity, whereupon the viral infection is inhibited. The anti-viral polypeptide can be derived from a griffithsin obtained from Griffithsia or recombinantly produced in accordance with the methods described above. Nonglycosylated anti-viral polypeptides can be produced in prokaryotic cells/organisms. Amino acids 45, 60, 71, and/or 104 in such nonglycosylated antiviral polypeptides can be deleted or substituted, for example, with alanine or glutamine. Nonglycosylated antiviral polypeptides also can be produced in eukaryotic cells/organisms by expressing a portion of a griffithsin, such as that of SEQ ID NO: 3, that does not contain a glycosylation site or all or a portion of a griffithsin, such as that of SEQ ID NO: 3, which contains a glycosylation site that has been rendered glycosylation-resistant as described and exemplified herein. When the viral infection is an influenza viral infection and the anti-viral polypeptide or anti-viral polypeptide conjugate is administered topically to the host, preferably the anti-viral protein or anti-viral peptide is administered to the respiratory system of the host, preferably as an aerosol or microparticulate powder.

[0075] The prophylactic and therapeutic treatment of many viral infections, including influenza virus infections, is complicated by appearance of virus forms resistant to currently employed medications, such as neurominidase inhibitors. The inventive method is particularly useful in this context, as the inventive anti-viral polypeptide or anti-viral polypeptide conjugate binds a wide range of glycoproteins present on the viral surface. Accordingly, the inventive anti-viral polypeptide or conjugate thereof can be administered to an animal, preferably a human, dog, cat, bird, cow, pig, horse, lamb, mouse, or rat, in combination with other anti-viral agents to guard against the propagation of anti-viral-resistant strains of virus. In addition, it is thought that during adaptive mutation (e.g., resistance to neuraminidase inhibitors), the level of glycosylation found at the viral surface increases in some viruses, such as influenza. Thus, in that the inventive anti-viral

agent binds sugars of viral surface glycoproteins, the inventive method provides a valuable complimentary therapy to current anti-viral regimens.

[0076] Griffithsins and conjugates thereof collectively comprise polypeptides and proteins, and, as such, are particularly susceptible to hydrolysis of amide bonds (e.g., catalyzed by peptidases) and disruption of essential disulfide bonds or formation of inactivating or unwanted disulfide linkages (Carone et al., J. Lab. Clin. Med., 100:1-14 (1982)). There are various ways to alter molecular structure, if necessary, to provide enhanced stability to the griffithsin or conjugate thereof (Wunsch, Biopolymers, 22: 493-505 (1983); and Samanen, in Polymeric Materials in Medication, Gebelein et al., eds., Plenum Press: New York (1985) pp. 227-242), which may be essential for preparation and use of pharmaceutical compositions containing griffithsins or conjugates thereof for therapeutic or prophylactic applications against viruses, e.g., HIV. Possible options for useful chemical modifications of a griffithsin or conjugate include, but are not limited to, the following (adapted from Samanen, J. M. (1985) supra): (a) olefin substitution, (b) carbonyl reduction, (c) D-amino acid substitution, (d) N-methyl substitution, (e) C-methyl substitution, (f) C-C'-methylene insertion, (g) dehydro amino acid insertion, (h) retro-inverso modification, (i) N-terminal to C-terminal cyclization, and (j) thiomethylene modification. Griffithsins and conjugates thereof also can be modified by covalent attachment of carbohydrate and polyoxyethylene derivatives, which are expected to enhance stability and resistance to proteolysis (Abuchowski et al., in Enzymes as Drugs, Holcenberg et al., eds., John Wiley: New York (1981), pp. 367-378).

[0077] Other important general considerations for design of delivery strategy systems and compositions, and for routes of administration, for protein and peptide drugs, such as griffithsins and conjugates thereof (Eppstein, CRC Crit. Rev. Therapeutic Drug Carrier Systems, 5: 99-139 (1988); Siddiqui et al., CRC Crit. Rev. Therapeutic Drug Carrier Systems, 3: 195-208 (1987); Banga et al., Int. J. Pharmaceutics, 48: 15-50 (1988); Sanders, Eur. J. Drug Metab. Pharmacokinetics, 15: 95-102 (1990); and Verhoef, Eur. J. Drug Metab. Pharmacokinetics, 15: 83-93 (1990)), also apply. The appropriate delivery system for a given griffithsin or conjugate thereof will depend upon its particular nature, the particular clinical application, and the site of drug action. As with any protein or peptide drug, oral delivery of a griffithsin or a conjugate thereof will likely present special problems, due primarily to instability in the gastrointestinal tract and poor absorption and bioavailability of intact, bioactive drug therefrom. Therefore, especially in the case of oral delivery, but also possibly in conjunction with other routes of delivery, it will be necessary to use an absorption-enhancing agent in combination with a given griffithsin or conjugate thereof A wide variety of absorption-enhancing agents have been investigated and/or applied in combination with protein and peptide drugs for oral delivery and for delivery by other routes (Verhoef (1990), supra; van Hoogdalem, Pharmac. Ther., 44: 407-443 (1989); and Davis, J. Pharm. Pharmacol, 44 (Suppl. 1): 186-190 (1992)). Most commonly, typical enhancers fall into the general categories of (a) chelators, such as EDTA, salicylates, and N-acyl derivatives of collagen, (b) surfactants, such as lauryl sulfate and polyoxyethylene-9-lauryl ether, (c) bile salts, such as glycholate and taurocholate, and derivatives, such as taurodihydrofusidate, (d) fatty acids, such as oleic acid and capric acid, and their derivatives, such as acylcarnitines, monoglycerides and diglycerides, (e) non-surfactants, such as unsaturated cyclic ureas, (f) saponins, (g) cyclodextrins, and (h) phospholipids.

[0078] Other approaches to enhancing oral delivery of protein and peptide drugs, such as the griffithsins and conjugates thereof, can include aforementioned chemical modifications to enhance stability to gastrointestinal enzymes and/or increased lipophilicity. Alternatively, or in addition, the protein or peptide drug can be administered in combination with other drugs or substances, which directly inhibit proteases and/or other potential sources of enzymatic degradation of proteins and peptides. Yet another alternative approach to prevent or delay gastrointestinal absorption of protein or peptide drugs, such as griffithsins or conjugates, is to incorporate them into a delivery system that is designed to protect the protein or peptide from contact with the proteolytic enzymes in the intestinal lumen and to release the intact protein or peptide only upon reaching an area favorable for its absorption. A more specific example of this strategy is the use of biodegradable microcapsules or microspheres, both to protect vulnerable drugs from degradation, as well as to effect a prolonged release of active drug (Deasy, in Microencapsulation and Related Processes, Swarbrick, ed., Marcell Dekker, Inc.: New York (1984), pp. 1-60, 88-89, 208-211). Microcapsules also can provide a useful way to effect a prolonged delivery of a protein and peptide drug, such as a griffithsin or conjugate thereof, after injection (Maulding, J. Controlled Release, 6: 167-176 (1987)).

[0079] Given the aforementioned potential complexities of successful oral delivery of a protein or peptide drug, it is fortunate that there are numerous other potential routes of delivery of a protein or peptide drug, such as a griffithsin or conjugate thereof. These routes include topical, subcutaneous, intravenous, intraarterial, intrathecal, intracisternal, buccal, rectal, nasal, pulmonary, transdermal, vaginal, ocular, and the like (Eppstein (1988), supra; Siddiqui et al. (1987), supra; Banga et al. (1988), supra; Sanders (1990), supra; Verhoef (1990), supra; Barry, in Delivery Systems for Peptide Drugs, Davis et al., eds., Plenum Press: New York (1986), pp. 265-275; and Patton et al., Adv. Drug Delivery Rev, 8: 179-196 (1992)). With any of these routes, or, indeed, with any other route of administration or application, a protein or peptide drug, such as a griffithsin or conjugate thereof, may initiate an immunogenic reaction. In such situations it may be necessary to modify the molecule in order to mask immunogenic groups. It also can be possible to protect against undesired immune responses by judicious choice of method of formulation and/or administration. For example, site-specific delivery can be employed, as well as masking of recognition sites from the immune system by use or attachment of a so-called tolerogen, such as polyethylene glycol, dextran, albumin, and the like (Abuchowski et al. (1981), supra; Abuchowski et al., J. Biol. Chem., 252: 3578-3581 (1977); Lisi et al., J. Appl. Biochem, 4: 19-33 (1982); and Wileman et al., J. Pharm. Pharmacol, 38: 264-271 (1986)). Such modifications also can have advantageous effects on stability and half-life both in vivo and ex vivo.

[0080] Procedures for covalent attachment of molecules, such as polyethylene glycol, dextran, albumin and the like, to proteins, such as griffithsins or conjugates thereof, are well-known to those skilled in the art, and are extensively documented in the literature (e.g., see Davis et al., in Peptide and Protein Drug Delivery, Lee, ed., Marcel Dekker: New York (1991), pp. 831-864).

[0081] Other strategies to avoid untoward immune reactions also can include the induction of tolerance by administration initially of only low doses. In any event, it will be apparent from the present disclosure to one skilled in the art that for any particular desired medical application or use of a griffithsin or conjugate thereof, the skilled artisan can select from any of a wide variety of possible compositions, routes of administration, or sites of application, what is advantageous.

[0082] Accordingly, the anti-viral griffithsins and conjugates thereof of the invention can be formulated into various compositions for use, for example, either in therapeutic treatment methods for infected individuals, or in prophylactic methods against viral, e.g., HIV and influenza virus, infection of uninfected individuals.

[0083] The invention also provides a composition, such as a pharmaceutical composition, which comprises an isolated and purified griffithsin, a griffithsin conjugate, a matrix-anchored griffithsin or a matrix-anchored griffithsin conjugate, such as an anti-viral effective amount thereof. The composition can further comprise a carrier, such as a pharmaceutically acceptable carrier. The composition can further comprise at least one additional anti-viral compound other than a griffithsin or conjugate thereof, such as in an anti-viral effective amount of an anti-viral compound. Suitable anti-viral compounds include cyanovirin, AZT, ddI, ddC, gancyclovir, fluorinated dideoxynucleosides, nevirapine, R82913, Ro 31-8959, BI-RJ-70, acyclovir, [alpha]-interferon, recombinant sCD4, michellamines, calanolides, nonoxynol-9, gossypol and derivatives thereof, neuroamidase inhibitors, amantatadine, rimantadine, enfurtide, and gramicidin. If the composition is to be used to induce an immune response, it comprises an immune response-inducing amount of a griffithsin or conjugate thereof and can further comprise an immunoadjuvant, such as polyphosphazene polyelectrolyte. The griffithsin used in the composition, e.g., pharmaceutical composition, can be isolated and purified from nature or genetically engineered. Similarly, the griffithsin conjugate can be genetically engineered or chemically coupled.

[0084] The inventive compositions can be administered to a host, such as a human, so as to inhibit viral infection in a prophylactic or therapeutic method. The compositions of the invention are particularly useful in inhibiting the growth or replication of a virus, such as influenza virus or a retrovirus, in particular an influenza virus or an immunodeficiency virus, such as HIV, specifically HIV-1 and HIV-2, inhibiting infectivity of the virus, inhibiting the binding of virus to a host cell, and the like. The compositions are useful in the therapeutic or prophylactic treatment of animals, such as humans, who are infected with a virus or who are at risk for viral infection, respectively. The compositions also can be used to treat objects or materials, such as medical equipment,

supplies, or fluids, including biological fluids, such as blood, blood products and vaccine formulations, cells, tissues and organs, to remove or inactivate virus in an effort to prevent or treat viral infection of an animal, such as a human. Such compositions also are useful to prevent sexual transmission of viral infections, e.g., HIV, which is the primary way in which the world's AIDS cases are contracted (Merson (1993), supra). Adherence of the inventive anti-viral polypeptide or conjugate thereof to a solid support, such as a filter, can be used in clinics to remove all or part of the viral content of a biological solution. For example, filters comprising the inventive anti-viral agents can be used to treat blood supplies prior to transfusion to reduce the risk of viral transmission. Such filters would find particular utility in clinics wherein risk of viral infection is high. It will be appreciated that total removal of the viral content of a biological solution is not required to achieve a beneficial effect. Removal of even a fraction of virus from a biological solution decreases the risk of infection of a patient.

[0085] Potential virucides used or being considered for use against sexual transmission of HIV are very limited; present agents in this category include, for example, nonoxynol-9 (Bird, AIDS, 5: 791-796 (1991)), gossypol and derivatives (Polsky et al., Contraception, 39: 579-587 (1989); Lin, Antimicrob. Agents Chemother, 33: 2149-2151 (1989); and Royer, Pharmacol. Res, 24: 407-412 (1991)), and gramicidin (Bourinbair, Life Sci./Pharmacol. Lett, 54: PL5-9 (1994); and Bourinbair et al., Contraception, 49: 131-137 (1994)). The method of prevention of sexual transmission of viral infection, e.g., HIV infection, in accordance with the invention comprises vaginal, rectal, oral, penile or other topical treatment with an anti-viral effective amount of a griffithsin and/or griffithsin conjugate, alone or in combination with another anti-viral compound as described herein.

[0086] In a novel approach to anti-HIV prophylaxis pursued under auspices of the U.S. National Institute of Allergy and Infectious Diseases (NIAID) (e.g., as conveyed by Painter, USA Today, Feb. 13, 1996), vaginal suppository instillation of live cultures of lactobacilli was being evaluated in a 900-woman study. This study was based especially upon observations of anti-HIV effects of certain H_2O_2-producing lactobacilli in vitro (e.g., see published abstract by Hilier, from NIAID-sponsored Conference on "Advances in AIDS Vaccine Development," Bethesda, Md., Feb. 11-15, 1996). Lactobacilli readily populate the vagina, and indeed are a predominant bacterial population in most healthy women (Redondo-Lopez et al., Rev. Infect. Dis., 12: 856-872 (1990); Reid et al., Clin. Microbiol. Rev., 3: 335-344 (1990); Bruce and Reid, Can. J. Microbiol., 34: 339-343 (1988); Reu et al., J. Infect. Dis., 171: 1237-1243 (1995); Hilier et al., Clin. Infect. Dis., 16 (Suppl 4): S273-S281; and Agnew et al., Sex. Transm. Dis., 22: 269-273 (1995)). Lactobacilli are also prominent, nonpathogenic inhabitants of other body cavities such as the mouth, nasopharynx, upper and lower gastrointestinal tracts, and rectum.

[0087] It is well-established that lactobacilli can be readily transduced using available genetic engineering techniques to incorporate a desired foreign DNA coding sequence, and that such lactobacilli can be made to express a corresponding desired foreign protein (see, e.g., Hols et al., Appl. and Environ. Microbiol., 60: 1401-1413 (1994)). Therefore, within the context of the present disclosure, it will be appreciated by one skilled in the art that viable host cells containing a DNA sequence or vector of the invention, and expressing a polypeptide or fusion protein of the invention, can be used directly as the delivery vehicle for a griffithsin or fusion protein thereof to the desired site(s) in vivo. Preferred host cells for such delivery of griffithsins or fusion proteins thereof directly to desired site(s), such as, for example, to a selected body cavity, can comprise bacteria or yeast. More specifically, such host cells can comprise suitably engineered strain(s) of lactobacilli, enterococci, or other common bacteria, such as E. coli, normal strains of which are known to commonly populate body cavities. More specifically yet, such host cells can comprise one or more selected nonpathogenic strains of lactobacilli, such as those described by Andreu et al. ((1995), supra), especially those having high adherence properties to epithelial cells, such as, for example, adherence to vaginal epithelial cells, and suitably transformed using the DNA sequences of the present invention.

[0088] As reviewed by McGroarty (FEMS Immunol. Med. Microbiol., 6: 251-264 (1993)) the "probiotic" or direct therapeutic application of live bacteria, particularly bacteria that occur normally in nature, more particularly lactobacilli, for treatment or prophylaxis against pathogenic bacterial or yeast infections of the urogenital tract, in particular the female urogenital tract, is a

well-established concept. Recently, the use of a conventional probiotic strategy, in particular the use of live lactobacilli, to inhibit sexual transmission of HIV has been suggested, based specifically upon the normal, endogenous production of virucidal levels of H2O2 and/or lactic acid and/or other potentially virucidal substances by certain normal strains of lactobacilli (e.g., Hilier (1996), supra). However, the inventive use of non-mammalian cells, particularly bacteria, more particularly lactobacilli, specifically engineered with a foreign gene, more specifically a griffithsin gene, to express an anti-viral substance, more specifically a protein, and even more specifically a griffithsin, is heretofore unprecedented as a method of treatment of an animal, specifically a human, to prevent infection by a virus, specifically a retrovirus, more specifically HIV-1 or HIV-2.

[0089] Elmer et al. (JAMA, 275: 870-876 (1996)) have recently speculated that "genetic engineering offers the possibility of using microbes to deliver specific actions or products to the colon or other mucosal surfaces . . . other fertile areas for future study include defining the mechanisms of action of various biotherapeutic agents with the possibility of applying genetic engineering to enhance activities." Elmer et al. ((1996), supra) further point out that the terms "probiotic" and "biotherapeutic agent" have been used in the literature to describe microorganisms that have antagonistic activity toward pathogens in vivo; those authors more specifically prefer the term "biotherapeutic agent" to denote "microorganisms having specific therapeutic properties."

[0090] In view of the present disclosure, one skilled in the art will appreciate that the invention teaches an entirely novel type of "probiotic" or "biotherapeutic" treatment using specifically engineered strains of microorganisms provided herein which do not occur in nature. Nonetheless, available teachings concerning selection of optimal microbial strains, in particular bacterial strains, for conventional probiotic or biotherapeutic applications can be employed in the context of the invention. For example, selection of optimal lactobacillus strains for genetic engineering, transformation, direct expression of griffithsins or conjugates thereof, and direct probiotic or biotherapeutic applications, to treat or prevent viral (e.g., HIV) infection, can be based upon the same or similar criteria, such as those described by Elmer et al. ((1996), supra), typically used to select normal, endogenous or "nonengineered" bacterial strains for conventional probiotic or biotherapeutic therapy. Furthermore, the recommendations and characteristics taught by McGroarty, particularly for selection of optimal lactobacillus strains for conventional probiotic use against female urogenital infections, are pertinent to the present invention: " . . . lactobacilli chosen for incorporation into probiotic preparations should be easy and, if possible, inexpensive to cultivate . . . strains should be stable, retain viability following freeze-drying and, of course, be non-pathogenic to the host . . . it is essential that lactobacilli chosen for use in probiotic preparations should adhere well to the vaginal epithelium . . . ideally, artificially implanted lactobacilli should adhere to the vaginal epithelium, integrate with the indigenous microorganisms present, and proliferate" (McGroarty (1993), supra). While McGroarty's teachings specifically address selections of "normal" lactobacillus strains for probiotic uses against pathogenic bacterial or yeast infections of the female urogenital tract, similar considerations will apply to the selection of optimal bacterial strains for genetic engineering and "probiotic" or "biotherapeutic" application against viral infections as particularly encompassed by the present invention.

[0091] Accordingly, the method of the invention for the prevention of sexual transmission of viral infection, e.g., HIV infection, comprises vaginal, rectal, oral, penile, or other topical, insertional, or instillational treatment with an anti-viral effective amount of a griffithsin, a griffithsin conjugate or fusion protein, a matrix-anchored griffithsin or conjugate or fusion protein thereof, and/or viable host cells transformed to express a griffithsin or conjugate or fusion protein thereof, alone or in combination with one or more other anti-viral compound (e.g., as described above). However, commensal organisms which produce griffithsin or a fragment, homolog, or conjugate thereof can inhibit viruses other than HIV. For example, commensal microorganisms that produce the inventive polypeptide can be instilled in mucosal tissue at the site of influenza contact, such as nasal or oral mucosa, to inhibit influenza infection of a host.

[0092] Compositions for use in the prophylactic or therapeutic treatment methods of the invention comprise one or more griffithsin(s) or conjugate(s) or fusion protein(s) thereof, either one of which can be matrix-anchored, and desirably a carrier therefor, such as a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well-known to those who are skilled in the art, as are suitable methods of administration. The choice of carrier will be determined in part by the

particular griffithsin or conjugate or fusion protein thereof, as well as by the particular method used to administer the composition.

[0093] One skilled in the art will appreciate that various routes of administering a drug are available, and, although more than one route can be used to administer a particular drug, a particular route can provide a more immediate and more effective reaction than another route. For example, the anti-viral agent of the invention can be inhaled in methods of prophylactically treating a subject for influenza infection. Delivery of the anti-viral agent to a location of initial viral contact, such as the nose or mouth, blocks the onset of infection. The anti-viral agent can be administered via subcutaneous injection. Alternatively, in acute or critical medical situations, the anti-viral agent can be administered intravenously. In many cases of infection, a patient generates an immune response to a virus. However, the effects of the viral infection so severely compromise the health of the patient that an effective immune response is not reached prior to death. Administration of the anti-viral agent can prolong the life of the patient until a patient's natural immune defense clears the virus. Furthermore, one skilled in the art will appreciate that the particular pharmaceutical carrier employed will depend, in part, upon the particular griffithsin or conjugate or fusion protein thereof employed, and the chosen route of administration. Accordingly, there is a wide variety of suitable formulations of the composition of the invention.

[0094] Formulations suitable for oral administration can consist of liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or fruit juice; capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solid, granules or freeze-dried cells; solutions or suspensions in an aqueous liquid; and oil-in-water emulsions or water-in-oil emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Suitable formulations for oral delivery can also be incorporated into synthetic and natural polymeric microspheres, or other means to protect the agents of the present invention from degradation within the gastrointestinal tract (see, for example, Wallace et al., Science, 260: 912-915 (1993)).

[0095] The anti-viral agent of the invention (e.g., griffithsin or conjugates thereof), alone or in combination with other anti-viral compounds, can be made into aerosol formulations or microparticulate powder formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0096] The anti-viral agent of the invention (e.g., griffithsin or conjugates thereof), alone or in combinations with other anti-viral compounds or absorption modulators, can be made into suitable formulations for transdermal application and absorption, such as a patch (Wallace et al. (1993), supra). Transdermal electroporation or iontophoresis also can be used to promote and/or control the systemic delivery of the compounds and/or compositions of the present invention through the skin (e.g., see Theiss et al., Meth. Find. Exp. Clin. Pharmacol., 13: 353-359 (1991)).

[0097] Formulations suitable for topical administration include lozenges comprising the active ingredient in a flavor, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier; as well as creams, emulsions, gels and the like containing, in addition to the active ingredient, such as, for example, freeze-dried lactobacilli or live lactobacillus cultures genetically engineered to directly produce a griffithsin or conjugate or fusion protein thereof of the present invention, such carriers as are known in the art. Topical administration is preferred for the prophylactic and therapeutic treatment of influenza viral infection, such as through the use of an inhaler, for example.

[0098] Formulations for rectal administration can be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate. Formulations suitable for vaginal administration can be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such as, for example, freeze-dried

lactobacilli or live lactobacillus cultures genetically engineered to directly produce a griffithsin or conjugate or fusion protein thereof of the present invention, such carriers as are known in the art to be appropriate. Similarly, the active ingredient can be combined with a lubricant as a coating on a condom. Indeed, preferably, the active ingredient is applied to any contraceptive device, including, but not limited to, a condom, a diaphragm, a cervical cap, a vaginal ring, and a sponge.

[0099] Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

[0100] Formulations comprising a griffithsin or griffithsin conjugate suitable for virucidal (e.g., HIV) sterilization of inanimate objects, such as medical supplies or equipment, laboratory equipment and supplies, instruments, devices, and the like, can, for example, be selected or adapted as appropriate, by one skilled in the art, from any of the aforementioned compositions or formulations. Preferably, the griffithsin is produced by recombinant DNA technology. The griffithsin conjugate can be produced by recombinant DNA technology or by chemical coupling of a griffithsin with an effector molecule as described above. Similarly, formulations suitable for ex vivo sterilization, inactivation, or removal of virus, such as infectious virus, from a sample, such as blood, blood products, sperm, or other bodily products, such as a fluid, cells, a tissue or an organ, or any other solution, suspension, emulsion, vaccine formulation (such as in the removal of infectious virus), or any other material which can be administered to a patient in a medical procedure, can be selected or adapted as appropriate by one skilled in the art, from any of the aforementioned compositions or formulations. However, suitable formulations for ex vivo sterilization or inactivation or removal of virus from a sample or on an inanimate object are by no means limited to any of the aforementioned formulations or compositions. For example, such formulations or compositions can comprise a functional griffithsin, such as that which is encoded by SEQ ID NO: 3, or anti-viral fragment thereof, such as a fragment comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids bind to a virus, or a conjugate or fusion protein of either of the foregoing, attached to a solid support matrix, to facilitate contacting or binding infectious virus in a sample or removing infectious virus from a sample as described above, e.g., a bodily product such as a fluid, cells, a tissue or an organ from an organism, in particular a mammal, such as a human, including, for example, blood, a component of blood (e.g., plasma, blood cells, and the like), or sperm. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3. Also preferably, the at least eight contiguous amino acids bind gp120 of HIV, in particular infectious HIV. As a more specific example, such a formulation or composition can comprise a functional griffithsin, or conjugate or fusion protein thereof, attached to (e.g., coupled to or immobilized on) a solid support matrix comprising magnetic beads, to facilitate contacting, binding and removal of infectious virus, and to enable magnet-assisted removal of the virus from a sample as described above, e.g., a bodily product such as a fluid, cells, a tissue or an organ, e.g., blood, a component of blood, or sperm. Alternatively, and also preferably, the solid support matrix comprises a contraceptive device, such as a condom, a diaphragm, a cervical cap, a vaginal ring, or a sponge. The anti-viral agent also can be encapsulated or dispersed within a solid matrix, such as a vaginal ring or sponge. Methods for encapsulating biotherapeutics into, for example, biocompatible sustained release devices, are known in the art.

[0101] As an even more specific illustration, such a composition (e.g., for ex vivo) can comprise a functional (e.g., gp120-binding, HIV-inactivating) griffithsin, or conjugate or fusion protein thereof, attached to a solid support matrix, such as magnetic beads or a flow-through matrix, by means of an anti-griffithsin antibody or at least one effector component, which can be the same or different, such as polyethylene glycol, albumin, or dextran. The conjugate can further comprise at least one effector component, which can be the same or different, selected from the group consisting of, for example, an immunological reagent and a toxin. A flow-through matrix would comprise, for instance, a configuration similar to an affinity column. The griffithsin can be covalently coupled to

a solid support matrix via an anti-griffithsin antibody, described below. Methods of attaching an antibody to a solid support matrix are well-known in the art (see, for example, Harlow and Lane. Antibodies: A Laboratory Manual, Cold Springs Harbor Laboratory: Cold Spring Harbor, N.Y. (1988)). Alternatively, the solid support matrix, such as magnetic beads, can be coated with streptavidin, in which case the griffithsin or fragment thereof (which comprises at least eight contiguous amino acids of SEQ ID NO: 3 or SEQ ID NO: 2), or a conjugate or fusion protein of either one, is biotinylated. The at least eight contiguous amino acids of SEQ ID NO: 2 desirably have anti-viral activity and preferably bind gp120 of HIV, which preferably is infectious. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3 or SEQ ID NO: 2. Such a composition can be prepared, for example, by biotinylating the griffithsin, or conjugate or fusion protein thereof, and then contacting the biotinylated protein or peptide with a (commercially available) solid support matrix, such as magnetic beads, coated with streptavidin. The use of biotinylation as a means to attach a desired biologically active protein or peptide to a streptavidin-coated support matrix, such as magnetic beads, is well-known in the art.

[0102] One skilled in the art will appreciate that a suitable or appropriate formulation can be selected, adapted or developed based upon the particular application at hand.

[0103] For ex vivo uses, such as virucidal treatments of inanimate objects or materials, blood or blood products, or tissues, the amount of griffithsin, conjugate thereof, fusion protein thereof, or composition of any of the foregoing, to be employed should be sufficient that any virus or virus-producing cells present will be rendered noninfectious or will be destroyed. For example, for HIV, this would require that the virus and/or the virus-producing cells be exposed to concentrations of griffithsin in the range of 0.1-1000 nM. Similar considerations apply to in vivo applications. Therefore, the designation of "anti-viral effective amount" is used generally to describe the amount of a particular griffithsin, conjugate, fusion protein, or composition thereof required for anti-viral efficacy in any given application.

[0104] In view of the above, the invention also provides a method of inhibiting prophylactically or therapeutically a viral infection of a host in which an anti-viral effective amount of an above-described anti-viral polypeptide, conjugate, or fusion protein is administered to the host. Upon administration of the anti-viral effective amount of the anti-viral polypeptide, conjugate, or fusion protein, the viral infection is inhibited.

[0105] The invention additionally provides a method of prophylactically or therapeutically inhibiting a viral infection of a host in which an anti-viral effective amount of a composition comprising an isolated and purified anti-viral polypeptide, or anti-viral polypeptide conjugate or fusion protein, either one of which comprises at least eight contiguous amino acids of SEQ ID NO: 3 having anti-viral activity, attached to or encapsulated within a solid support matrix is administered to the host. By "therapeutically" is meant that the host already has been infected with the virus. By "prophylactically" is meant that the host has not yet been infected with the virus but is at risk of being infected with the virus. Prophylactic treatment is intended to encompass any degree of inhibition of viral infection, including, but not limited to, complete inhibition, as one of ordinary skill in the art will readily appreciate that any degree in inhibition of viral infection is advantageous. Preferably, the inventive active agent is administered before viral infection or immediately upon determination of viral infection and is continuously administered until the virus is undetectable. The method optionally further comprises the prior, simultaneous or subsequent administration, by the same route or a different route, of an antiviral agent or another agent that is efficacious in inhibiting the viral infection. Upon administration of the anti-viral effective amount of the composition, the viral infection is inhibited. Preferably, the solid support matrix is a contraceptive device, such as a condom, diaphragm, cervical cap, vaginal ring, or sponge. In an alternative embodiment, a solid support matrix can be surgically implanted and later removed.

[0106] For in vivo uses, the dose of a griffithsin, or conjugate or composition thereof, administered to an animal, particularly a human, in the context of the invention should be sufficient to effect a prophylactic or therapeutic response in the individual over a reasonable time frame. The dose used to achieve a desired anti-viral concentration in vivo (e.g., 0.1-1000 nM) will be determined by the potency of the particular griffithsin or conjugate employed, the severity of the disease state of infected individuals, as well as, in the case of systemic administration, the body weight and age of

the infected individual. The size of the dose also will be determined by the existence of any adverse side effects that may accompany the particular griffithsin, or conjugate or composition thereof, employed. It is always desirable, whenever possible, to keep adverse side effects to a minimum.

[0107] The invention also provides a method of removing virus, such as infectious virus, from a sample. The method comprises contacting the sample with a composition comprising an isolated and purified anti-viral polypeptide or conjugate or fusion protein thereof, comprising at least eight contiguous amino acids of SEQ ID NO: 3 (or SEQ ID NO: 2). The at least eight contiguous amino acids desirably have anti-viral activity and bind to the virus and the anti-viral polypeptide (or conjugate or fusion protein of either of the foregoing) is attached to a solid support matrix, such as a magnetic bead. "Attached" is used herein to refer to attachment to (or coupling to) and immobilization in or on a solid support matrix. While any means of attachment can be used, preferably, attachment is by covalent bonds. The method further comprises separating the sample and the composition by any suitable means, whereupon the virus, such as infectious virus, is removed from the sample. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3 (or SEQ ID NO: 2). In one embodiment, the anti-viral polypeptide is conjugated with an anti-griffithsin antibody or at least one effector component, which can be the same or different, selected from polyethylene glycol, dextran and albumin, in which case the anti-viral polypeptide is desirably attached to the solid support matrix through at least one effector component. The anti-viral polypeptide can be further conjugated with at least one effector component, which can be the same or different, selected from the group consisting of an immunological reagent and a toxin. In another embodiment, the solid support matrix is coated with streptavidin and the anti-viral polypeptide is biotinylated. Through biotin, the biotinylated anti-viral polypeptide is attached to the streptavidin-coated solid support matrix. Other types of means, as are known in the art, can be used to attach a functional griffithsin (i.e., an anti-viral polypeptide or conjugate as described above) to a solid support matrix, such as a magnetic bead, in which case contact with a magnet is used to separate the sample and the composition. Similarly, other types of solid support matrices can be used, such as a matrix comprising a porous surface or membrane, over or through which a sample is flowed or percolated, thereby selectively entrapping or removing infectious virus from the sample. The choice of solid support matrix, means of attachment of the functional griffithsin to the solid support matrix, and means of separating the sample and the matrix-anchored griffithsin will depend, in part, on the sample (e.g., fluid vs. tissue) and the virus to be removed. It is expected that the use of a selected coupling molecule can confer certain desired properties to a matrix, comprising a functional griffithsin coupled therewith, that may have particularly advantageous properties in a given situation. Preferably, the sample is blood, a component of blood, sperm, cells, tissue or an organ. Also, preferably the sample is a vaccine formulation, in which case the virus that is removed is infectious, such as HIV, although HIV, in particular infectious HIV, can be removed from other samples in accordance with this method.

[0108] For instance, the skilled practitioner might select a poly(ethylene glycol) molecule for attaching a functional griffithsin to a solid support matrix, thereby to provide a matrix-anchored griffithsin, wherein the griffithsin is attached to the matrix by a longer "tether" than would be feasible or possible for other attachment methods, such as biotinylation/streptavidin coupling. A griffithsin coupled by a poly(ethylene glycol) "tether" to a solid support matrix (such as magnetic beads, porous surface or membrane, and the like) can permit optimal exposure of a binding surface, epitope, hydrophobic or electrophilic focus, and/or the like, on a functional griffithsin in a manner that, in a given situation and/or for a particular virus, facilitates the binding and/or inactivation of the virus. A preferred solid support matrix is a magnetic bead such that separation of the sample and the composition is effected by a magnet. In a preferred embodiment of the method, the at least eight contiguous amino acids bind gp120 of HIV and HIV is removed from the sample.

[0109] Similarly, other types of solid support matrices can be used, such as a matrix comprising a porous surface or membrane, over or through which a sample is flowed or percolated, thereby selectively inhibiting infectious virus (e.g., HIV or influenza) in the sample. The choice of solid support matrix, means of attachment of the functional griffithsin to the solid support matrix, and means of separating the sample and the matrix-anchored griffithsin will depend, in part, on the sample (e.g., fluid vs. tissue) and the virus to be inhibited. It is expected that the use of a selected coupling molecule can confer certain desired properties to a matrix, comprising a functional

griffithsin coupled therewith, that may have particularly advantageous properties in a given situation.

[0110] The methods described herein also have utility in real time ex vivo inhibition of virus or virus infected cells in a bodily fluid, such as blood, e.g., in the treatment of viral infection, or in the inhibition of virus in blood or a component of blood, e.g., for transfusion, in the inhibition or prevention of viral infection. Such methods also have potential utility in dialysis, such as kidney dialysis, and in inhibiting virus in sperm obtained from a donor for in vitro and in vivo fertilization. The methods also have applicability in the context of tissue and organ transplantations.

[0111] In summary, a griffithsin attached to a solid support matrix, such as a magnetic bead, can be used to remove virus, in particular infectious virus, including immunodeficiency virus, such as HIV, e.g., HIV-1 or HIV-2, from a sample, such as a sample comprising both infectious and noninfectious virus. The inventive method also can be used to remove viral glycoprotein presenting cells, e.g., infected cells that have, for example, gp120 on their surfaces, from a sample.

[0112] The invention, therefore, further provides a composition comprising naturally-occurring, non-infectious virus, such as a composition produced as described above. The composition can further comprise a carrier, such as a biologically or pharmaceutically acceptable carrier, and an immuno-adjuvant. Preferably, the noninfectious virus is an influenza or an immunodeficiency virus, such as HIV, e.g., HIV-1 or HIV-2. Alternatively, and also preferably, the noninfectious virus is FIV. A composition comprising only naturally-occurring, non-infectious virus has many applications in research and the prophylactic treatment of a viral infection. In terms of prophylactic treatment of a viral infection, the skilled artisan will appreciate the need to eliminate completely all infectious virus from the composition. If desired, further treatment of the composition comprising non-infectious particles with virus-inactivating chemicals, such as imines or psoralens, and/or pressure or heat inactivation, will further the non-infectious nature of the composition. For example, an immune response-inducing amount of the inventive composition can be administered to an animal at risk for a viral infection in order to induce an immune response. The skilled artisan will appreciate that such a composition is a significant improvement over previously disclosed compositions in that the virus is non-infectious and naturally-occurring. Thus, there is no risk of inadvertent infection, greater doses can be administered in comparison to compositions comprising infectious viral particles, and the subsequent immune response will assuredly be directed to antigens present on naturally-occurring virus. The composition comprising naturally-occurring, non-infectious virus can be administered in any manner appropriate to induce an immune response. Preferably, the virus is administered, for example, intramuscularly, mucosally, intravenously, subcutaneously, or topically. Preferably, the composition comprises naturally-occurring, non-infectious human immunodeficiency virus comprising gp120.

[0113] The composition comprising naturally-occurring, non-infectious virus can be combined with various carriers, adjuvants, diluents or other anti-viral therapeutics, if desired. Appropriate carriers include, for example, ovalbumin, albumin, globulins, hemocyanins, and the like. Adjuvants or immuno-adjuvants are incorporated in most cases to stimulate further the immune system. Any physiologically appropriate adjuvant can be used. Suitable adjuvants for inclusion in the inventive composition include, for example, aluminum hydroxide, beryllium sulfate, silica, kaolin, carbon, bacterial endotoxin, saponin, and the like.

[0114] Thus, the invention also provides a method of inducing an immune response to a virus in an animal. The method comprises administering to the animal an immune response-inducing amount of a composition comprising naturally-occurring, non-infectious virus as described above.

[0115] The appropriate dose of a composition comprising naturally-occurring, non-infectious virus required to induce an immune response to the virus in an animal is dependent on numerous factors, such as size of the animal and immune competency. The amount of composition administered should be sufficient to induce a humoral and/or cellular immune response. The amount of non-infectious virus in a particular composition can be determined using routine methods in the art, such as the Coulter HIV p24 antigen assay (Coulter Corp., Hialeah, Fla.). Any suitable dose of a composition comprising non-infectious virus is appropriate so long as an immune response is

induced, desirably without the appearance of harmful side effects to the host. In this regard, compositions comprising from about 10<1 >to about 10<5 >particles, preferably from about 10<2 >to about 10<4 >particles, most preferably about 10<3 >particles, are suitable for inducing an immune response.

[0116] One of ordinary skill can determine the effectiveness of the composition to induce an immune response using routine methods known in the art. Cell-mediated response can be determined by employing, for example, a virus antigen-stimulated T-cell proliferation assay. The presence of a humoral immune response can be determined, for instance, with the Enzyme Linked Immunosorbent Assay (ELISA). The skilled artisan will appreciate that there are numerous other suitable assays for evaluating induction of an immune response. To the extent that a dose is inadequate to induce an appropriate immune response, "booster" administrations can subsequently be administered in order to prompt a more effective immune response.

[0117] In terms of administration of the inventive anti-viral agents or conjugates thereof, the dosage can be in unit dosage form, such as a tablet or capsule. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a griffithsin or conjugate thereof, alone or in combination with other anti-viral agents, calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier, or vehicle.

[0118] The specifications for the unit dosage forms of the invention depend on the particular griffithsin, or conjugate or composition thereof, employed and the effect to be achieved, as well as the pharmacodynamics associated with each griffithsin, or conjugate or composition thereof, in the host. The dose administered should be an "anti-viral effective amount" or an amount necessary to achieve an "effective level" in the individual patient.

[0119] Since the "effective level" is used as the preferred endpoint for dosing, the actual dose and schedule can vary, depending upon interindividual differences in pharmacokinetics, drug distribution, and metabolism. The "effective level" can be defined, for example, as the blood or tissue level (e.g., 0.1-1000 nM) desired in the patient that corresponds to a concentration of one or more griffithsin or conjugate thereof, which inhibits a virus, such as HIV, in an assay known to predict for clinical anti-viral activity of chemical compounds and biological agents. The "effective level" for agents of the invention also can vary when the griffithsin, or conjugate or composition thereof, is used in combination with AZT or other known anti-viral compounds or combinations thereof.

[0120] One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired "effective concentration" in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the "effective concentration" of the compounds of the invention by a direct (e.g., analytical chemical analysis) or indirect (e.g., with surrogate indicators such as p24 or RT) analysis of appropriate patient samples (e.g., blood and/or tissues).

[0121] In the treatment of some virally infected individuals, it can be desirable to utilize a "mega-dosing" regimen, wherein a large dose of the griffithsin or conjugate thereof is administered, time is allowed for the drug to act, and then a suitable reagent is administered to the individual to inactivate the drug.

[0122] The pharmaceutical composition can contain other pharmaceuticals, in conjunction with the griffithsin or conjugate thereof, when used to therapeutically treat a viral infection, such as an influenza infection or an HIV infection which results in AIDS. Representative examples of these additional pharmaceuticals include anti-viral compounds, virucides, immunomodulators, immunostimulants, antibiotics and absorption enhancers. Exemplary anti-viral compounds include cyanovirin, AZT, ddI, ddC, gancylclovir, fluorinated dideoxynucleosides, nonnucleoside analog compounds, such as nevirapine (Shih et al., PNAS, 88: 9878-9882 (1991)), TIBO derivatives, such as R82913 (White et al., Anti-viral Res., 16: 257-266 (1991)), BI-RJ-70 (Merigan, Am. J. Med., 90 (Suppl. 4A): 8S-17S (1991)), michellamines (Boyd et al., J. Med. Chem., 37: 1740-1745 (1994)) and calanolides Kashman et al., J. Med. Chem., 35: 2735-2743 (1992)), nonoxynol-9, gossypol and

derivatives, gramicidin (Bourinbair et al. (1994), supra), neuraminidase inhibitors, amantadine, enfurtide, and the like. Exemplary immunomodulators and immunostimulants include various interleukins, sCD4, cytokines, antibody preparations, blood transfusions, and cell transfusions. Exemplary antibiotics include antifungal agents, antibacterial agents, and anti-Pneutnoeystitis carnii agents. Exemplary absorption enhancers include bile salts and other surfactants, saponins, cyclodextrins, and phospholipids (Davis (1992), supra).

[0123] Administration of a griffithsin or conjugate or fusion protein thereof with other anti-retroviral agents and particularly with known RT inhibitors, such as ddC, AZT, ddI, ddA, or other inhibitors that act against other HIV proteins, such as anti-TAT agents, is expected to inhibit most or all replicative stages of the viral life cycle. The dosages of ddC and AZT used in AIDS or ARC patients have been published. A virustatic range of ddC is generally between 0.05 [mu]M to 1.0 [mu]M. A range of about 0.005-0.25 mg/kg body weight is virustatic in most patients. The preliminary dose ranges for oral administration are somewhat broader, for example 0.001 to 0.25 mg/kg given in one or more doses at intervals of 2, 4, 6, 8, 12, etc. hours. Currently, 0.01 mg/kg body weight ddC given every 8 hrs is preferred. When given in combined therapy, the other anti-viral compound, for example, can be given at the same time as the griffithsin or conjugate thereof or the dosing can be staggered as desired. The two drugs also can be combined in a composition. Doses of each can be less when used in combination than when either is used alone.

[0124] It will also be appreciated by one skilled in the art that a DNA sequence of a griffithsin or conjugate thereof of the invention can be inserted ex vivo into mammalian cells previously removed from a given animal, in particular a human, host. Such cells can be employed to express the corresponding griffithsin or conjugate or fusion protein in vivo after reintroduction into the host. Feasibility of such a therapeutic strategy to deliver a therapeutic amount of an agent in close proximity to the desired target cells and pathogens, i.e., virus, more particularly retrovirus, specifically HIV and its envelope glycoprotein gp120, has been demonstrated in studies with cells engineered ex vivo to express sCD4 (Morgan et al. (1994), supra). It is also possible that, as an alternative to ex vivo insertion of the DNA sequences of the invention, such sequences can be inserted into cells directly in vivo, such as by use of an appropriate viral vector. Such cells transfected in vivo are expected to produce anti-viral amounts of griffithsin or a conjugate or fusion protein thereof directly in vivo.

[0125] Given the present disclosure, it will be additionally appreciated that a DNA sequence corresponding to a griffithsin or conjugate thereof can be inserted into suitable nonmammalian host cells, and that such host cells will express therapeutic or prophylactic amounts of a griffithsin or conjugate or fusion protein thereof directly in vivo within a desired body compartment of an animal, in particular a human. Example 5 illustrates the transformation and expression of effective virucidal amounts of a griffithsin in a non-mammalian cell, more specifically a bacterial cell. In a preferred embodiment of the invention, a method of female-controllable prophylaxis against HIV infection comprises the intravaginal administration and/or establishment of, in a female human, a persistent intravaginal population of lactobacilli that have been transformed with a coding sequence of the invention to produce, over a prolonged time, effective virucidal levels of a griffithsin or conjugate thereof, directly on or within the vaginal and/or cervical and/or uterine mucosa. It is noteworthy that both the World Health Organization (WHO), as well as the U.S. National Institute of Allergy and Infectious Diseases, have pointed to the need for development of female-controlled topical microbicides, suitable for blocking the transmission of HIV, as an urgent global priority (Lange et al., Lancet, 341: 1356 (1993); Fauci, NIAID News, Apr. 27, 1995). A composition comprising the inventive anti-viral agent and a solid-support matrix is particularly useful in this regard, particularly when the solid-support matrix is a contraceptive device, such as a condom, a diaphragm, a cervical cap, a vaginal ring, or a sponge. In another embodiment, a colony of commensal organisms transduced with the nucleic acid of the invention and producing the inventive anti-viral agent is applied to mucosal tissue associated with the onset of influenza infection, such as respiratory or oral mucosal.

[0126] The invention also provides antibodies directed to the polypeptides of the invention. The availability of antibodies to any given protein is highly advantageous, as it provides the basis for a wide variety of qualitative and quantitative analytical methods, separation and purification methods, and other useful applications directed to the subject polypeptides. Accordingly, given the

present disclosure and the polypeptides of the invention, it will be readily apparent to one skilled in the art that antibodies, in particular antibodies specifically binding to a polypeptide of the invention, can be prepared using well-established methodologies (e.g., such as the methodologies described in detail by Harlow and Lane, in Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (1988), pp. 1-725). Such antibodies can comprise both polyclonal and monoclonal antibodies. Furthermore, such antibodies can be obtained and employed either in solution-phase or coupled to a desired solid-phase matrix, such as magnetic beads or a flow through matrix. Having in hand such antibodies as provided by the invention, one skilled in the art will further appreciate that such antibodies, in conjunction with well-established procedures (e.g., such as described by Harlow and Lane (1988), supra) comprise useful methods for the detection, quantification, or purification of a griffithsin, conjugate thereof, or host cell transformed to produce a griffithsin or conjugate or fusion protein thereof. Example 6 further illustrates an antibody that specifically binds to a griffithsin. Accordingly, the invention further provides a composition comprising an anti-griffithsin antibody bound to the anti-viral agent of the invention, preferably an anti-viral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3.

[0127] Matrix-anchored anti-griffithsin antibodies also can be used in a method to remove virus in a sample. Preferably, the antibody binds to an epitope of an anti-viral polypeptide of SEQ ID NO: 2 or SEQ ID NO: 3. Preferably, the matrix is a solid support matrix, such as a magnetic bead or a flow-through matrix. If the solid support matrix to which the anti-griffithsin antibody is attached comprises magnetic beads, removal of the antibody-griffithsin-virus complex can be readily accomplished using a magnet.

[0128] In view of the above, the invention provides a method of removing virus from a sample. The method comprises (a) contacting the sample with a composition comprising an isolated and purified anti-viral polypeptide or conjugate or fusion protein thereof, wherein (i) the anti-viral polypeptide comprises at least eight contiguous amino acids of SEQ ID NO: 3, and (ii) the at least eight contiguous amino acids bind to the virus, and (b) contacting the sample with an anti-griffithsin antibody attached to a solid support matrix, whereupon the anti-griffithsin antibody binds to the anti-viral polypeptide or conjugate or fusion protein thereof to which is bound the virus, and (c) separating the solid support matrix from the sample, whereupon the virus is removed from the sample. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3. Desirably, the virus that is removed is infectious, such as HIV. The sample can be blood, a component of blood, sperm, cells, tissue or an organ.

[0129] The antibody for use in the aforementioned method is an antibody that binds to a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, and, which polypeptide can bind to and inactivate a virus. The antibody can be coupled to the solid support matrix using similar methods and with similar considerations as described above for attaching a griffithsin to a solid support matrix. For example, coupling methods and molecules employed to attach an anti-griffithsin antibody to a solid support matrix, such as magnetic beads or a flow-through matrix, can employ biotin/streptavidin coupling or coupling through molecules, such as polyethylene glycol, albumin or dextran. Also analogously, it can be shown that, after such coupling, the matrix-anchored anti-griffithsin antibody retains its ability to bind to a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, which polypeptide can bind to and inactivate a virus.

[0130] The invention also provides an anti-griffithsin antibody that is anti-idiotypic in respect to a viral glycoprotein, such as gp120, i.e., has an internal image of gp120 of a primate immunodeficiency virus. Preferably, the antibody can compete with gp120 of a primate immunodeficiency virus for binding to a griffithsin. In this regard, the primary immunodeficiency virus preferably is HIV-1 or HIV-2 and the griffithsin preferably consists essentially of SEQ ID NO: 2 or SEQ ID NO: 3. Anti-idiotypic antibodies can be generated in accordance with methods known in the art (see, for example, Benjamin, in Immunology: a short course, Wiley-Liss, N Y (1996), pp. 436-437; Kuby, in Immunology, 3rd ed., Freeman, N.Y. (1997), pp. 455-456; Greenspan et al., FASEB J., 7: 437-443 (1993); and Poskitt, Vaccine, 9: 792-796 (1991)). Such an anti-idiotypic (in respect to gp120) anti-griffithsin antibody is useful in a method of inhibiting infection of an animal with a virus as provided herein.

[0131] In view of the above, a griffithsin can be administered to an animal, the animal generates anti-griffithsin antibodies, among which are antibodies that have an internal image of a viral glycoprotein, such as gp120. In accordance with well-known methods, polyclonal or monoclonal antibodies can be obtained, isolated, and selected. Selection of an anti-griffithsin antibody that has an internal image of gp120 can be based upon competition between the anti-griffithsin antibody and gp120 for binding to a griffithsin, or upon the ability of the anti-griffithsin antibody to bind to a free griffithsin as opposed to a griffithsin bound to gp120. Such an anti-griffithsin antibody can be administered to an animal to inhibit a viral infection in accordance with methods provided herein. Although nonhuman anti-idiotypic antibodies, such as an anti-griffithsin antibody that has an internal image of gp120 and, therefore, is anti-idiotypic to gp120, are proving useful as vaccine antigens in humans, their favorable properties might, in certain instances, be further enhanced and/or their adverse properties further diminished, through "humanization" strategies, such as those recently reviewed by Vaughan (Nature Biotech., 16: 535-539 (1998)). Alternatively, a griffithsin can be directly administered to an animal to inhibit a viral infection in accordance with methods provided herein such that the treated animal, itself, generates an anti-griffithsin antibody that has an internal image of gp120. The production of anti-idiotypic antibodies, such as anti-griffithsin antibody that has an internal image of gp120 and, therefore, is anti-idiotypic to gp120, in an animal to be treated is known as "anti-idiotype induction therapy," and is described by Madiyalakan et al. (Hybridoma, 14: 199-203 (1995)), for example.

[0132] In view of the above, the invention enables another method of inhibiting infection of an animal, such as a mammal, in particular a human, with a virus. The method comprises administering to the animal an anti-griffithsin antibody, or a composition comprising same, in an amount sufficient to induce in the animal an immune response to the virus, whereupon the infection of the animal with the virus is inhibited. Preferably, the anti-griffithsin antibody has an internal image of a viral glycoprotein, such as gp120 of an immunodeficiency virus with which the animal can be infected, such as a primate immunodeficiency virus. Preferably, the antibody can compete with, for example, gp120 of a primate immunodeficiency virus for binding to a griffithsin. In this regard, the primate immunodeficiency virus preferably is HIV-1 or HIV-2 and the griffithsin preferably consists essentially of SEQ ID NO: 3 or SEQ ID NO: 2. The method can further comprise the administration of an immunostimulant.

[0133] Also enabled by the invention is yet another method of inhibiting infection of an animal, such as a mammal, in particular a human, with a virus. The method comprises administering to the animal a griffithsin, which binds a viral glycoprotein, such as gp120 of an immunodeficiency virus with which the animal can be infected, in an amount sufficient to induce in the animal an anti-griffithsin antibody in an amount sufficient to induce an immune response to a virus sufficient to inhibit infection of the animal with the virus. Preferably, the anti-griffithsin antibody has an internal image of gp120 of an immunodeficiency virus with which the animal can be infected, such as a primate immunodeficiency virus. Preferably, the antibody can compete with gp120 of a primate immunodeficiency virus for binding to a griffithsin. In this regard, the primate immunodeficiency virus preferably is HIV-1 or HIV-2 and the griffithsin preferably consists essentially of SEQ ID NO: 2 or SEQ ID NO: 3.

[0134] With respect to the above methods, sufficient amounts can be determined in accordance with methods known in the art. Similarly, the sufficiency of an immune response in the inhibition of a viral infection in an animal also can be assessed in accordance with methods known in the art.

[0135] Either one of the above methods can further comprise concurrent, pre- or post-treatment with an adjuvant to enhance the immune response, such as the prior, simultaneous or subsequent administration, by the same or a different route, of an antiviral agent or another agent that is efficacious in inducing an immune response to the virus, such as an immunostimulant. See, for example, Harlow et al. (1988), supra.

[0136] The inventive griffithsins, conjugates, host cells, antibodies, compositions and methods are further described in the context of the following examples. These examples serve to illustrate further the present invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

[0137] This example illustrates a method of isolating and purifying griffithsin from Griffithsin sp. and elucidating the griffithsin amino acid sequence.

[0138] Anti-HIV bioassay guided fractionation was used to track the isolation of the griffithsin polypeptide. In brief, the cellular mass from Griffithsia sp. was harvested by filtration, freeze-dried, and extracted first with H2O followed by (1:1) MeOH-CH2Cl2. Individual aliquots of the organic and aqueous extracts were tested for cytoprotective properties in the NCI primary anti-HIV screen (Weislow et al. J. Natl. Cancer Inst., 81: 577-586 (1989)). Only the H2O extract showed anti-HIV activity.

[0139] A freeze-dried aqueous extract (10 g) was brought to a concentration of 50 mg/ml by addition of DDH2O and maintained on ice. Crystalline ammonium sulfate (Sigma, St. Louis, Mo.; molecular biology grade) was added to the solution such that the final concentration of the mixture was 75% saturation. The mixture was allowed to precipitate on ice over night, and was then centrifuged at 3000 rpm for 50 min. The resulting pellets were set aside. The supernatant was brought to 1 M ammonium sulfate followed by another round of precipitation and centrifugation. The pellets from the second centrifugation were saved, and the resulting supernatant was filtered using a 0.22 [mu]m filter and subjected to hydrophobic interaction chromatography. A BioCad workstation (Perseptive Biosystems) was used for the following column chromatographies. The protein solution from the centrifugation and filtration steps was injected onto a Poros PE column (10*100 mm, Perseptive Biosystems) pre-equilibrated with a starting buffer of 50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.5. The column was eluted at a flow rate of 15 ml/min over the following gradient: (1) 7 column volumes (CV, equal to 7.85 ml) of the starting buffer; (2) 1.5-0 M ammonium sulfate over 2 CV; (3) 0 M ammonium sulfate for 15 CV. The eluate was monitored for both conductivity and absorbance (280 nm). Ammonium sulfate was added to the void fraction possessing anti-HIV activity to bring the final concentration to 75% saturation. The mixture was allowed to precipitate on ice overnight, and was then centrifuged at 3000 rpm for 50 min. DDH2O-resuspended pellets were first concentrated using a 10 kDa molecular weight limit membrane, dialyzed against 0.02% sodium azide, and then brought up to a concentration of 25 mM Tris-HCl, pH 8.5. The resulting protein solution was injected onto a Poros HQ anion exchange column (10*100 mm, Perseptive Biosystems) pre-equilibrated with a starting buffer of 25 mM Tris-HCl, pH 8.5. The column was eluted at a flow rate of 15 ml/min using the following gradient: (1) 5 CV of the starting buffer; (2) 0-1 M sodium chloride over 20 CV; (3) 1 M sodium chloride for 5 CV. The eluate was monitored for absorbance (280 nm). Active fractions from the HQ column were concentrated and desalted using a 10 kDa molecular weight limit membrane and subjected to a Bio-RP C4 reverse phase column (4.6*100 mm, Covance, Princeton, N.J.) and eluted at a flow rate of 4 ml/min using the following gradient: (1) 10 CV of the starting buffer of 5% acetonitrile in H2O; (2) 5-95% acetonitrile in H2O over 2.5 CV; (3) 95% acetonitrile in H2O for 5 CV. The eluate was monitored for absorbance (280 nm), and the active fraction was pooled, lyophilized, and resuspended in phosphate-buffered saline (PBS), pH 7.4. The protein solution was injected onto a G3000PW gel permeation column (21.5*600 mm, TosoHaas, Montgomeryville, Pa.) and eluted with PBS, pH 7.4, at a flow rate of 5 ml/min.

[0140] Molecular mass and purity (>99%) of griffithsin were confirmed by Electrospray ionization mass spectrometry (ESI-MS), and the protein concentrations were determined by amino acid analysis. Native molecular weight was determined by calibrating standard proteins (albumin (68 kDa), cytochrome c (12.5 kDa), and aprotinin (6.5 kDa)) by their retention time (as measured by absorbance at 280 nm) and comparing the resulting calibration curve to the retention time of the active protein. Amino acid analysis was accomplished using a Beckman Model 6300 Automated Amino Acid Analyzer according to manufacturer protocols. N-terminal amino acid sequencing was performed using an Applied Biosystems Model 4774A Sequencer according to manufacturer protocols. Matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS) was performed using a Kratos Kompact Maldi III instrument (Shimadzu, Columbia, Md.) operated in a linear mode using sinapinic acid as a matrix and trypsin as an external standard. ESI-MS was performed with a JEOL SX102 equipped with an Analytica electrospray source. The spectrometer was calibrated using a lysozyme standard (molecular weight=14305.2)

prior to each analysis. Samples were injected into the source in a 1:1 solution of hexafluorosopropanol and 2% acetic acid. The masses reported were averages calculated from the various charged states observed.

[0141] Griffithsin was subjected to digestion with cyanogen bromide (CNBr) and a variety of endoproteinases (Lys-C, Arg-C, and Asp-N) per manufacturer's instructions. The cleaved peptide products were purified by reversed-phase HPLC using a gradient of 0.05% aqueous trifluoroacetic acid for 20 min, then increasing to 60% acetonitrile in 0.05% aqueous trifluoroacetic acid over 100 min. Amino acid sequences were determined by sequential Edman degradation using an Applied Biosystems Model 494 sequencer according to the protocols of the manufacturer, and the masses of cleaved peptides were analyzed by MALDI-TOF mass spectrometer. The amino acid sequence of the native griffithsin polypeptide is set forth as SEQ ID NO: 3.

[0142] In summary, the preliminary analysis of the crude aqueous extract of algae Griffithsia sp. in the NCI's primary in vitro anti-HIV screening assay (Weislow et al., supra) identified a protein that bound soluble gp120. The process described herein is illustrated in FIG. 1. Anti-HIV bioassay-guided fractionation of the aqueous resulted in the isolation of griffithsin. The aqueous extract was subjected to ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, reversed-phase chromatography, and size exclusion chromatography to produce a homogeneous protein fraction. SDS-PAGE analysis showed a single protein band with a relative molecular mass of approximately 13 kDa, named griffithsin. Purified griffithsin exhibited a single band by immunoblotting with anti-griffithsin polyclonal antibodies. The amino acid sequence of the purified griffithsin was established by N-terminal Edman degradation of the intact protein and by N-terminal sequencing of peptide fragments cleaved by CNBr and a variety of endopeptidases (Lys-C, Arg-C, and Asp-N) followed by reversed phase purification and MALDI-TOF mass spectrometric analysis. The entire 121 amino acid sequence was established except for a single amino acid at position 31, which does not match any of the common amino acids. Electrospray ionization mass spectrometric analysis of isolated griffithsin showed a molecular ion with m/z 12,770.05, and the calculated value for the deduced amino acid sequence without amino acid at position 31 was m/z 12619.00. It was deduced that the molecular mass of the amino acid at position 31 was 151.05. The amino acid analysis of griffithsin also agreed with the deduced primary sequence. These data fully support the proposed primary amino acid sequence of griffithsin. A search of the BLAST database (Altschul et al., Nucleic Acids Res, 25 (17), 3389-3402 (1997)) for identification of protein sequence similarities did not reveal any homologies of greater than eight contiguous amino acids nor >30% total sequence homology between griffithsin and any amino acid sequences of known proteins or transcription products of known nucleotide sequences, including the anti-HIV proteins cyanovirin-N and scytovirin.

Example 2

[0143] This example demonstrates the synthesis of griffithsin genes. The methods described herein are illustrated in FIG. 2.

[0144] The chemically deduced amino acid sequence of griffithsin was back-translated to elucidate the corresponding DNA coding sequence. Since amino acid residue 31 of native griffithsin did not appear to be one of the twenty common amino acids, alanine was substituted in this position (SEQ ID NO: 2). In order to facilitate initial production and purification of recombinant griffithsin, a commercial expression vector pET-26b(+), from Novagen, Inc., Madison, Wis., for which reagents were available for affinity purification and detection, was selected. Appropriate restriction sites for ligation to pET-26b(+), and a stop codon, were included in the DNA sequence. SEQ ID NO: 1 is an example of a DNA sequence encoding a synthetic griffithsin gene. A flowchart illustrating a method of synthesizing of a griffithsin gene is shown in FIG. 2.

[0145] A griffithsin-encoding DNA sequence was synthesized as 13 overlapping, complementary oligonucleotides and assembled to form the double-stranded coding sequence. Oligonucleotide elements of the synthetic DNA coding sequence were synthesized using a nucleic acid synthesizer (model 394, Applied Biosystems Inc., Foster City, Calif.). The purified 13 oligonucleotides were individually treated with T4 polynucleotide kinase, and 1 nM quantities of each were pooled and boiled for 10 minutes to ensure denaturation. The temperature of the mixture was then reduced to

70[deg.] C. for annealing of the complementary strands for 15 minutes, and further reduced to 60[deg.] C. for 15 minutes. The reaction was cooled on ice and T4 DNA ligase (2,000 units) additional ligase buffer was added to the reaction. Ligation of the oligonucleotides was performed with T4 DNA ligase overnight at 16[deg.] C. The resulting DNA was recovered and purified from the reaction buffer by phenol:chloroform extraction, ethanol precipitation, and further washing with ethanol.

[0146] The purified, double-stranded synthetic DNA was then used as a template in a polymerase chain reaction (PCR). One [mu]l of the DNA solution obtained after purification of the ligation reaction mixture was used as a template. Thermal cycling was performed using a Perkin-Elmer instrument. "Pfu" thermostable DNA polymerase, restriction enzymes, T4 DNA ligase, and polynucleotide kinase were obtained from Stratagene, La Jolla, Calif. Pfu polymerase was selected for this application because of its claimed superiority in fidelity compared to the usual Taq enzyme. The PCR reaction product was run on a 2% agarose gel in TAE buffer. The 465 base pair DNA construct was cut from the gel and purified. The purified DNA, which was digested with Nde I and Xho I restriction enzymes, was then ligated into the multicloning site of the pet-26b(+) vector.

[0147] E. coli were transfected with the generated pET-26b(+)-construct, and recombinant clones were identified by analysis of restriction digests of plasmid DNA. Sequence analysis of one of these selected clones indicated that three bases deviated from the intended coding sequence. These "mutations," which presumably arose during the PCR amplification of the synthetic template, were corrected by a site-directed mutagenesis kit from Stratagene, La Jolla, Calif. The repair was confirmed by DNA sequence analysis.

[0148] For preparation of a DNA sequence encoding a griffithsin polypeptide tagged with a penta-His peptide at the C-terminal end of griffithsin (e.g., SEQ ID NO: 4), the aforementioned recombinant griffithsin construct was subjected to site-directed mutagenesis to eliminate stop codons located between the griffithsin coding sequence and the penta-His peptide coding sequence using a site-directed mutagenesis kit from Stratagene, La Jolla, Calif. A pair of mutagenic oligonucleotide primers were synthesized, which included portions of the codons encoding the griffithsin polypeptide and penta-His peptide, but lacked the stop codons. Annealing of these mutagenic primers with the template DNA and extension by DNA polymerase resulted in the generation of a DNA construct encoding a fusion protein comprising the griffithsin amino acid sequence linked to a penta-His peptide tag. DNA sequencing verified the presence of the intended sequence.

Example 3

[0149] This example demonstrates the expression of an N-terminal His-tagged-griffithsin gene.

[0150] A recombinant griffithsin protein and a C-terminal, His-tagged griffithsin protein encoded by the nucleic acids of Example 2 did not efficiently translocate to the periplasmic fraction of E. coli following protein expression. In addition, the majority of the produced proteins accumulated in the inclusion bodies of E. coli without the cleavage of a pelB signal sequence located at the N-terminus of the griffithsin protein. Thus, steps were taken to express griffithsin in the cytosolic fraction of E. coli.

[0151] The pET-26b(+)-griffithsin DNA construct was used as a template PCR using a pair of appropriate primers. The PCR product was designed to have a "penta-His" peptide and thrombin recognition site at the N-terminal end of the griffithsin polypeptide, providing for production of a N-terminal, His-tagged-griffithsin fusion protein. The PCR reaction product was purified from an agarose gel. The purified DNA, which was digested with Nco I and Xho I restriction enzymes, was ligated into the expression vector pET-28a(+) vector (Novagen, Inc., Madison, Wis.).

[0152] E. coli (strain BL21 (DE3)) were transfected with the pET-28a(+) vector containing the nucleic acid coding sequence for the His-tagged-griffithsin fusion protein (see SEQ ID NO: 4). Selected clones were seeded into small-scale shake flasks containing LB growth medium with 30 [mu]g/ml kanamycin and expanded by incubation at 37[deg.] C. Larger-scale Erlenmeyer flasks (0.5-3.0 liters) were then seeded. The culture was allowed to grow to a density of 0.5-0.7 OD600

units. The expression of the His-tagged-griffithsin fusion protein was induced by adding IPTG to a final concentration of 1 mM and continuing incubation at 37[deg.] C. for 3-6 hrs. Bacteria were harvested by centrifugation, and the soluble fraction was obtained using BugBuster(TM) reagent and Benzonase nuclease (Novagen, Inc., Madison, Wis.). Crude soluble fractions showed both anti-HIV activity and presence of a His-tagged-griffithsin fusion protein by Western-blotting. In addition, the His-tagged-griffithsin protein accumulated in the inclusion bodies of E. coli. A flowchart illustrating a method of expressing and purifying recombinant His-tagged-griffithsin is shown in FIG. 3.

[0153] The purity (~98%) of recombinant His-tagged griffithsin was confirmed by SDS-PAGE on 16% Tricine gel stained by Coomassie Blue staining. The protein showed the expected molecular mass for griffithsin (i.e., 14.6 kDa). Protein concentrations were determined based on extinction coefficient at 280 nm of the protein. Approximately 1.6 mg of recombinant His-tagged griffithsin was purified from 1 L of E. coli culture. The purified protein demonstrated gp120-binding and anti-viral activity equivalent to that of native griffithsin.

[0154] This example illustrates a method of producing recombinant griffithsin, which displays physical and functional properties similar, if not identical, to that of natural griffithsin.

Example 4

[0155] This example describes a method of purifying a recombinant His-tagged-griffithsin protein.

[0156] Using an immobilized metal affinity chromatography set-up including Ni-NTA agarose (QIAGEN Inc., Valencia, Calif.), a His-tagged-griffithsin fusion protein (as described in Example 3) was purified.

[0157] The soluble fraction described in Example 3 was loaded onto 20 ml gravity columns containing affinity matrix. The columns were washed extensively with washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0) to remove contaminating proteins. Since His-tagged griffithsin cannot compete for binding sites on the Ni-NTA resin if the imidazole concentration is increased to 100-250 mM, the His-tagged griffithsin protein was eluted by applying elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0) through the column. Column fractions and wash volumes were monitored by Western-blot analysis using Penta-His(TM) antibody (QIAGEN Inc., Valencia, Calif.) or anti-griffithsin antibody. Fractions containing the purified His-tagged griffithsin protein were pooled, dialyzed extensively against distilled water, and lyophilized.

[0158] Potent cytoprotective and anti-replicative activities of both natural and His-tagged recombinant griffithsin were observed using the HIV-1RF strain of HIB in CEM-SS cells. Both the natural and recombinant griffithsin polypeptides demonstrated a concentration-dependent inhibition of virus-induced cell killing. Griffithsin treatment also resulted in concomitant decreases in supernatant reverse transcriptase and viral core antigen, p24. Mid-to-high picomolar concentrations of griffithsin exhibited comparably potent activity against all of the representative T-tropic laboratory strains and primary isolates as well as M-tropic primary isolates. In the antiviral assays, there was little or no evidence of direct cytotoxicity of griffithsin to the uninfected control cells at the highest tested concentrations of griffithsin (78.3 to 783 nM). Griffithsin-pretreated uninfected CEM-SS cells retained normal susceptibility to HIV infection after the removal of griffithsin. In contrast, infectivity of cell-free virus was abolished after pretreatment and removal of griffithsin. These results indicate that griffithsin is a virucide. Cocultivation of uninfected and chronically infected CEM-SS with griffithsin resulted in concentration-dependent inhibition of cell-cell fusion. Additional binding and fusion inhibition assay using [beta]-gal indicator cells showed similar results. Griffithsin inhibited fusion of CD4 [beta]-gal cells with HL [2/3] cells and also inhibited cell-free HIV-1IIIB fusion and infection of [beta]-gal cells in a concentration-dependent manner.

Example 5

[0159] This example illustrates the anti-HIV activity of natural griffithsin polypeptide and His-

tagged griffithsin polypeptide.

[0160] Pure proteins were initially evaluated for antiviral activity using an XTT-tetrazolium anti-HIV assay described previously (Boyd, in Aids, Etiology, Diagnosis, Treatment And Prevention (1988), supra; Gustafson et al., J. Med. Chem., 35: 1978-1986 (1992); Weislow (1989), supra; Gulakowski (1991), supra). A CEM-SS human lymphocytic target cell line was used in all assays maintained in RPMI 1650 medium (Gibco, Grand Island, N.Y.), without phenol red, supplemented with 5% fetal bovine serum, 2 mM L-Glutamine, and 50 mg/ml Gentamicin (complete medium).

[0161] Exponentially growing cells were pelleted and resuspended at a concentration of 2.0*10<5 >cells/ml in complete medium. The Haitian variant of HIV, HTLV-IIIRF (3.54*10<6 >SFU/ml), was used throughout. Frozen virus stock solutions were thawed immediately before use and resuspended in complete medium to yield 1.2*10<5>SFU/ml. The appropriate amounts of the pure proteins for anti-HIV evaluations were dissolved in H2O-DMSO (3:1), then diluted in complete medium to the desired initial concentration. All serial drug dilutions, reagent additions, and plate-to-plate transfers were carried out with an automated Biomek 1000 Workstation (Beckman Instruments, Palo Alto, Calif.).

[0162] FIG. 4 summarizes the observed antiviral activities of native griffithsin from Griffithsia sp. (FIG. 4a) and recombinant His-tagged-fusion griffithsin (FIG. 4b). Effects of a range of concentrations of native griffithsin and HIS-tagged-griffithsin upon CEM-SS cells infected with HIV-1, as determined after 6 days in culture is illustrated in FIG. 6. Data points represent the percent of the respective uninfected, nondrug-treated control values. The two griffithsin polypeptides demonstrated potent anti-HIV activity with an EC50 in the low nanomolar range and no significant evidence of direct cytotoxicity to the host cells at the highest tested concentrations (up to 1 mM).

Example 6

[0163] This example demonstrates that HIV viral envelope gp120 is the principal target for griffithsin.

[0164] To determine the affinity of griffithsin for a series of protein standards, 100 ng each of gp160, gp120, gp41, sCD4, bovine IgG, [alpha]-acid glycoprotein, and aprotinin were subjected to ELISA as previously described (Bokesch et al., Biochemistry, 42: 2578-2584 (2003)). Briefly, the protein standards were bound to a 96-well plate, which was rinsed with PBST (three times) and blocked with BSA. Between each step of the protocol, the plate was rinsed with PBST (three times). The protein standards were incubated with griffithsin (100 ng/well), followed by incubation with a 1:500 dilution of an anti-griffithsin rabbit polyclonal antibody preparation. Griffithsin bound to the protein standards was detected by adding goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, Ind.). Upon addition of alkaline phosphatase substrate buffer, absorbance was measured at 405 nm for each well. Glycosylation-dependent binding of griffithsin to gp120 was examined using an ELISA as above, with glycosylated and nonglycosylated gp120 (HIV-1SF2 gp120) added to the 96-well plate and incubated with serial dilutions of griffithsin.

[0165] Griffithsin was tested for its ability to bind HIV envelope glycoproteins. Evidence for direct interaction of griffithsin with gp120, gp160, and to a lesser degree, gp41 was obtained from ELISA experiments (FIG. 5a). There was little or no detectable interaction between griffithsin and cCD4 or other reference proteins, including bovine IgG, [alpha]-acid glycoprotein, and aprotinin. An additional ELISA experiment showed that binding of griffithsin to sgp120 is both concentration-dependent and glycosylation-dependent (FIG. 5b).

[0166] To undertake preliminary mapping studies to define griffithsin-binding site on the gp120, we evaluated the effect of griffithsin on the reactivity of soluble CD4 (sCD4), cyanovirin-N, and a panel of monoclonal antibodies (mAb) with soluble gp120 (sgp120) in an ELISA format assay. These studies demonstrated that griffithsin interfered strongly with recognition of sgp120 by the mAbs 48d and 2G12. Griffithsin moderately interfered with sCD4 and mAb IgG1b12 binding to sgp120. Griffithsin had little or no effect on the recognition of sgp120 by mAbs that recognize the C1 region

(or V3 loop), and the mAb 17b. However, additional studies demonstrated that pretreatment of sgp120 with sCD4 and the mAbs IgG 1b12, 48d, and 2G12 did not block subsequent binding of griffithsin to sgp120. Cyanovirin-N interfered strongly with the recognition of sgp120 by griffithsin. On the other hand, griffithsin pretreatment of sgp120 did not block subsequent binding of cyanovirin-N to sgp120.

[0167] Since griffithsin inhibited viral entry, we compared matched control and griffithsin-treated sgp120 preparations in a flow cytometric sgp120/CD4-expressing cell binding assay to determine whether griffithsin inhibits viral attachment or subsequent fusion events. The CEM-SS cell line expresses CD4, as demonstrated by the binding of target cells with both anti-Leu3a and anti-OKT4 monoclonal antibodies. After incubation of CEM-SS cells with sgp120, the cells were stained by anti-gp120 mAb-FITC. A concomitant decrease in the availability of the Leu3a epitope (i.e., the gp120-binding site on target cells) was observed. In other words, the sgp120 bound to the gp120 binding site on the target cells. As expected, little change in the staining specific for the OKT4 epitope (i.e., a non-gp120 binding site) was observed. These results are consistent with sgp120 binding of CD4 on the target cells. Pretreatment of sgp120 with griffithsin substantially recovered the availability of the Leu3a epitope, indicating that griffithsin completely blocked CD4-dependent sgp120 binding. However, overall sgp120 binding showed two peaks in the flow cytometry data when griffithsin-treated sgp120 was added to the cells. The decreased signal suggests inhibition of sgp120 binding to CD4 by griffithsin, which was consistent with the recovery of the availability of the Leu3a epitope. The increased signal suggests that the griffithsin/sgp120 complex also non-specifically bound to target cells.

[0168] This example demonstrates that griffithsin binds to a region of gp120 that recognizes CD4 on host cells.

Example 7

[0169] This example illustrates the broad-range anti-HIV activity of griffithsin.

[0170] Anti-viral assays used to study the activities of laboratory strains and primary isolates of virus have been previously published (Buckheit et al., Antiviral Res., 21: 247-265 (1993)). The low passage HIV-1 pediatric isolate ROJO was derived as previously described (Buckheit et al., AIDS Res. Hum. Retroviruses, 10: 1497-1506 (1994)). Peripheral blood mononuclear cells (PBMC) and macrophages were isolated from hepatitis and HIV sero-negative donors following Ficoll-Hypaque centrifugation as described elsewhere (Gartner and Popovic, Techniques in HIV Research, Aldovini, A. and Walker, B., eds., Stockton Press, New York (1994) pp. 59-63). Mean EC50 values were determined from concentration-response curves from eight dilutions of griffithsin (triplicate wells/concentration); assays for HIV-1 RF/CEM-SS employed XTT-tetrazolium; HIV-1 ROJO were tested in human PBMC cultures by supernatant reverse transcriptase activity; HIV-1 Ba-L and ADA were tested in human primary macrophage cultures by p24 ELISA assay. Standard errors averaged less than 10% of the respective means. The results of this study are summarized in Table 1 below.

[0000]

TABLE 1

Virus	Target Cell	Tropism	EC50 (nM)
HIV-1 Laboratory Strain			
RF	CEM-SS	T	0.043
HIV Primary Isolates			
ROJO	PBMC	T	0.63
ADA	Macrophage	M	0.50
Ba-L	Macrophage	M	0.098

[0171] The results show that griffithsin is potently active (sub-nanomolar EC50 values) against a broad range of HIV isolates including T-tropic viruses (utilizing CCR5 as a co-receptor) and M-tropic viruses (utilizing CXCR4 as a co-receptor). This picomolar level of activity is more potent

than that described for most of the current anti-HIV agents utilized in therapy or in development, including the entry inhibitors cyanovirin-N and Enfurtide(R). The data also show that griffithsin is effective at inhibiting infection by both laboratory-adapted strains and, more importantly, primary clinical isolates of HIV (e.g., ROJO, ADA, and Ba-L). Finally, the results indicate that griffithsin is active regardless of the cell type used in the assay, having potent activity whether the cells were T-lymphocytes (CEM-SS), PBMCs, or macrophages. Griffithsin did not show any toxicity against any of the cell lines even at concentrations 1000-fold higher than the EC50 values.

Example 8

[0172] This example describes the production of anti-griffithsin polyclonal antibodies. A flow diagram illustrating a method of producing anti-griffithsin antibodies is provided in FIG. 6.

[0173] A New Zealand white rabbit was immunized with 100 [mu]g of griffithsin in Freund's complete adjuvant. Booster injections of 50 [mu]g of griffithsin in Freund's incomplete adjuvant were administered on days 13, 29, 51, 64, 100, and 195. On days 7, 21, 42, 63, 78, and 112, 10 mL of blood was removed from the rabbit. On day 112 the rabbit was sacrificed and bled out. The IgG fraction of the immune sera of the rabbit was isolated by protein-A Sepharose affinity chromatography (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. Reactivity of the polyclonal antibodies for griffithsin was demonstrated by immunoblot and ELISA studies with 1:500 to 1:3000 dilution of the rabbit immunoglobulin fractions.

[0174] For immunoblotting, samples were transferred to PVDF membranes following SDS-PAGE according to standard procedures. The membranes were incubated for 1 hour with anti-griffithsin polyclonal antibodies, washed three times with PBS containing 0.05% Tween 20 (PBST), and then treated with goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, Mo.). After three washes with PBST, bound antibodies were visualized by incubating membranes in a solution of 0.05% 3,3'-diaminobenzidine and 0.003% H_2O_2.

[0175] The IgG fraction of rabbit polyclonal anti-griffithsin antibodies were purified after the final boost and animal sacrifice by using protein-A Sepharose chromatography on the 57 mL of rabbit serum collected. Following purification, 78 mL of purified anti-griffithsin IgGs were produced. The final concentration of protein was 335 micrograms/mL for a total yield of 27.3 mg of anti-griffithsin IgG. To analyze the specificity of the resulting antibody preparation, Western blot analysis was performed and resulted in the clear determination of specificity and avidity for griffithsin by the purified antibodies. A 1:250 dilution of the purified antibodies clearly visualized only the griffithsin from a mixture of griffithsin and other proteins. The response to griffithsin by the anti-griffithsin antibodies was also shown to be concentration-dependent.

Example 9

[0176] This example illustrates the anti-influenza virus activity of griffithsin.

[0177] All examined influenza viruses were passaged in Madin Darby canine kidney (MDCK) cells to prepare viral stocks. MDCK cells (from ATCC, Manassas, Va.) were grown in antibiotic-free minimum essential medium (MEM) with non-essential amino acids (Gibco, Long Island, N.Y.) containing 5% fetal bovine serum (FBS, HyClone Laboratories, Logan, Utah) and 0.1% $NaHCO_3$. Test medium consisted of MEM with 0.18% $NaHCO_3$, 10 units/mL trypsin, 1 [mu]g of ethylenediaminetetraacetate (EDTA) per ml, and 50 [mu]g gentamicin/mL.

[0178] Inhibition of virus-induced cytopathic effect (CPE) as determined by visual (microscopic) examination of infected cells and confirmed by increase in neutral red (NR) dye uptake into infected cells was used as an indicator of griffithsin antiviral activity. The CPE inhibition method was reported previously by Smee et al. (Antiviral Res., 5: 251-259 (2001)). Seven concentrations of griffithsin were screened for antiviral activity against each virus in 96-well flat-bottomed microplates of cells. The griffithsin protein was added 5-10 minutes prior to addition of virus to the cells. The concentration of virus correspond to approximately 50% infection of cells in culture (CCID50) per well. The virus challenge dose equals a multiplicity of infection of approximately 0.001 infectious particles per cell. The reaction proceeded at 37[deg.] C. for 72 hr. To perform the

NR uptake assay for confirmation of antiviral activity, dye (0.34% concentration in medium) was added to the plates used to obtain visual scores of CPE. After 2 hours, color intensity of the dye absorbed by and subsequently eluted from the cells was determined by the method of Finter et al., J. Gen. Virol., 5, 419-427 (1969) using a computerized EL-309 microplate autoreader (Bio-Tek Instruments, Winooski, Vt.). Antiviral activity was expressed as the 50% effective (virus-inhibitory) concentration (EC50 value) determined by plotting griffithsin concentration versus percent inhibition on semi-logarithmic graph paper. Cytotoxicity of compounds was assessed in parallel with the antiviral determinations in the same microplates, except in the absence of virus. From these, 50% cytotoxic endpoints (IC50 values) were determined. The results of this study are summarized in Table 2.

[0000]

TABLE 2

Influenza Virus Strain	EC50 ([mu]g/ml)
Beijing/262/95 (H1N1)	0.07
Texas/36/91 (H1N1)	0.06
Los Angeles/2/87 (H3N2)	0.037
Panama/2007/99 (H3N2)	0.006
Shandong/09/93 (H3N2)	0.018
Sydney/5/97 (H3N2)	0.016
Washington/05/96 (H3N2)	0.016

[0179] Similar to the results with HIV, griffithsin was found to be potently active against a wide spectrum of influenza A viruses. These viruses included both H1N1 strains and H3N2 strains of influenza, which is especially significant in light of the fact that the highly virulent Fijian strain of influenza A that afflicted the United States in 2003/2004 was also a H3N2 strain. Griffithsin was not found to be toxic to the MDCK cell line utilized for these experiments, even when the cells were exposed to a high dose of griffithsin (10 micrograms/mL).

Example 10

[0180] This example describes a method of producing recombinant griffithsin.

[0181] Recombinant expression of His-tagged griffithsin in E. coli was optimized using a fermenter in combination with an auto-induction media. A seed culture was grown in LB media containing 30 [mu]g/ml kanamycin in a shaker flask at 37[deg.] C. and 150 rpm for 17 hours. In addition, a fermenter containing an auto-induction media was inoculated with the seed culture. The ratio of auto-induction media to seed culture was approximately 50:1. The culture was grown at 37[deg.] C. for 24 hours. The final culture density was approximately 8.6 OD600 units. The final culture was harvested by centrifugation, and the soluble fraction was obtained as described above.

[0182] Crude soluble fractions contained His-tagged-griffithsin fusion protein, which was detected by Western-blotting with anti-griffithsin polyclonal antibodies. The ratio of soluble:insoluble protein at approximately 15 kDa was 50:50. The ratio indicates that more griffithsin protein was produced in soluble fraction in this fermentation procedure compared with protein expression achieved using a shaker flask procedure. In addition, the fermentation procedure provided approximately 30-fold higher quantities of griffithsin protein than the shaker flask procedure. Approximately 50 mg of purified recombinant griffithsin was isolated from 1 L of the fermentation. The purified protein existed as a homodimer and demonstrated gp120 binding and anti-viral activity equivalent to that of native griffithsin.

[0183] The results of this example confirm a method of producing recombinant, anti-viral griffithsin protein.

[0184] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference: Birren et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1997),

Birren et al., Genome Analysis: A Laboratory Manual Series, Volume 2, Detecting Genes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998),
Birren et al., Genome Analysis: A Laboratory Manual Series, Volume 3, Cloning Systems, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999),
Birren et al., Genome Analysis: A Laboratory Manual Series, Volume 4, Mapping Genomes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999),
Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988),
Harlow et al., Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999), and
Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

US8088729
Anti-viral griffithsin compounds, compositions, and methods of use

A method of inhibiting a viral infection of a host comprising administering to the host an anti-viral griffithsin polypeptide comprising SEQ ID NO: 3 or a fragment thereof comprising at least eight contiguous amino acids, a nucleic acid encoding the anti-viral polypeptide, or an antibody to the anti-viral polypeptide. A method of inhibiting a virus in a sample comprising contacting the sample with an anti-viral griffithsin polypeptide or antibody thereto also is provided.

TECHNICAL FIELD OF THE INVENTION

The invention relates to an anti-viral Griffithsin polypeptide related conjugates, compositions, nucleic acids, vectors, host cells, antibodies, and methods for their production and use.

BACKGROUND OF THE INVENTION

Although the field of viral therapeutics has advanced in response to the need for treatments and prophylactics effective against diverse classes of viruses, the threat of viruses remain among several human populations across the world.

Retroviruses, such as HIV, continue to pose a threat to humans. There are many ways in which an agent can exhibit anti-retroviral activity (e.g., see DeClercq, Adv. Virus Res., 42: 1-55 (1993); DeClercq, J. Acquir. Immun. Def. Synd., 4: 207-218 (1991); and Mitsuya et al., Science, 249: 1533-1544 (1990). Nucleoside derivatives, such as AZT, which inhibit the viral reverse transcriptase, were among the first clinically active agents available commercially for anti-HIV therapy. Although very useful in some patients, the utility of AZT and related compounds is limited by toxicity and insufficient therapeutic indices for fully adequate therapy. Also, given the subsequent revelations about the true dynamics of HIV infection (Coffin, Science, 267: 483-489 (1995); and Cohen, Science, 267: 179 (1995)), it has become increasingly apparent that agents acting as early as possible in the viral replicative cycle are needed to inhibit infection of newly produced, uninfected immune cells generated in the body in response to the virus-induced killing of infected cells. Also, it is essential to neutralize or inhibit new infectious virus produced by infected cells.

Effective means for preventing HIV infection also are needed as a global priority. Heterosexual transmission accounts for the majority of new cases of HIV infection each year. Current reports from the World Health Organization estimate that a total of more than 40 million people are now infected with HIV. HIV prevention research has to date focused predominantly on vaccine development. However, no effective preventative or therapeutic vaccine has been identified thus far. New approaches to vaccine development, as well as entirely different strategies and agents for preventing person-to-person transmission of HIV infection, are needed. One approach showing great promise is the development and use of topical microbicides. In this approach, a suitable antiviral agent is applied directly at the potential site of virus exposure, e.g., the genital mucosa in the case of HIV. A suitable antiviral agent is one which inactivates or inhibits infectivity of a virus upon contact of the antiviral agent with the virus. Suitable animal models are available for

demonstrating in vivo efficacy of such approaches for preventing transmission of immunodeficiency viruses, such as HIV. For instance, the HIV-inactivating protein, cyanovirin-N, has been shown to inhibit the sexual transmission of a chimeric simian/human immunodeficiency virus (SHIV) infection in a primate model employing macaques exposed to the virus vaginally or rectally (C-C Tsai et al., AIDS Res. Hum. Retroviruses, 19, 535-541 (2003) and C-C Tsai et al., AIDS Res. Hum. Retroviruses, 20, 11-18 (2004)).

Infection of people by influenza viruses is also a major cause of pandemic illness, morbidity and mortality worldwide. The adverse economic consequences, as well as human suffering, are enormous. Available treatments for established infection by this virus are either minimally effective or ineffective; these treatments employ amantatadine, rimantadine and neuraminidase inhibitors. Of these drugs, only the neuraminidase inhibitors are substantially active against multiple strains of influenza virus that commonly infect humans, yet these drugs still have limited utility or efficacy against pandemic disease.

Currently, the only effective preventative treatment against influenza viral infection is vaccination. However, this, like the drug treatments, is severely limited by the propensity of influenza viruses to mutate rapidly by genetic exchange, resulting in the emergence of highly resistant viral strains that rapidly infect and spread throughout susceptible populations. In fact, a vaccination strategy is only effective from year-to-year if the potential pandemic strains can be identified or predicted, and corresponding vaccines prepared and administered early enough that the year's potential pandemic can be aborted or attenuated. Thus, new preventative and therapeutic interventions and agents are urgently needed to combat influenza viruses.

New agents with broad anti-influenza virus activity against diverse strains, clinical isolates and subtypes of influenza virus would be highly useful, since such agents would most likely remain active against the mutating virus. The two major types of influenza virus that infect humans are influenza A and B, both of which cause severe acute illness that may include both respiratory and gastrointestinal distress, as well as other serious pathological sequellae. An agent that has anti-influenza virus activity against diverse strains and isolates of both influenza A and B, including recent clinical isolates thereof, would be particularly advantageous for use in prevention or treatment of hosts susceptible to influenza virus infection.

The predominant mode of transmission of influenza viral infection is respiratory, i.e., transmission via inhalation of virus-laden aerosolized particles generated through coughing, sneezing, breathing, etc., of an influenza-infected individual. Transmission of infectious influenza virions may also occur through contact (e.g., through inadvertent hand-to-mouth contact, kissing, touching, etc.) with saliva or other bodily secretions of an infected individual. Thus, the primary first points of contact of infectious influenza virions within a susceptible individual are the mucosal surfaces within the oropharyngeal mucosa, and the mucosal surfaces within the upper and lower respiratory tracts. Not only do these sites comprise first points of virus contact for initial infection of an individual, they are also the primary sites for production and exit (e.g., by coughing, sneezing, salivary transmission, etc.) of bodily fluids containing infectious influenza viral particles. Therefore, availability of a highly potent anti-influenza virus agent, having broad-spectrum activity against diverse strains and isolates of influenza viruses A and B, which could be applied or delivered topically to the aforementioned mucosal sites of contact and infection and transmission of infectious influenza viruses, would be highly advantageous for therapeutic and preventative inhibition of influenza viral infection, either in susceptible uninfected or infected hosts.

Highly pathogenic avian H5N1 influenza A viruses have been of widespread concern in recent years. The H5N1 virus can be highly lethal to birds and humans raising concerns of a possible pandemic (Hatta and Kawaoka, Uirusu. 55(1):55-61 (2005)). The well-established pathogenicity of these avian influenza viruses makes evident the need in the art for the development of effective anti-H5N1 drugs and vaccines.

Infection with hepatitis C virus (HCV) also represents an important public health problem. HCV is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Verslype et al., Acta Gastroenterol Belg. 68(3):314-318 (2005)). Patients with HCV are mainly treated today with interferon, alone or in combination with ribavirin. However, such treatments eliminate the virus

from only about one half of the patients (Watashi and Shimotohno, Uirusu. 55(1):105-110 (2005)). Therefore, a more effective approach to the treatment of HCV infection is needed.

In the latter part of 2002, a new disease, severe acute respiratory syndrome (SARS), emerged in China, and an animal coronavirus that had crossed the species barrier through close contact of humans with infected animals was later identified as the etiological agent. The coronavirus rapidly adapted to the new host and not only became readily transmissible between humans but also more pathogenic. Air travel spread the virus rapidly around the world and ultimately the virus infected 8096 people and caused 774 deaths in 26 countries on 5 continents. Aggressive quarantine measures successfully terminated SARS (Stadler and Rappuoli, Curr Mol Med. 5(7):677-697 (2005)). However, a resurgence of SARS is still a threat, because the causative agent remaining in animal reservoirs is not fully understood, and sporadic cases continue to be reported (Lu et al., Acta Pharmacol Sin. 26(12):1479-1484 (2005)). Therefore, there is a need in the art to develop antiviral drugs and vaccines specific for the SARS virus.

The Zaire ebola virus has caused large outbreaks of severe and usually fatal hemorrhagic disease in humans for which there is no effective treatment or cure (Towner et al., Virology 332(1):20-27 (2005)). Thus, there is a need in the art for effective methods of treating or preventing ebola viral infections in humans.

In this regard, new classes of anti-viral agents, to be used alone or in combination existing anti-viral agents, are needed for effective anti-viral therapy. New agents are also important for the prophylactic inhibition of viral infection. In both areas of need, the ideal new agent(s) would act as early as possible in the viral life cycle; be as virus-specific as possible (i.e., attack a molecular target specific to the virus but not the host); render the intact virus noninfectious; prevent the death or dysfunction of virus-infected cells; prevent further production of virus from infected cells; prevent spread of virus infection to uninfected cells; be highly potent and active against the broadest possible range of strains and isolates of a given virus; be resistant to degradation under physiological and rigorous environmental conditions; and be readily and inexpensively produced. In view of the foregoing, there is a need in the art for new methods and compositions for inhibiting viral infection. The invention provides such methods. These and other advantages of the invention, as well as additional inventive features, will become apparent from the description provided herein.

BRIEF SUMMARY OF THE INVENTION

The invention provides, among other things, an isolated and purified nucleic acid molecule that encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, optionally as part of an encoded fusion protein. In this regard, the invention also provides an isolated and purified nucleic acid molecule that encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO: 3 which have been rendered glycosylation-resistant and wherein the at least eight contiguous amino acids have antiviral activity, optionally as part of an encoded fusion protein. Further provided are vectors comprising an aforementioned isolated and purified nucleic acid molecule and a host cell or organism comprising such a vector.

Accordingly, the invention also provides a method of producing an anti-viral polypeptide, which method comprises expressing the nucleic acid molecule, optionally in the form of a vector, in a host cell or organism. Thus, an anti-viral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids have anti-viral activity, and an antiviral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO: 3, which have been rendered glycosylation-resistant and wherein the at least eight contiguous amino acids have antiviral activity, are also provided, as are conjugates comprising an aforementioned anti-viral polypeptide and at least one effector component. Compositions comprising an effective amount of an aforementioned anti-viral polypeptide or anti-viral polypeptide conjugate are also provided.

The invention further provides a method of inhibiting prophylactically or therapeutically a viral infection of a host, such as a retroviral infection of a host (e.g., human immunodeficiency virus

(HIV), e.g., HIV-1 or HIV-2) or, especially, a viral infection by an influenza virus (e.g., an H5N1 virus), Severe Acute Respiratory Syndrome (SARS) virus, Hepatitis C virus, or Ebola virus. The method comprises administering to the host an effective amount of an anti-viral polypeptide or anti-viral polypeptide conjugate as described herein (e.g., comprising SEQ ID NO:3 or antiviral fragment thereof comprising at least eight contiguous amino acids of SEQ ID NO: 3), whereupon the viral infection is inhibited.

Still further provided is a method of inhibiting prophylactically or therapeutically a viral infection of a host, e.g., an animal, comprising transforming host cells in vivo with a nucleic acid molecule encoding an above-described polypeptide. Even still further provided is a method of inhibiting prophylactically or therapeutically a viral infection of a host, e.g., an animal, comprising transforming host cells with a nucleic acid molecule encoding an above-described polypeptide and placing the transformed host cells into or onto the host.

The present invention also provides a method of removing virus from a sample. The method comprises contacting the sample with a composition comprising an anti-viral polypeptide or conjugate or fusion protein thereof, wherein the anti-viral polypeptide comprises at least eight contiguous amino acids of SEQ ID NO: 3, which at least eight contiguous amino acids of SEQ ID NO: 3 bind to the virus.

An antibody that binds Griffithsin is provided as is a composition comprising the same. Similarly, an anti-Griffithsin antibody is provided as is a composition comprising the same. A method of administering an anti-Griffithsin antibody or a composition comprising the same to a mammal so as to inhibit infection of the mammal with a virus is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow diagram illustrating an anti-HIV bioassay-guided method of isolating, purifying, and elucidating the amino acid sequence of Griffithsin.

FIG. 2 is a flow diagram illustrating a method of synthesizing a recombinant Griffithsin gene.

FIG. 3 is a flow diagram illustrating a method of expressing a synthetic Griffithsin gene encoding a His-tagged Griffithsin polypeptide protein and purification of the recombinant His-tagged Griffithsin.

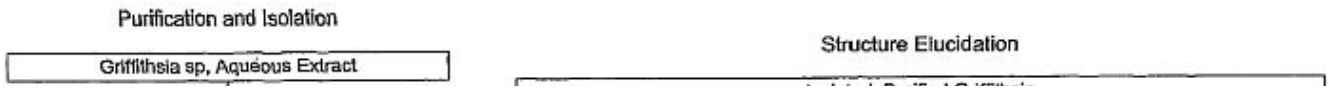
FIG. 4a is a line graph illustrating the anti-HIV activity of native Griffithsin, in terms of concentration of Griffithsin (nM) (X-axis) versus % control (Y-axis). FIG. 4b is a line graph illustrating the anti-HIV activity of recombinant, His-tagged Griffithsin in terms of concentration of Griffithsin (nM) (X-axis) versus % control (Y-axis).

FIG. 5a is a bar graph comparing test proteins bound by Griffithsin (Y-axis) and absorbance of the Griffithsin-test protein complex at 405 nm (X-axis). FIG. 5b illustrates the concentration-dependent binding of Griffithsin to gp120 by comparing Griffithsin (GRFT) concentration (pmol) and absorbance of Griffithsin-gp120 complexes at 405 nm.

FIG. 6 is a flow diagram illustrating a method of producing anti-Griffithsin antibodies.

FIG. 7 is a graph of the anti-viral effect (% virus control; -) or the cytotoxic effect (% cell viability; -) of Griffithsin at different concentrations.

FIG. 1



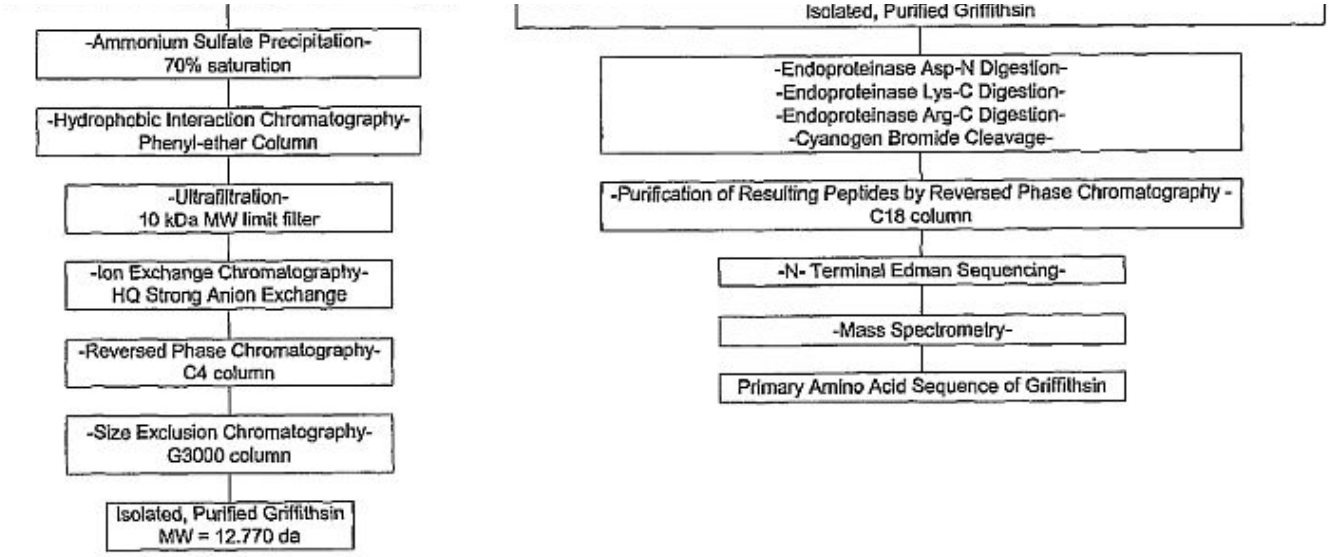

-Ammonium Sulfate Precipitation-
70% saturation
-Hydrophobic Interaction Chromatography-
Phenyl-ether Column
-Ultrafiltration-
10 kDa MW limit filter
-Ion Exchange Chromatography-
HQ Strong Anion Exchange
-Reversed Phase Chromatography-
C4 column
-Size Exclusion Chromatography-
G3000 column
Isolated, Purified Griffithsin
MW = 12.770 da
Isolated, Purified Griffithsin
-Endoproteinase Asp-N Digestion-
-Endoproteinase Lys-C Digestion-
-Endoproteinase Arg-C Digestion-
-Cyanogen Bromide Cleavage-
-Purification of Resulting Peptides by Reversed Phase Chromatography -
C18 column
-N- Terminal Edman Sequencing-
-Mass Spectrometry-
Primary Amino Acid Sequence of Griffithsin

FIG. 2

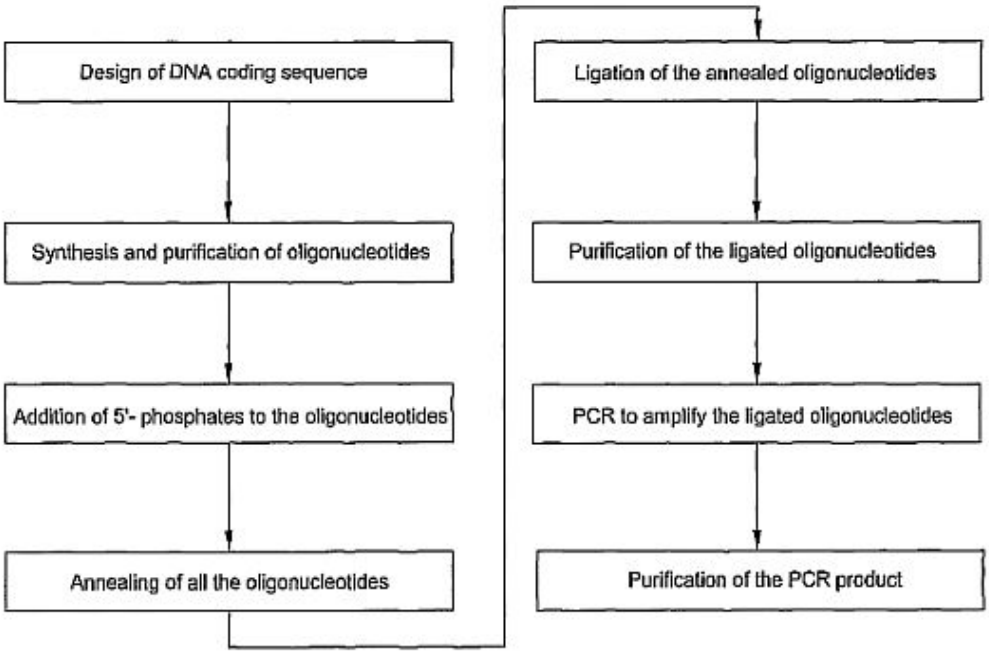
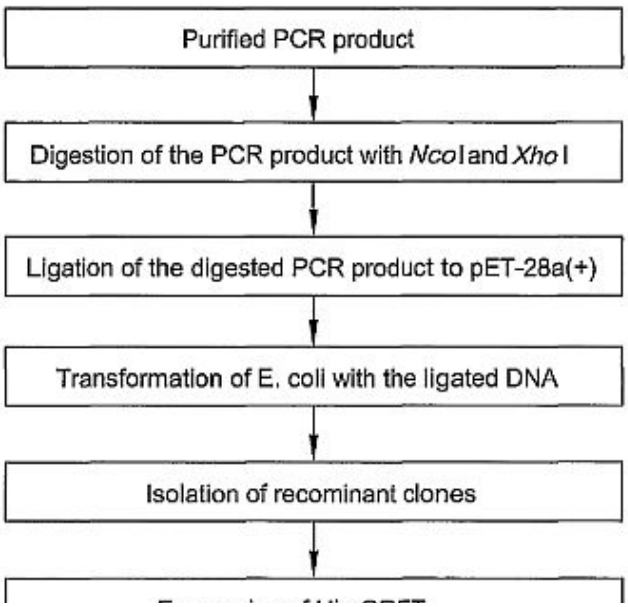

Design of DNA coding sequence
Synthesis and purification of oligonucleotides
Addition of 5'- phosphates to the oligonucleotides
Annealing of all the oligonucleotides
Ligation of the annealed oligonucleotides
Purification of the ligated oligonucleotides
PCR to amplify the ligated oligonucleotides
Purification of the PCR product

FIG. 3


Purified PCR product
Digestion of the PCR product with NcoI and XhoI
Ligation of the digested PCR product to pET-28a(+)
Transformation of E. coli with the ligated DNA
Isolation of recominant clones

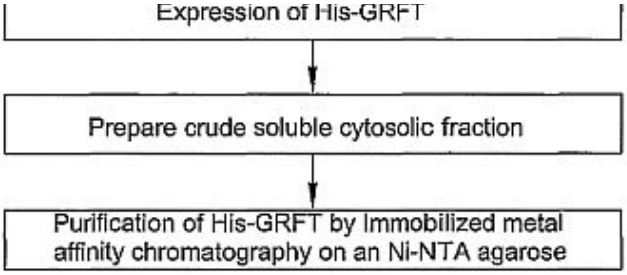


FIG. 4a

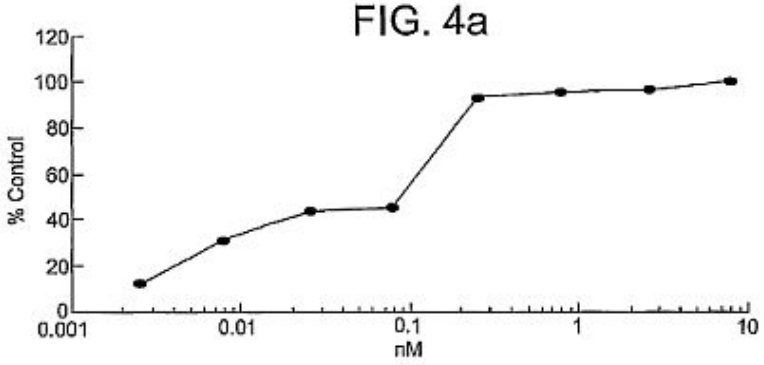


FIG. 4b

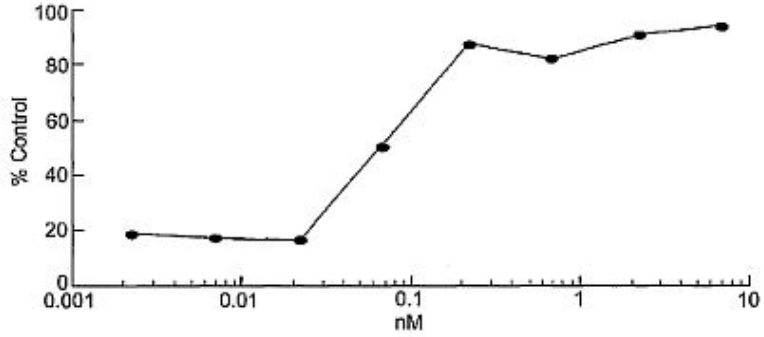


FIG. 5a

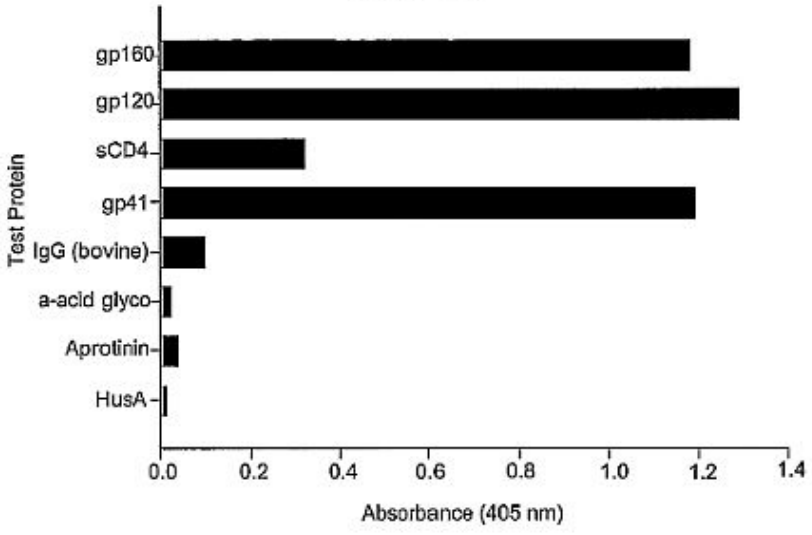


FIG. 5b

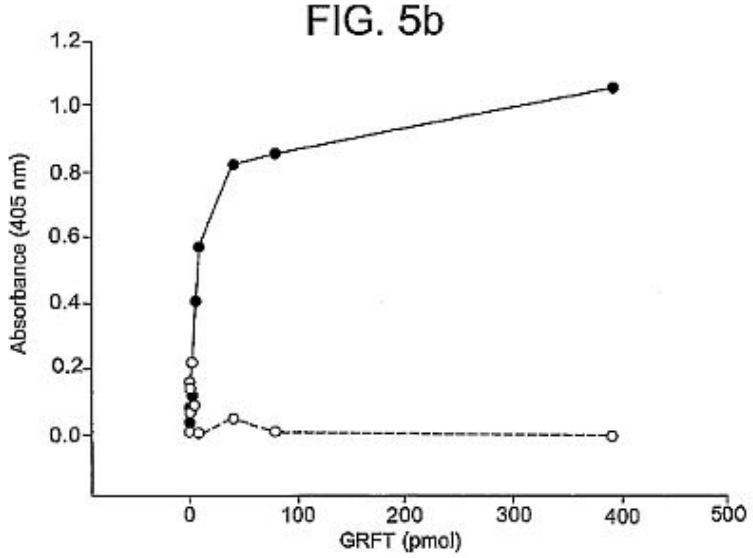


FIG. 6

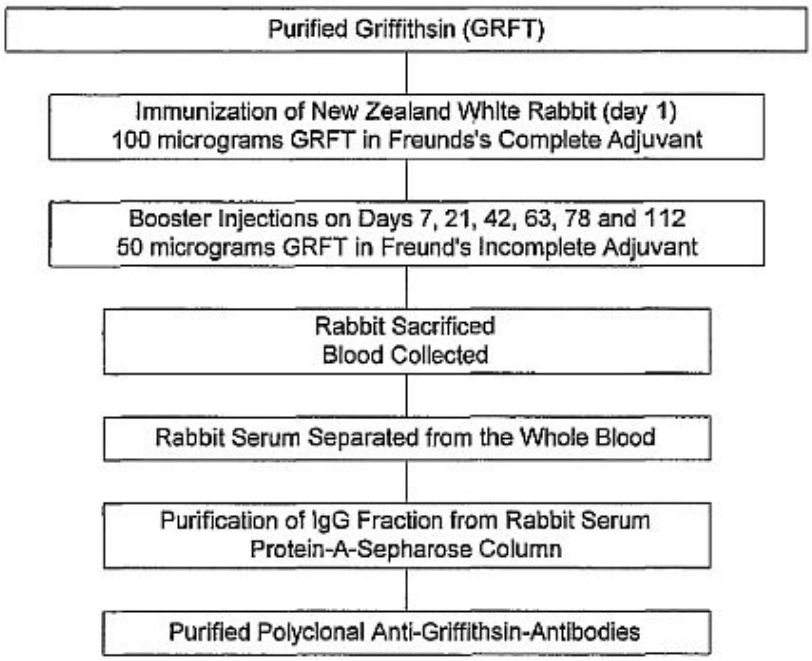
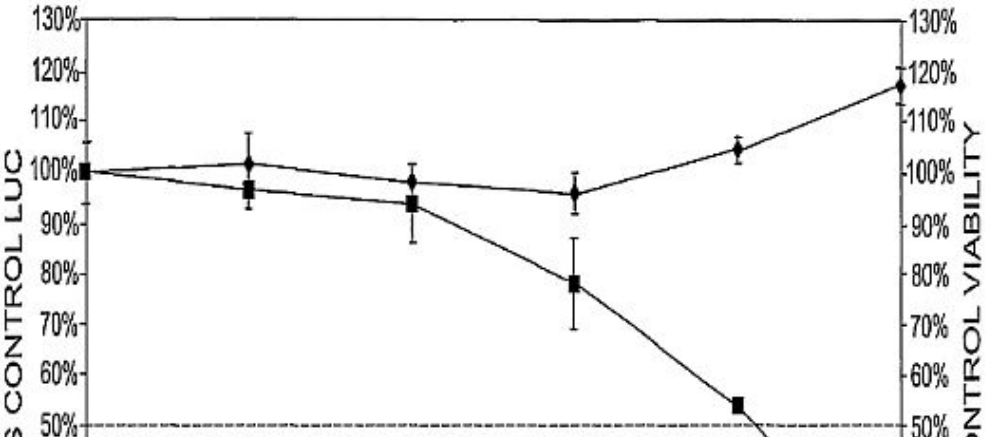
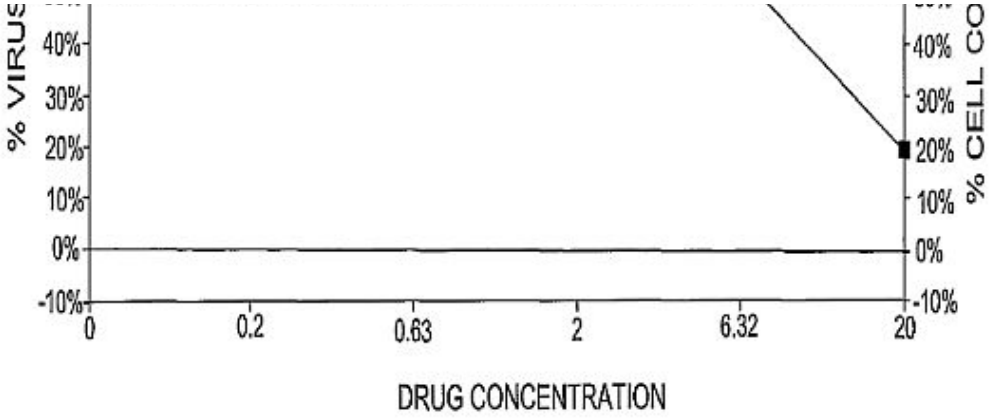


FIG. 7





DETAILED DESCRIPTION OF THE PRESENT INVENTION

The principal overall objective of the invention is to provide an anti-viral polypeptide and derivatives thereof, and broad uses thereof (e.g., medical and research uses), including prophylactic and/or therapeutic applications against viruses. An initial observation, which led to the invention, was anti-viral activity of certain extracts from a marine organism, namely Rhodophyte (Griffithsia sp.), originally collected in the territorial waters of New Zealand. Low picomolar concentrations of a protein isolated from the extracts, referred to herein as Griffithsin, irreversibly inactivated human clinical isolates of HIV. Its HIV molecular target is high mannose-comprised oligosaccharide constituents of Env glycoproteins. Upon binding, Griffithsin inhibits viral binding, fusion, and entry. Griffithsin also targets other viruses, such as other retroviruses, e.g., EV, SIV and HTLV, and non-retroviruses, such as measles and, especially, influenza (e.g., H5N1 virus), Ebola, Hepatitis C, and SARS virus.

Accordingly, the invention provides an isolated and purified anti-viral polypeptide of SEQ ID NO: 3 from Griffithsia sp. and functional homologs thereof, referred to collectively as "Griffithsin." Herein the term "Griffithsin" is used generically to refer to a natural Griffithsin or any related, functionally equivalent (i.e., anti-viral) polypeptide or derivative thereof. By definition, in this context, a related, functionally equivalent polypeptide or derivative thereof (a) contains a sequence of at least eight contiguous amino acids directly identical to a sub-sequence of eight contiguous amino acids contained within a natural Griffithsin, and (b) can specifically bind to a virus, in particular an influenza virus (e.g., H5N1), Hepatitis C, or SARS, Ebola, a retrovirus, more specifically a primate immunodeficiency virus, more specifically HIV-1, HIV-2 or SIV, or to an infected host cell expressing one or more viral antigen(s), more specifically an envelope glycoprotein, such as gp120, of the respective virus. In addition, such a functionally equivalent polypeptide or derivative thereof can comprise the amino acid sequence of a natural Griffithsin (see SEQ ID NO: 3), in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been removed from one or both ends, preferably from only one end, e.g., removed from the amino-terminal end, of natural Griffithsin. Alternatively, a functionally equivalent polypeptide or derivative thereof can comprise the amino acid sequence of a native Griffithsin (see SEQ ID NO: 3), in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been added to one or both ends, preferably from only one end, e.g., the amino-terminal end, of the native Griffithsin.

The invention further provides an isolated and purified polypeptide encoded by a nucleic acid molecule comprising a sequence of SEQ ID NO: 1 or a nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3. Upon examination of the antiviral Griffithsin polypeptide, the amino acid at position 31 of SEQ ID NO: 3 (represented as Xaa) was found not to be a familiar amino acid residue. Placement of an alanine at position 31, such as achieved in the recombinant Griffithsin polypeptide described herein (SEQ ID NO: 2), results in a polypeptide exhibiting equivalent activity as the natural Griffithsin polypeptide. If desired, the amino acid at position 31 can be substituted with any other amino acid to facilitate protein production. Ideally, the substitution at position 31 of SEQ ID NO: 3 does not diminish the anti-viral activity of the protein (e.g., does not diminish the anti-viral activity more than 50%, more than 30% or more than 10%) as compared to the anti-viral activity of the native protein. Preferably, the aforementioned nucleic acid molecules encode at least eight (e.g., at least 10, at least 20, at least 30, at least 50, at

least 70, at least 80, at least 90, or at least 100) contiguous amino acids of the amino acid sequence of SEQ ID NO: 3, which desirably have anti-viral activity. If the at least eight contiguous amino acids of SEQ ID NO: 3 comprise amino acids 1-121, desirably amino acid residue 45, 60, 71, and/or 104 has been rendered glycosylation resistant, while maintaining antiviral activity of the polypeptide.

The term "isolated" as used herein means having been removed from its natural environment. The term "purified" as used herein means having been increased in purity, wherein "purity" is a relative term and not to be construed as absolute purity. By "antiviral" is meant that the polypeptide or fragment thereof can inhibit a virus (e.g., inhibit entry of a virus into a host cell, limit the spread of viral infection by inhibiting cell to cell fusion, and the like), in particular an influenza virus, such as influenza virus of a strain A or strain B, or an H5N1 influenza virus, a retrovirus, specifically a primate immunodeficiency virus (e.g., an HIV virus such as HIV-1, HIV-2 or SW), a SARS coronavirus, Ebola, or a Hepatitis C virus.

Preferably, the polypeptide or derivative thereof comprises an amino acid sequence that is substantially homologous to that of an anti-viral protein from Griffithsia sp. By "substantially homologous" is meant sufficient homology to render the polypeptide or derivative thereof anti-viral, with anti-viral activity characteristic of an anti-viral protein isolated from Griffithsia sp. At least about 50% homology (e.g., at least about 60% homology, at least about 65% homology, or at least about 70% homology), preferably at least about 75% homology (e.g., at least about 80% homology or at least about 85% homology), and most preferably at least about 90% homology (e.g., at least about 95% homology) should exist.

Alterations of the natural amino acid sequence to produce variant polypeptides can be done by a variety of means known to those skilled in the art. For instance, amino acid substitutions can be conveniently introduced into the polypeptides at the time of synthesis. Alternatively, site-specific mutations can be introduced by ligating into an expression vector a synthesized oligonucleotide comprising the modified site. Alternately, oligonucleotide-directed, site-specific mutagenesis procedures can be used, such as disclosed in Walder et al., Gene, 42: 133 (1986); Bauer et al., Gene, 37: 73 (1985); Craik, Biotechniques, 12-19 (January 1995); and U.S. Pat. Nos. 4,518,584 and 4,737,462.

It is within the skill of the ordinary artisan to select synthetic and naturally-occurring amino acids that effect conservative or neutral substitutions for any particular naturally-occurring amino acids. The ordinarily skilled artisan desirably will consider the context in which any particular amino acid substitution is made, in addition to considering the hydrophobicity or polarity of the side-chain, the general size of the side chain and the pK value of side-chains with acidic or basic character under physiological conditions. For example, lysine, arginine, and histidine are often suitably substituted for each other, and more often arginine and histidine. As is known in the art, this is because all three amino acids have basic side chains, whereas the pK value for the side-chains of lysine and arginine are much closer to each other (about 10 and 12) than to histidine (about 6). Similarly, glycine, alanine, valine, leucine, and isoleucine are often suitably substituted for each other, with the proviso that glycine is frequently not suitably substituted for the other members of the group. This is because each of these amino acids are relatively hydrophobic when incorporated into a polypeptide, but glycine's lack of an [alpha]-carbon allows the phi and psi angles of rotation (around the [alpha]-carbon) so much conformational freedom that glycinyl residues can trigger changes in conformation or secondary structure that do not often occur when the other amino acids are substituted for each other. Other groups of amino acids frequently suitably substituted for each other include, but are not limited to, the group consisting of glutamic and aspartic acids; the group consisting of phenylalanine, tyrosine and tryptophan; and the group consisting of serine, threonine and, optionally, tyrosine. Additionally, the ordinarily skilled artisan can readily group synthetic amino acids with naturally-occurring amino acids.

The ordinarily skilled artisan can generate Griffithsin mutants or variants by, for example, substituting or mutating amino acids which are not critical for the anti-viral function of the polypeptide. Ideally, mutations that do not modify the electronic or structural environment of the peptide are generated to retain optimal antiviral activity. For example, natural Griffithsin forms dimers, which can be advantageous in some embodiments. Therefore, alterations which do not

disrupt dimer formation can be preferred. Amino acid residues which are not responsible for folding or stability of the three-dimensional conformation of the Griffithsin polypeptide are candidate residues for mutation. Alternatively or in addition, amino acids which are not involved in glycoprotein binding can be mutated or replaced. It is understood that surface hydrophobicity plays a key role in protein-protein interactions and surface electrophilicity is important to protein-sugar interactions, such as the interaction between Griffithsin and viral proteins. Hydrophobic surface clusters and electrophilic surface clusters on the Griffithsin peptide or homologs which suggest regions critical for interaction with the viral envelope can be mapped using routine methods such as those disclosed in Bewley et al., Nature Structural Biology, 5(7): 571-578 (1998). Amino acid residues not found either in electrophilic or hydrophobic surface clusters are likely not critical for hydrophobicity or electrophilicity of these clusters and, thus, are appropriate targets for mutation to create Griffithsin fragments (e.g., anti-viral polypeptides comprising at least about eight contiguous amino acids of SEQ ID NO: 2 or SEQ ID NO: 3), variants, mutants, or homologs (e.g., Griffithsin variants having 80%, 85%, or 90% homology to SEQ ID NO: 2 or SEQ ID NO: 3) which retain antiviral activity. If desired, amino acid residues which are responsible for binding to high-mannose oligosaccharide-containing glycoproteins on the viral surface can be mutated to increase the specificity or affinity of glycoprotein binding.

If desired, the proteins and peptides of the invention (including antiviral fragments, variant polypeptides, fusion proteins, and conjugates) can be modified, for instance, by glycosylation, amidation, carboxylation, or phosphorylation, or by the creation of acid addition salts, amides, esters, in particular C-terminal esters, and N-acyl derivatives of the proteins of the invention. The polypeptides also can be modified to create protein derivatives by forming covalent or noncovalent complexes with other moieties in accordance with methods known in the art. Covalently-bound complexes can be prepared by linking the chemical moieties to functional groups on the side chains of amino acids comprising the proteins, or at the N- or C-terminus. Desirably, such modifications and conjugations do not adversely affect the activity of the polypeptides (and variants thereof). While such modifications and conjugations can have greater or lesser activity, the activity desirably is not negated and is characteristic of the unaltered polypeptide.

The polypeptides (and fragments, homologs, variants, and fusion proteins) can be prepared by any of a number of conventional techniques. The polypeptide can be isolated or purified from a naturally occurring source or from a recombinant source. For instance, in the case of recombinant proteins, a DNA fragment encoding a desired polypeptide can be subcloned into an appropriate vector using well-known molecular genetic techniques (see, e.g., Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory (1989)) and other references cited herein under "EXAMPLES"). The fragment can be transcribed and the polypeptide subsequently translated in vitro. Commercially available kits also can be employed (e.g., such as manufactured by Clontech, Palo Alto, Calif.; Amersham Life Sciences, Inc., Arlington Heights, Ill.; InVitrogen, San Diego, Calif.; and the like). The polymerase chain reaction optionally can be employed in the manipulation of nucleic acids.

Such polypeptides also can be synthesized using an automated peptide synthesizer in accordance with methods known in the art. Alternately, the polypeptide (and fragments, homologs, variants, and fusion proteins) can be synthesized using standard peptide synthesizing techniques well-known to those of skill in the art (e.g., as summarized in Bodanszky, Principles of Peptide Synthesis, (Springer-Verlag, Heidelberg: 1984)). In particular, the polypeptide can be synthesized using the procedure of solid-phase synthesis (see, e.g., Merrifield, J. Am. Chem. Soc., 85: 2149-54 (1963); Barany et al., Int. J. Peptide Protein Res., 30: 705-739 (1987); and U.S. Pat. No. 5,424,398). If desired, this can be done using an automated peptide synthesizer. Removal of the t-butyloxycarbonyl (t-BOC) or 9-fluorenylmethyloxycarbonyl (Fmoc) amino acid blocking groups and separation of the polypeptide from the resin can be accomplished by, for example, acid treatment at reduced temperature. The protein-containing mixture then can be extracted, for instance, with diethyl ether, to remove non-peptidic organic compounds, and the synthesized polypeptide can be extracted from the resin powder (e.g., with about 25% w/v acetic acid). Following the synthesis of the polypeptide, further purification (e.g., using HPLC) optionally can be preformed in order to eliminate any incomplete proteins, polypeptides, peptides or free amino acids. Amino acid and/or HPLC analysis can be performed on the synthesized polypeptide to validate its identity. For other applications according to the invention, it may be preferable to

produce the polypeptide as part of a larger fusion protein, either by chemical conjugation or through genetic means, such as are known to those skilled in the art. In this regard, the invention also provides a fusion protein comprising the isolated or purified antiviral polypeptide (or fragment thereof) or variant thereof and one or more other protein(s) having any desired properties or effector functions, such as cytotoxic or immunological properties, or other desired properties, such as to facilitate isolation, purification, analysis, or stability of the fusion protein.

A Griffithsin conjugate comprising a Griffithsin coupled to at least one effector component, which can be the same or different, is also provided. The effector component can be polyethylene glycol, dextran, albumin, an immunological reagent, a toxin, an antiviral agent, or a solid support matrix. "Immunological reagent" will be used to refer to an antibody, an antibody fragment (e.g., an F(ab')2, an Fab', an Fab, an Fv, an sFv, a dsFv, or an Fc antibody fragment), an immunoglobulin, and an immunological recognition element. An immunological recognition element is an element, such as a peptide, e.g., the FLAG sequence of a recombinant Griffithsin-FLAG fusion protein, which facilitates, through immunological recognition, isolation and/or purification and/or analysis of the protein or peptide to which it is attached. An immunological reagent also can be an immunogenic peptide, which can be fused to Griffithsin for enhancing an immune response. In this respect, the invention provides an anti-viral conjugate comprising a Griffithsin polypeptide or fragment thereof bound to a virus or viral envelope glycoprotein. A Griffithsin fusion protein is a type of Griffithsin conjugate, wherein a Griffithsin is coupled to one or more other protein(s) having any desired properties or effector functions, such as cytotoxic or immunological properties, or other desired properties, such as to facilitate isolation, purification or analysis of the fusion protein or increase the stability or in vivo half-life of the fusion protein. Griffithsin also can be attached to a chemical moiety which allows recognition, isolation, purification, and/or analysis of the protein or peptide. An example of such a chemical moiety is a His tag of a recombinant Griffithsin-His fusion protein.
A "toxin" can be, for example, Pseudomonas exotoxin. An "antiviral agent" can be AZT, ddI, ddC, 3TC gancyclovir, fluorinated dideoxynucleosides, nevirapine, R82913, Ro 31-8959, BI-RJ-70, acyclovir, [alpha]-interferon, recombinant sCD4, michellamines, calanolides, nonoxynol-9, gossypol and derivatives thereof, gramicidin, amantatadine, rimantadine, and neuraminidase inhibitors, and cyanovirin-N or a functional homolog or derivative thereof (see, for example, U.S. Pat. No. 5,843,882). A "solid support matrix" can be a magnetic bead, a flow-through matrix, a sponge, a stent, a culture plate, or a matrix comprising a contraceptive device, such as a condom, diaphragm, cervical cap, vaginal ring or contraceptive sponge. In an alternative embodiment, a solid support matrix can be an implant for surgical implantation in a host and, if appropriate, later removal.
In view of the foregoing, the invention further provides a composition comprising (i) the isolated or purified antiviral polypeptide (or fragment thereof), a variant thereof, a fusion protein of the antiviral polypeptide (or fragment thereof) or variant thereof, and a conjugate of the antiviral polypeptide (or fragment thereof) or variant thereof, and/or (ii) a carrier, excipient or adjuvant therefor. Preferably, component (i) of the composition is present in an antiviral effective amount and the carrier is pharmaceutically acceptable. By "antiviral effective amount" is meant an amount sufficient to inhibit the infectivity of the virus.

The carrier can be any of those conventionally used and is limited only by chemico-physical considerations, such as solubility and lack of reactivity with the active agent of the invention, and by the route of administration. It is preferred that the pharmaceutically acceptable carrier be one which is chemically inert to the active agent and one which has no detrimental side effects or toxicity under the conditions of use. The pharmaceutically acceptable carriers described herein, for example, vehicles, adjuvants, excipients, and diluents, are well-known to those ordinarily skilled in the art and are readily available to the public. Typically, the composition, such as a pharmaceutical composition, can comprise a physiological saline solution; dextrose or other saccharide solution; or ethylene, propylene, polyethylene, or other glycol. The pharmaceutical composition preferably does not comprise mannose or N-acetyl-glucosamine, as these molecules may interfere with the functioning of the antiviral agent.

The invention also provides a method of obtaining a Griffithsin from Griffithsia sp. Such a method comprises (a) identifying an extract of Griffithsia sp. containing anti-viral activity, (b) optionally removing high molecular weight biopolymers from the extract, (c) anti-viral bioassay-guided fractionating the extract to obtain a crude extract of Griffithsin, and (d) purifying the crude extract

by reverse-phase HPLC to obtain Griffithsin (see, also, Example 1). More specifically, the method involves the use of ethanol to remove high molecular weight biopolymers from the extract and the use of an anti-HIV bioassay to guide fractionation of the extract.

Griffithsin (a polypeptide of exactly SEQ ID NO: 3), which was isolated and purified using the aforementioned method, was subjected to conventional procedures typically used to determine the amino acid sequence of a given pure protein. Thus, the Griffithsin was initially sequenced by N-terminal Edman degradation of intact protein and numerous overlapping peptide fragments generated by endoproteinase digestion. Amino acid analysis was in agreement with the deduced sequence. ESI mass spectrometry of reduced, HPLC-purified Griffithsin showed a molecular ion consistent with the calculated value. These studies indicated that Griffithsin from Griffithsia was comprised of a unique sequence of 121 amino acids having little or no significant homology or identity to previously described proteins or transcription products of known nucleotide sequences. No more than eight contiguous amino acids from Griffithsin were found in any amino acid sequences from known proteins, nor were there any known proteins from any source having significant sequence identity with Griffithsin. Given the chemically deduced amino acid sequence of Griffithsin, a corresponding recombinant Griffithsin (r-Griffithsin) was created and used to establish definitively that the deduced amino acid sequence was, indeed, active against virus, such as HIV and influenza.

Accordingly, the invention provides isolated and purified nucleic acid molecules and synthetic nucleic acid molecules, which comprise a coding sequence for a Griffithsin, such as an isolated and purified nucleic acid molecule comprising a sequence of SEQ ID NO: 1, an isolated and purified nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2, an isolated and purified nucleic acid sequence encoding an amino acid sequence SEQ ID NO: 3, an isolated and purified nucleic acid molecule comprising a sequence of SEQ ID NO: 4, an isolated and purified nucleic acid sequence encoding an amino acid sequence of SEQ ID NO: 5, and a nucleic acid molecule that is substantially homologous or substantially identical to any one of the aforementioned nucleic acid molecules. By "substantially homologous" is meant sufficient homology to render the polypeptide or derivative thereof anti-viral, with anti-viral activity characteristic of an anti-viral protein isolated from Griffithsia. At least about 50% homology or identity (e.g., at least about 60%, at least about 65%, or at least about 70% homology or identity), preferably at least about 75% homology or identity (e.g., at least about 80% or at least about 85% homology or identity), and most preferably at least about 90% homology or identity (e.g., at least about 95% homology or identity) should exist.

The inventive nucleic acid molecule preferably comprises a nucleic acid sequence encoding at least eight (preferably at least 10, more preferably at least 20, and most preferably at least 30) contiguous amino acids of the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 2. The inventive nucleic acid molecule also comprises a nucleic acid sequence encoding a polypeptide comprising the amino acid sequence of a native Griffithsin, in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been removed from one or both ends, preferably from only one end, e.g., removed from the amino-terminal end, of the native Griffithsin. Alternatively, the nucleic acid molecule can comprise a nucleic acid sequence encoding a polypeptide comprising the amino acid sequence of a natural Griffithsin (see SEQ ID NO: 3), in which 1-20, preferably 1-10, more preferably 1, 2, 3, 4, or 5, and most preferably 1 or 2, amino acids have been added to one or both ends, preferably from only one end, e.g., the amino-terminal end, of the native Griffithsin. Preferably, the isolated and purified nucleic acid molecule encodes a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, which desirably have anti-viral activity. If the at least eight contiguous amino acids comprise amino acids 1-121 of SEQ ID NO: 3, desirably amino acids 46, 60, 71, and/or 104 have been rendered glycosylation resistant, while maintaining antiviral activity of the polypeptide. Deletions and substitutions of SEQ ID NO: 2 or SEQ ID NO: 3 are within the skill in the art.

Given the present disclosure, it will be apparent to one skilled in the art that a partial Griffithsin gene sequence will likely suffice to code for a fully functional, i.e., anti-viral, such as anti-influenza or anti-HIV, Griffithsin. A minimum essential DNA coding sequence(s) for a functional Griffithsin can readily be determined by one skilled in the art, for example, by synthesis and evaluation of sub-sequences comprising the native Griffithsin, and by site-directed mutagenesis studies of the

Griffithsin DNA coding sequence.

Using an appropriate DNA coding sequence, a recombinant Griffithsin can be made by genetic engineering techniques (for general background see, e.g., Nicholl, in An Introduction to Genetic Engineering, Cambridge University Press: Cambridge (1994), pp. 1-5 & 127-130; Steinberg et al., in Recombinant DNA Technology Concepts and Biomedical Applications, Prentice Hall: Englewood Cliffs, N.J. (1993), pp. 81-124 & 150-162; Sofer in Introduction to Genetic Engineering, Butterworth-Heinemann, Stoneham, Mass. (1991), pp. 1-21 & 103-126; Old et al., in Principles of Gene Manipulation, Blackwell Scientific Publishers: London (1992), pp. 1-13 & 108-221; and Emtage, in Delivery Systems for Peptide Drugs, Davis et al., eds., Plenum Press: New York (1986), pp. 23-33). For example, a Griffithsia gene or cDNA encoding a Griffithsin can be identified and subcloned. The gene or cDNA then can be incorporated into an appropriate expression vector and delivered into an appropriate polypeptide-synthesizing organism (e.g., E. coli, S. cerevisiae, P. pastoris, or other bacterial, yeast, insect, plant or mammalian cells), where the gene, under the control of an endogenous or exogenous promoter, can be appropriately transcribed and translated. Alternatively, the expression vector can be administered to a plant or animal, for example, for large-scale production (see, e.g., Fischer et al., Transgenic Res., 9(4-5): 279-299 (2000); Fischer et al., J. Biol. Regul. Homeost. Agents, 14: 83-92 (2000); deWilde et al., Plant Molec. Biol., 43: 347-359 (2000); Houdebine, Transgenic Research, 9: 305-320 (2000); Brink et al., Theriogenology, 53: 139-148 (2000); Pollock et al., J. Immunol. Methods, 231: 147-157 (1999); Conrad et al., Plant Molec. Biol., 38: 101-109 (1998); Staub et al., Nature Biotech., 18: 333-338 (2000); McCormick et al., PNAS USA, 96: 703-708 (1999); Zeitlin et al., Nature Biotech., 16: 1361-1364 (1998); Tacker et al., Microbes and Infection, 1: 777-783 (1999); Tacket et al., Nature Med., 4(5): 607-609 (1998); and Methods in Biotechnology, Recombinant Proteins from Plants, Production and Isolation of Clinically Useful Compounds, Cunningham and Porter, eds., Humana Press: Totowa, N.J. (1998)). Such expression vectors (including, but not limited to, phage, cosmid, viral, and plasmid vectors) are known to those skilled in the art, as are reagents and techniques appropriate for gene transfer (e.g., transfection, electroporation, transduction, micro-injection, transformation, etc.). If a Griffithsin is to be recombinantly produced in isolated eukaryotic cells or in a eukaryotic organism, such as a plant (see above references and also Methods in Biotechnology, Recombinant Proteins from Plants, Production and Isolation of Clinically Useful Compounds, Cunningham and Porter, eds., Humana Press: Totowa, N.J. (1998)), desirably the N-linked glycosylation sites at positions 45, 60, 71, and/or 104 is rendered glycosylation-resistant, such as in accordance with the methods described herein. Subsequently, the recombinantly produced polypeptide can be isolated and purified using standard techniques known in the art (e.g., chromatography, centrifugation, differential solubility, isoelectric focusing, etc.), and assayed for anti-viral activity.
Alternatively, a natural Griffithsin can be obtained from Griffithsia by non-recombinant methods, and sequenced by conventional techniques. The sequence can then be used to synthesize the corresponding DNA, which can be subcloned into an appropriate expression vector and delivered into a polypeptide-producing cell for en mass recombinant production of the desired polypeptide.

In this regard, the invention also provides a vector comprising a DNA sequence, e.g., a Griffithsia gene sequence for Griffithsin, a cDNA encoding a Griffithsin, or a synthetic DNA sequence encoding Griffithsin. The vector can be targeted to a cell-surface receptor if so desired. A nucleic acid molecule as described above can be cloned into any suitable vector and can be used to transform or transfect any suitable host. The selection of vectors and methods to construct them are commonly known to persons of ordinary skill in the art and are described in general technical references (see, in general, "Recombinant DNA Part D," Methods in Enzymology, Vol. 153, Wu and Grossman, eds., Academic Press (1987) and the references cited herein under "EXAMPLES"). Desirably, the vector comprises regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of host (e.g., bacterium, fungus, plant or animal) into which the vector is to be introduced, as appropriate and taking into consideration whether the vector is DNA or RNA. Preferably, the vector comprises regulatory sequences that are specific to the genus of the host. Most preferably, the vector comprises regulatory sequences that are specific to the species of the host.
Constructs of vectors, which are circular or linear, can be prepared to contain an entire nucleic acid as described above or a portion thereof ligated to a replication system functional in a prokaryotic or eukaryotic host cell. Replication systems can be derived from ColE1, 2 m[mu] plasmid, [lambda], SV40, bovine papilloma virus, and the like.

In addition to the replication system and the inserted nucleic acid, the construct can include one or more marker genes, which allow for selection of transformed or transfected hosts. Marker genes include biocide resistance, e.g., resistance to antibiotics, heavy metals, etc., complementation in an auxotrophic'host to provide prototrophy, and the like.

One of ordinary skill in the art will appreciate that any of a number of vectors known in the art are suitable for use in the invention. Suitable vectors include those designed for propagation and expansion or for expression or both. Examples of suitable vectors include, for instance, plasmids, plasmid-liposome complexes, and viral vectors, e.g., parvoviral-based vectors (i.e., adeno-associated virus (AAV)-based vectors), retroviral vectors, herpes simplex virus (HSV)-based vectors, and adenovirus-based vectors. Any of these expression constructs can be prepared using standard recombinant DNA techniques described in, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 2<nd >edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and John Wiley & Sons, New York, N.Y. (1994); Fischer et al., Transgenic Res., 9(4-5): 279-299 (2000); Fischer et al., J. Biol. Regul. Homeost. Agents, 14: 83-92 (2000); deWilde et al., Plant Molec. Biol., 43: 347-359 (2000); Houdebine, Transgenic Research, 9: 305-320 (2000); Brink et al., Theriogenology, 53: 139-148 (2000); Pollock et al., J. Immunol. Methods, 231: 147-157 (1999); Conrad et al., Plant Molec. Biol., 38: 101-109 (1998); Staub et al., Nature Biotech., 18: 333-338 (2000); McCormick et al., PNAS USA, 96: 703-708 (1999); Zeitlin et al., Nature Biotech., 16: 1361-1364 (1998); Tacker et al., Microbes and Infection, 1: 777-783 (1999); and Tacket et al., Nature Med., 4(5): 607-609 (1998). Examples of cloning vectors include the pUC series, the pBluescript series (Stratagene, LaJolla, Calif.), the pET series (Novagen, Madison, Wis.), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clonetech, Palo Alto, Calif.). Bacteriophage vectors, such as [lambda]GT10, [lambda]GT11, [lambda]ZapII (Stratagene), [lambda] EMBL4, and [lambda] NM1149, also can be used. Examples of plant expression vectors include pBI101, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clonetech, Palo Alto, Calif.). Examples of animal expression vectors include pEUK-C1, pMAM and pMAMneo (Clonetech).
An expression vector can comprise a native or normative promoter operably linked to an isolated or purified nucleic acid as described above. The selection of promoters, e.g., strong, weak, inducible, tissue-specific and developmental-specific, is within the skill in the art. Similarly, the combining of a nucleic acid molecule as described above with a promoter is also within the skill in the art.

The DNA, whether isolated and purified or synthetic, or cDNA encoding a Griffithsin can encode for either the entire Griffithsin or a portion thereof. Where the DNA or cDNA does not comprise the entire coding sequence of the native Griffithsin, the DNA or cDNA can be subcloned as part of a gene fusion. In a transcriptional gene fusion, the DNA or cDNA will contain its own control sequence directing appropriate production of protein (e.g., ribosome binding site, translation initiation codon, etc.), and the transcriptional control sequences (e.g., promoter elements and/or enhancers) will be provided by the vector. In a translational gene fusion, transcriptional control sequences as well as at least some of the translational control sequences (i.e., the translational initiation codon) will be provided by the vector. In the case of a translational gene fusion, a chimeric protein will be produced.

Genes also can be constructed for specific fusion proteins containing a functional Griffithsin component plus a fusion component conferring additional desired attribute(s) to the composite protein. For example, a fusion sequence for a toxin or immunological reagent can be added to facilitate purification and analysis of the functional protein.

Genes can be specifically constructed to code for fusion proteins, which contain a Griffithsin coupled to an effector protein, such as a toxin or immunological reagent, for specific targeting to a virus or viral-infected cells, e.g., HIV and/or HIV-infected cells or influenza and/or influenza-infected cells. In these instances, the Griffithsin moiety serves not only as a neutralizing agent but also as a targeting agent to direct the effector activities of these molecules selectively against a given virus, such as HIV or influenza. Thus, for example, a therapeutic agent can be obtained by combining the HIV-targeting function or influenza-targeting function of a functional Griffithsin with a toxin aimed at neutralizing infectious virus and/or by destroying cells producing infectious virus, such as HIV or influenza. Similarly, a therapeutic agent can be obtained, which combines the

viral-targeting function of a Griffithsin with the multivalency and effector functions of various immunoglobulin subclasses. Example 6 further illustrates the viral-targeting, specifically gp120-targeting, properties of a Griffithsin.

Similar rationales underlie extensive developmental therapeutic efforts exploiting the HIV gp120-targeting properties of sCD4. For example, sCD4-toxin conjugates have been prepared in which sCD4 is coupled to a Pseudomonas exotoxin component (Chaudhary et al., in The Human Retrovirus, Gallo et al., eds., Academic Press: San Diego, Calif. (1991), pp. 379-387; and Chaudhary et al., Nature, 335: 369-372 (1988)), or to a diphtheria toxin component (Aullo et al., EMBO J., 11: 575-583 (1992)) or to a ricin A-chain component (Till et al., Science, 242: 1166-1167 (1988)). Likewise, sCD4-immunoglobulin conjugates have been prepared in attempts to decrease the rate of in vivo clearance of functional sCD4 activity, to enhance placental transfer, and to effect a targeted recruitment of immunological mechanisms of pathogen elimination, such as phagocytic engulfment and killing by antibody-dependent cell-mediated cytotoxicity, to kill and/or remove HIV-infected cells and virus (Capon et al., Nature, 337: 525-531 (1989); Traunecker et al., Nature, 339: 68-70 (1989); and Langner et al. (1993), supra). While such CD4-immunoglobulin conjugates (sometimes called "immunoadhesins") have, indeed, shown advantageous pharmacokinetic and distributional attributes in vivo, and anti-HIV effects in vitro, clinical results have been discouraging (Schooley et al. (1990), supra; Husson et al. (1992), supra; and Langner et al. (1993), supra). This is not surprising since clinical isolates of HIV, as opposed to laboratory strains, are highly resistant to binding and neutralization by sCD4 (Orloff et al. (1995), supra; and Moore et al. (1992), supra). The Griffithsin polypeptide binds to a wide range of sugars present on viral glycoproteins and, therefore, can inhibit a wide range of viruses which display those glycoproteins. The extraordinarily broad targeting properties of a functional Griffithsin to viruses, e.g., primate retroviruses, in general, and clinical and laboratory strains, in particular, can be especially advantageous for combining with toxins, immunoglobulins and other selected effector proteins.

Viral-targeted conjugates can be prepared either by genetic engineering techniques (see, for example, Chaudhary et al. (1988), supra) or by chemical coupling of the targeting component with an effector component. The most feasible or appropriate technique to be used to construct a given Griffithsin conjugate or fusion protein will be selected based upon consideration of the characteristics of the particular effector molecule selected for coupling to a Griffithsin. For example, with a selected non-proteinaceous effector molecule, chemical coupling, rather than genetic engineering techniques, may be the only feasible option for creating the desired Griffithsin conjugate.

Accordingly, the invention also provides nucleic acid molecules encoding Griffithsin fusion proteins. In particular, the invention provides a nucleic acid molecule comprising SEQ ID NO: 4 and substantially homologous sequences thereof. Also provided is a vector comprising a nucleic acid sequence encoding a Griffithsin fusion protein and a method of obtaining a Griffithsin fusion protein by expression of the vector encoding a Griffithsin fusion protein in a protein-synthesizing organism as described above. Accordingly, Griffithsin fusion proteins are also provided.

In view of the above, the invention further provides an isolated and purified nucleic acid molecule, which comprises a Griffithsin coding sequence, such as one of the aforementioned nucleic acids, namely a nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3 or a nucleic acid molecule comprising a sequence of SEQ ID NO: 1 coupled to a second nucleic acid encoding an effector protein. The first nucleic acid preferably comprises a nucleic acid sequence encoding at least eight contiguous amino acids of the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, which encodes a functional Griffithsin, and the second nucleic acid preferably encodes an effector protein, such as a toxin or immunological reagent as described herein.

Accordingly, the invention also further provides an isolated and purified fusion protein encoded by a nucleic acid molecule comprising a sequence of SEQ ID NO: 1 or a nucleic acid molecule encoding an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, either one of which is coupled to a second nucleic acid encoding an effector protein. Preferably, the aforementioned nucleic acid molecules encode at least eight contiguous amino acids of the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, which desirably have anti-viral activity, coupled to an effector molecule, such as a toxin or immunological reagent as described above. Preferably, the effector

molecule targets a virus, such as any one or more of the viruses identified herein, especially an HIV virus, influenza virus (e.g., an H5N1 virus), Severe Acute Respiratory Syndrome (SARS) virus, Hepatitis C virus, or Ebola virus. When HIV or influenza is targeted, the effector molecule preferably targets glycoprotein gp120 of HIV or hemaglutinin of influenza. If the at least eight contiguous amino acids of SEQ ID NO: 3 (or SEQ ID NO: 2) comprise amino acids 1-121, desirably amino acids 46, 60, 71, and/or 104 have been rendered glycosylation-resistant, yet maintain antiviral activity by substitution of the asparagine at those positions with, for example, an alanine or a glutamine residue.

The coupling can be effected at the DNA level or by chemical coupling as described above. For example, a Griffithsin-effector protein conjugate of the invention can be obtained by (a) selecting a desired effector protein or peptide; (b) synthesizing a composite DNA coding sequence comprising a first DNA coding sequence comprising one of the aforementioned nucleic acid sequences, which codes for a functional Griffithsin, coupled to a second DNA coding sequence for an effector protein or peptide, e.g., a toxin or immunological reagent; (c) expressing said composite DNA coding sequence in an appropriate protein-synthesizing organism; and (d) purifying the desired fusion protein to substantially pure form. Alternatively, a Griffithsin-effector molecule conjugate of the invention can be obtained by (a) selecting a desired effector molecule and a Griffithsin or Griffithsin fusion protein; (b) chemically coupling the Griffithsin or Griffithsin fusion protein to the effector molecule; and (c) purifying the desired Griffithsin-effector molecule conjugate to substantially pure form.
Conjugates comprising a functional Griffithsin (e.g., an anti-viral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, as previously described, coupled to an anti-Griffithsin antibody, a virus, a viral glycoprotein, or at least one effector component, which can be the same or different, such as a toxin, an immunological reagent, an antiviral agent, or other functional reagent, can be designed even more specifically to exploit the unique viral targeting properties of Griffithsins.

Other functional reagents that can be used as effector components in the inventive conjugates can include, for example, polyethylene glycol, dextran, albumin, a solid support matrix, and the like, whose intended effector
functions may include one or more of the following: to improve stability of the conjugate; to increase the half-life of the conjugate; to increase resistance of the conjugate to proteolysis; to decrease the immunogenicity of the conjugate; to provide a means to attach or immobilize a functional Griffithsin onto a solid support matrix (e.g., see, for example, Harris, in Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Harris, ed., Plenum Press: New York (1992), pp. 1-14). Conjugates furthermore can comprise a functional Griffithsin coupled to more than one effector molecule, each of which, optionally, can have different effector functions (e.g., such as a toxin molecule (or an immunological reagent) and a polyethylene glycol (or dextran or albumin) molecule). Diverse applications and uses of functional proteins and peptides, such as in the present instance a functional Griffithsin, attached to or immobilized on a solid support matrix, are exemplified more specifically for poly(ethylene glycol) conjugated proteins or peptides in a review by Holmberg et al. (In Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Harris, ed., Plenum Press: New York (1992), pp. 303-324). Preferred examples of solid support matrices include magnetic beads, a flow-through matrix, and a matrix comprising a contraceptive device, such as a condom, a diaphragm, a cervical cap, a vaginal ring or a sponge.

Example 4 reveals novel gp120-directed effects of Griffithsins. Solid-phase ELISA experiments show that Griffithsin is capable of global conformational effects on gp120, as observed as a decrease of immunoreactivity at multiple, distinct, non-overlapping epitopes.

The range of anti-viral activity of Griffithsin against diverse CD4<+>-tropic immunodeficiency virus strains in various target cells is remarkable; virtually all tested strains of HIV-1, HIV-2 and SIV were similarly sensitive to Griffithsin; clinical isolates and laboratory strains showed essentially equivalent sensitivity. Cocultivation of chronically infected and uninfected CEM-SS cells with Griffithsin did not inhibit viral replication, but did cause a concentration-dependent inhibition of cell-to-cell fusion and virus transmission; similar results from binding and fusion inhibition assays employing HeLa-CD4-LTR-[beta]-galactosidase cells were consistent with Griffithsin inhibition of virus-cell and/or cell-cell binding.

The anti-viral, e.g., anti-HIV, activity of the Griffithsins and conjugates thereof of the invention can be further demonstrated in a series of interrelated in vitro anti-viral assays (Gulakowski et al., J. Virol. Methods, 33: 87-100 (1991)), which accurately predict for anti-viral activity in humans. These assays measure the ability of compounds to prevent the replication of HIV and/or the cytopathic effects of HIV on human target cells. These measurements directly correlate with the pathogenesis of HIV-induced disease in vivo. The results of the analysis of the anti-viral activity of Griffithsins or conjugates, as set forth in Examples 5-7 and 9, predict accurately the anti-viral activity of these products in vivo in humans and, therefore, establish the utility of the invention. Furthermore, since the invention also provides methods of ex vivo use of Griffithsins and conjugates, the utility of Griffithsins and conjugates thereof is even more certain.

The Griffithsins and conjugates thereof of the invention can be used to inhibit a broad range of viruses (see, e.g., Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint et al., eds., ASM Press: Washington, D.C. (2000), particularly Chapter 19). Examples of viruses that may be treated in accordance with the invention include, but are not limited to, Type C and Type D retroviruses, HTLV-1, HTLV-2, HIV, FIV, FLV, MLV, BLV, BIV, equine infectious virus, anemia virus, avian sarcoma viruses, such as Rous sarcoma virus (RSV), hepatitis type A, B, C, non-A and non-B viruses, arboviruses, varicella viruses, human herpes virus (e.g., HHV-6), measles, mumps, filovirus (e.g., Ebola, such as Ebola strains Sudan, Zaire, Cote d'Ivoire, and Reston), SARS virus, and rubella viruses. Griffithsins and conjugates thereof also can be used to inhibit influenza viral infection, such as an H5N1 viral infection, i.e., a Bird flu viral infection, (see, e.g., Fields Virology, third edition, Fields et al., eds., Lippincott-Raven Publishers: Philadelphia, Pa. (1996), particularly Chapter 45) prophylactically and therapeutically in accordance with the methods set forth herein.

The methods, compounds, and compositions described herein can be used to inhibit any of the foregoing viruses, as well as other viruses not specifically listed. However, the methods, compounds, and compositions described herein are especially useful for the inhibition of the H5N1 virus, SARS virus, Hepatitis C virus, and Ebola virus as well as retroviruses such as HIV.

Thus, the invention further provides a composition comprising (i) one or more of an above-described purified or isolated nucleic acid or variant thereof, optionally as part of an encoded fusion protein, and (ii) a carrier, excipient or adjuvant. Preferably, (i) is present in an antiviral effective amount and the composition is pharmaceutically acceptable. The composition can further comprise at least one additional active agent, such as an antiviral agent other than a Griffithsin (or antiviral fragment, fusion protein or conjugate thereof), in an antiviral effective amount. Suitable antiviral agents include AZT, ddA, ddI, ddC, 3TC gancyclovir, fluorinated dideoxynucleosides, acyclovir, [alpha]-interferon, nonnucleoside analog compounds, such as nevirapine (Shih et al., PNAS, 88: 9878-9882, (1991)), TIBO derivatives, such as R82913 (White et al., Antiviral Res., 16: 257-266 (1991)), Ro31-8959, BI-RJ-70 (Merigan, Am. J. Med., 90 (Suppl. 4A): 8S-17S (1991)), michellamines (Boyd et al., J. Med. Chem., 37: 1740-1745 (1994)) and calanolides (Kashman et al., J. Med. Chem., 35: 2735-2743 (1992)), nonoxynol-9, gossypol and derivatives, gramicidin, Enfurtide (i.e., T20), cyanovirin-N and functional homologs thereof (Boyd et al. (1997), supra). Other exemplary antiviral compounds include protease inhibitors (see R. C. Ogden and C. W. Flexner, eds., Protease Inhibitors in AIDS Therapy, Marcel Dekker, NY (2001)), such as saquinavir (see I. B. Duncan and S. Redshaw, in R. C. Ogden and C. W. Flexner, supra, pp. 27-48), ritonavir (see D. J. Kempf, in R. C. Ogden and C. W. Flexner, supra, pp. 49-64), indinavir (see B. D. Dorsey and J. P. Vacca, in R. C. Ogden and C. W. Flexner, supra, pp. 65-84), nelfinavir (see S. H. Reich, in R. C. Ogden and C. W. Flexner, supra, pp. 85-100), amprenavir (see R. D. Tung, in R. C. Ogden and C. W. Flexner, supra, pp. 101-118), and anti-TAT agents. If the composition is to be used to induce an immune response, it comprises an immune response-inducing amount of the inventive agent and can further comprise an immunoadjuvant, such as polyphosphazene polyelectrolyte.

The pharmaceutical composition can contain other pharmaceuticals, such as virucides, immunomodulators, immunostimulants, antibiotics and absorption enhancers.

Exemplary immunomodulators and immunostimulants include various interleukins, sCD4, cytokines, antibody preparations, blood transfusions, and cell transfusions. Exemplary antibiotics include antifungal agents, antibacterial agents, and anti-Pneumocystitis carnii agents. Exemplary absorption enhancers include bile salts and other surfactants, saponins, cyclodextrins, and

phospholipids (Davis (1992), supra).

An isolated cell comprising an above-described purified or isolated nucleic acid or variant thereof, optionally in the form of a vector, which is optionally targeted to a cell-surface receptor, is also provided. Examples of host cells include, but are not limited to, a human cell, a human cell line, E. coli, B. subtilis, P. aerugenosa, S. cerevisiae, and N. crassa. E. coli, in particular E. coli TB-1, TG-2, DH5[alpha], XL-Blue MRF' (Stratagene), SA2821 and Y1090. Preferably, the cell is a mammalian cell, bacterium, or yeast. A preferred bacterium is lactobacillus or other commensal microorganism. The above-described nucleic acid or variant thereof, optionally in the form of a vector, can be introduced into a host cell using such techniques as transfection, electroporation, transduction, micro-injection, transformation, and the like.

Accordingly, the invention provides a method of inhibiting prophylactically or therapeutically a viral infection, in particular an influenza viral infection (e.g., an H5N1 viral infection), a SARS infection, an Ebola infection, a Hepatitis C viral infection, or HIV infection, of a host. The method comprises administering to the host an effective amount of an anti-viral polypeptide, a variant thereof, an anti-viral polypeptide conjugate or an anti-viral fusion protein comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids are nonglycosylated and have anti-viral activity, whereupon the viral infection is inhibited. The anti-viral polypeptide can be derived from a Griffithsin obtained from Griffithsia or recombinantly produced in accordance with the methods described above. Nonglycosylated anti-viral polypeptides can be produced in prokaryotic cells/organisms. Amino acids 45, 60, 71, and/or 104 in such nonglycosylated antiviral polypeptides can be deleted or substituted, for example, with alanine or glutamine. Nonglycosylated antiviral polypeptides also can be produced in eukaryotic cells/organisms by expressing a portion of a Griffithsin, such as that of SEQ ID NO: 3, that does not contain a glycosylation site or all or a portion of a Griffithsin, such as that of SEQ ID NO: 3, which contains a glycosylation site that has been rendered glycosylation-resistant as described and exemplified herein. When the viral infection is an influenza viral infection and the anti-viral polypeptide, variant thereof, anti-viral polypeptide conjugate, or anti-viral fusion protein is administered topically to the host, preferably the anti-viral protein or anti-viral peptide is administered to the respiratory system of the host, preferably as an inhalant, e.g., an inhalant comprising an aerosol or microparticulate powder.

The prophylactic and therapeutic treatment of many viral infections, including influenza virus infections, is complicated by appearance of virus forms resistant to currently employed medications, such as neuromimidase inhibitors. The inventive method is particularly useful in this context, as the inventive anti-viral polypeptide or anti-viral polypeptide conjugate binds a wide range of glycoproteins present on the viral surface. Accordingly, the inventive anti-viral polypeptide, variant, conjugate, or fusion protein thereof, can be administered to an animal, preferably a human, dog, cat, bird, cow, pig, horse, lamb, mouse, or rat, in combination with other anti-viral agents to guard against the propagation of anti-viral-resistant strains of virus. In addition, it is thought that during adaptive mutation (e.g., resistance to neuraminidase inhibitors), the level of glycosylation found at the viral surface increases in some viruses, such as influenza. Thus, in that the inventive anti-viral agent binds sugars of viral surface glycoproteins, the inventive method provides a valuable complimentary therapy to current anti-viral regimens.

Griffithsins and conjugates thereof collectively comprise polypeptides and proteins, and, as such, are particularly susceptible to hydrolysis of amide bonds (e.g., catalyzed by peptidases) and disruption of essential disulfide bonds or formation of inactivating or unwanted disulfide linkages (Carone et al., J. Lab. Clin. Med., 100:1-14 (1982)). There are various ways to alter molecular structure, if necessary, to provide enhanced stability to the Griffithsin, variant, fusion protein, or conjugate thereof (Wunsch, Biopolymers, 22: 493-505 (1983); and Samanen, in Polymeric Materials in Medication, Gebelein et al., eds., Plenum Press: New York (1985) pp. 227-242), which may be essential for preparation and use of pharmaceutical compositions containing Griffithsins, or variants, fusion proteins, or conjugates thereof, for therapeutic or prophylactic applications against viruses, e.g., HIV, influenza, (e.g. H5N1), Hepatitis C, Ebola, or SARS. Possible options for useful chemical modifications of a Griffithsin, or variant, fusion protein or conjugate thereof, include, but are not limited to, the following (adapted from Samanen, J. M. (1985) supra): (a) olefin substitution, (b) carbonyl reduction, (c) D-amino acid substitution, (d) N-methyl substitution, (e) C-

methyl substitution, (f) C-C'-methylene insertion, (g) dehydro amino acid insertion, (h) retro-inverso modification, (i) N-terminal to C-terminal cyclization, and (j) thiomethylene modification. Griffithsins, variants, fusion proteins, and conjugates thereof also can be modified by covalent attachment of carbohydrate and polyoxyethylene derivatives, which are expected to enhance stability and resistance to proteolysis (Abuchowski et al., in Enzymes as Drugs, Holcenberg et al., eds., John Wiley: New York (1981), pp. 367-378).

Other important general considerations for design of delivery strategy systems and compositions, and for routes of administration, for protein and peptide drugs, such as Griffithsins, variants, fusion proteins, and conjugates thereof (Eppstein, CRC Crit. Rev. Therapeutic Drug Carrier Systems, 5: 99-139 (1988); Siddiqui et al., CRC Crit. Rev. Therapeutic Drug Carrier Systems, 3: 195-208 (1987); Banga et al., Int. J. Pharmaceutics, 48: 15-50 (1988); Sanders, Eur. I Drug Metab. Pharmacokinetics, 15: 95-102 (1990); and Verhoef, Eur. J. Drug Metab. Pharmacokinetics, 15: 83-93 (1990)), also apply. The appropriate delivery system for a given Griffithsin, variant, fusion protein, or conjugate thereof will depend upon its particular nature, the particular clinical application, and the site of drug action. As with any protein or peptide drug, oral delivery of a Griffithsin, variant, fusion protein, or a conjugate thereof will likely present special problems, due primarily to instability in the gastrointestinal tract and poor absorption and bioavailability of intact, bioactive drug therefrom. Therefore, especially in the case of oral delivery, but also possibly in conjunction with other routes of delivery, it will be necessary to use an absorption-enhancing agent in combination with a given Griffithsin, variant, fusion protein, or conjugate thereof. A wide variety of absorption-enhancing agents have been investigated and/or applied in combination with protein and peptide drugs for oral delivery and for delivery by other routes (Verhoef (1990), supra; van Hoogdalem, Pharmac. Ther., 44: 407-443 (1989); and Davis, J. Pharm. Pharmacol, 44(Suppl. 1): 186-190 (1992)). Most commonly, typical enhancers fall into the general categories of (a) chelators, such as EDTA, salicylates, and N-acyl derivatives of collagen, (b) surfactants, such as lauryl sulfate and polyoxyethylene-9-lauryl ether, (c) bile salts, such as glycolate and taurocholate, and derivatives, such as taurodihydrofusidate, (d) fatty acids, such as oleic acid and capric acid, and their derivatives, such as acylcarnitines, monoglycerides and diglycerides, (e) non-surfactants, such as unsaturated cyclic ureas, (f) saponins, (g) cyclodextrins, and (h) phospholipids.

Other approaches to enhancing oral delivery of protein and peptide drugs, such as the Griffithsins, variants, fusion proteins, and conjugates thereof, can include aforementioned chemical modifications to enhance stability to gastrointestinal enzymes and/or increased lipophilicity. Alternatively, or in addition, the protein or peptide drug can be administered in combination with other drugs or substances, which directly inhibit proteases and/or other potential sources of enzymatic degradation of proteins and peptides. Yet another alternative approach to prevent or delay gastrointestinal absorption of protein or peptide drugs, such as Griffithsins, variants, fusion proteins, or conjugates thereof, is to incorporate them into a delivery system that is designed to protect the protein or peptide from contact with the proteolytic enzymes in the intestinal lumen and to release the intact protein or peptide only upon reaching an area favorable for its absorption. A more specific example of this strategy is the use of biodegradable microcapsules or microspheres, both to protect vulnerable drugs from degradation, as well as to effect a prolonged release of active drug (Deasy, in Microencapsulation and Related Processes, Swarbrick, ed., Marcell Dekker, Inc.: New York (1984), pp. 1-60, 88-89, 208-211). Microcapsules also can provide a useful way to effect a prolonged delivery of a protein and peptide drug, such as a Griffithsin, variant, fusion protein, or conjugate thereof, after injection (Maulding, J. Controlled Release, 6: 167-176 (1987)).

Given the aforementioned potential complexities of successful oral delivery of a protein or peptide drug, it is fortunate that there are numerous other potential routes of delivery of a protein or peptide drug, such as a Griffithsin, variant, fusion protein, or conjugate thereof. These routes include topical, subcutaneous, intravenous, intraarterial, intrathecal, intracisternal, buccal, rectal, nasal, pulmonary, transdermal, vaginal, ocular, and the like (Eppstein (1988), supra; Siddiqui et al. (1987), supra; Banga et al. (1988), supra; Sanders (1990), supra; Verhoef (1990), supra; Barry, in Delivery Systems for Peptide Drugs, Davis et al., eds., Plenum Press: New York (1986), pp. 265-275; and Patton et al., Adv. Drug Delivery Rev, 8: 179-196 (1992)). With any of these routes, or, indeed, with any other route of administration or application, a protein or peptide drug, such as a Griffithsin, variant, fusion protein, or conjugate thereof, may initiate an immunogenic reaction. In such situations it may be necessary to modify the molecule in order to mask immunogenic groups.

It also can be possible to protect against undesired immune responses by judicious choice of method of formulation and/or administration. For example, site-specific delivery can be employed, as well as masking of recognition sites from the immune system by use or attachment of a so-called tolerogen, such as polyethylene glycol, dextran, albumin, and the like (Abuchowski et al. (1981), supra; Abuchowski et al., J. Biol. Chem., 252: 3578-3581 (1977); Lisi et al., J. Appl. Biochem, 4: 19-33 (1982); and Wileman et al., J. Pharm. Pharmacol, 38: 264-271 (1986)). Such modifications also can have advantageous effects on stability and half-life both in vivo and ex vivo.

Procedures for covalent attachment of molecules, such as polyethylene glycol, dextran, albumin and the like, to proteins, such as Griffithsins, variants, fusion proteins, or conjugates thereof, are well-known to those skilled in the art, and are extensively documented in the literature (e.g., see Davis et al., in Peptide and Protein Drug Delivery, Lee, ed., Marcel Dekker: New York (1991), pp. 831-864). Other strategies to avoid untoward immune reactions also can include the induction of tolerance by administration initially of only low doses. In any event, it will be apparent from the present disclosure to one skilled in the art that for any particular desired medical application or use of a Griffithsin or conjugate thereof, the skilled artisan can select from any of a wide variety of possible compositions, routes of administration, or sites of application, what is advantageous.

Accordingly, the anti-viral Griffithsins, variants, fusion proteins, and conjugates thereof of the invention can be formulated into various compositions for use, for example, either in therapeutic treatment methods for infected individuals, or in prophylactic methods against viral, e.g., HIV and influenza virus, infection of uninfected individuals.

The invention also provides a composition, such as a pharmaceutical composition, which comprises an isolated and purified Griffithsin, a variant thereof or fusion protein comprising the same, a Griffithsin conjugate, a matrix-anchored Griffithsin or a matrix-anchored Griffithsin conjugate, such as an anti-viral effective amount thereof. The composition can further comprise a carrier, such as a pharmaceutically acceptable carrier. The composition can further comprise at least one additional anti-viral compound other than a Griffithsin, variant, fusion protein, or conjugate thereof, such as in an anti-viral effective amount of an anti-viral compound. Suitable anti-viral compounds include cyanovirin, AZT, ddI, ddC, gancyclovir, fluorinated dideoxynucleosides, nevirapine, R82913, Ro 31-8959, BI-RJ-70, acyclovir, [alpha]-interferon, recombinant sCD4, michellamines, calanolides, nonoxynol-9, gossypol and derivatives thereof, neuroamidase inhibitors, amantatadine, rimantadine, enfurtide, and gramicidin. If the composition is to be used to induce an immune response, it comprises an immune response-inducing amount of a Griffithsin, variant, fusion protein, or conjugate thereof and can further comprise an immunoadjuvant, such as polyphosphazene polyelectrolyte. The Griffithsin used in the composition, e.g., pharmaceutical composition, can be isolated and purified from nature or genetically engineered. Similarly, the Griffithsin conjugate can be genetically engineered or chemically coupled.

The inventive compositions can be administered to a host, such as a human, so as to inhibit viral infection in a prophylactic or therapeutic method. The compositions of the invention are particularly useful in inhibiting the growth or replication of a virus, such as influenza virus (e.g., a H5N1 virus), a SARS virus, an Ebola virus, a Hepatitis C virus, or a retrovirus, in particular an influenza virus or an immunodeficiency virus, such as HIV, specifically HIV-1 and HIV-2, inhibiting infectivity of the virus, inhibiting the binding of virus to a host cell, and the like. The compositions are useful in the therapeutic or prophylactic treatment of hosts, e.g., animals, such as humans, who are infected with a virus or who are at risk for viral infection, respectively. The compositions also can be used to treat objects or materials, such as medical equipment, supplies, or fluids, including biological fluids, such as blood, blood products and vaccine formulations, cells, tissues and organs, to remove or inactivate virus in an effort to prevent or treat viral infection of a host, e.g., animal, such as a human. Such compositions also are useful to prevent sexual transmission of viral infections, e.g., HIV, which is the primary way in which the world's AIDS cases are contracted (Merson (1993), supra). Adherence of the inventive anti-viral polypeptide, variant, fusion protein, or conjugate thereof to a solid support, such as a filter, can be used in clinics to remove all or part of the viral content of a biological solution. For example, filters comprising the inventive anti-viral agents can be used to treat blood supplies prior to transfusion to reduce the risk of viral transmission. Such filters would find particular utility in clinics wherein risk of viral infection is high. It will be appreciated that total removal of the viral content of a biological solution

is not required to achieve a beneficial effect. Removal of even a fraction of virus from a biological solution decreases the risk of infection of a patient.

Potential virucides used or being considered for use against sexual transmission of HIV are very limited; present agents in this category include, for example, nonoxynol-9 (Bird, AIDS, 5: 791-796 (1991)), gossypol and derivatives (Polsky et al., Contraception, 39: 579-587 (1989); Lin, Antimicrob. Agents Chemother, 33: 2149-2151 (1989); and Royer, Pharmacol. Res, 24: 407-412 (1991)), and gramicidin (Bourinbair, Life Sci./Pharmacol. Lett, 54: PL5-9 (1994); and Bourinbair et al., Contraception, 49: 131-137 (1994)). The method of prevention of sexual transmission of viral infection, e.g., HIV infection, in accordance with the invention comprises vaginal, rectal, oral, penile or other topical treatment with an anti-viral effective amount of a Griffithsin and/or Griffithsin conjugate, alone or in combination with another anti-viral compound as described herein.

In a novel approach to anti-HIV prophylaxis pursued under auspices of the U.S. National Institute of Allergy and Infectious Diseases (NIAID) (e.g., as conveyed by Painter, USA Today, Feb. 13, 1996), vaginal suppository instillation of live cultures of lactobacilli was being evaluated in a 900-woman study. This study was based especially upon observations of anti-HIV effects of certain H_2O_2-producing lactobacilli in vitro (e.g., see published abstract by Hilier, from NIAID-sponsored Conference on "Advances in AIDS Vaccine Development," Bethesda, Md., Feb. 11-15, 1996). Lactobacilli readily populate the vagina, and indeed are a predominant bacterial population in most healthy women (Redondo-Lopez et al., Rev. Infect. Dis., 12: 856-872 (1990); Reid et al., Clin. Microbiol. Rev., 3: 335-344 (1990); Bruce and Reid, Can. J. Microbiol., 34: 339-343 (1988); Reu et al., J. Infect. Dis., 171: 1237-1243 (1995); Hilier et al., Clin. Infect. Dis., 16(Suppl 4): S273-S281; and Agnew et al., Sex. Transm. Dis., 22: 269-273 (1995)). Lactobacilli are also prominent, nonpathogenic inhabitants of other body cavities such as the mouth, nasopharynx, upper and lower gastrointestinal tracts, and rectum.

It is well-established that lactobacilli can be readily transduced using available genetic engineering techniques to incorporate a desired foreign DNA coding sequence, and that such lactobacilli can be made to express a corresponding desired foreign protein (see, e.g., Hols et al., Appl. and Environ. Microbiol., 60: 1401-1413 (1994)). Therefore, within the context of the present disclosure, it will be appreciated by one skilled in the art that viable host cells containing a DNA sequence or vector of the invention, and expressing a polypeptide, variant, or fusion protein of the invention, can be used directly as the delivery vehicle for a Griffithsin, variant, or fusion protein thereof to the desired site(s) in vivo. Preferred host cells for such delivery of Griffithsins, variants, or fusion proteins thereof directly to desired site(s), such as, for example, to a selected body cavity, can comprise bacteria or yeast. More specifically, such host cells can comprise suitably engineered strain(s) of lactobacilli, enterococci, or other common bacteria; such as E. coli, normal strains of which are known to commonly populate body cavities. More specifically yet, such host cells can comprise one or more selected nonpathogenic strains of lactobacilli, such as those described by Andreu et al. ((1995), supra), especially those having high adherence properties to epithelial cells, such as, for example, adherence to vaginal epithelial cells, and suitably transformed using the DNA sequences of the present invention.

As reviewed by McGroarty (FEMS Immunol. Med. Microbiol., 6: 251-264 (1993)) the "probiotic" or direct therapeutic application of live bacteria, particularly bacteria that occur normally in nature, more particularly lactobacilli, for treatment or prophylaxis against pathogenic bacterial or yeast infections of the urogenital tract, in particular the female urogenital tract, is a well-established concept. Recently, the use of a conventional probiotic strategy, in particular the use of live lactobacilli, to inhibit sexual transmission of HIV has been suggested, based specifically upon the normal, endogenous production of virucidal levels of H_2O_2 and/or lactic acid and/or other potentially virucidal substances by certain normal strains of lactobacilli (e.g., Hilier (1996), supra). However, the inventive use of non-mammalian cells, particularly bacteria, more particularly lactobacilli, specifically engineered with a foreign gene, more specifically a Griffithsin gene, to express an anti-viral substance, more specifically a protein, and even more specifically a Griffithsin, is heretofore unprecedented as a method of treatment of an animal, specifically a human, to prevent infection by a virus, specifically a retrovirus, more specifically HIV-1 or HIV-2.

Elmer et al. (JAMA, 275: 870-876 (1996)) have recently speculated that "genetic engineering offers the possibility of using microbes to deliver specific actions or products to the colon or other mucosal surfaces . . . other fertile areas for future study include defining the mechanisms of action of various biotherapeutic agents with the possibility of applying genetic engineering to enhance activities." Elmer et al. ((1996), supra) further point out that the terms "probiotic" and "biotherapeutic agent" have been used in the literature to describe microorganisms that have antagonistic activity toward pathogens in vivo; those authors more specifically prefer the term "biotherapeutic agent" to denote "microorganisms having specific therapeutic properties."

In view of the present disclosure, one skilled in the art will appreciate that the invention teaches an entirely novel type of "probiotic" or "biotherapeutic" treatment using specifically engineered strains of microorganisms provided herein which do not occur in nature. Nonetheless, available teachings concerning selection of optimal microbial strains, in particular bacterial strains, for conventional probiotic or biotherapeutic applications can be employed in the context of the invention. For example, selection of optimal lactobacillus strains for genetic engineering, transformation, direct expression of Griffithsins or conjugates thereof, and direct probiotic or biotherapeutic applications, to treat or prevent viral (e.g., HIV) infection, can be based upon the same or similar criteria, such as those described by Elmer et al. ((1996), supra), typically used to select normal, endogenous or "nonengineered" bacterial strains for conventional probiotic or biotherapeutic therapy. Furthermore, the recommendations and characteristics taught by McGroarty, particularly for selection of optimal lactobacillus strains for conventional probiotic use against female urogenital infections, are pertinent to the present invention: " . . . lactobacilli chosen for incorporation into probiotic preparations should be easy and, if possible, inexpensive to cultivate . . . strains should be stable, retain viability following freeze-drying and, of course, be non-pathogenic to the host . . . it is essential that lactobacilli chosen for use in probiotic preparations should adhere well to the vaginal epithelium . . . ideally, artificially implanted lactobacilli should adhere to the vaginal epithelium, integrate with the indigenous microorganisms present, and proliferate" (McGroarty (1993), supra). While McGroarty's teachings specifically address selections of "normal" lactobacillus strains for probiotic uses against pathogenic bacterial or yeast infections of the female urogenital tract, similar considerations will apply to the selection of optimal bacterial strains for genetic engineering and "probiotic" or "biotherapeutic" application against viral infections as particularly encompassed by the present invention.

Accordingly, the method of the invention for the prevention of sexual transmission of viral infection, e.g., HIV infection, comprises vaginal, rectal, oral, penile, or other topical, insertional, or instillational treatment with an anti-viral effective amount of a Griffithsin, a Griffithsin conjugate or fusion protein, a matrix-anchored Griffithsin or conjugate or fusion protein thereof, and/or viable host cells transformed to express a Griffithsin or conjugate or fusion protein thereof, alone or in combination with one or more other anti-viral compound (e.g., as described above). However, commensal organisms which produce Griffithsin or a fragment, homolog, or conjugate thereof can inhibit viruses other than HIV. For example, commensal microorganisms that produce the inventive polypeptide can be instilled in mucosal tissue at the site of influenza contact, such as nasal or oral mucosa, to inhibit influenza infection of a host.

Compositions for use in the prophylactic or therapeutic treatment methods of the invention comprise one or more Griffithsin(s), variant(s), or conjugate(s) or fusion protein(s) thereof, either one of which can be matrix-anchored, and desirably a carrier therefor, such as a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well-known to those who are skilled in the art, as are suitable methods of administration. The choice of carrier will be determined in part by the particular Griffithsin or conjugate or fusion protein thereof, as well as by the particular method used to administer the composition.

One skilled in the art will appreciate that various routes of administering a drug are available, and, although more than one route can be used to administer a particular drug, a particular route can provide a more immediate and more effective reaction than another route. For example, the anti-viral agent of the invention can be inhaled in methods of prophylactically treating a subject for influenza infection (e.g., an H5N1 infection). Delivery of the anti-viral agent to a location of initial viral contact, such as the nose or mouth, blocks the onset of infection. The anti-viral agent can be administered via subcutaneous injection. Alternatively, in acute or critical medical situations, the

anti-viral agent can be administered intravenously. In many cases of infection, a patient generates an immune response to a virus. However, the effects of the viral infection so severely compromise the health of the patient that an effective immune response is not reached prior to death. Administration of the anti-viral agent can prolong the life of the patient until a patient's natural immune defense clears the virus. Furthermore, one skilled in the art will appreciate that the particular pharmaceutical carrier employed will depend, in part, upon the particular Griffithsin or conjugate or fusion protein thereof employed, and the chosen route of administration. Accordingly, there is a wide variety of suitable formulations of the composition of the invention.

Formulations suitable for oral administration can consist of liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or fruit juice; capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solid, granules or freeze-dried cells; solutions or suspensions in an aqueous liquid; and oil-in-water emulsions or water-in-oil emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Suitable formulations for oral delivery can also be incorporated into synthetic and natural polymeric microspheres, or other means to protect the agents of the present invention from degradation within the gastrointestinal tract (see, for example, Wallace et al., Science, 260: 912-915 (1993)).

The anti-viral agent of the invention (e.g., Griffithsin, variants, fusion proteins, or conjugates thereof), alone or in combination with other anti-viral compounds, can be made into aerosol formulations or microparticulate powder formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

The anti-viral agent of the invention (e.g., Griffithsin, variants, fusion proteins, or conjugates thereof), alone or in combinations with other anti-viral compounds or absorption modulators, can be made into suitable formulations for transdermal application and absorption, such as a patch (Wallace et al. (1993), supra). Transdermal electroporation or iontophoresis also can be used to promote and/or control the systemic delivery of the compounds and/or compositions of the present invention through the skin (e.g., see Theiss et al., Meth. Find. Exp. Clin. Pharmacol., 13: 353-359 (1991)).

Formulations suitable for topical administration include lozenges comprising the active ingredient in a flavor, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier; as well as creams, emulsions, gels and the like containing, in addition to the active ingredient, such as, for example, freeze-dried lactobacilli or live lactobacillus cultures genetically engineered to directly produce a Griffithsin, variant, conjugate, or fusion protein thereof of the present invention, such carriers as are known in the art. Topical administration is preferred for the prophylactic and therapeutic treatment of influenza viral infection, e.g., H5N1 infection, such as through the use of an inhaler, for example.

Formulations for rectal administration can be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate. Formulations suitable for vaginal administration can be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such as, for example, freeze-dried lactobacilli or live lactobacillus cultures genetically engineered to directly produce a Griffithsin or conjugate or fusion protein thereof of the present invention, such carriers as are known in the art to be appropriate. Similarly, the active ingredient can be combined with a lubricant as a coating on a condom. Indeed, preferably, the active ingredient is applied to any contraceptive device, including, but not limited to, a condom, a diaphragm, a cervical cap, a vaginal ring, and a sponge.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-

aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

Formulations comprising a Griffithsin, variant, or fusion protein thereof, or Griffithsin conjugate suitable for virucidal (e.g., HIV, Hepatitis C, Ebola, SARS and H5N1) sterilization of inanimate objects, such as medical supplies or equipment, laboratory equipment and supplies, instruments, devices, and the like, can, for example, be selected or adapted as appropriate, by one skilled in the art, from any of the aforementioned compositions or formulations. Preferably, the Griffithsin, variant, or fusion protein thereof, is produced by recombinant DNA technology. The Griffithsin conjugate can be produced by recombinant DNA technology or by chemical coupling of a Griffithsin, variant, or fusion protein thereof, with an effector molecule as described above. Similarly, formulations suitable for ex vivo sterilization, inactivation, or removal of virus, such as infectious virus, from a sample, such as blood, blood products, sperm, or other bodily products, such as a fluid, cells, a tissue or an organ, or any other solution, suspension, emulsion, vaccine formulation (such as in the removal of infectious virus), or any other material which can be administered to a patient in a medical procedure, can be selected or adapted as appropriate by one skilled in the art, from any of the aforementioned compositions or formulations. However, suitable formulations for ex vivo sterilization or inactivation or removal of virus from a sample or on an inanimate object are by no means limited to any of the aforementioned formulations or compositions. For example, such formulations or compositions can comprise a functional Griffithsin, such as that which is encoded by SEQ ID NO: 3, or anti-viral fragment thereof, such as a fragment comprising at least eight contiguous amino acids of SEQ ID NO: 3, wherein the at least eight contiguous amino acids bind to a virus, or a variant, conjugate, or fusion protein of either of the foregoing, attached to a solid support matrix, to facilitate contacting or binding infectious virus in a sample or removing infectious virus from a sample as described above, e.g., a bodily product such as a fluid, cells, a tissue or an organ from an organism, in particular a mammal, such as a human, including, for example, blood, a component of blood (e.g., plasma, blood cells, and the like), or sperm. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3. Also preferably, the at least eight contiguous amino acids bind gp120 of HIV, in particular infectious HIV. As a more specific example, such a formulation or composition can comprise a functional Griffithsin, variant, conjugate or fusion protein thereof, attached to (e.g., coupled to or immobilized on) a solid support matrix comprising magnetic beads, to facilitate contacting, binding and removal of infectious virus, and to enable magnet-assisted removal of the virus from a sample as described above, e.g., a bodily product such as a fluid, cells, a tissue or an organ, e.g., blood, a component of blood, or sperm. Alternatively, and also preferably, the solid support matrix comprises a contraceptive device, such as a condom, a diaphragm, a cervical cap, a vaginal ring, or a sponge. The anti-viral agent also can be encapsulated or dispersed within a solid matrix, such as a vaginal ring or sponge. Methods for encapsulating biotherapeutics into, for example, biocompatible sustained release devices, are known in the art.

As an even more specific illustration, such a composition (e.g., for ex vivo) can comprise a functional (e.g., gp120-binding, HIV-inactivating) Griffithsin, variant, conjugate, or fusion protein thereof, attached to a solid support matrix, such as magnetic beads or a flow-through matrix, by means of an anti-Griffithsin antibody or at least one effector component, which can be the same or different, such as polyethylene glycol, albumin, or dextran. The conjugate can further comprise at least one effector component, which can be the same or different, selected from the group consisting of, for example, an immunological reagent and a toxin. A flow-through matrix would comprise, for instance, a configuration similar to an affinity column. The Griffithsin can be covalently coupled to a solid support matrix via an anti-Griffithsin antibody, described below. Methods of attaching an antibody to a solid support matrix are well-known in the art (see, for example, Harlow and Lane. Antibodies: A Laboratory Manual, Cold Springs Harbor Laboratory: Cold Spring Harbor, N.Y. (1988)). Alternatively, the solid support matrix, such as magnetic beads, can be coated with streptavidin, in which case the Griffithsin or fragment thereof (which comprises at least eight contiguous amino acids of SEQ ID NO: 3 or SEQ ID NO: 2) or variant thereof, or a conjugate or fusion protein of either one, is biotinylated. The at least eight contiguous amino acids of SEQ ID

NO: 2 desirably have anti-viral activity and preferably bind gp120 of HIV, which preferably is infectious. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3 or SEQ ID NO: 2. Such a composition can be prepared, for example, by biotinylating the Griffithsin, variant, conjugate, or fusion protein thereof, and then contacting the biotinylated protein or peptide with a (commercially available) solid support matrix, such as magnetic beads, coated with streptavidin. The use of biotinylation as a means to attach a desired biologically active protein or peptide to a streptavidin-coated support matrix, such as magnetic beads, is well-known in the art.

One skilled in the art will appreciate that a suitable or appropriate formulation can be selected, adapted or developed based upon the particular application at hand.

For ex vivo uses, such as virucidal treatments of inanimate objects or materials, blood or blood products, or tissues, the amount of Griffithsin, variant, conjugate thereof, fusion protein thereof, or composition of any of the foregoing, to be employed should be sufficient that any virus or virus-producing cells present will be rendered noninfectious or will be destroyed. For example, for HIV, this would require that the virus and/or the virus-producing cells be exposed to concentrations of Griffithsin in the range of 0.1-1000 nM. Similar considerations apply to in vivo applications. Therefore, the designation of "anti-viral effective amount" is used generally to describe the amount of a particular Griffithsin, variant, conjugate, fusion protein, or composition thereof required for anti-viral efficacy in any given application.

In view of the above, the invention also provides a method of inhibiting prophylactically or therapeutically a viral infection of a host in which an anti-viral effective amount of an above-described anti-viral polypeptide, variant, conjugate, or fusion protein is administered to the host. Upon administration of the anti-viral effective amount of the anti-viral polypeptide, variant, conjugate, or fusion protein, the viral infection is inhibited.

The invention additionally provides a method of prophylactically or therapeutically inhibiting a viral infection of a host in which an anti-viral effective amount of a composition comprising an isolated and purified anti-viral polypeptide, variant, or anti-viral polypeptide conjugate or fusion protein, either one of which comprises at least eight contiguous amino acids of SEQ ID NO: 3 having anti-viral activity, attached to or encapsulated within a solid support matrix is administered to the host. By "therapeutically" is meant that the host already has been infected with the virus. By "prophylactically" is meant that the host has not yet been infected with the virus but is at risk of being infected with the virus. Prophylactic treatment is intended to encompass any degree of inhibition of viral infection, including, but not limited to, complete inhibition, as one of ordinary skill in the art will readily appreciate that any degree in inhibition of viral infection is advantageous. Preferably, the inventive active agent is administered before viral infection or immediately upon determination of viral infection and is continuously administered until the virus is undetectable. The method optionally further comprises the prior, simultaneous or subsequent administration, by the same route or a different route, of an antiviral agent or another agent that is efficacious in inhibiting the viral infection. Upon administration of the anti-viral effective amount of the composition, the viral infection is inhibited. Preferably, the solid support matrix is a contraceptive device, such as a condom, diaphragm, cervical cap, vaginal ring, or sponge. In an alternative embodiment, a solid support matrix can be surgically implanted and later removed.

For in vivo uses, the dose of a Griffithsin, variant, conjugate, fusion protein, or composition thereof, administered to an animal, particularly a human, in the context of the invention should be sufficient to effect a prophylactic or therapeutic response in the individual over a reasonable time frame. The dose used to achieve a desired anti-viral concentration in vivo (e.g., 0.1-1000 nM) will be determined by the potency of the particular Griffithsin, variant, fusion protein, or conjugate employed, the severity of the disease state of infected individuals, as well as, in the case of systemic administration, the body weight and age of the infected individual. The size of the dose also will be determined by the existence of any adverse side effects that may accompany the particular Griffithsin, variant, fusion protein, or conjugate or composition thereof, employed. It is always desirable, whenever possible, to keep adverse side effects to a minimum.

The invention also provides a method of removing virus, such as infectious virus, from a sample. The method comprises contacting the sample with a composition comprising an isolated and

purified anti-viral polypeptide, variant, conjugate, or fusion protein thereof, comprising at least eight contiguous amino acids of SEQ ID NO: 3 (or SEQ ID NO: 2). The at least eight contiguous amino acids desirably have anti-viral activity and bind to the virus and the anti-viral polypeptide (or conjugate or fusion protein of either of the foregoing) is attached to a solid support matrix, such as a magnetic bead. "Attached" is used herein to refer to attachment to (or coupling to) and immobilization in or on a solid support matrix. While any means of attachment can be used, preferably, attachment is by covalent bonds. The method further comprises separating the sample and the composition by any suitable means, whereupon the virus, such as infectious virus, is removed from the sample. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3 (or SEQ ID NO: 2). In one embodiment, the anti-viral polypeptide is conjugated with an anti-Griffithsin antibody or at least one effector component, which can be the same or different, selected from polyethylene glycol, dextran and albumin, in which case the anti-viral polypeptide is desirably attached to the solid support matrix through at least one effector component. The anti-viral polypeptide can be further conjugated with at least one effector component, which can be the same or different, selected from the group consisting of an immunological reagent and a toxin. In another embodiment, the solid support matrix is coated with streptavidin and the anti-viral polypeptide is biotinylated. Through biotin, the biotinylated anti-viral polypeptide is attached to the streptavidin-coated solid support matrix. Other types of means, as are known in the art, can be used to attach a functional Griffithsin (i.e., an anti-viral polypeptide, variant, conjugate, or fusion protein, as described above) to a solid support matrix, such as a magnetic bead, in which case contact with a magnet is used to separate the sample and the composition. Similarly, other types of solid support matrices can be used, such as a matrix comprising a porous surface or membrane, over or through which a sample is flowed or percolated, thereby selectively entrapping or removing infectious virus from the sample. The choice of solid support matrix, means of attachment of the functional Griffithsin to the solid support matrix, and means of separating the sample and the matrix-anchored Griffithsin will depend, in part, on the sample (e.g., fluid vs. tissue) and the virus to be removed. It is expected that the use of a selected coupling molecule can confer certain desired properties to a matrix, comprising a functional Griffithsin coupled therewith, that may have particularly advantageous properties in a given situation. Preferably, the sample is blood, a component of blood, sperm, cells, tissue or an organ. Also, preferably the sample is a vaccine formulation, in which case the virus that is removed is infectious, such as HIV, although HIV, in particular infectious HIV, can be removed from other samples in accordance with this method. In another embodiment, the virus that is removed is a SARS, Ebola, Hepatitis C, or H5N1 virus.

For instance, the skilled practitioner might select a poly(ethylene glycol) molecule for attaching a functional Griffithsin to a solid support matrix, thereby to provide a matrix-anchored Griffithsin, wherein the Griffithsin is attached to the matrix by a longer "tether" than would be feasible or possible for other attachment methods, such as biotinylation/streptavidin coupling. A Griffithsin coupled by a poly(ethylene glycol) "tether" to a solid support matrix (such as magnetic beads, porous surface or membrane, and the like) can permit optimal exposure of a binding surface, epitope, hydrophobic or electrophilic focus, and/or the like, on a functional Griffithsin in a manner that, in a given situation and/or for a particular virus, facilitates the binding and/or inactivation of the virus. A preferred solid support matrix is a magnetic bead such that separation of the sample and the composition is effected by a magnet. In a preferred embodiment of the method, the at least eight contiguous amino acids bind gp120 of HIV and HIV is removed from the sample.

Similarly, other types of solid support matrices can be used, such as a matrix comprising a porous surface or membrane, over or through which a sample is flowed or percolated, thereby selectively inhibiting infectious virus (e.g., HIV or influenza) in the sample. The choice of solid support matrix, means of attachment of the functional Griffithsin to the solid support matrix, and means of separating the sample and the matrix-anchored Griffithsin will depend, in part, on the sample (e.g., fluid vs. tissue) and the virus to be inhibited. It is expected that the use of a selected coupling molecule can confer certain desired properties to a matrix, comprising a functional Griffithsin coupled therewith, that may have particularly advantageous properties in a given situation.

The methods described herein also have utility in real time ex vivo inhibition of virus or virus infected cells in a bodily fluid, such as blood, e.g., in the treatment of viral infection, or in the inhibition of virus in blood or a component of blood, e.g., for transfusion, in the inhibition or prevention of viral infection. Such methods also have potential utility in dialysis, such as kidney

dialysis, and in inhibiting virus in sperm obtained from a donor for in vitro and in vivo fertilization. The methods also have applicability in the context of tissue and organ transplantations.

In summary, a Griffithsin attached to a solid support matrix, such as a magnetic bead, can be used to remove virus, in particular infectious virus, including immunodeficiency virus, such as HIV, e.g., HIV-1 or HIV-2, SARS, Ebola, H5N1, and Hepatitis C, from a sample, such as a sample comprising both infectious and noninfectious virus. The inventive method also can be used to remove viral glycoprotein presenting cells, e.g., infected cells that have, for example, gp120 on their surfaces, from a sample.

The invention, therefore, further provides a composition comprising naturally-occurring, non-infectious virus, such as a composition produced as described above. The composition can further comprise a carrier, such as a biologically or pharmaceutically acceptable carrier, and an immuno-adjuvant. Preferably, the noninfectious virus is an influenza (e.g., H5N1), an Ebola virus, a SARS virus, a Hepatitis C virus, or an immunodeficiency virus, such as HIV, e.g., HIV-1 or HIV-2. Alternatively, and also preferably, the noninfectious virus is FIV. A composition comprising only naturally-occurring, non-infectious virus has many applications in research and the prophylactic treatment of a viral infection. In terms of prophylactic treatment of a viral infection, the skilled artisan will appreciate the need to eliminate completely all infectious virus from the composition. If desired, further treatment of the composition comprising non-infectious particles with virus-inactivating chemicals, such as imines or psoralens, and/or pressure or heat inactivation, will further the non-infectious nature of the composition. For example, an immune response-inducing amount of the inventive composition can be administered to an animal at risk for a viral infection in order to induce an immune response. The skilled artisan will appreciate that such a composition is a significant improvement over previously disclosed compositions in that the virus is non-infectious and naturally-occurring. Thus, there is no risk of inadvertent infection, greater doses can be administered in comparison to compositions comprising infectious viral particles, and the subsequent immune response will assuredly be directed to antigens present on naturally-occurring virus. The composition comprising naturally-occurring, non-infectious virus can be administered in any manner appropriate to induce an immune response. Preferably, the virus is administered, for example, intramuscularly, mucosally, intravenously, subcutaneously, or topically. Preferably, the composition comprises naturally-occurring, non-infectious human immunodeficiency virus comprising gp120.

The composition comprising naturally-occurring, non-infectious virus can be combined with various carriers, adjuvants, diluents or other anti-viral therapeutics, if desired. Appropriate carriers include, for example, ovalbumin, albumin, globulins, hemocyanins, and the like. Adjuvants or immuno-adjuvants are incorporated in most cases to stimulate further the immune system. Any physiologically appropriate adjuvant can be used. Suitable adjuvants for inclusion in the inventive composition include, for example, aluminum hydroxide, beryllium sulfate, silica, kaolin, carbon, bacterial endotoxin, saponin, and the like.

Thus, the invention also provides a method of inducing an immune response to a virus in an animal. The method comprises administering to the animal an immune response-inducing amount of a composition comprising naturally-occurring, non-infectious virus as described above.

The appropriate dose of a composition comprising naturally-occurring, non-infectious virus required to induce an immune response to the virus in an animal is dependent on numerous factors, such as size of the animal and immune competency. The amount of composition administered should be sufficient to induce a humoral and/or cellular immune response. The amount of non-infectious virus in a particular composition can be determined using routine methods in the art, such as the Coulter HIV p24 antigen assay (Coulter Corp., Hialeah, Fla.). Any suitable dose of a composition comprising non-infectious virus is appropriate so long as an immune response is induced, desirably without the appearance of harmful side effects to the host. In this regard, compositions comprising from about 10<1 >to about 10<5 >particles, preferably from about 10<2 >to about 10<4 >particles, most preferably about 10<3 >particles, are suitable for inducing an immune response.

One of ordinary skill can determine the effectiveness of the composition to induce an immune response using routine methods known in the art. Cell-mediated response can be determined by employing, for example, a virus antigen-stimulated T-cell proliferation assay. The presence of a humoral immune response can be determined, for instance, with the Enzyme Linked Immunosorbent Assay (ELISA). The skilled artisan will appreciate that there are numerous other suitable assays for evaluating induction of an immune response. To the extent that a dose is inadequate to induce an appropriate immune response, "booster" administrations can subsequently be administered in order to prompt a more effective immune response.

In terms of administration of the inventive anti-viral agents or conjugates thereof, the dosage can be in unit dosage form, such as a tablet or capsule. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of a Griffithsin or conjugate thereof, alone or in combination with other anti-viral agents, calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier, or vehicle.

The specifications for the unit dosage forms of the invention depend on the particular Griffithsin, variant, fusion protein, conjugate, or composition thereof, employed and the effect to be achieved, as well as the pharmacodynamics associated with each Griffithsin, variant, fusion protein, conjugate, or composition thereof, in the host. The dose administered should be an "anti-viral effective amount" or an amount necessary to achieve an "effective level" in the individual patient.

Since the "effective level" is used as the preferred endpoint for dosing, the actual dose and schedule can vary, depending upon interindividual differences in pharmacokinetics, drug distribution, and metabolism. The "effective level" can be defined, for example, as the blood or tissue level (e.g., 0.1-1000 nM) desired in the patient that corresponds to a concentration of one or more Griffithsin or conjugate thereof, which inhibits a virus, such as HIV, in an assay known to predict for clinical anti-viral activity of chemical compounds and biological agents. The "effective level" for agents of the invention also can vary when the Griffithsin, or conjugate or composition thereof, is used in combination with AZT or other known anti-viral compounds or combinations thereof.

One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired "effective concentration" in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the "effective concentration" of the compounds of the invention by a direct (e.g., analytical chemical analysis) or indirect (e.g., with surrogate indicators such as p24 or RT) analysis of appropriate patient samples (e.g., blood and/or tissues).

In the treatment of some virally infected individuals, it can be desirable to utilize a "mega-dosing" regimen, wherein a large dose of the Griffithsin or conjugate thereof is administered, time is allowed for the drug to act, and then a suitable reagent is administered to the individual to inactivate the drug.

The pharmaceutical composition can contain other pharmaceuticals, in conjunction with the Griffithsin, variant, fusion protein, or conjugate thereof, when used to therapeutically treat a viral infection, such as an influenza infection or an HIV infection which results in AIDS. Representative examples of these additional pharmaceuticals include anti-viral compounds, virucides, immunomodulators, immuno stimulants, antibiotics and absorption enhancers. Exemplary anti-viral compounds include cyanovirin, AZT, ddI, ddC, gancylclovir, fluorinated dideoxynucleosides, nonnucleoside analog compounds, such as nevirapine (Shih et al., PNAS, 88: 9878-9882 (1991)), TIBO derivatives, such as R82913 (White et al., Anti-viral Res., 16: 257-266 (1991)), BI-RJ-70 (Merigan, Am. J. Med., 90 (Suppl. 4A): 8S-17S (1991)), michellamines (Boyd et al., J. Med. Chem., 37: 1740-1745 (1994)) and calanolides (Kashman et al., J. Med. Chem., 35: 2735-2743 (1992)), nonoxynol -9, gossypol and derivatives, gramicidin (Bourinbair et al. (1994), supra), neuraminidase inhibitors, amantadine, enfurtide, and the like. Exemplary immunomodulators and immunostimulants include various interleukins, sCD4, cytokines, antibody preparations, blood transfusions, and cell transfusions. Exemplary antibiotics include antifungal agents, antibacterial agents, and anti-Pneumocystitis carnii agents. Exemplary absorption enhancers include bile salts and other surfactants, saponins, cyclodextrins, and phospholipids (Davis (1992), supra).

Administration of a Griffithsin, variant, conjugate, or fusion protein thereof with other anti-retroviral agents and particularly with known RT inhibitors, such as ddC, AZT, ddI, ddA, or other inhibitors that act against other viral, e.g., HIV, proteins, such as anti-TAT agents, is expected to inhibit most or all replicative stages of the viral life cycle. The dosages of ddC and AZT used in AIDS or ARC patients have been published. A virustatic range of ddC is generally between 0.05 [mu]M to 1.0 [mu]M. A range of about 0.005-0.25 mg/kg body weight is virustatic in most patients. The preliminary dose ranges for oral administration are somewhat broader, for example 0.001 to 0.25 mg/kg given in one or more doses at intervals of 2, 4, 6, 8, 12, etc. hours. Currently, 0.01 mg/kg body weight ddC given every 8 hrs is preferred. When given in combined therapy, the other anti-viral compound, for example, can be given at the same time as the Griffithsin or conjugate thereof or the dosing can be staggered as desired. The two drugs also can be combined in a composition. Doses of each can be less when used in combination than when either is used alone.

It will also be appreciated by one skilled in the art that a DNA sequence of a Griffithsin, variant, conjugate, or fusion protein thereof of the invention can be inserted ex vivo into mammalian cells previously removed from a given animal, in particular a human, host. Such cells can be employed to express the corresponding Griffithsin, variant, conjugate or fusion protein in vivo after reintroduction into the host. Feasibility of such a therapeutic strategy to deliver a therapeutic amount of an agent in close proximity to the desired target cells and pathogens, i.e., virus, more particularly SARS, Ebola, Hepatitis C, H5N1, and retrovirus, specifically HIV and its envelope glycoprotein gp120, has been demonstrated in studies with cells engineered ex vivo to express sCD4 (Morgan et al. (1994), supra). It is also possible that, as an alternative to ex vivo insertion of the DNA sequences of the invention, such sequences can be inserted into cells directly in vivo, such as by use of an appropriate viral vector. Such cells transfected in vivo are expected to produce anti-viral amounts of Griffithsin, variant, conjugate, or fusion protein thereof directly in vivo.

Given the present disclosure, it will be additionally appreciated that a DNA sequence corresponding to a Griffithsin, variant, fusion protein, or conjugate thereof can be inserted into suitable nonmammalian host cells, and that such host cells will express therapeutic or prophylactic amounts of a Griffithsin, variant, conjugate, or fusion protein thereof directly in vivo within a desired body compartment of an animal, in particular a human. Example 5 illustrates the transformation and expression of effective virucidal amounts of a Griffithsin in a non-mammalian cell, more specifically a bacterial cell. In a preferred embodiment of the invention, a method of female-controllable prophylaxis against HIV infection comprises the intravaginal administration and/or establishment of, in a female human, a persistent intravaginal population of lactobacilli that have been transformed with a coding sequence of the invention to produce, over a prolonged time, effective virucidal levels of a Griffithsin, variant, fusion protein, or conjugate thereof, directly on or within the vaginal and/or cervical and/or uterine mucosa. It is noteworthy that both the World Health Organization (WHO), as well as the U.S. National Institute of Allergy and Infectious Diseases, have pointed to the need for development of female-controlled topical microbicides, suitable for blocking the transmission of HIV, as an urgent global priority (Lange et al., Lancet, 341: 1356 (1993); Fauci, NIAID News, Apr. 27, 1995). A composition comprising the inventive anti-viral agent and a solid-support matrix is particularly useful in this regard, particularly when the solid-support matrix is a contraceptive device, such as a condom, a diaphragm, a cervical cap, a vaginal ring, or a sponge. In another embodiment, a colony of commensal organisms transduced with the nucleic acid of the invention and producing the inventive anti-viral agent is applied to mucosal tissue associated with the onset of influenza infection, such as respiratory or oral mucosal.

The invention also provides antibodies directed to the polypeptides of the invention. The availability of antibodies to any given protein is highly advantageous, as it provides the basis for a wide variety of qualitative and quantitative analytical methods, separation and purification methods, and other useful applications directed to the subject polypeptides. Accordingly, given the present disclosure and the polypeptides of the invention, it will be readily apparent to one skilled in the art that antibodies, in particular antibodies specifically binding to a polypeptide of the invention, can be prepared using well-established methodologies (e.g., such as the methodologies described in detail by Harlow and Lane, in Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (1988), pp. 1-725). Such antibodies can comprise both polyclonal and monoclonal antibodies. Furthermore, such antibodies can be obtained and employed either in solution-phase or coupled to a desired solid-phase matrix, such as magnetic beads or a flow through

matrix. Having in hand such antibodies as provided by the invention, one skilled in the art will further appreciate that such antibodies, in conjunction with well-established procedures (e.g., such as described by Harlow and Lane (1988), supra) comprise useful methods for the detection, quantification, or purification of a Griffithsin, conjugate thereof, or host cell transformed to produce a Griffithsin or conjugate or fusion protein thereof. Example 6 further illustrates an antibody that specifically binds to a Griffithsin. Accordingly, the invention further provides a composition comprising an anti-Griffithsin antibody bound to the anti-viral agent of the invention, preferably an anti-viral polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3.

Matrix-anchored anti-Griffithsin antibodies also can be used in a method to remove virus in a sample. Preferably, the antibody binds to an epitope of an anti-viral polypeptide of SEQ ID NO: 2 or SEQ ID NO: 3. Preferably, the matrix is a solid support matrix, such as a magnetic bead or a flow-through matrix. If the solid support matrix to which the anti-Griffithsin antibody is attached comprises magnetic beads, removal of the antibody-Griffithsin-virus complex can be readily accomplished using a magnet.

In view of the above, the invention provides a method of removing virus from a sample. The method comprises (a) contacting the sample with a composition comprising an isolated and purified anti-viral polypeptide, variant, or conjugate or fusion protein thereof, wherein (i) the anti-viral polypeptide comprises at least eight contiguous amino acids of SEQ ID NO: 3, and (ii) the at least eight contiguous amino acids bind to the virus, and (b) contacting the sample with an anti-Griffithsin antibody attached to a solid support matrix, whereupon the anti-Griffithsin antibody binds to the anti-viral polypeptide or conjugate or fusion protein thereof to which is bound the virus, and (c) separating the solid support matrix from the sample, whereupon the virus is removed from the sample. Preferably, the anti-viral polypeptide comprises SEQ ID NO: 3. The virus that is removed can be any virus, e.g., a Hepatitis C, SARS, Ebola, and H5N1 virus. Desirably, the virus that is removed is infectious, such as HIV, Hepatitis C, Ebola, SARS, and H5N1. The sample can be blood, a component of blood, sperm, cells, tissue or an organ.

The antibody for use in the aforementioned method is an antibody that binds to a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, and, which polypeptide can bind to and inactivate a virus. The antibody can be coupled to the solid support matrix using similar methods and with similar considerations as described above for attaching a Griffithsin to a solid support matrix. For example, coupling methods and molecules employed to attach an anti-Griffithsin antibody to a solid support matrix, such as magnetic beads or a flow-through matrix, can employ biotin/streptavidin coupling or coupling through molecules, such as polyethylene glycol, albumin or dextran. Also analogously, it can be shown that, after such coupling, the matrix-anchored anti-Griffithsin antibody retains its ability to bind to a polypeptide comprising at least eight contiguous amino acids of SEQ ID NO: 3, which polypeptide can bind to and inactivate a virus.

The invention also provides an anti-Griffithsin antibody that is anti-idiotypic in respect to a viral glycoprotein, such as gp120, i.e., has an internal image of gp120 of a primate immunodeficiency virus. Preferably, the antibody can compete with gp120 of a primate immunodeficiency virus for binding to a Griffithsin. In this regard, the primary immunodeficiency virus preferably is HIV-1 or HIV-2 and the Griffithsin preferably consists essentially of SEQ ID NO: 2 or SEQ ID NO: 3. Anti-idiotypic antibodies can be generated in accordance with methods known in the art (see, for example, Benjamin, in Immunology: a short course, Wiley-Liss, NY (1996), pp. 436-437; Kuby, in Immunology, 3rd ed., Freeman, N.Y. (1997), pp. 455-456; Greenspan et al., FASEB J., 7: 437-443 (1993); and Poskitt, Vaccine, 9: 792-796 (1991)). Such an anti-idiotypic (in respect to gp120) anti-Griffithsin antibody is useful in a method of inhibiting infection of an animal with a virus as provided herein.

In view of the above, a Griffithsin can be administered to an animal, the animal generates anti-Griffithsin antibodies, among which are antibodies that have an internal image of a viral glycoprotein, such as gp120. In accordance with well-known methods, polyclonal or monoclonal antibodies can be obtained, isolated, and selected. Selection of an anti-Griffithsin antibody that has an internal image of gp120 can be based upon competition between the anti-Griffithsin antibody

and gp120 for binding to a Griffithsin, or upon the ability of the anti-Griffithsin antibody to bind to a free Griffithsin as opposed to a Griffithsin bound to gp120. Such an anti-Griffithsin antibody can be administered to an animal to inhibit a viral infection in accordance with methods provided herein. Although nonhuman anti-idiotypic antibodies, such as an anti-Griffithsin antibody that has an internal image of gp120 and, therefore, is anti-idiotypic to gp120, are proving useful as vaccine antigens in humans, their favorable properties might, in certain instances, be further enhanced and/or their adverse properties further diminished, through "humanization" strategies, such as those recently reviewed by Vaughan (Nature Biotech., 16: 535-539 (1998)). Alternatively, a Griffithsin can be directly administered to an animal to inhibit a viral infection in accordance with methods provided herein such that the treated animal, itself, generates an anti-Griffithsin antibody that has an internal image of gp120. The production of anti-idiotypic antibodies, such as anti-Griffithsin antibody that has an internal image of gp120 and, therefore, is anti-idiotypic to gp120, in an animal to be treated is known as "anti-idiotype induction therapy," and is described by Madiyalakan et al. (Hybridoma, 14: 199-203 (1995)), for example.

In view of the above, the invention enables another method of inhibiting infection of an animal, such as a mammal, in particular a human, with a virus. The method comprises administering to the animal an anti-Griffithsin antibody, or a composition comprising same, in an amount sufficient to induce in the animal an immune response to the virus, whereupon the infection of the animal with the virus is inhibited. Preferably, the anti-Griffithsin antibody has an internal image of a viral glycoprotein, such as gp120 of an immunodeficiency virus with which the animal can be infected, such as a primate immunodeficiency virus. Preferably, the antibody can compete with, for example, gp120 of a primate immunodeficiency virus for binding to a Griffithsin. In this regard, the primate immunodeficiency virus preferably is HIV-1 or HIV-2 and the Griffithsin preferably consists essentially of SEQ ID NO: 3 or SEQ ID NO: 2. The method can further comprise the administration of an immunostimulant.

Also enabled by the invention is yet another method of inhibiting infection of an animal, such as a mammal, in particular a human, with a virus. The method comprises administering to the animal a Griffithsin, which binds a viral glycoprotein, such as gp120 of an immunodeficiency virus with which the animal can be infected, in an amount sufficient to induce in the animal an anti-Griffithsin antibody in an amount sufficient to induce an immune response to a virus sufficient to inhibit infection of the animal with the virus. Preferably, the anti-Griffithsin antibody has an internal image of gp120 of an immunodeficiency virus with which the animal can be infected, such as a primate immunodeficiency virus. Preferably, the antibody can compete with gp120 of a primate immunodeficiency virus for binding to a Griffithsin. In this regard, the primate immunodeficiency virus preferably is HIV-1 or HIV-2 and the Griffithsin preferably consists essentially of SEQ ID NO: 2 or SEQ ID NO: 3.

With respect to the above methods, sufficient amounts can be determined in accordance with methods known in the art. Similarly, the sufficiency of an immune response in the inhibition of a viral infection in an animal also can be assessed in accordance with methods known in the art.

Either one of the above methods can further comprise concurrent, pre- or post-treatment with an adjuvant to enhance the immune response, such as the prior, simultaneous or subsequent administration, by the same or a different route, of an antiviral agent or another agent that is efficacious in inducing an immune response to the virus, such as an immunostimulant. See, for example, Harlow et al. (1988), supra.
The inventive Griffithsins, conjugates, host cells, antibodies, compositions and methods are further described in the context of the following examples. These examples serve to illustrate further the present invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

This example illustrates a method of isolating and purifying Griffithsin from Griffithsia sp. and elucidating the Griffithsin amino acid sequence.

Anti-HIV bioassay guided fractionation was used to track the isolation of the Griffithsin polypeptide. In brief, the cellular mass from Griffithsia sp. was harvested by filtration, freeze-dried, and extracted first with H2O followed by (1:1) MeOH-CH2O2. Individual aliquots of the organic and aqueous extracts were tested for cytoprotective properties in the NCI primary anti-HIV screen (Weislow et al. J. Natl. Cancer Inst., 81: 577-586 (1989)). Only the H2O extract showed anti-HIV activity.

A freeze-dried aqueous extract (10 g) was brought to a concentration of 50 mg/ml by addition of DDH2O and maintained on ice. Crystalline ammonium sulfate (Sigma, St. Louis, Mo.; molecular biology grade) was added to the solution such that the final concentration of the mixture was 75% saturation. The mixture was allowed to precipitate on ice over night, and was then centrifuged at 3000 rpm for 50 min. The resulting pellets were set aside. The supernatant was brought to 1 M ammonium sulfate followed by another round of precipitation and centrifugation. The pellets from the second centrifugation were saved, and the resulting supernatant was filtered using a 0.22 [mu]m filter and subjected to hydrophobic interaction chromatography. A BioCad workstation (Perseptive Biosystems) was used for the following column chromatographies. The protein solution from the centrifugation and filtration steps was injected onto a Poros PE column (10*100 mm, Perseptive Biosystems) pre-equilibrated with a starting buffer of 50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.5. The column was eluted at a flow rate of 15 ml/min over the following gradient: (1) 7 column volumes (CV, equal to 7.85 ml) of the starting buffer; (2) 1.5-0 M ammonium sulfate over 2 CV; (3) 0 M ammonium sulfate for 15 CV. The eluate was monitored for both conductivity and absorbance (280 nm). Ammonium sulfate was added to the void fraction possessing anti-HIV activity to bring the final concentration to 75% saturation. The mixture was allowed to precipitate on ice overnight, and was then centrifuged at 3000 rpm for 50 min. DDH2O-resuspended pellets were first concentrated using a 10 kDa molecular weight limit membrane, dialyzed against 0.02% sodium azide, and then brought up to a concentration of 25 mM Tris-HCl, pH 8.5. The resulting protein solution was injected onto a Poros HQ anion exchange column (10*100 mm, Perseptive Biosystems) pre-equilibrated with a starting buffer of 25 mM Tris-HCl, pH 8.5. The column was eluted at a flow rate of 15 ml/min using the following gradient: (1) 5 CV of the starting buffer; (2) 0-1 M sodium chloride over 20 CV; (3) 1 M sodium chloride for 5 CV. The eluate was monitored for absorbance (280 nm). Active fractions from the HQ column were concentrated and desalted using a 10 kDa molecular weight limit membrane and subjected to a Bio-RP C4 reverse phase column (4.6*100 mm, Covance, Princeton, N.J.) and eluted at a flow rate of 4 ml/min using the following gradient: (1) 10 CV of the starting buffer of 5% acetonitrile in H2O; (2) 5-95% acetonitrile in H2O over 2.5 CV; (3) 95% acetonitrile in H2O for 5 CV. The eluate was monitored for absorbance (280 nm), and the active fraction was pooled, lyophilized, and resuspended in phosphate-buffered saline (PBS), pH 7.4. The protein solution was injected onto a G3000PW gel permeation column (21.5*600 mm, TosoHaas, Montgomeryville, Pa.) and eluted with PBS, pH 7.4, at a flow rate of 5 ml/min.

Molecular mass and purity (>99%) of Griffithsin were confirmed by Electrospray ionization mass spectrometry (ESI-MS), and the protein concentrations were determined by amino acid analysis. Native molecular weight was determined by calibrating standard proteins (albumin (68 kDa), cytochrome c (12.5 kDa), and aprotinin (6.5 kDa)) by their retention time (as measured by absorbance at 280 nm) and comparing the resulting calibration curve to the retention time of the active protein. Amino acid analysis was accomplished using a Beckman Model 6300 Automated Amino Acid Analyzer according to manufacturer protocols. N-terminal amino acid sequencing was performed using an Applied Biosystems Model 4774A Sequencer according to manufacturer protocols. Matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS) was performed using a Kratos Kompact Maldi III instrument (Shimadzu, Columbia, Md.) operated in a linear mode using sinapinic acid as a matrix and trypsin as an external standard. ESI-MS was performed with a JEOL SX102 equipped with an Analytica electrospray source. The spectrometer was calibrated using a lysozyme standard (molecular weight=14305.2) prior to each analysis. Samples were injected into the source in a 1:1 solution of hexafluorosopropanol and 2% acetic acid. The masses reported were averages calculated from the various charged states observed.

Griffithsin was subjected to digestion with cyanogen bromide (CNBr) and a variety of endoproteinases (Lys-C, Arg-C, and Asp-N) per manufacturer's instructions. The cleaved peptide

products were purified by reversed-phase HPLC using a gradient of 0.05% aqueous trifluoroacetic acid for 20 min, then increasing to 60% acetonitrile in 0.05% aqueous trifluoroacetic acid over 100 min. Amino acid sequences were determined by sequential Edman degradation using an Applied Biosystems Model 494 sequencer according to the protocols of the manufacturer, and the masses of cleaved peptides were analyzed by MALDI-TOF mass spectrometer. The amino acid sequence of the native Griffithsin polypeptide is set forth as SEQ ID NO: 3.

In summary, the preliminary analysis of the crude aqueous extract of algae Griffithsia sp. in the NCI's primary in vitro anti-HIV screening assay (Weislow et al., supra) identified a protein that bound soluble gp120. The process described herein is illustrated in FIG. 1. Anti-HIV bioassay-guided fractionation of the aqueous resulted in the isolation of Griffithsin. The aqueous extract was subjected to ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange chromatography, reversed-phase chromatography, and size exclusion chromatography to produce a homogeneous protein fraction. SDS-PAGE analysis showed a single protein band with a relative molecular mass of approximately 13 kDa, named Griffithsin. Purified Griffithsin exhibited a single band by immunoblotting with anti-Griffithsin polyclonal antibodies. The amino acid sequence of the purified Griffithsin was established by N-terminal Edman degradation of the intact protein and by N-terminal sequencing of peptide fragments cleaved by CNBr and a variety of endopeptidases (Lys-C, Arg-C, and Asp-N) followed by reversed phase purification and MALDI-TOF mass spectrometric analysis. The entire 121 amino acid sequence was established except for a single amino acid at position 31, which does not match any of the common amino acids. Electrospray ionization mass spectrometric analysis of isolated Griffithsin showed a molecular ion with m/z 12,770.05, and the calculated value for the deduced amino acid sequence without amino acid at position 31 was m/z 12619.00. It was deduced that the molecular mass of the amino acid at position 31 was 151.05. The amino acid analysis of Griffithsin also agreed with the deduced primary sequence. These data fully support the proposed primary amino acid sequence of Griffithsin. A search of the BLAST database (Altschul et al., Nucleic Acids Res, 25(17), 3389-3402 (1997)) for identification of protein sequence similarities did not reveal any homologies of greater than eight contiguous amino acids nor >30% total sequence homology between Griffithsin and any amino acid sequences of known proteins or transcription products of known nucleotide sequences, including the anti-HIV proteins cyanovirin-N and scytovirin.

Example 2

This example demonstrates the synthesis of Griffithsin genes. The methods described herein are illustrated in FIG. 2.

The chemically deduced amino acid sequence of Griffithsin was back-translated to elucidate the corresponding DNA coding sequence. Since amino acid residue 31 of native Griffithsin did not appear to be one of the twenty common amino acids, alanine was substituted in this position (SEQ ID NO: 2). In order to facilitate initial production and purification of recombinant Griffithsin, a commercial expression vector pET-26b(+), from Novagen, Inc., Madison, Wis., for which reagents were available for affinity purification and detection, was selected. Appropriate restriction sites for ligation to pET-26b(+), and a stop codon, were included in the DNA sequence. SEQ ID NO: 1 is an example of a DNA sequence encoding a synthetic Griffithsin gene. A flowchart illustrating a method of synthesizing of a Griffithsin gene is shown in FIG. 2.

A Griffithsin-encoding DNA sequence was synthesized as 13 overlapping, complementary oligonucleotides and assembled to form the double-stranded coding sequence. Oligonucleotide elements of the synthetic DNA coding sequence were synthesized using a nucleic acid synthesizer (model 394, Applied Biosystems Inc., Foster City, Calif.). The purified 13 oligonucleotides were individually treated with T4 polynucleotide kinase, and 1 nM quantities of each were pooled and boiled for 10 minutes to ensure denaturation. The temperature of the mixture was then reduced to 70[deg.] C. for annealing of the complementary strands for 15 minutes, and further reduced to 60[deg.] C. for 15 minutes. The reaction was cooled on ice and T4 DNA ligase (2,000 units) additional ligase buffer was added to the reaction. Ligation of the oligonucleotides was performed with T4 DNA ligase overnight at 16[deg.] C. The resulting DNA was recovered and purified from the reaction buffer by phenol:chloroform extraction, ethanol precipitation, and further washing with ethanol.

The purified, double-stranded synthetic DNA was then used as a template in a polymerase chain reaction (PCR). One [mu]l of the DNA solution obtained after purification of the ligation reaction mixture was used as a template. Thermal cycling was performed using a Perkin-Elmer instrument. "Pfu" thermostable DNA polymerase, restriction enzymes, T4 DNA ligase, and polynucleotide kinase were obtained from Stratagene, La Jolla, Calif. Pfu polymerase was selected for this application because of its claimed superiority in fidelity compared to the usual Taq enzyme. The PCR reaction product was run on a 2% agarose gel in TAE buffer. The 465 base pair DNA construct was cut from the gel and purified. The purified DNA, which was digested with Nde I and Xho I restriction enzymes, was then ligated into the multicloning site of the pet-26b(+) vector.

E. coli were transfected with the generated pET-26b(+)-construct, and recombinant clones were identified by analysis of restriction digests of plasmid DNA. Sequence analysis of one of these selected clones indicated that three bases deviated from the intended coding sequence. These "mutations," which presumably arose during the PCR amplification of the synthetic template, were corrected by a site-directed mutagenesis kit from Stratagene, La Jolla, Calif. The repair was confirmed by DNA sequence analysis.

For preparation of a DNA sequence encoding a Griffithsin polypeptide tagged with a penta-His peptide at the C-terminal end of Griffithsin (e.g., SEQ ID NO: 4), the aforementioned recombinant Griffithsin construct was subjected to site-directed mutagenesis to eliminate stop codons located between the Griffithsin coding sequence and the penta-His peptide coding sequence using a site-directed mutagenesis kit from Stratagene, La Jolla, Calif. A pair of mutagenic oligonucleotide primers were synthesized, which included portions of the codons encoding the Griffithsin polypeptide and penta-His peptide, but lacked the stop codons. Annealing of these mutagenic primers with the template DNA and extension by DNA polymerase resulted in the generation of a DNA construct encoding a fusion protein comprising the Griffithsin amino acid sequence linked to a penta-His peptide tag. DNA sequencing verified the presence of the intended sequence.

Example 3

This example demonstrates the expression of an N-terminal His-tagged-Griffithsin gene.

A recombinant Griffithsin protein and a C-terminal, His-tagged Griffithsin protein encoded by the nucleic acids of Example 2 did not efficiently translocate to the periplasmic fraction of E. coli following protein expression. In addition, the majority of the produced proteins accumulated in the inclusion bodies of E. coli without the cleavage of a pelB signal sequence located at the N-terminus of the Griffithsin protein. Thus, steps were taken to express Griffithsin in the cytosolic fraction of E. coli.

The pET-26b(+)-Griffithsin DNA construct was used as a template PCR using a pair of appropriate primers. The PCR product was designed to have a "penta-His" peptide and thrombin recognition site at the N-terminal end of the Griffithsin polypeptide, providing for production of a N-terminal, His-tagged-Griffithsin fusion protein. The PCR reaction product was purified from an agarose gel. The purified DNA, which was digested with Nco I and Xho I restriction enzymes, was ligated into the expression vector pET-28a(+) vector (Novagen, Inc., Madison, Wis.).

E. coli (strain BL21(DE3)) were transfected with the pET-28a(+) vector containing the nucleic acid coding sequence for the His-tagged-Griffithsin fusion protein (see SEQ ID NO: 4). Selected clones were seeded into small-scale shake flasks containing LB growth medium with 30 [mu]g/ml kanamycin and expanded by incubation at 37[deg.] C. Larger-scale Erlenmeyer flasks (0.5-3.0 liters) were then seeded. The culture was allowed to grow to a density of 0.5-0.7 OD600 units. The expression of the His-tagged-Griffithsin fusion protein was induced by adding IPTG to a final concentration of 1 mM and continuing incubation at 37[deg.] C. for 3-6 hrs. Bacteria were harvested by centrifugation, and the soluble fraction was obtained using BugBuster(TM) reagent and Benzonase nuclease (Novagen, Inc., Madison, Wis.). Crude soluble fractions showed both anti-HIV activity and presence of a His-tagged-Griffithsin fusion protein by Western-blotting. In addition, the His-tagged-Griffithsin protein accumulated in the inclusion bodies of E. coli. A flowchart illustrating a method of expressing and purifying recombinant His-tagged-Griffithsin is shown in FIG. 3.

The purity (~98%) of recombinant His-tagged Griffithsin was confirmed by SDS-PAGE on 16% Tricine gel stained by Coomassie Blue staining. The protein showed the expected molecular mass for Griffithsin (i.e., 14.6 kDa). Protein concentrations were determined based on extinction coefficient at 280 nm of the protein. Approximately 1.6 mg of recombinant His-tagged Griffithsin was purified from 1 L of E. coli culture. The purified protein demonstrated 120-binding and anti-viral activity equivalent to that of native Griffithsin.

This example illustrates a method of producing recombinant Griffithsin, which displays physical and functional properties similar, if not identical, to that of natural Griffithsin.

Example 4

This example describes a method of purifying a recombinant His-tagged-Griffithsin protein.

Using an immobilized metal affinity chromatography set-up including Ni-NTA agarose (QIAGEN Inc., Valencia, Calif.), a His-tagged-Griffithsin fusion protein (as described in Example 3) was purified.
The soluble fraction described in Example 3 was loaded onto 20 ml gravity columns containing affinity matrix. The columns were washed extensively with washing buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 8.0) to remove contaminating proteins. Since His-tagged Griffithsin cannot compete for binding sites on the Ni-NTA resin if the imidazole concentration is increased to 100-250 mM, the His-tagged Griffithsin protein was eluted by applying elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0) through the column. Column fractions and wash volumes were monitored by Western-blot analysis using Penta-His(TM) antibody (QIAGEN Inc., Valencia, Calif.) or anti-Griffithsin antibody. Fractions containing the purified His-tagged Griffithsin protein were pooled, dialyzed extensively against distilled water, and lyophilized.
Potent cytoprotective and anti-replicative activities of both natural and His-tagged recombinant Griffithsin were observed using the HIV-1RF strain of HIB in CEM-SS cells. Both the natural and recombinant Griffithsin polypeptides demonstrated a concentration-dependent inhibition of virus-induced cell killing. Griffithsin treatment also resulted in concomitant decreases in supernatant reverse transcriptase and viral core antigen, p24. Mid-to-high picomolar concentrations of Griffithsin exhibited comparably potent activity against all of the representative T-tropic laboratory strains and primary isolates as well as M-tropic primary isolates. In the antiviral assays, there was little or no evidence of direct cytotoxicity of Griffithsin to the uninfected control cells at the highest tested concentrations of Griffithsin (78.3 to 783 nM). Griffithsin-pretreated uninfected CEM-SS cells retained normal susceptibility to HIV infection after the removal of Griffithsin. In contrast, infectivity of cell-free virus was abolished after pretreatment and removal of Griffithsin. These results indicate that Griffithsin is a virucide. Cocultivation of uninfected and chronically infected CEM-SS with Griffithsin resulted in concentration-dependent inhibition of cell-cell fusion. Additional binding and fusion inhibition assay using [beta]-gal indicator cells showed similar results. Griffithsin inhibited fusion of CD4 [beta]-gal cells with HL 2/3 cells and also inhibited cell-free HIV-1IIIB fusion and infection of [beta]-gal cells in a concentration-dependent manner.

Example 5

This example illustrates the anti-HIV activity of natural Griffithsin polypeptide and His-tagged Griffithsin polypeptide.

Pure proteins were initially evaluated for antiviral activity using an XTT-tetrazolium anti-HIV assay described previously (Boyd, in Aids, Etiology, Diagnosis, Treatment And Prevention (1988), supra; Gustafson et al., J. Med. Chem., 35: 1978-1986 (1992); Weislow (1989), supra; Gulakowski (1991), supra). A CEM-SS human lymphocytic target cell line was used in all assays maintained in RPMI 1650 medium (Gibco, Grand Island, N.Y.), without phenol red, supplemented with 5% fetal bovine serum, 2 mM L-Glutamine, and 50 mg/ml Gentamicin (complete medium).

Exponentially growing cells were pelleted and resuspended at a concentration of 2.0*10<5 >cells/ml in complete medium. The Haitian variant of HIV, HTLV-IIIRF (3.54*10<6 >SFU/ml), was used

throughout. Frozen virus stock solutions were thawed immediately before use and resuspended in complete medium to yield 1.2*10<5 >SFU/ml. The appropriate amounts of the pure proteins for anti-HIV evaluations were dissolved in H2O-DMSO (3:1), then diluted in complete medium to the desired initial concentration. All serial drug dilutions, reagent additions, and plate-to-plate transfers were carried out with an automated Biomek 1000 Workstation (Beckman Instruments, Palo Alto, Calif.).

FIG. 4 summarizes the observed antiviral activities of native Griffithsin from Griffithsia sp. (FIG. 4a) and recombinant His-tagged-fusion Griffithsin (FIG. 4b). Effects of a range of concentrations of native Griffithsin and HIS-tagged-Griffithsin upon CEM-SS cells infected with HIV-1, as determined after days in culture is illustrated in FIG. 6. Data points represent the percent of the respective uninfected, nondrug-treated control values. The two Griffithsin polypeptides demonstrated potent anti-HIV activity with an EC50 in the low nanomolar range and no significant evidence of direct cytotoxicity to the host cells at the highest tested concentrations (up to 1 mM).

Example 6

This example demonstrates that HIV viral envelope gp120 is the principal target for Griffithsin.

To determine the affinity of Griffithsin for a series of protein standards, 100 ng each of gp160, gp120, gp41, sCD4, bovine IgG, [alpha]-acid glycoprotein, and aprotinin were subjected to ELISA as previously described (Bokesch et al., Biochemistry, 42: 2578-2584 (2003)). Briefly, the protein standards were bound to a 96-well plate, which was rinsed with PBST (three times) and blocked with BSA. Between each step of the protocol, the plate was rinsed with PBST (three times). The protein standards were incubated with Griffithsin (100 ng/well), followed by incubation with a 1:500 dilution of an anti-Griffithsin rabbit polyclonal antibody preparation. Griffithsin bound to the protein standards was detected by adding goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, Ind.). Upon addition of alkaline phosphatase substrate buffer, absorbance was measured at 405 nm for each well. Glycosylation-dependent binding of Griffithsin to gp120 was examined using an ELISA as above, with glycosylated and nonglycosylated gp120 (HIV-1SF2 gp120) added to the 96-well plate and incubated with serial dilutions of Griffithsin.

Griffithsin was tested for its ability to bind HIV envelope glycoproteins. Evidence for direct interaction of Griffithsin with gp120, gp160, and to a lesser degree, gp41 was obtained from ELISA experiments (FIG. 5a). There was little or no detectable interaction between Griffithsin and cCD4 or other reference proteins, including bovine IgG, [alpha]-acid glycoprotein, and aprotinin. An additional ELISA experiment showed that binding of Griffithsin to sgp120 is both concentration-dependent and glycosylation-dependent (FIG. 5b).

To undertake preliminary mapping studies to define Griffithsin-binding site on the gp120, we evaluated the effect of Griffithsin on the reactivity of soluble CD4 (sCD4), cyanovirin-N, and a panel of monoclonal antibodies (mAb) with soluble gp120 (sgp120) in an ELISA format assay. These studies demonstrated that Griffithsin interfered strongly with recognition of sgp120 by the mAbs 48d and 2G12. Griffithsin moderately interfered with sCD4 and mAb IgG 1b12 binding to sgp120. Griffithsin had little or no effect on the recognition of sgp120 by mAbs that recognize the C1 region (or V3 loop), and the mAb 17b. However, additional studies demonstrated that pretreatment of sgp120 with sCD4 and the mAbs IgG1b12, 48d, and 2G12 did not block subsequent binding of Griffithsin to sgp120. Cyanovirin-N interfered strongly with the recognition of sgp120 by Griffithsin. On the other hand, Griffithsin pretreatment of sgp120 did not block subsequent binding of cyanovirin-N to sgp120.

Since Griffithsin inhibited viral entry, we compared matched control and Griffithsin-treated sgp120 preparations in a flow cytometric sgp120/CD4-expressing cell binding assay to determine whether Griffithsin inhibits viral attachment or subsequent fusion events. The CEM-SS cell line expresses CD4, as demonstrated by the binding of target cells with both anti-Leu3a and anti-OKT4 monoclonal antibodies. After incubation of CEM-SS cells with sgp120, the cells were stained by anti-gp120 mAb-FITC. A concomitant decrease in the availability of the Leu3a epitope (i.e., the gp120-binding site on target cells) was observed. In other words, the sgp120 bound to the gp120

binding site on the target cells. As expected, little change in the staining specific for the OKT4 epitope (i.e., a non-gp120 binding site) was observed. These results are consistent with sgp120 binding of CD4 on the target cells. Pretreatment of sgp120 with Griffithsin substantially recovered the availability of the Leu3a epitope, indicating that Griffithsin completely blocked CD4-dependent sgp120 binding. However, overall sgp120 binding showed two peaks in the flow cytometry data when Griffithsin-treated sgp120 was added to the cells. The decreased signal suggests inhibition of sgp120 binding to CD4 by Griffithsin, which was consistent with the recovery of the availability of the Leu3a epitope. The increased signal suggests that the Griffithsin/sgp120 complex also non-specifically bound to target cells.

This example demonstrates that Griffithsin binds to a region of gp120 that recognizes CD4 on host cells.

Example 7

This example illustrates the broad-range anti-HIV activity of Griffithsin.

Anti-viral assays used to study the activities of laboratory strains and primary isolates of virus have been previously published (Buckheit et al., Antiviral Res., 21: 247-265 (1993)). The low passage HIV-1 pediatric isolate ROJO was derived as previously described (Buckheit et al., AIDS Res. Hum. Retroviruses, 10: 1497-1506 (1994)). Peripheral blood mononuclear cells (PBMC) and macrophages were isolated from hepatitis and HIV sero-negative donors following Ficoll-Hypaque centrifugation as described elsewhere (Gartner and Popovic, Techniques in HIV Research, Aldovini, A. and Walker, B., eds., Stockton Press, New York (1994) pp. 59-63). Mean EC50 values were determined from concentration-response curves from eight dilutions of Griffithsin (triplicate wells/concentration); assays for HIV-1 RF/CEM-SS employed XTT-tetrazolium; HIV-1 ROJO were tested in human PBMC cultures by supernatant reverse transcriptase activity; HIV-1 Ba-L and ADA were tested in human primary macrophage cultures by p24 ELISA assay. Standard errors averaged less than 10% of the respective means. The results of this study are summarized in Table 1 below.

TABLE 1

Virus	Target Cell	Tropism	EC50 (nM)
HIV-1 Laboratory Strain			
RF	**CEM-SS**	**T**	**0.043**
HIV Primary Isolates			
ROJO	**PBMC**	**T**	**0.63**
ADA	**Macrophage**	**M**	**0.50**
Ba-L	**Macrophage**	**M**	**0.098**

The results show that Griffithsin is potently active (sub-nanomolar EC50 values) against a broad range of HIV isolates including T-tropic viruses (utilizing CCR5 as a co-receptor) and M-tropic viruses (utilizing CXCR4 as a co-receptor). This picomolar level of activity is more potent than that described for most of the current anti-HIV agents utilized in therapy or in development, including the entry inhibitors cyanovirin-N and Enfurtide(R). The data also show that Griffithsin is effective at inhibiting infection by both laboratory-adapted strains and, more importantly, primary clinical isolates of HIV (e.g., ROJO, ADA, and Ba-L). Finally, the results indicate that Griffithsin is active regardless of the cell type used in the assay, having potent activity whether the cells were T-lymphocytes (CEM-SS), PBMCs, or macrophages. Griffithsin did not show any toxicity against any of the cell lines even at concentrations 1000-fold higher than the EC50 values.

Example 8

This example describes the production of anti-Griffithsin polyclonal antibodies. A flow diagram illustrating a method of producing anti-Griffithsin antibodies is provided in FIG. 6.

A New Zealand white rabbit was immunized with 100 [mu]g of Griffithsin in Freund's complete adjuvant. Booster injections of 50 [mu]g of Griffithsin in Freund's incomplete adjuvant were

administered on days 13, 29, 51, 64, 100, and 195. On days 7, 21, 42, 63, 78, and 112, 10 mL of blood was removed from the rabbit. On day 112 the rabbit was sacrificed and bled out. The IgG fraction of the immune sera of the rabbit was isolated by protein-A Sepharose affinity chromatography (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. Reactivity of the polyclonal antibodies for Griffithsin was demonstrated by immunoblot and ELISA studies with 1:500 to 1:3000 dilution of the rabbit immunoglobulin fractions.

For immunoblotting, samples were transferred to PVDF membranes following SDS-PAGE according to standard procedures. The membranes were incubated for 1 hour with anti-Griffithsin polyclonal antibodies, washed three times with PBS containing 0.05% Tween 20 (PBST), and then treated with goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Sigma, St. Louis, Mo.). After three washes with PBST, bound antibodies were visualized by incubating membranes in a solution of 0.05% 3,3'-diaminobenzidine and 0.003% H2O2.

The IgG fraction of rabbit polyclonal anti-Griffithsin antibodies were purified after the final boost and animal sacrifice by using protein-A Sepharose chromatography on the 57 mL of rabbit serum collected. Following purification, 78 mL of purified anti-Griffithsin IgGs were produced. The final concentration of protein was 335 micrograms/mL for a total yield of 27.3 mg of anti-Griffithsin IgG. To analyze the specificity of the resulting antibody preparation, Western blot analysis was performed and resulted in the clear determination of specificity and avidity for Griffithsin by the purified antibodies. A 1:250 dilution of the purified antibodies clearly visualized only the Griffithsin from a mixture of Griffithsin and other proteins. The response to Griffithsin by the anti-Griffithsin antibodies was also shown to be concentration-dependent.

Example 9

This example illustrates the anti-influenza virus activity of Griffithsin.

All examined influenza viruses were passaged in Madin Darby canine kidney (MDCK) cells to prepare viral stocks. MDCK cells (from ATCC, Manassas, Va.) were grown in antibiotic-free minimum essential medium (MEM) with non-essential amino acids (Gibco, Long Island, N.Y.) containing 5% fetal bovine serum (FBS, HyClone Laboratories, Logan, Utah) and 0.1% NaHCO3. Test medium consisted of MEM with 0.18% NaHCO3, 10 units/mL trypsin, 1 [mu]g of ethylenediaminetetraacetate (EDTA) per ml, and 50 [mu]g gentamicin/mL.

Inhibition of virus-induced cytopathic effect (CPE) as determined by visual (microscopic) examination of infected cells and confirmed by increase in neutral red (NR) dye uptake into infected cells was used as an indicator of Griffithsin antiviral activity. The CPE inhibition method was reported previously by Smee et al. (Antiviral Res., 5: 251-259 (2001)). Seven concentrations of Griffithsin were screened for antiviral activity against each virus in 96-well flat-bottomed microplates of cells. The Griffithsin protein was added 5-10 minutes prior to addition of virus to the cells. The concentration of virus correspond to approximately 50% infection of cells in culture (CCID50) per well. The virus challenge dose equals a multiplicity of infection of approximately 0.001 infectious particles per cell. The reaction proceeded at 37[deg.] C. for 72 hr. To perform the NR uptake assay for confirmation of antiviral activity, dye (0.34% concentration in medium) was added to the plates used to obtain visual scores of CPE. After 2 hours, color intensity of the dye absorbed by and subsequently eluted from the cells was determined by the method of Finter et al., J. Gen. Virol., 5, 419-427 (1969) using a computerized EL-309 microplate autoreader (Bio-Tek Instruments, Winooski, Vt.). Antiviral activity was expressed as the 50% effective (virus-inhibitory) concentration (EC50 value) determined by plotting Griffithsin concentration versus percent inhibition on semi-logarithmic graph paper. Cytotoxicity of compounds was assessed in parallel with the antiviral determinations in the same microplates, except in the absence of virus. From these, 50% cytotoxic endpoints (IC50 values) were determined. The results of this study are summarized in Table 2.

TABLE 2
Influenza Virus Strain EC50 ([mu]g/ml)

Beijing/262/95 (H1N1) 0.07

Texas/36/91 (H1N1) 0.06
Los Angeles/2/87 (H3N2) 0.037
Panama/2007/99 (H3N2) 0.006
Shandong/09/93 (H3N2) 0.018
Sydney/5/97 (H3N2) 0.016
Washington/05/96 (H3N2) 0.016

Similar to the results with HIV, Griffithsin was found to be potently active against a wide spectrum of influenza A viruses. These viruses included both H1N1 strains and H3N2 strains of influenza, which is especially significant in light of the fact that the highly virulent Fijian strain of influenza A that afflicted the United States in 2003/2004 was also a H3N2 strain. Griffithsin was not found to be toxic to the MDCK cell line utilized for these experiments, even when the cells were exposed to a high dose of Griffithsin (10 micrograms/mL).

Example 10

This example describes a method of producing recombinant Griffithsin.

Recombinant expression of His-tagged Griffithsin in E. coli was optimized using a fermenter in combination with an auto-induction media. A seed culture was grown in LB media containing 30 [mu]g/ml kanamycin in a shaker flask at 37[deg.] C. and 150 rpm for 17 hours. In addition, a fermenter containing an auto-induction media was inoculated with the seed culture. The ratio of auto-induction media to seed culture was approximately 50:1. The culture was grown at 37[deg.] C. for 24 hours. The final culture density was approximately 8.6 OD600 units. The final culture was harvested by centrifugation, and the soluble fraction was obtained as described above.

Crude soluble fractions contained His-tagged-Griffithsin fusion protein, which was detected by Western-blotting with anti-Griffithsin polyclonal antibodies. The ratio of soluble:insoluble protein at approximately 15 kDa was 50:50. The ratio indicates that more Griffithsin protein was produced in soluble fraction in this fermentation procedure compared with protein expression achieved using a shaker flask procedure. In addition, the fermentation procedure provided approximately 30-fold higher quantities of Griffithsin protein than the shaker flask procedure. Approximately 50 mg of purified recombinant Griffithsin was isolated from 1 L of the fermentation. The purified protein existed as a homodimer and demonstrated gp120 binding and anti-viral activity equivalent to that of native Griffithsin.

The results of this example confirm a method of producing recombinant, anti-viral Griffithsin protein.

Example 11

This example demonstrates the anti-Hepatitis C(HCV) activity of Griffithsin.

The anti-HCV activity of Griffithsin was analyzed as generally described in Krieger et al., J. Virol. 75: 4614-4624 (2001), but using the Huh7 ET (luc-ubi-neo/ET) cell line, which contains a new HCV RNA replicon with a stable luciferase (LUC) reporter. The HCV RNA replicon ET contains the 5' N-terminal repeat (IRES) of HCV (5') which drives the production of a firefly LUC, ubiquitin, and neomycin phosphotransferase (Neo) fusion protein. Ubiquitin cleavage releases the LUC and Neo genes. The encephalomycarditis virus (EMCV) IRES element controls the translation of the HCV structural proteins NS3-NS5. The NS3 protein cleaves the HCV polyprotein to release the mature NS3, NS4A, NS4B, NS5A, and NS5B proteins that are required for HCV replication. At the 3' end of the replicon is the authentic 3' NTR of HCV. The LUC reporter is used as an indirect measure of HCV replication. The activity of the LUC reporter is directly proportional to HCV RNA levels and positive control antiviral compounds behave comparably using either LUC or RNA endpoints.

The effect of a His-tagged Griffithsin (SEQ ID NO: 5) added in triplicate at a single high-test concentration of 20 [mu]M on HCV RNA-derived LUC activity and cytotoxicity was examined. Human IFN[alpha]-2b was included in each run as a positive control compound. Subconfluent cultures of the Huh7 ET cell line were plated out into 96-well plates that were dedicated for the

analysis of cell numbers (cytotoxicity) or antiviral activity and, on the next day, Griffithsin or IFN[alpha]-2b was added to the appropriate wells. Cells were processed 72 hr later when the cells were still subconfluent. Compound cytotoxicity was assessed as the percent viable cells relative to the untreated cell controls.

As shown in Table 3, the Griffithsin protein demonstrated 60% inhibition of viral replication, and an overall 25% reduction in cell growth.

TABLE 3

Compound	Inhibition of Viral (% reduction Luc)	Cytotoxicity Selective Activity (% reduction in rRNA)*	Index (SI) (IC50/EC50)
Griffithsin	60	74.5	>1
IFN-[alpha]	98.6	98.4	>1

***Reduction as compared to control cell**

The HCV RNA replicon confirmatory assay was then used to examine the effects of Griffithsin at different concentrations. Specifically, 0, 0.20, 0.63, 6.32, and 20 [mu]g/ml Griffithsin was tested. Human IFN[alpha]-2b was included in each run as a positive control compound. Subconfluent cultures of the Huh7 ET cell line were plated out into 96-well plates that were dedicated for the analysis of cell numbers (cytotoxicity) or antiviral activity and, on the next day, each dose of Griffithsin was added to the appropriate wells. Cells were processed 72 hr later when the cells were still subconfluent. Compound EC50 and EC90 values (antiviral activity) were derived from HCV RNA levels assessed as either HCV RNA replicon-derived LUC activity or as HCV RNA using TaqMan RT-PCR. Compound IC50 and IC90 values (cytotoxicity) were calculated where applicable using CytoTox-1 (Promega), a colorimetric assay used as an indicator of cell numbers and cytotoxicity when the LUC assay system was employed, while ribosomal (rRNA) levels determined via TaqMan RT-PCR were used as an indication of cell numbers in the RNA-based assay. Compound selectivity indices SI50 and SI90 values also were calculated from the spreadsheets.

The results are presented in FIG. 7. As shown in FIG. 7, Griffithsin demonstrated anti-HCV activity in a dose-dependent manner. The EC50 of Griffithsin was 7.17 [mu]g/ml indicating substantial anti-viral potency. Griffithsin never reached a toxicity level, even at the highest test concentration of 10 [mu]g/ml. Therefore, the IC50 and IC90 could not be determined.

This example demonstrates that Griffithsin can be used to effectively inhibit HCV.

Example 12

This example demonstrates the anti-SARS activity of Griffithsin.

The medium from an 18 h monolayer (80-100% confluent) of Vero76 cells was drained and a His-tagged Griffithsin (SEQ ID NO: 5) at 0.1, 0.3, 1.0, 3.2, 10.1, 31.8, or 100 [mu]g/ml was added, followed within 15 min by the SARS virus or virus diluent. The plate of treated cells was sealed and incubated for the standard time period required to induce near-maximal viral CPE. The plate of cells was then stained with neutral red as described by Smee et al., Antimicrob. Agents Chemother. 45: 743-748 (2001) and McManus, Appl. Environment. Microbiol. 31: 35-38 (1976). Cells not damaged by virus took up a greater amount of dye. The percentage of neutral red uptake indicating viable cells was read on a microplate autoreader at dual wavelengths of 405 and 540 nm, with the difference taken to eliminate background. An approximated virus-inhibitory concentration at the 50% endpoint (EC50) and cell-inhibitory concentration at the 50% endpoint (IC50) was determined, and a general selectivity index was calculated from these values: SI= (IC50)/(EC50). The virus inhibitory EC50 and IC50 values and the SI values from the neutral red assay are provided in Table 4.

The effect on reduction of virus yield was determined by assaying frozen and thawed eluates from

each cup for virus titer using serial dilution onto monolayers of susceptible cells. The development of viral cytopathic effect (CPE) in these cells was an indication of the presence of infectious virus. The 90% virus-inhibitory effective concentration (EC90) of Griffithsin, which is the concentration of Griffithsin at which the virus yield was inhibited by 1 log 10, was determined from these data. The EC90 value from the virus yield assay is provided in Table 4.

The visual appearance of treated infected cells was compared to that of treated uninfected cells. Specifically, changes such as enlargement, granularity, development of ragged edges, filmy appearance, rounding, and detachment from the surface of the well were detected by visual observation. Based on these observations, the cells were given a designation of T (100% toxic), PVH (partially toxic-very heavy-80%), PH (partially toxic-heavy-60%), P (partially toxic-40%), Ps (partially toxic-slight-20%), or 0 (no toxicity-0%) conforming to the degree of cytotoxicity visually present. A 50% virus inhibitory concentration (EC50) and 50% cell inhibitory (cytotoxic) concentration (IC50) was determined by regression analysis of these data. This assay was repeated to confirm the results. The EC50 and IC50 values and corresponding SI values obtained in the visual assay and the visual confirmation assay are provided in Table 4.

TABLE 4

EC50 IC50 EC90
Compound Vehicle Assay ([mu]g/ml) ([mu]g/ml) ([mu]g/ml) SI

Griffithsin water Neutral Red 14 >100 7
water Virus Yield 5 >20
water Visual 4 >100 >25
water Visual- 2 >100 >50

Confirmation

As shown in Table 4, the Griffithsin protein inhibited the SARS virus with an average EC50 of 3 [mu]g/ml as determined by visual assay, and an EC90 of 5 [mu]g/ml as determined by virus yield assay indicating substantial antiviral potency. The EC50 of 14 [mu]g/ml as determined by the neutral red assay provides a third measure of anti-viral activity for this protein. The IC50 of Griffithsin in each assay was greater than 100 [mu]g/ml, indicating good cell viability at effective concentrations.

This example demonstrates that Griffithsin can be used to effectively inhibit the SARS virus.

Example 13

This example demonstrates the anti-H5N1 activity of Griffithsin.

Madin Darby canine kidney (MDCK) cells, obtained from the American Type Culture Collection (Manassas, Va.), were grown in antibiotic-free minimum essential medium with non-essential amino acids (MEM) (Hyclone Labs, Logan, Utah) containing 5% fetal bovine serum (FBS) and 0.18% NaHCO3. The test medium was the above MEM without FBS, with added 10 units/ml trypsin (Sigma, St. Louis, Mo.), 1 [mu]g of ethylenediaminetetraacetate (EDTA) per ml, and 50 [mu]g gentamicin/ml. MDCK cells were used for the following cell culture antiviral studies.

An influenza A (H5N1) hybrid virus was kindly provided by Medimmune, Inc. (Mountain View, Calif.). The virus consisted of the core (6 genes) of influenza A/Ann Arbor/6/60 with the H5 and Ni components from A/Vietnam/1203/2004. The virus was attenuated and was resistant to amantadine.

Two methods were used to assay the antiviral activity of His-tagged Griffithsin (SEQ ID NO: 5) against the H5N1 virus in vitro: inhibition of virus-induced cytopathic effect (CPE) determined by visual (microscopic) examination of the cells, and increase in neutral red (NR) dye uptake into cells, as previously described in Smee et al., Antimicrob. Agents Chemother. 45: 743-748 (2001).

In the CPE inhibition test, eight concentrations of Griffithsin or Ribavirin (a positive control; ICN

Pharmaceuticals (Costa Mesa, Calif.)) was added to 96-well flat-bottomed microplates containing a cell monolayer. The compound was added 5-10 minutes prior to virus, which was used at a concentration of approximately 50% cell culture and 50% infectious doses (CCID50) per well. The viral dose equated to a multiplicity of infection of approximately 0.001 infectious particles per cell. The plate was sealed and incubated at 37[deg.] C. The CPE values were read microscopically after 72 h of incubation. Antiviral activity expressed as the 50% effective (virus-inhibitory) concentration (EC50) is provided in Table 5.

The NR assay was performed as reported in Smee et al., supra. In the NR uptake assay, dye (0011% final concentration in medium) was added to the same set of plates used to obtain the visual scores. After 2 hours, color intensity of the dye absorbed by and subsequently eluted from the cells was determined spectrophotometrically. Antiviral activity was determined by plotting compound concentration against percent inhibition. The results expressed as the 50% effective (virus-inhibitory) concentration (EC50) are provided in Table 5.

Cytotoxicity of compounds was assessed in parallel with the above antiviral determinations using the same microplates in the absence of virus. After three days, the percent inhibition of cell proliferation was assessed by visual and neutral red assays as described above. From this data, 50% virus inhibitor concentration (EC50) and 50% cytotoxic endpoints (IC50) values were determined. Using both antiviral and cytotoxicity data, selectivity index values (IC50 divided by EC50) could be calculated for each set of data. These results are provided in Table 5.

TABLE 5
Visual (CPE) Assay Neutral Red Assay

EC50 IC50 EC50

Compound ([mu]g/ml) ([mu]g/ml) SI ([mu]g/ml) IC50 ([mu]g/ml) SI
Griffithsin 0.65 >10 >15 1.2 >10 >8
Ribavirin 1.8 >100 >56 1.8 >100 >56

As shown in Table 5, Griffithsin demonstrated potent anti-H5N1 activity, exhibiting an EC50 of 0.65 [mu]g/ml, as determined by the visual assay, and an EC50 of 1.2 [mu]g/ml, as determined by the neutral red assay. The IC50 values demonstrate cytotoxic tolerance at effective concentrations.

This example shows that Griffithsin can be used to effectively inhibit the anti-H5N1 virus.

Example 14

This example demonstrates the anti-ebola virus activity of Griffithsin.

Griffithsin is tested for anti-ebola virus activity by using the Zaire ebola virus engineered to express Green Fluorescence Protein (GFP), which is described in Towner et al., Virology. 332(1):20-27 (2005).

A His-tagged Griffithsin (SEQ ID NO: 5) or a positive control is added to a cell monolayer. Thereafter, the cells are challenged with the engineered Zaire ebola virus. Inhibition of ebola viral replication, and thus anti-viral activity, is detected on the basis of expression of GFP through means such as flow cytometry analysis. Griffithsin is found to have anti-ebola virus activity in the low [mu]g/ml range.

The results indicated that Griffithsin can be used to effectively inhibit the ebola virus.

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

Birren et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1997),
Birren et al., Genome Analysis: A Laboratory Manual Series, Volume 2, Detecting Genes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1998),

Birren et al., Genome Analysis: A Laboratory Manual Series, Volume 3, Cloning Systems, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999),
Birren et al., Genome Analysis: A Laboratory Manual Series, Volume 4, Mapping Genomes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999),
Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1988),
Harlow et al., Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1999), and
Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

Scytonema varium red algae
Scytovirin

SCYTOVIRIN DOMAIN 1 RELATED POLYPEPTIDES
US8481255

ANTIVIRAL ACTIVITY OF THE PROTEIN SCYTOVIRIN AND METHODS OF USE
US2011183894

Scytovirins and related conjugates, fusion proteins, nucleic acids, vectors, host cells, compositions, antibodies and methods of using scytovirins
US7491798

Nostoc ellipsosporum

Cyanovirin-N

Coded sequence CVN (Cyanovirin-N) mutant with high expression quantity and high activity and application of coded sequence
CN103255151

CYANOVIRIN VARIANT-POLYMER CONJUGATE
JP4903891

A CYANOVIRIN N MUTANT, MODIFIED DERIVATIVE AND USES THEREOF
WO2011026351

ANTI-H5N1 INFLUENZA ACTIVITY OF THE ANTIVIRAL PROTEIN CYANOVIRIN
US2010240578

Antiviral drug combination for livestock
CN101612389

Modified Cyanovirin-N Polypeptide
US2009155304

GENOMIC NUCLEIC ACID SEQUENCE FOR CYANOVIRIN-N AND SIGNAL PEPTIDE THEREOF
WO2007005766

Anti-cyanovirin antibody
US5998587

Cyanovirin conjugates and matrix-anchored cyanovirin and related compositions and methods of use
US7105169

Obligate domain-swapped dimer of cyanovirin with enhanced anti-viral activity
US7276227

Cyanovirin conjugates and matrix-anchored cyanovirin and related compositions and methods of use
US7048935

Anti-cyanovirin antibody with an internal image of gp120, a method of use thereof, and a method of using a cyanovirin to induce an immune response to gp120
US6193982

Cyanovirin conjugates, matrix -anchored cyanovirin and anti-cyanovirin antibody, and related compositions and methods of use
AU2003252207
