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The T4 Gene Encodes the AIDS Virus Receptor and Is Expressed in the Immune System and the Brain

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Summary

The isolation of clones encoding the human surface protein T4, and the expression of the T4 gene in new cellular environments, have enabled us to examine the role of this protein in the pathogenesis of AIDS. Our studies support a mechanism of AIDS virus infection that initially involves the specific interaction of the AIDS virus with T4 molecules on the cell surface. This association can be demonstrated on $T4^+$ transformed T and B lymphocytes as well as epithelial cells. Furthermore, the presence of T4 on the surface of all human cells examined is sufficient to render these cells susceptible to AIDS virus infection. Our data suggest that the T4-AIDS virus complex is then internalized by receptor-mediated endocytosis. Finally, we find that the T4 gene is expressed in the brain as well as in lymphoid cells, providing an explanation for the dual neurotropic and lymphotropic character of the AIDS virus. In this manner, a T lymphocyte surface protein important in mediating effector cell–target cell interactions has been exploited by a human retrovirus to specifically target the AIDS virus to populations of $T4^+$ cells.

Introduction

The human acquired immune deficiency syndrome (AIDS) is characterized by a depletion of $T4^+$ T lymphocytes, a subpopulation of peripheral lymphocytes composed of the majority of helper T cells. As a consequence, T-cell-mediated immunity is impaired in AIDS patients, resulting in the occurrence of severe opportunistic infections and unusual neoplasms (reviewed in Lane and Fauci, 1985; Weiss, 1986). AIDS results from the infection of T lymphocytes with a collection of closely related T lymphotropic retroviruses (LAV, HTLV-III, or ARV), now termed human immunodeficiency virus, HIV (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984). The range of infectivity of these agents is restricted to cells expressing the T4 glycoprotein on their surface (Klatzman et al., 1984a; Dalgleish et al., 1984; McDougal et al., 1985b).

The T4 molecule is one of several non-polymorphic T lymphocyte surface proteins that have been implicated in the mediation of efficient T cell–target cell interactions. Analysis of these surface proteins indicates that mature T lymphocytes segregate into one of two classes: those that express the surface glycoprotein T4 and those that express the glycoprotein T8 (Reinherz and Schlossman, 1980). The T4 molecule is predominantly expressed on helper T lymphocytes whereas T8 is expressed on cytotoxic and suppressor T cells (Reinherz and Schlossman, 1980). This functional distinction is not absolute since some $T4^+$ T lymphocytes can effect cytotoxicity and suppression (Thomas et al., 1981; Meuer et al., 1982). A more stringent relationship, however, exists between T cell subsets and the major histocompatibility complex (MHC) gene products expressed by the target cells. $T4^+$ T lymphocytes interact with target cells expressing MHC class II gene products whereas $T8^+$ T cells interact with targets expressing class I MHC molecules (Engleman et al., 1981a; Krensky et al., 1982; Meuer et al., 1982; Biddison et al., 1982; Wilde et al., 1983; Swain et al., 1983). Moreover, monoclonal antibodies directed against T4 and T8 inhibit T cell function in vitro (Engleman et al., 1981b; Swain et al., 1981; Biddison et al., 1982; Landegren et al., 1982; Wilde et al., 1983; Marrack et al., 1983; Rognozinski et al., 1984). These observations suggest that the specificity of interaction of subpopulations of T lymphocytes with different target cells results from the association of T4 and T8 with class II MHC and class I MHC proteins, respectively, and that this recognition may be essential for T cell function.

The T4 glycoprotein may not only serve as a receptor for molecules on the surface of target cells, but also as a receptor for the AIDS virus. Monoclonal antibodies directed against T4 block AIDS virus infection of $T4^+$ cells in vitro (Dalgleish et al., 1984; Klatzman et al., 1984b; McDougal et al., 1985b). Furthermore, recent studies have demonstrated that when $T4^+$ T lymphocytes are exposed to AIDS virus, the 110 kd exterior envelope glycoprotein of the virus is associated with the T4 molecule on the host cell (McDougal et al., 1986). The lymphotropic character of the virus could therefore be explained by the restricted expression of its receptor, T4, in subpopulations of T lymphocytes.

The depletion of $T4^+$ T lymphocytes in AIDS results in the impairment of the cellular immune response. In addition, AIDS is frequently accompanied by central nervous system (CNS) dysfunction, most often the consequence of a subacute encephalitis (Snider et al., 1983; Nielsen et al., 1984; reviewed in Johnson and McArthur, 1986). AIDS virus RNA and DNA have been identified in affected brains

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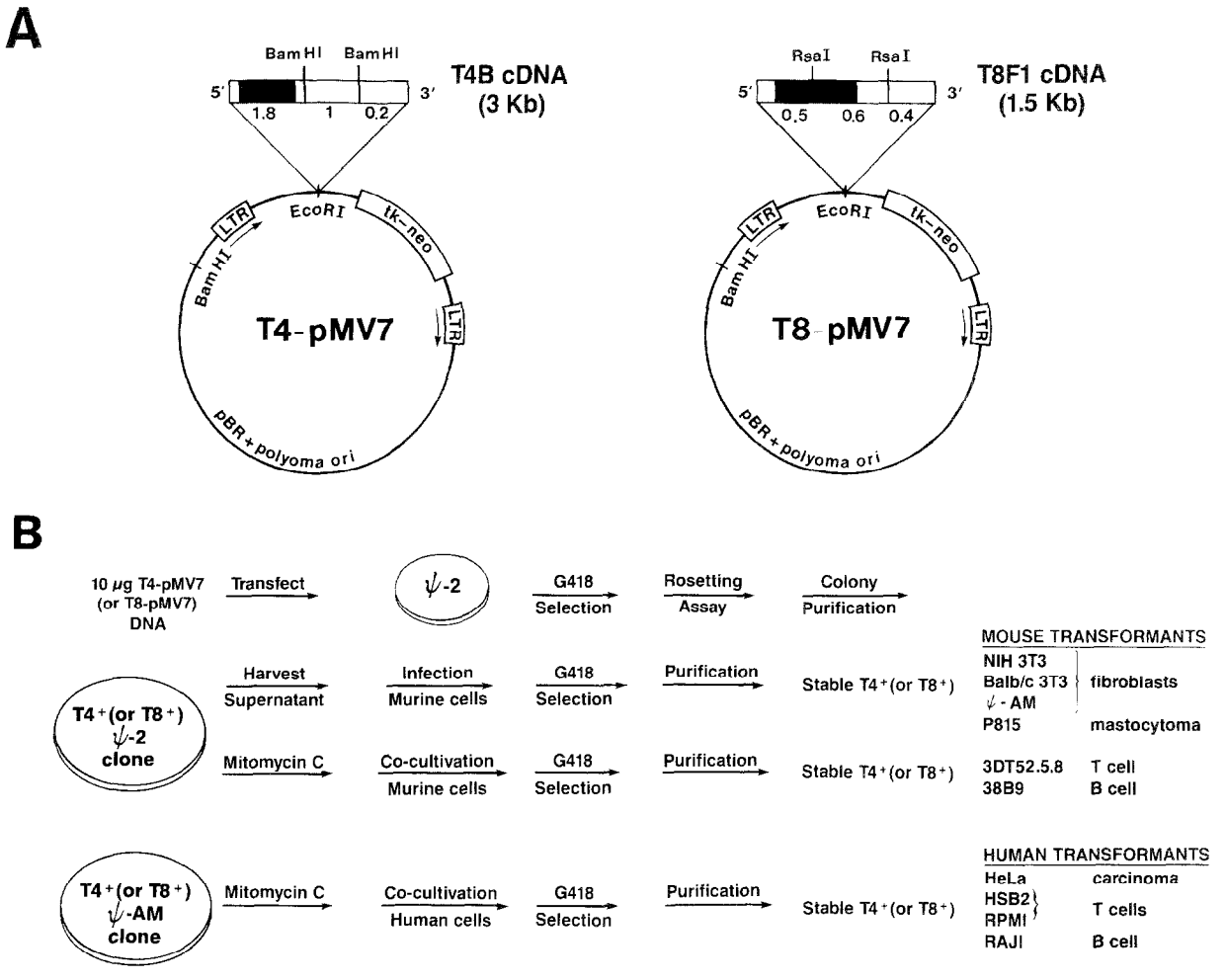

Figure 1. Recombinant Retroviral Expression Vectors and Construction of Transformed Cells

(A) Recombinant retroviral expression vectors. pMV7 contains two directly repeated Moloney murine sarcoma virus long terminal repeats (LTRs) in the orientation indicated by arrows. pMV7 also contains the bacterial neomycin phosphotransferase gene (neo) fused to the HSV thymidine kinase promoter (tk). Full-length cDNA inserts encoding T4 (T4B; Maddon et al., 1985) or T8 (T8F1; Littman et al., 1985) were subcloned into the EcoRI site in the orientation indicated by arrows, generating T4-pMV7 and T8-pMV7, respectively. The coding sequences are shown as shaded regions. The indicated sizes are in kilobases.

(B) Retrovirus-mediated gene transfer strategy. For explanation see text and Experimental Procedures.

(Shaw et al., 1985; Sharer et al., 1986), virus has been located within macrophages and astrocytes (Epstein et al., 1985; Koenig et al., 1986), and virus has been isolated from both brain and cerebrospinal fluid of patients with neurological disorders (Levy et al., 1985; Ho et al., 1985). These observations suggest that the AIDS virus infects cells in the brain and is directly responsible for the CNS lesions observed in AIDS patients. Thus, the AIDS virus may be neurotropic as well as lymphotropic. It is therefore important to ask whether T4 is also expressed in the CNS or whether additional brain-specific surface molecules may serve as receptors for the AIDS virus.

We have previously isolated the cDNA and gene encoding the T4 molecule and have deduced the protein sequence from the nucleotide sequence (Maddon et al., 1985). The availability of clones encoding T4 has enabled us to express the T4 gene in new cellular environments and to examine the role of this molecule in the pathogenesis of AIDS. In this study, we have performed genetic and biochemical experiments which demonstrate that the T4 glycoprotein is the receptor for AIDS virus and that the mere presence of T4 on the cell surface is sufficient to render both human lymphoid and non-lymphoid cells susceptible to AIDS virus infection in vitro. Moreover, the T4 gene is expressed in the brain as well as in lymphocytes, providing a possible explanation for the dual neurotropic and lymphotropic character of the virus.

Results

Construction of T4$^+$ and T8$^+$ Transformed Cells

The experimental approach we have used to study the role of T4 in AIDS virus infection initially involved the introduction of the T4 gene into T4$^-$ cell lines incapable of supporting viral infection. The transformed cells were then tested for susceptibility to AIDS virus infection and the mechanism by which T4 mediates viral infection was then studied.

Table 1. Susceptibility of $T4^+$ and $T8^+$ Human Transformants to AIDS Virus Infection

Human Cell	Maximum Reverse Transcriptase	Cytoplasmic Virus	Supernate Viral Ag	Supernate Subculture	Syncytium Induction	VSV(AIDS) Pseudotype Infection	Virus Binding
CEM($T4^+$)	675023	+	+	+	+	+	+
HSB2	4245	–	–	–	–	–	–
HSB2-$T8^+$	4460	–	–	–	–	–	–
HSB2-$T4^+$	190915	+	+	+	+	+	+
Raji	ND	ND	ND	ND	–	–	ND
Raji-$T8^+$	5595	–	–	–	–	–	–
Raji-$T4^+$	103500	+	+	+	+	+	+
HeLa	6438	–	–	–	–	–	–
HeLa-$T8^+$	4875	–	–	–	–	ND	–
HeLa-$T4^+$	48125	+	+	+	+	+	+

5×10^6 cells were inoculated with AIDS virus, incubated at 37°C for 24 hr, washed, and replated in fresh media. Cells and supernatants were removed at days 3, 6, 9, 12, 16, 20, 24, and 28 and used in four virus detection assays: reverse transcriptase, cytoplasmic virus, supernate viral antigen, and supernate subculture (see Experimental Procedures).

Syncytium induction, VSV(AIDS) pseudotype infection, and AIDS virus binding assays were performed as described in Experimental Procedures. The reverse transcriptase activity is expressed in cpm/ml. The results of the pseudotype infection experiments are expressed as follows: +, $\geq 10^3$ PFU/ml; –, 10 PFU/ml. ND, not determined.

A full-length cDNA clone encoding the surface protein T4 (Maddon et al., 1985) was subcloned into the retroviral expression vector, pMV7 (Figure 1A; Kirschmeier et al., submitted). The expression vector pMV7 contains two directly repeated Moloney murine sarcoma virus long terminal repeats (LTR) which flank a single Eco RI cloning site. The 5′-LTR constitutively promotes transcription through the cloning site whereas the 3′-LTR provides sequences necessary for cleavage and polyadenylation of the RNA. In addition, pMV7 contains the herpes virus thymidine kinase promoter (tk) fused to the coding region of the bacterial neomycin phosphotransferase gene (*neo*), a dominant selectable marker, permitting linked cotransformation and infection.

T4-pMV7 was then introduced into ψ-2 and ψ-AM cells, NIH 3T3 cell lines containing defective murine ecotropic and amphotropic proviruses, respectively (Figure 1B; Mann et al., 1983; Cone and Mulligan, 1984). Both cell lines are incapable of encapsidating endogenous viral RNA, but can provide all obligate *trans* viral functions. The introduction of T4-pMV7 DNA into these cell lines results in the production of replication-defective recombinant retroviral stocks encoding T4 which are free of helper virus. These pure viral stocks can then be used to efficiently introduce T4 sequences into both mouse and human cells without the production of retrovirus by the target cell.

Briefly, T4-pMV7 DNA was introduced into ψ-2 cells using the procedure of DNA-mediated gene transfer (Figure 1B; Graham and van der Eb, 1973; Wigler et al., 1977). Neo^+ colonies, selected by their ability to grow in media containing the neomycin analogue G418 (geneticin), were screened for the expression of T4 on the cell surface using an in situ rosetting assay (Littman et al., 1985; Maddon et al., 1985). Colonies of transfected ψ-2 cells expressing T4 were then identified, which produce recombinant retrovirus in titers of 10^5 cfu/ml. These viruses were used to infect ψ-AM cells to generate ψ-AM clones expressing T4, which yield recombinant retroviral titers of 10^4 cfu/ml. $T4^+$ ψ-2 and ψ-AM clones were then used to generate retroviruses capable of infecting both lymphoid and nonlymphoid mouse and human cells. $T4^+$ transformants were generated either by exposure of cells to culture supernatants or by co-cultivation of cells with mitomycin-C-treated ψ-2 or ψ-AM clones (Figure 1B). $T4^+$ transformants were subsequently analyzed by Northern blot analysis and flow cytometry to confirm that T4 is expressed and present on the cell surface. Control cell lines expressing the surface protein T8 were constructed in an analogous manner.

T4 Is Essential for AIDS Virus Infection

In an initial series of experiments, we asked whether the presence of the T4 protein on the surface of a human lymphocyte is sufficient to render the cell susceptible to AIDS virus infection. To this end, we chose a primitive T cell leukemic line, HSB2 (Adams et al., 1968), which expresses only the early T lymphocyte proteins T1 and T11 on its surface. HSB2 expresses neither T4 nor T8, nor does it express the T cell antigen receptor or the associated complex of T3 proteins. We then constructed transformants of HSB2 which express either the T4 or T8 proteins on the cell surface and determined the susceptibility of these cell lines to AIDS virus infection. We have employed several different experimental approaches to assess AIDS virus infection, including expression of reverse transcriptase activity (Poiesz et al., 1980), expression of virus in the cytoplasm of the cell by immunofluorescence microscopy (McDougal et al., 1985b), detection of viral antigen in the culture supernatant using an immunoassay (McDougal et al., 1985a), and production of infectious virions by supernate subculture with phytohemagglutinin (PHA)-stimulated peripheral lymphocytes (McDougal et al., 1985b). Using these assays, we have not observed evidence for AIDS virus infection of the parental HSB2 cell line (Table 1).

In addition, it has been previously demonstrated that

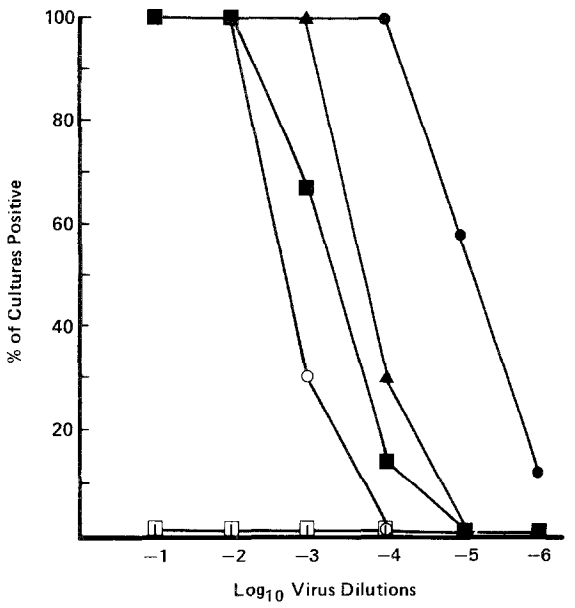

Figure 2. The Efficiency of Infection of Naturally Isolated and Transformed T4+ Cells

Cells were inoculated with serial 10-fold dilutions of AIDS virus, incubated for 18 hr at 37°C, washed, and plated in microculture. The frequency of infected cultures was determined by an enzyme-linked immunoabsorbent assay (ELISA) 12 days after infection (McDougal et al., 1985a). The results were plotted as % positive cultures vs. log virus dilution. Infectious virus titer (ID-50) is defined as the reciprocal of the dilution at which 50% of the cultures are positive for virus. Naturally isolated T4+ cells include phytohemagglutinin (PHA)-stimulated normal peripheral lymphocytes (●——●) and the T cell line CEM (○——○). T4+ transfected cell lines include HSB2-T4+ T cells (▲——▲) and Raji-T4+ B cells (■——■). The T8+ transfected cell lines HSB2-T8+ and Raji-T8+ (□——□) served as controls in these studies.

extensive cell fusion occurs when uninfected human cells bearing receptors for AIDS virus are co-cultivated with cells producing AIDS virus (Dalgleish et al., 1984). In this assay, there is no induction of syncytia when HSB2 cells are mixed with AIDS virus-producing H9 cells (Table 1), although abundant syncytia are formed with HTLV-I and HTLV-II producing cells (data not shown).

Finally, we tested for viral entry using pseudotypes of vesicular stomatitis virus (VSV) bearing the envelope glycoproteins of the AIDS virus (Clapham et al., 1984; Dalgleish et al., 1984). When cells infected with AIDS virus are superinfected with VSV, a proportion of the progeny VSV assemble sufficient AIDS virus envelope glycoprotein to resist neutralization by hyperimmune anti-VSV serum. The host range of these VSV(AIDS) pseudotype virions is restricted to cells expressing receptors specific to the AIDS virus. Following penetration of the cell and uncoating of the virion, the transcapsidated VSV genome replicates to produce nonpseudotype particles. During the secondary infection, progeny VSV released from infected cells penetrate and destroy neighboring indicator cells resistant to VSV(AIDS) pseudotype infection (mink CCL64 or bovine MDBK cells), resulting in the formation of VSV plaques which are then scored. Thus, infection with VSV(AIDS) pseudotypes provides a quantitative cytopathic plaque assay for viral entry (Dalgleish et al., 1984). In this assay, no plaques over background are observed when HSB2 cells are exposed to VSV(AIDS) pseudotypes (Table 1). In control experiments with pseudotypes of VSV RNA encapsidated in an HTLV-I envelope (VSV(HTLV-I)), numerous plaques are observed, demonstrating that the HSB2 cell, which bears HTLV-I receptors, is capable of replicating VSV efficiently (data not shown). These observations demonstrate that the VSV genome encapsidated in an AIDS virus envelope is incapable of entering HSB2 cells.

We now tried to determine whether the introduction of a functional T4 cDNA into HSB2 renders this cell susceptible to AIDS virus infection (Figure 1). Exposure of HSB2-T4+ transformants to AIDS virus results in a productive viral infection as determined by expression of reverse transcriptase activity (Poiesz et al., 1980), expression of virus in the cytoplasm of the cell by immunofluorescence microscopy (McDougal et al., 1985b), detection of viral antigen in the culture supernatant using an immunoassay (McDougal et al., 1985a), and production of infectious virions by supernate subculture with PHA-stimulated peripheral lymphocytes (McDougal et al., 1985b; Table 1). Control HSB2-T8+ cells are consistently negative in each of these assays (Table 1).

We have examined the efficiency with which different T4+ T cells are infected with AIDS virus. HSB2-T4+ and HSB2-T8+ transformants and the naturally isolated T4+ T cell line CEM, as well as PHA-stimulated peripheral lymphocytes, were exposed to serial 10-fold dilutions of AIDS virus, washed, and plated in microculture. The frequency of infected cultures was determined using an immunoassay 12 days after exposure to virus (McDougal et al., 1985a; Figure 2). In this manner, we defined the infectious virus titer, ID-50, as the reciprocal of the dilution required to infect 50% of the exposed cell cultures. The ID-50 of PHA-stimulated peripheral lymphocytes is two to three orders of magnitude greater than that observed for either naturally isolated or transformed T4+ T cell lines. The efficiency of infection of HSB2-T4+ cells is about 10-fold higher than that observed for the naturally isolated T4+ T cell line CEM. Control HSB2-T8+ cells are not susceptible to infection even at the highest virus titers examined (Figure 2).

We have also studied the ability of HSB2-T4+ cells to support both syncytia formation and the replication of VSV(AIDS) pseudotypes. When HSB2-T4+ cells are cocultivated with AIDS-virus-producing H9 cells, syncytia formation is readily observed within 18 hr (Tables 1 and 2). Moreover, syncytium induction is abolished by pretreating cultures with anti-T4A monoclonal antibody (Table 2). Finally, when HSB2-T4+ cells are exposed to VSV(AIDS) pseudotypes, infectious VSV particles are produced that destroy neighboring indicator cells (Tables 1 and 3). Plaque formation is inhibited by pretreatment with either anti-AIDS virus antibody or anti-T4A monoclonal antibody (Table 3). Control HSB2-T8+ cells are consistently negative in each of these assays (Tables 1, 2, and 3). These observations

Table 2. Induction of Syncytia in $T4^+$ Human Transformants

Human Cells	Syncytium Induction: H9/AIDS	Syncytium Induction: H9/AIDS + αT4A
JM($T4^+$)	+ + + + +	–
8166($T4^+$)	+ + + + +	–
HSB2	–	ND
HSB2-$T8^+$	–	ND
HSB2-$T4^+$	+ +	–
Raji	–	ND
Raji-$T8^+$	–	ND
Raji-$T4^+$	+ + +	–
HeLa	–	ND
HeLa-$T8^+$	–	ND
HeLa-$T4^+$	+ + + + +	–

2×10^5 cells were co-cultivated with 2×10^4 AIDS-virus-producing H9 cells (H9/AIDS) and incubated at 37°C. The cultures were examined for syncytia formation after 18 hr. The results are expressed as the approximate percentage of nuclei contained within syncytia: –, no syncytia; + +, 25%; + + +, 50%; + + + + +, ⩾90%; ND, not determined. Syncytium inhibition was assayed by adding anti-T4A monoclonal antibody (αT4A; 1:20) to the mixed cultures at the time of seeding. The naturally isolated $T4^+$ T cell lines JM and 8166 served as positive controls in these studies.

provide genetic evidence that in an immature human T lymphocyte, the mere presence of the T4 protein provides an essential function required for AIDS virus infection.

AIDS Virus Infection Is Not Restricted to T Lymphocytes

We next asked whether infection by AIDS virus is restricted to $T4^+$ T lymphocytes or whether the introduction of T4 into either human B lymphocytes or non-lymphoid cells would render these cells susceptible to AIDS virus infection. We therefore introduced the functional T4 cDNA into two human non-T cell lines: HeLa, an epithelial cell line derived from a cervical carcinoma (Gey et al., 1951), and Raji, a B lymphoblastoid cell line derived from a patient with Burkitt's lymphoma (Pulvertaft, 1963) (Figure 1). Prior to retrovirus-mediated gene transfer, these cell lines do not express surface T4 protein or T4 mRNA nor are they susceptible to AIDS virus infection (Table 1). In addition, the parental cell lines do not support the induction of syncytium nor the plating of VSV(AIDS) pseudotypes (Table 1).

In contrast, $T4^+$ Raji and HeLa transformants support AIDS virus infection by all of the criteria previously described (Table 1). The efficiency with which Raji-$T4^+$ cells can be infected with AIDS virus approximates that of HSB2-$T4^+$ cells and is about 10-fold higher than the efficiency of infection of the naturally isolated $T4^+$ T cell line CEM (Figure 2). Moreover, upon co-cultivation with AIDS-virus-producing H9 cells, Raji-$T4^+$ and HeLa-$T4^+$ cells support the induction of syncytia that is abolished by pretreating cultures with anti-T4A monoclonal antibody (Tables 1 and 2; Figure 3). In addition, exposure of these cells to VSV(AIDS) pseudotypes results in the production of infectious VSV and the formation of plaques which are inhibited by pretreatment with anti-AIDS virus antibody or anti-T4A monoclonal antibody (Tables 1 and 3). Control Raji-$T8^+$ and HeLa-$T8^+$ transformants are consistently negative in each of the seven assays employed to detect AIDS virus infection (Tables 1, 2, and 3).

Therefore, the introduction of a functional T4 gene into either human T lymphocytes, B lymphocytes, or epithelial cells is sufficient to render these cells susceptible to AIDS virus infection. Taken together, these observations demonstrate that the $T4^+$ T cell tropism observed in vivo is a consequence of the restricted expression of the T4 molecule and not the nature of the cell type in which it is expressed.

AIDS Virus Binds to Surface T4 Protein

The previous experiments provide genetic evidence that T4 expression is required for AIDS virus infection but do not provide information on the role of this molecule in the viral life cycle. The observation that surface expression of

Table 3. VSV Pseudotype Cytopathic Plaque Assay on $T4^+$ and $T8^+$ Human Transformants

Human Cells	VSV Pseudotype Titer (PFU/ml): VSV(HTLV-I)	VSV(HTLV-I) + αHTLV-I	VSV(AIDS)	VSV(AIDS) + αAIDS	VSV(AIDS) + αT4A
CEM($T4^+$)	20,000	50	42,000	50	200
HSB2-$T8^+$	10,000	50	0	ND	ND
HSB2-$T4^+$	12,000	50	1,000	100	300
Raji-$T8^+$	5,000	ND	0	ND	ND
Raji-$T4^+$	5,000	50	1,500	25	150
HeLa	10,000	ND	0	ND	ND
HeLa-$T4^+$	10,000	50	17,000	50	200

2×10^5 cells were incubated with VSV(AIDS) pseudotypes for 1 hr at 37°C. The cells were then washed with 1×10^6 mink CCL64 or bovine MDBK plaque indicator cells, permissive to VSV infection but resistant to VSV(AIDS), were added to each well. The cultures were then overlaid with agar medium and scored for VSV plaques two days after infection. Anti-T4A monoclonal antibody (αT4A; 1:20) or anti-AIDS virus serum (αAIDS; 1:10) were used to inhibit VSV(AIDS) pseudotype plaque formation by pretreatment of cells 30 min before exposure to pseudotypes. VSV(HTLV-I) pseudotypes, which plate on a wide variety of human cell types, were used as controls in these experiments. Anti-HTLV-I serum (1:10) was used to block VSV(HTLV-I) pseudotype plaque formation. The results are expressed as PFU/ml; ND, not determined.

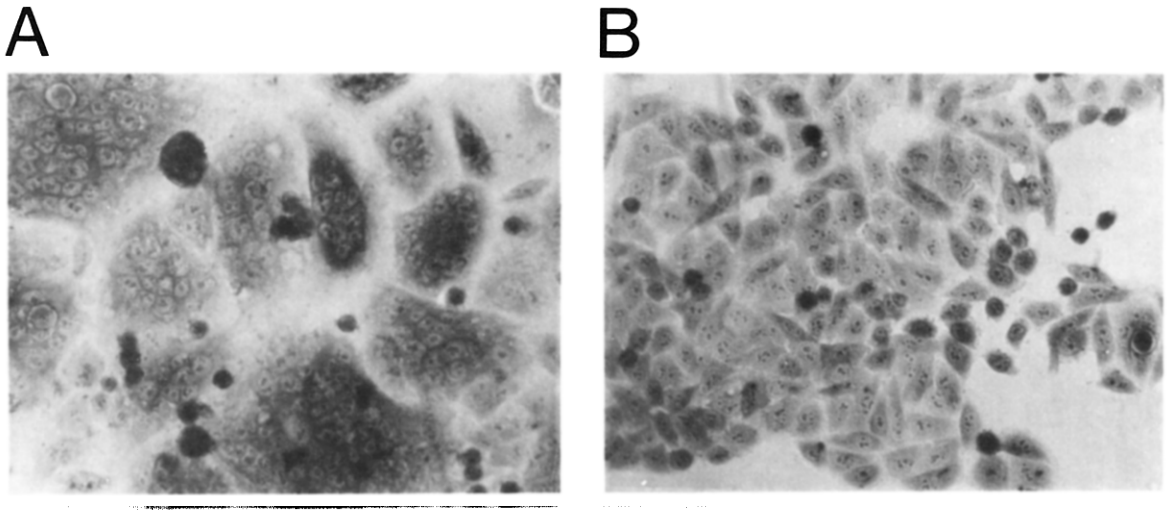

Figure 3. Formation of Syncytia in $T4^+$ HeLa Transformants

(A) 2×10^5 monolayer HeLa-$T4^+$ transformants were mixed with 2×10^4 AIDS-virus-producing H9 cells and incubated at 37°C. Inspection of the cultures after 18 hr revealed that over 90% of nuclei in the monolayer sheet were contained within syncytia.

(B) Anti-T4A monoclonal antibody (1:20) was added to the mixed cultures at the time of seeding. Inspection of the cultures after 18 hr revealed a complete absence of cell fusion. Cultures were photographed at 160× magnification.

T4 is necessary for AIDS virus infection immediately suggests that T4 is the AIDS virus receptor. We used cytofluorometry to examine the binding of AIDS virus to the surfaces of $T4^+$ and $T8^+$ transformed human cells (Figure 4; Table 1). HSB2, Raji, and HeLa cells, and the $T4^+$ or $T8^+$ transformants, were incubated with AIDS virus. Following viral absorption, the cells were washed, exposed to fluorescein-conjugated anti-AIDS virus antibody, and analyzed by flow cytometry. This assay demonstrates that the AIDS virus binds efficiently and specifically to the human transformants expressing surface T4, but not to the $T4^-$ parental cells or the $T8^+$ transformants (Figure 4, column B; Table 1). The binding of AIDS virus to the $T4^+$ cells is abolished by preincubation with anti-T4A monoclonal antibody but not by preincubation with anti-T8 monoclonal antibody (Figure 4, column C). Moreover, we have demonstrated (see below) that when $T4^+$ transformed cells are exposed to AIDS virus, the T4 glycoprotein coprecipitates with the viral envelope glycoprotein gp110, suggesting a direct physical association between these molecules. These studies strongly suggest that the AIDS virus binds to the T4 molecule on the cell surface and that this binding is independent of other T-cell-specific proteins since binding occurs to all $T4^+$ cell types examined.

Evidence for Endocytosis of AIDS Virus

We have performed preliminary experiments to determine how the AIDS virus, bound to T4 on the cell surface, introduces its RNA into the cytoplasm. Previous studies have described two distinct pathways of entry for enveloped viruses (reviewed in Dimmock, 1982; White et al., 1983; Marsh, 1984; Kielian and Helenius, 1986). Some viruses fuse directly with the plasma membrane, releasing their nucleocapsids into the cytoplasm, whereas others are internalized by receptor-mediated endocytosis. The acidic environment of the endosome then facilitates fusion of the viral envelope with the limiting membrane of the vacuole. Infection by viruses that enter cells via the endocytic pathway can be inhibited by treating cells with agents such as weak bases which deacidify the endosome (Ohkuma and Poole, 1978; Maxfield, 1982; Helenius et al., 1980, 1982). In the presence of ammonium chloride, fusion is blocked in the endosome but lysosomal degradation still proceeds at a reduced rate (Helenius et al., 1982).

In preliminary experiments, we have examined the effect of ammonium chloride on AIDS virus infection of the $T4^+$ T cell line JM (Table 4). In the absence of ammonium chloride, over 50% of JM cells exposed to AIDS virus express viral antigens five days after infection as determined by immunofluorescence microscopy. If JM cells are exposed to ammonium chloride (for 6 hr) either at the time of addition of virus or within 30 min after the addition of virus, we observe greater than 95% inhibition of viral infection (Table 4). However, if cells are treated with ammonium chloride 1 hr after the addition of virus, no inhibition of infection is observed, a finding consistent with the kinetics of viral entry described for other viruses which enter cells via receptor-mediated endocytosis (Helenius et al., 1980, 1982). Finally, the effect of ammonium chloride is completely reversible. Cells exposed to ammonium chloride for 1 hr and then washed free of the compound and exposed to AIDS virus support control levels of viral infection. These results are consistent with previous observations that upon removal of ammonium chloride, the pH of the endosome returns to the original low values within 1–2 min (Ohkuma and Poole, 1978; Helenius et al., 1982). We have also obtained similar results with amantadine, a compound that deacidifies the endosome.

These results are consistent with a mechanism of viral entry which involves endocytosis of the T4-AIDS virus complex and low pH-induced fusion of the viral envelope with the limiting membrane of the endosome, releasing

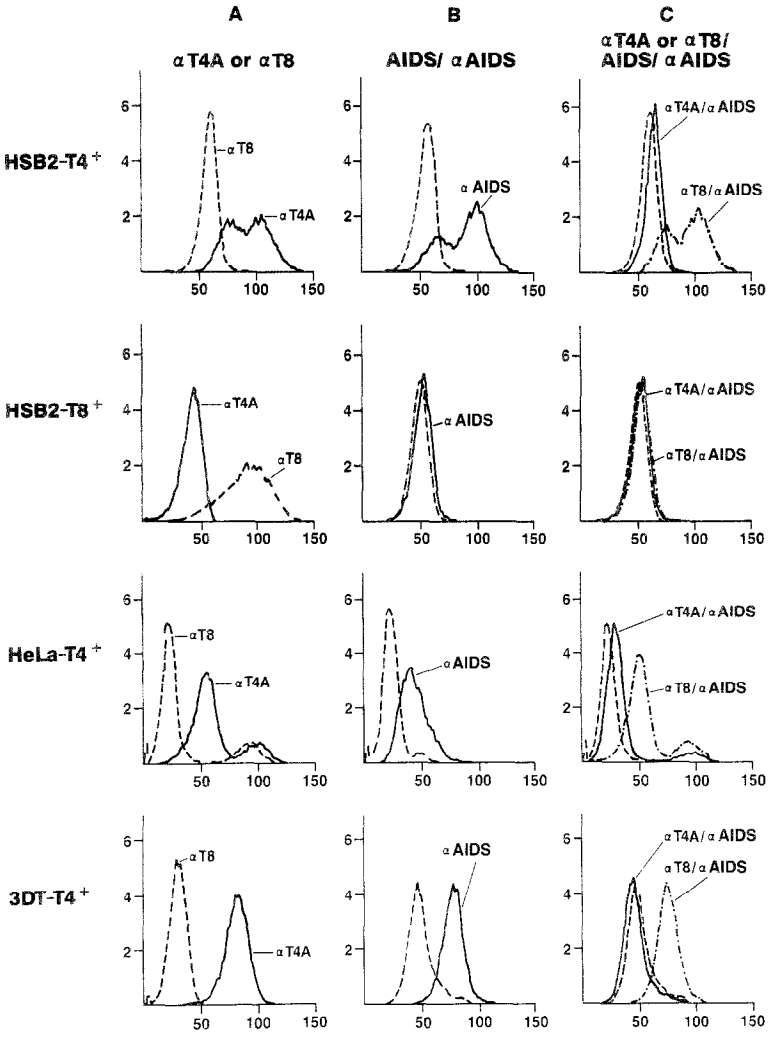

Figure 4. Flow Cytometry Analysis of AIDS Virus Binding to T4+ Transformed Cells

(Column A) Cells (5 × 10^5) were incubated with fluorescein-conjugated anti-T4A (———) or anti-T8 (– – –) monoclonal antibodies, washed, and analyzed by cytofluorometry.

(Column B) Cells (5 × 10^5) were incubated with buffer (– – –) or AIDS virus (———), washed, incubated with fluorescein-conjugated anti-AIDS virus antibody, and analyzed by cytofluorometry.

(Column C) Cells (5 × 10^5) were incubated with buffer (– – –), or with anti-T4A monoclonal antibody followed by AIDS virus (———), or with anti-T8 monoclonal antibody followed by AIDS virus (–·–·–). After a wash, fluorescein-conjugated anti-AIDS virus antibody was added and the cells were analyzed by cytofluorometry. Fluorescence histograms (cell number vs. fluorescence intensity) of each cell line are arranged horizontally.

the viral nucleocapsid into the cytoplasm of the cell. It should be noted, however, that ammonium chloride and amantadine may exert other inhibitory effects on virus production (Coombs et al., 1981; Cassell et al., 1984) and additional experiments are required before we may conclude with certainty that the primary route of entry of the AIDS virus is via receptor-mediated endocytosis.

T4+ Mouse Cells Do Not Support AIDS Virus Infection

Given that the AIDS virus infects all human cells expressing surface T4 protein, we next asked whether the presence of T4 on the surface of mouse cells would render these cells susceptible to AIDS virus infection. We therefore introduced the functional T4 gene into several lymphoid and non-lymphoid murine cells: 3DT52.5.8, an L3T4− T cell hybridoma (Greenstein et al., 1984); 38B9, an Abelson murine leukemia virus-transformed pre-B cell line (Alt et al., 1984); P815, a mastocytoma cell line (Lundak and Raidt, 1973); and NIH 3T3 and L, fibroblast cell lines (Figure 1). Using the criteria for viral infection described earlier, we observe that both the T4− mouse cell lines (including B and T cells) as well as their T4+ transformed counterparts are not susceptible to AIDS virus infection (Table 5). Moreover, T4+ mouse cells do not support the induction of syncytia upon co-cultivation with AIDS-virus-producing H9 cells (Table 5). Therefore, unlike human cells, the mere presence of T4 on the surface of mouse cells does not render these cells permissive to AIDS virus infection.

Table 4. Ammonium Chloride Inhibition of AIDS Virus Infection of T4+ JM T Cells

Time of NH_4Cl Addition after Virus Exposure	% Cells Infected	Syncytium Formation
0	<1	–
30 min	<1	–
60 min	40	+ + +
no inhibitor	>50	+ + +

2 × 10^5 T4+ JM T cells were exposed to AIDS virus at 0 min. Ammonium chloride (20 mM) was added to the cultures at various times during the course of virus infection (0 min, 30 min, and 60 min). After 6 hr, the cells were washed, replated in fresh media, and assayed five days after infection. The fraction of infected cells in the cultures expressing viral antigens was determined by immunofluorescence microscopy. The cultures were also examined for syncytium formation (see legend to Table 1).

Table 5. Susceptibility of $T4^+$ and $T8^+$ Mouse Transformants to AIDS Virus Infection

Mouse Cell	Maximum Reverse Transcriptase	Cytoplasmic Virus	Supernate Viral Ag	Supernate Subculture	Syncytium Induction	VSV(AIDS) Pseudotype Infection	Virus Binding
3DT($L3T4^+$)	4575	–	–	–	ND	ND	–
3DT($L3T4^-$)	3922	–	–	–	ND	ND	–
3DT-$T8^+$	3122	–	–	–	ND	ND	–
3DT-$T4^+$	4195	–	–	–	–	–	+
38B9	5607	–	–	–	ND	ND	–
38B9-$T4^+$	5211	–	–	–	–	–	+
P815-$T8^+$	4575	–	–	–	ND	ND	–
P815-$T4^+$	7210	–	–	–	–	–	+
3T3	3725	–	–	–	ND	ND	–
3T3-$T8^+$	2680	–	–	–	ND	ND	–
3T3-$T4^+$	3355	–	–	–	–	–	+
L	3948	–	–	–	ND	ND	–
L-$T8^+$	2490	–	–	–	ND	ND	–
L-$T4^+$	3671	–	–	–	–	–	+

The experimental strategy is described in the legend to Table 1 and in Experimental Procedures. 3DT($L3T4^+$) is a T cell expressing the murine homologue of T4, L3T4. 3DT($L3T4^-$) is a spontaneous variant no longer expressing L3T4.

We have performed a series of experiments to determine the nature of the block to infection. If AIDS virus DNA is introduced into mouse fibroblasts by DNA-mediated gene transfer, the virus replicates and infectious virions are produced (Levy et al., 1986). These results suggest that the block to infection of $T4^+$ mouse cells occurs at an event prior to viral replication. In order to define which of these early events in the viral life cycle is defective in mouse cells, we first asked whether $T4^+$ mouse cells are capable of binding AIDS virus. Parental $T4^-$ mouse cells, and the $T4^+$ or $T8^+$ transformants, were incubated with AIDS virus. Following viral absorption, the cells were washed, exposed to fluorescein-conjugated anti-AIDS virus antibody, and analyzed by flow cytometry. Using this assay, we observed that the AIDS virus binds to the surface of mouse cells bearing T4, but not to the $T4^-$ parental cells or the $T8^+$ transformants (Figure 4, column B; Table 5). The level of binding to the surface of $T4^+$ transformed mouse cells is comparable to that observed with $T4^+$ human cells. Moreover, the binding of AIDS virus to the $T4^+$ mouse cells is abolished by preincubation with anti-T4A monoclonal antibody but not by preincubation with anti-T8 monoclonal antibody (Figure 4, column C).

We have also performed biochemical experiments that demonstrate the direct interaction of the AIDS virus with the T4 protein on the surface of mouse cells. To this end, AIDS virus was incubated with $T4^+$ NIH 3T3 cells whose surface was radiolabeled with ^{125}I (Figure 5). The cells were then lysed in non-ionic detergent and the lysates were exposed to anti-AIDS virus antibody. Anti-AIDS virus antibody coprecipitates a 55 kd surface protein (lane 2) which migrates to the identical relative molecular mass (M_r) as the protein precipitated directly by anti-T4A monoclonal antibody (lane 3). If cells are incubated with anti-T4A monoclonal antibody prior to AIDS virus exposure, the 55 kd protein is not detected in anti-AIDS virus precipitates (lane 1). These observations demonstrate that the AIDS virus binds to the T4 protein on the surface of mouse transformed cells and that the binding does not require additional proteins restricted to human cells.

These results suggest that the block to infection of $T4^+$ mouse cells is likely to result from the inability of mouse cells to internalize the T4-AIDS virus complex, to deliver the viral nucleocapsid from the endosome into the cytoplasm, or to uncoat the viral nucleocapsid. We have provided support for a block in either endocytosis or fusion within the endosome by performing experiments in which $T4^+$ mouse cells are exposed to VSV(AIDS) pseudotypes (Tables 5 and 6). In control experiments, we have shown that several $T4^+$ mouse cells produce infectious VSV when exposed to pseudotypes of VSV bearing the envelope antigens of Moloney murine leukemia virus (VSV(Mo-MuLV); Table 6). These data indicate that mouse cells, which bear Mo-MuLV receptors, are capable of uncoating the VSV nucleocapsid and replicating VSV efficiently. However, no VSV plaques over background are observed when $T4^+$ lymphoid or non-lymphoid mouse cells are exposed to VSV(AIDS) pseudotypes (Tables 5 and 6). Taken together, these studies demonstrate that $T4^+$ mouse cells bind AIDS virus efficiently but fail to deliver the viral nucleocapsid into the cytoplasm. $T4^+$ mouse cells therefore do not support viral entry and are likely to be defective in either endocytosis or fusion within the endosome.

T4 mRNA Is Expressed in the Brain

In addition to the disruption of the cellular immune system, AIDS is frequently accompanied by central nervous system (CNS) disorders which are thought to be a consequence of the direct infection of cells in the brain by the AIDS virus (reviewed in Johnson and McArthur, 1986). It was of interest to determine whether T4 is expressed in cells within the CNS, thereby providing an explanation for the neurotropic properties of the virus. We therefore performed Northern blot analysis of RNA prepared from both human and mouse brains to determine whether T4 mRNA

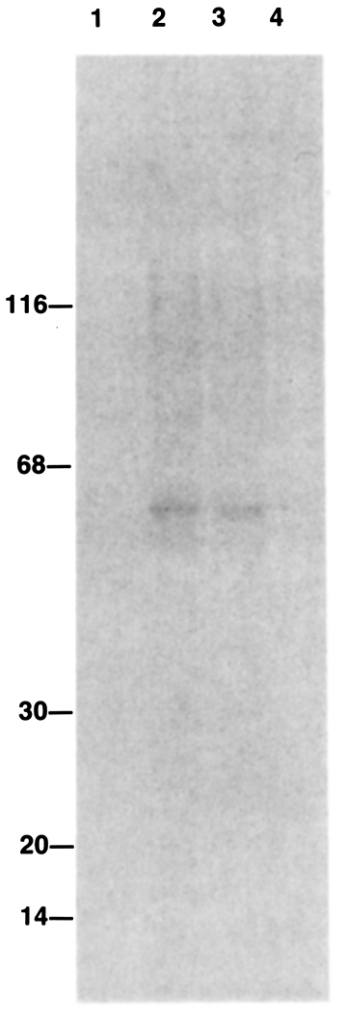

Figure 5. Coprecipitation of the T4 Glycoprotein with the AIDS Virus

Lane 1: $T4^+$ NIH 3T3 transformants (1×10^7) were surface radiolabeled with ^{125}I by the lactoperoxidase technique and incubated with anti-T4A monoclonal antibody. Labeled cells were then exposed to unlabeled AIDS virus, washed, and lysed in non-ionic detergent buffer. The lysate was precleared and immunoprecipitated with anti-AIDS virus antibody-Sepharose conjugates.

Lane 2: Same as Lane 1 except that cells were pretreated with anti-T8 monoclonal antibody instead of anti-T4A antibody before virus addition.

Lane 3: $T4^+$ NIH 3T3 cells (1×10^7) were surface radiolabeled with ^{125}I, washed, and lysed in non-ionic detergent buffer. The lysate was precleared and immunoprecipitated with anti-T4A monoclonal antibody-Sepharose conjugates.

Lane 4: Same as Lane 3 except that the lysate was immunoprecipitated with anti-T8 monoclonal antibody-Sepharose conjugates. Sepharose absorbants were washed, dissolved in sample buffer, and electrophoresed through a 3.3%–20% gradient polyacrylamide gel under reducing conditions. Relative molecular masses (M_r) are given in kilodaltons.

Table 6. VSV Pseudotype Cytopathic Plaque Assay on $T4^+$ Mouse Transformants

	VSV Pseudotype Titer (PFU/ml)	
Mouse Cells	VSV(Mo-MuLV)	VSV(AIDS)
P815	30,000	30
P815-$T4^+$	30,000	50
3T3-$T4^+$	22,000	30
3DT-$T4^+$	ND	100

2×10^5 cells were incubated with VSV(AIDS) pseudotypes for 1 hr at 37°C and plaques were scored on indicator cells as described in the legend to Table 3. VSV(Mo-MuLV) pseudotypes, which plate on a wide variety of mouse cell types, were used as controls in these experiments. The results are expressed as PFU/ml; ND, not determined.

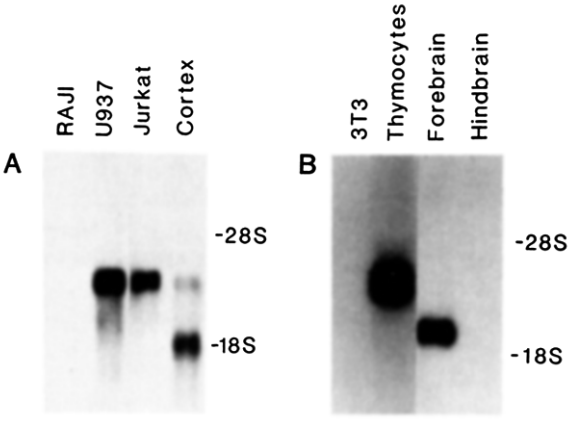

Figure 6. Northern Blot Analysis of RNA Derived from Human and Mouse Brain, Lymphoid, and Myeloid Cells

(A) Northern blot analysis of human RNA samples. One microgram of poly(A)$^+$ RNA from Raji ($T4^-$ B cell line), U937 ($T4^+$ monocytic cell line), and Jurkat ($T4^+$ T cell line), and 5 μg of poly(A)$^+$ RNA from cerebral cortex were electrophoresed through a 1% agarose-formaldehyde gel, blotted onto Hybond (Amersham), and probed with a ^{32}P-labeled T4 cDNA insert, pT4B (Maddon et al., 1985).

(B) Northern blot analysis of mouse RNA samples. Five micrograms of poly(A)$^+$ RNA from 3T3 cells (fibroblast cell line), forebrain, and hindbrain, and 20 μg of total RNA from thymocytes were electrophoresed through a 1% agarose-formaldehyde gel, transferred onto Hybond, and probed with a ^{32}P-labeled L3T4 cDNA insert, pL3T4B (P. J. Maddon, D. E. Maddon, and R. Axel, unpublished data).

sequences are expressed in the CNS (Figure 6). Interestingly, poly(A)$^+$ RNA derived from human cerebral cortex contains two distinct T4 mRNAs with molecular weights of approximately 3 and 1.8 kb (Figure 6A). The weaker 3 kb RNA is identical in size to the mRNA expressed by two $T4^+$ leukemic cell lines, U937 (monocytic cell line) and Jurkat (T cell line), as well as that expressed by peripheral T lymphocytes. The smaller, more abundant 1.8 kb mRNA absent from T lymphocytes could result from alternative splicing or alternative 5′ or 3′ termini.

A more careful analysis of the localization of T4 mRNA has been performed by isolating poly(A)$^+$ RNA from specific regions of the mouse brain (Figure 6B). Hybridization with radiolabeled cDNA encoding the murine homologue of T4, L3T4 (P. J. Maddon, D. E. Maddon, and R. Axel, unpublished data), reveals an intense 2.2 kb mRNA in mouse forebrain which is absent from hindbrain samples. The 2.2 kb L3T4 mRNA is detectable in the cortex and is most abundant in the striatum, but is absent from the cerebellum, brain stem, and spinal cord (data not shown; Molineaux, Maddon, and Axel, unpublished data). This 2.2 kb mRNA present in the CNS is approximately 1 kb smaller than the 3.2 kb mRNA encoding L3T4 in thymocytes (Figure 6B). These results suggest that the neuro-

tropism displayed by the AIDS virus is likely to be the result of surface expression of the T4 molecule on cells in the brain. The level of mRNA detected in forebrain is about 1/30th the level of thymocytes. This may reflect low-level expression by a large number of cells or higher levels of expression by a small subpopulation of cells. We do not know at present whether T4 is expressed by neurons or supporting cells. The presence of a variant transcript in the CNS, however, makes it unlikely that all of the T4 mRNA in brain is expressed by the rare invading hematopoietic cell.

Discussion

These studies support a mechanism of AIDS virus infection that initially involves the specific interaction of the AIDS virus with T4 molecules on the cell surface. This association can be demonstrated on both T and B lymphocytes as well as epithelial cells, and therefore does not require the participation of additional T-cell-specific proteins. Our data suggest that the T4-AIDS virus complex is internalized via receptor-mediated endocytosis and the viral envelope then fuses with the limiting membrane of the endosome, releasing the nucleocapsid into the cytoplasm. Viral replication and transcription can then occur in both lymphoid and non-lymphoid cell lines. Moreover, the T4 gene is expressed in the brain as well as in lymphocytes, providing an explanation for the dual neurotropic and lymphotropic character of the AIDS virus. In this manner, a T lymphocyte surface protein important in mediating effector cell–target cell interactions has been exploited by a human retrovirus to specifically target the AIDS virus to populations of $T4^+$ cells.

Cell surface receptors have been identified for a number of enveloped viruses and the pattern of expression of these receptors is often responsible for the host range and tropic properties of specific viruses (reviewed in Dimmock, 1982; Marsh, 1984). Some viruses will infect only a narrow range of cell types, reflecting the expression of the viral receptor on specific populations of target cells. Rabies virus, for example, interacts with the nicotinic acetylcholine receptor (Lentz et al., 1982) and infects largely skeletal muscle and neurons, whereas Epstein-Barr virus interacts with the C3d complement receptor type 2 (Fingeroth et al., 1984) and infects B lymphocytes. Other viruses, such as the myxoviruses, interact with ubiquitously distributed sialic acid residues on the cell surface and infect a much broader range of cell types.

The restricted expression of cell surface receptors provides only one explanation for viral tropism. Some viruses will replicate only in a restricted set of differentiated cell types whereas others will only be efficiently transcribed in specific cell types. Hence, the Moloney murine leukemia virus (Mo-MuLV) induces T cell lymphomas in newborn mice, yet the closely related Friend helper murine leukemia virus (Fr-MuLV) induces primarily erythroleukemias (Tambourin et al., 1979; Oliff et al., 1981; Silver and Fredrickson, 1983). This tropism is thought to result from differences in the long terminal repeats (LTR) which facilitate the efficient transcription of the Mo-MuLV genome in T lymphocytes and the Fr-MuLV genome in erythroid precursors (Chatis et al., 1983, 1984; Bosze et al., 1986).

We have demonstrated that the primary tropic determinant of the AIDS virus is the expression of the T4 protein on the surface of the target cell. In vivo infection is restricted to lymphoid and myeloid cells as well as brain cells, and we have shown that each of these cell types expresses T4 (Figure 6; Maddon and Axel, unpublished data). In vitro we have demonstrated that the introduction of T4 into $T4^-$ human B lymphocytes and epithelial cells, cells which are not natural targets for AIDS virus, now renders these cells susceptible to productive infection by AIDS virus.

It is significant that the AIDS virus has evolved a mechanism whereby it specifically infects $T4^+$ T lymphocytes, a population of cells which is largely composed of helper T cells. Moreover, unlike most retroviruses, the AIDS virus has a cytopathic effect upon its target cell. Thus, the T4 tropism the AIDS virus has evolved to enter the target cell also serves to eliminate the helper T lymphocyte population and suppress the cellular immune response. This is only one of several alternative strategies that viruses have evolved to evade both humoral and cellular immune defenses.

AIDS Virus gp110 Shares Homology with Immunoglobulins

The T4 glycoprotein consists of four tandem extracellular domains which contain significant sequence and structural homology with the variable (V) and joining (J) regions of immunoglobulin gene family members (Maddon et al., 1985; Barclay et al., 1986; Maddon and Axel, unpublished data). The T4 molecule on the surface of T lymphocytes is thought to interact with class II major histocompatibility complex (MHC) proteins expressed by antigen-bearing target cells. This presumed interaction between class II MHC proteins and T4, two members of the immunoglobulin gene family, may be essential for the appropriate function of the T cell. Recent studies provide several additional examples in which members of the immunoglobulin gene family interact with one another in the immune system. Such interactions not only occur between immunoglobulin chains, but also involve class I MHC proteins and β_2-microglobulin, the individual subunits of class II MHC proteins, the chains of the T cell antigen receptor, and presumably the T cell antigen receptor and class I or class II proteins.

The observation that T4 interacts with the exterior envelope glycoprotein gp110 of the AIDS virus prompted us to examine the sequence of gp110 for homology to members of the immunoglobulin gene family. Interestingly, two regions in gp110 share significant homology with human immunoglobulin heavy chain constant (C) regions (Figure 7). A 44 amino acid stretch surrounding cysteine 61 of gp110 is homologous at 15 positions (34% homology without gaps) to a region surrounding cysteine 84 of the first constant domain (C_H1) of human immunoglobulin $\gamma2$ heavy chains; similar homology is shared with human immunoglobulin $\gamma1$ and $\gamma4$ heavy chains (Figure 7A). Statistical analysis (Dayhoff et al., 1983) indicates that the ho-

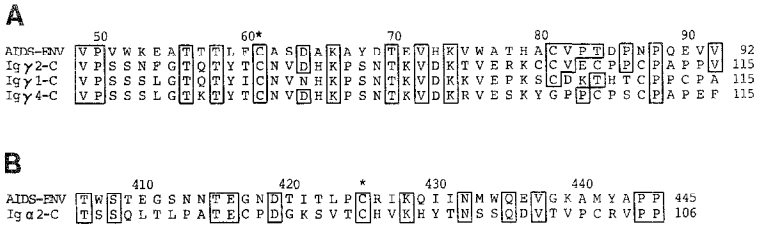

Figure 7. Alignment of AIDS Envelope Glycoprotein gp110 with Immunoglobulin Heavy Chain Constant Regions

(A) Alignment of the conserved exterior envelope glycoprotein (gp110) sequence of the AIDS virus (AIDS-ENV; Starcich et al., 1986) with first constant region (C_H1) of human immunoglobulin γ heavy chains: γ2 (Ellison and Hood, 1982), γ1 (Ellison et al., 1982), and γ4 (Ellison et al., 1981). Numbers shown above the single letter code amino acid sequence designate positions in gp110. The numbers on the right show amino acid residue positions for each protein. Conserved cysteine residues of the immunoglobulin disulphide bridge are marked by stars. Identities appear as boxed residues.

(B) Alignment of the conserved exterior envelope glycoprotein (gp110) sequence of the AIDS virus (AIDS-ENV; Starcich et al., 1986) with the first constant region (C_H1) of human immunoglobulin α2 heavy chains (Torano and Putman, 1978).

mologies between this region of gp110 and human immunoglobulin γ heavy chains are 7–8.94 standard deviations from chance scores. In addition, we observed a second region surrounding cysteine 425 of gp110 and cysteine 86 of the C_H1 domain of human immunoglobulin α1 heavy chains that revealed a 30% homology without gaps (8 standard deviations from random scores) (Figure 7B). Moreover, when analyzed using the algorithm of Chou and Fasman (1978), these regions in gp110 are capable of forming β-strands that align with the β-strands formed in this region of immunoglobulin C domains. Thus, two blocks of over 40 amino acid residues in gp110 share significant sequence and structural homology with immunoglobulin C domains. The tendency of immunoglobulin family members to associate with one another suggests the possibility that these immunoglobulin-like regions of gp110 may interact directly with T4.

Receptor-Mediated Endocytosis: A Possible Mechanism of Viral Entry

We performed a series of experiments to determine the mechanisms the AIDS virus uses to introduce its genetic material into the cytoplasm of the cell. Previous studies with enveloped viruses have defined two distinct pathways by which the virus can enter the cell (reviewed in Dimmock, 1982; White et al., 1983; Marsh, 1984; Kielian and Helenius, 1986). One mechanism, exemplified by paramyxoviruses, involves the direct fusion of the viral envelope with the plasma membrane. The second mechanism, employed by orthomyxoviruses for example, involves the association of virus with a specific cell surface receptor and the internalization of the complex by receptor-mediated endocytosis. In the low pH environment of the endosome, the viral envelope fuses with the limiting membrane of the vacuole and the nucleocapsid enters the cytoplasm of the cell.

Recent observations suggest that the complex of AIDS virus and T4 on the cell surface is internalized via endocytosis. First, exposure of $T4^+$ T cells to phorbol esters results in rapid internalization of surface T4 protein into the cytoplasm (Hoxie et al., 1986). Moreover, immunoelectronmicroscopy data demonstrate that in resting T lymphocytes, the T4 molecule resides on the cell surface. Upon activation of T cells with phytohemagglutinin or phorbol esters, most of the T4 protein is present within endocytotic vesicles in the cytoplasm (Lederman and Chess, unpublished data). These observations suggest that when $T4^+$ T lymphocytes are exposed to AIDS virus, the virus is internalized via its T4 receptor molecule. Finally, infection by viruses which enter the cell via receptor-mediated endocytosis can be inhibited by treating cells with agents that deacidify the endosome. We have demonstrated that exposure of cells to the weak bases ammonium chloride or amantadine results in greater than 95% inhibition of AIDS virus infection in vitro (Table 4).

Although the expression of T4 is sufficient for AIDS virus infection of all human cell lines examined, we have not observed infection of a number of transformed mouse cell lines expressing the T4 protein on their surface. Our data demonstrating the efficient binding of the AIDS virus to $T4^+$ mouse cells and the inability of VSV(AIDS) pseudotypes to infect these cells, taken together with the observation that AIDS virus DNA transfected into mouse cells produces infectious virions (Levy et al., 1986), indicates that the block to infection resides in viral entry, most likely the result of a defect either in endocytosis or in fusion within the endosome. In support of this suggestion, Godfrey and Chess (unpublished data) have demonstrated that murine T cell clones bearing the murine T4 homologue, L3T4, internalize L3T4 efficiently. However, murine T cell transformants expressing human T4 internalize the human protein far less efficiently than the mouse protein L3T4.

Why is a mouse T cell unable to efficiently internalize surface T4 when it internalizes the mouse homologue L3T4 efficiently? Species-specific endocytosis has not been generally observed when surface molecules are transferred across species by gene transfer (Davis et al.,

1986). However, it is possible that the cytoplasmic domain of T4, which differs from that of the mouse L3T4 at 8 of 38 positions (P. J. Maddon, D. E. Maddon, and R. Axel, unpublished data), is not efficiently recognized by either mouse clathrin or other molecules important in the internalization process. Alternatively, endocytosis may require the participation of additional molecules not present in a mouse cell which either interact with T4 or modify T4 to provide a signal for internalization. Whatever the mechanism, our data suggest that mouse cells do not efficiently internalize the human T4 molecule and, as a consequence, mouse transformants bearing surface T4 protein are resistant to AIDS virus infection.

T4 Is Expressed in CNS

The observations that AIDS virus infects the central nervous system (CNS) prompted us to ask whether T4, a receptor for AIDS virus in lymphocytes, is also expressed in cells in the brain. We have shown that the human cerebral cortex expresses T4 mRNA, suggesting that T4 or a variant form of T4 serves as a viral receptor on cells in the brain, thereby providing an explanation for the neurotropic property of the virus. The human forebrain expresses two species of T4 mRNA: a 3 kb mRNA identical in size to that expressed by T lymphocytes and a predominant 1.8 kb mRNA (Figure 6A). Whereas the 3 kb mRNA encodes a receptor for the AIDS virus, we have not yet determined the structure of the smaller mRNA and therefore do not know whether this RNA species is capable of encoding a virus receptor. We have also detected a variant form of mouse L3T4 mRNA in the cortex and striatum. Interestingly, this mRNA is most prevalent in the striatum, a frequent site of lesions in AIDS patients with neurological symptoms (Sharer et al., 1986). These studies provide gross regional distribution of T4 in the CNS, but do not allow us to define the precise cells expressing the T4 protein. AIDS virus has been identified by electron microscopy in macrophages and astrocytes in the brain (Epstein et al., 1985; Koenig et al., 1986). Macrophages express the 3 kb T4 mRNA and surface T4 protein and are susceptible to AIDS virus infection (Figure 6; Maddon and Axel, unpublished data; Nicholson et al., 1986). It is likely that other cell types in the brain also express T4 or its variant form since the macrophage expresses only the larger of the mRNAs whereas the predominant T4 mRNA in the CNS is the smaller 1.8 kb variant. It will therefore be of interest to determine what additional cell types express T4 and whether these cells are susceptible to AIDS virus infection.

The observation that T4 is expressed in the brain raises the question of the possible function of T4 in the CNS. A number of members of the immunoglobulin gene family, including Thy-1 (Barclay and Hyden, 1978), OX-2 (Webb and Barclay, 1984), and now, T4, have been identified in both the immune system and the CNS. While the functions of OX-2 and Thy-1 remain unknown, recent data suggest that the specificity of interaction of $T4^+$ T lymphocytes with a restricted subset of antigen-bearing target cells may result from the interaction of the T4 protein with the products of class II MHC genes. It is therefore possible that T4 serves a similar function as a recognition molecule governing specific cell–cell interaction in the central nervous system.

Experimental Procedures

Cotransformation and Rosetting Assay

Mouse ψ-2 cells (a generous gift from Richard Mulligan, MIT) were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% calf serum (CS; Gibco). ψ-2 cells were plated out at a density of 5×10^5 cells per 10 cm dish, two days before transformation. Calcium phosphate precipitates were prepared by the method of Graham and van der Eb (1973), as modified by Wigler et al. (1977). Precipitates were applied to the cells using 10 μg of carrier DNA and either 10 μg of T4-pMV7 or 10 μg of T8-pMV7. After two days, the cells were placed under selection in DME/10% CS and 500 μg/ml G418 (Geneticin; Gibco).

Rosetting assays to identify $T4^+$ or $T8^+$ colonies were performed on surviving colonies one week after growth in selective medium. After one rinse with phosphate-buffered saline (PBS), the plates were incubated with 2.5 ml of the purified monoclonal antibody OKT4A or OKT8 (1 mg/ml; Ortho) diluted at 1/500 in PBS containing 5% fetal calf serum (FCS) for 45 min at room temperature. Free antibody was removed from the plates with three gentle rinses in PBS. Six milliliters of human erythrocytes conjugated with purified rabbit anti-mouse IgG antibody (2% v/v stock suspension, diluted 1/10 in PBS/5% FCS) was added and the plates were left at room temperature. After 45 min, free erythrocytes were gently aspirated and PBS was added prior to inspection. $T4^+$ and $T8^+$ ψ-2 clones were purified by colony isolation and characterized by flow cytometry and Northern blot analysis.

Recombinant Retrovirus Production and Infection

$T4^+$ and $T8^+$ ψ-2 clones were isolated that produce recombinant retrovirus stocks with titers of 10^5 cfu/ml. Viral stocks were prepared by adding 10 ml of fresh DME/10% CS to a near confluent monolayer of the $T4^+$ or $T8^+$ ψ-2 clones. After 24 hr, the medium was removed and filtered through a 0.45 μm filter (Millipore). For infection, 5×10^5 cells were incubated with 2 ml of viral supernatant (or a dilution) in the presence of 8 μg/ml polybrene (Aldrich). After 3 hr, 8 ml of fresh medium was added. Three days after infection the cells were reseeded into DME/10% CS containing 500 μg/ml G418, grown for two weeks, scored for $G418^r$ colonies, and screened for surface T4 or T8 expression using the in situ rosetting procedure or flow cytometry.

ψ-2 culture supernatants were used to infect mouse fibroblasts (NIH 3T3, ψ-AM [a gift from Richard Mulligan, MIT]) and mastocytoma cells (P815) as described above. Mouse T (3DT52.5.8, a gift from Philippa Marrack and John Kappler, National Jewish Hospital) and B (38B9, a gift from Fred Alt, Columbia) cell lines were infected by co-cultivation with a 10-fold excess of $T4^+$ or $T8^+$ ψ-2 clones (pretreated with 10 μg/ml mitomycin-C for 2 hr; Sigma) for two days in the presence of 2 μg/ml polybrene. $T4^+$ or $T8^+$ adherent transformants (fibroblasts) were purified by the in situ rosetting assay followed by colony isolation; $T4^+$ or $T8^+$ nonadherent transformants (mastocytoma, lymphocytes) were purified by fluorescence-activated cell sorting (FACS). Nonadherent human lymphoid cell lines (HSB2, RPMI–T cells; Raji–B cell) and adherent epithelial cells (HeLa) were infected by co-cultivation with $T4^+$ or $T8^+$ ψ-AM clones and purified in a similar manner.

Cell lines were selected for G418 resistance at a concentration of 1.5 mg/ml, except for HeLa cells, which require 1 mg/ml, and fibroblasts, which require 0.5 mg/ml. All cell cultures producing recombinant amphotrophic viruses (ψ-AM) were maintained under P3 containment conditions.

AIDS Virus

The prototype LAV strain of HTLV-III/LAV was obtained from J.-C. Chermann (Institut Pasteur, Paris; Barre-Sinoussi et al., 1983). Virus inocula used in these studies were from the second to fifth passages of virus in our laboratory. Inocula are culture supernatants from HTLV-III/LAV-infected, phytohemagglutinin (PHA)-stimulated peripheral lymphocytes (see below) which were harvested by sequential centrifugation ($300 \times g$ for 7 min followed by $1500 \times g$ for 20 min) and stored in liquid nitrogen. For binding studies, virus was concentrated from culture su-

pernatants, harvested as above, by ultracentrifugation at 90,000 × g for 90 min over a 15% cushion of Renograffin (E. R. Squibb) in 0.01 M Tris, 0.15 M NaCl, 1 mM EDTA (pH 8.0).

Anti-HTLV-III/LAV Reagents

Serum with high levels of antibody to HTLV-III/LAV was obtained from a homosexual man with chronic lymphadenopathy, and its specificity by immunofluorescence (McDougal et al., 1985b), Western blot analysis (McDougal et al., 1985a), and radioimmunoprecipitation (McDougal et al., 1986) has been described. Portions of the IgG fraction were coupled with fluorescein isothiocyanate (FITC; FITC:protein ratio of 10.7 μg/ml), horseradish peroxidase (HPO; type VI; Sigma) and agarose as described (McDougal et al., 1985a; Reimer et al., 1978; Wilson and Nakane, 1978; Porath et al., 1967). Conjugates of IgG from a nonimmune serum were prepared in parallel.

Reverse Transcriptase Assay

Magnesium-dependent, particulate reverse transcriptase (RT) activity was measured with a template primer of $(A)_n(dT)_{12\text{-}18}$ (or $(dA)_n(dT)_{12\text{-}18}$ as the negative control) in the presence of 7.5 mM Mg^{2+} (Poiesz et al., 1980).

Immunofluorescence Detection of Cytoplasmic AIDS Virus

Cultured cells (1 × 10^5 in 0.1 ml) were centrifuged onto glass slides (Shandon Cytocentrifuge), fixed in 95% ethanol and 5% acetic acid at −20°C for 30 min, and rehydrated with three 10 min changes of PBS (0.01 M PO_4, 0.15 M NaCl [pH 8.0]). Slides were exposed to a 1/500 dilution of FITC-anti-HTLV-III/LAV (19 μg/ml) for 30 min at room temperature. The slides were then washed (three changes, 10 min each) and mounted under a coverslip with 50% glycerol in PBS. The slides were examined with an epi-illuminated Leitz Orthoplan microscope at 630 × power. Under these conditions, the FITC-anti-HTLV-III/LAV reagent is specific for HTLV-III/LAV. Uninfected PHA-stimulated cells, Epstein Barr (EB) virus-infected B cell lines, an adenovirus-infected cell line, several T cell lines, and HTLV-I and HTLV-II infected cell lines were not stained.

AIDS Virus Immunoassay (Antigen Capture Assay)

This is a sandwich immunoassay that has been described in detail (McDougal et al., 1985a). Briefly, culture supernatant is added to microtiter plate wells coated with anti-HTLV-III/LAV IgG. After the plates are washed, bound virus antigen is detected with HPO-anti-HTLV-III/LAV. This assay, which is at least as sensitive as the RT assay, is negative with culture supernatants from PHA-stimulated lymphocytes from numerous donors, EB virus-infected B cell lines, several T cell lines, polyclonal and cloned IL-2 dependent T cell lines, the myeloid line K562, and cell lines that harbor HTLV-I or HTLV-II. The cutoff OD_{490} for discriminating a positive from a negative supernatant was determined in each run from the mean plus 2 SD of at least ten replicative determinations on control (uninfected cell culture) supernatants harvested at the same time.

AIDS Virus Infectivity (ID-50) Assay

The microculture assay for the titration of infectious HTLV-III/LAV has been described in detail (McDougal et al., 1985a). Briefly, PHA-stimulated lymphocytes or cell lines (2 × 10^6 cells/ml) are inoculated with serial 10-fold dilutions of virus inoculum and incubated for 18 hr at 37°C. The cells were then washed and plated in microculture (10 to 20 cultures per dilution: 1 × 10^5 cells per culture in 0.25 ml medium). Every four days, 100 μl of supernatant was removed and replaced with fresh medium. Supernatants were then assayed for viral antigen by the antigen capture assay as described above. Infectious virus titer (ID-50) is defined as the reciprocal of the dilution at which 50% of the cultures are positive for virus (McDougal et al., 1985a).

VSV Pseudotype Assay

Vesicular stomatitis virus (VSV, Indiana strain, wild type) was propagated in cells producing the retrovirus required for the envelope pseudotype as described by Clapham et al. (1984). Hyperimmune neutralizing sheep anti-VSV serum (a gift from J. Zavada, Bratislava, Czechoslovakia) was added to the harvested VSV to inactivate nonpseudotype virions. The pseudotype titers ranged between 10^4 and 10^5 PFU/ml. For the assay, 2 × 10^5 cells to be infected with VSV pseudotypes were plated in 30 mm diameter tissue culture wells. HeLa, NIH 3T3, and L cells were naturally adherent; all other cells types were attached by pretreatment of the substratum with 50 μg/ml poly-L-lysine. After virus adsorption for 1 hr, the cells were washed and 10^6 mink CCL64 or bovine MDBK cells were added to each well. These cells provide excellent plaques for secondary VSV infection but are resistant to infection by pseudotype virions. After allowing the plaque indicator cells to settle and spread (approximately 90 min), the monolayers were overlaid with agar medium. VSV plaques were counted two days after infection. Anti-T4A monoclonal antibody (1:20), anti-HTLV-III serum (1:10), or anti-HTLV-I serum (1:10) were used to inhibit pseudotype plaque formation by pretreatment of cells 30 min before addition of pseudotypes as described by Dalgleish et al. (1984).

Syncytium Induction Assay

2 × 10^5 cells were co-cultivated with 2 × 10^4 H9 cells infected by and producing HTLV-III (Popovic et al., 1984) in 10 mm diameter wells. The cultures were incubated at 37°C and examined for syncytia formation after 18 hr as previously described (Nagy et al., 1983; Dalgleish et al., 1984). Cells with five or more syncytia were scored as positive. Syncytium inhibition was assayed by adding anti-T4A monoclonal antibody (1:20) to the mixed cultures at the time of seeding.

Cytofluorometric Analysis and AIDS Virus Binding

The method has been described in detail (McDougal et al., 1985b). Briefly, cell surface T4 or T8 expression was detected by direct immunofluorescence with fluorescein-conjugated anti-T4A or anti-T8 monoclonal antibodies (OKT4A, OKT8). The diluent/wash buffer was 0.01 M PO_4, 0.15 M NaCl (pH 7.4) containing 0.1% bovine serum albumin, 2% v/v AB^+ human serum, and 0.01% NaN_3. All reagents were pretitered for optimal (saturating) binding. Cells (5 × 10^5) were incubated in a 25 μl dilution of monoclonal antibody for 30 min at 4°C. The cells were washed by centrifugation (300 × g for 7 min), resuspended in 0.5 ml of 1% paraformaldehyde in saline, and analyzed with a fluorescence-activated cell sorter (FACS IV, Becton Dickinson). For HTLV-III/LAV binding, 5 × 10^5 cells were incubated with HTLV-III/LAV (500 ng in 10 μl) for 30 min at 37°C. Washed cells were resuspended in 25 μl of fluorescein-conjugated anti-HTLV-III/LAV for 30 min at 4°C. The cells were washed, resuspended in 1% paraformaldehyde, and analyzed by FACS as above. For inhibition of HTLV-III/LAV binding, cells were preincubated with anti-T4A or anti-T8 (20 ng in 20 μl) for 30 min at 4°C followed by addition of HTLV-III/LAV (500 ng in 10 μl) for 30 min at 37°C. The cells were washed, incubated with fluorescein-conjugated anti-HTLV-III/LAV, washed, resuspended in paraformaldehyde, and analyzed by FACS as above.

Cell Surface Radioiodination, Immunoprecipitation, and Gel Electrophoresis

$T4^+$ NIH 3T3 transformants were surface radioiodinated by the lactoperoxidase technique (Acuto et al., 1983) as follows: 4 × 10^7 cells were suspended in 1 ml of PBS containing 0.5 mM EDTA, 2 mCi $Na^{125}I$, and 20 μg lactoperoxidase. At times 0, 1, 5, 10, 15 min, 10 μl of 0.03% H_2O_2 was added. The reaction was carried out at 23°C and was stopped at 20 min by two centrifugations in 50 vol of cold PBS containing 10 mM NaI. Labeled cells were split into four tubes and incubated, as indicated, with HTLV-III/LAV (2 μg in 20 μl) for 30 min at 37°C. Subsequent washes and manipulations were performed at 0°C to 4°C. Washed cells were lysed by adding 1 ml of detergent lysing buffer (LB; 0.02 M Tris, 0.12 M NaCl [pH 8.0], containing 0.2 mM phenylethylsulfonylfluoride, 5 μg/ml aprotinin, 0.2 mM EGTA, 0.2 mM NaF, 0.2% sodium deoxycholate, and 0.5% (v/v) Nonidet P-40). Tubes were held on ice for 15 min, and nuclei were removed by centrifugation at 3000 × g for 20 min.

For absorptions, we prepared Sepharose conjugates of human anti-HTLV-III/LAV IgG, human nonimmune IgG, anti-T4A, and anti-T8 antibodies as described (McDougal et al., 1986). Lysates were preabsorbed with 200 μl of Sepharose-nonimmune human IgG for 1.5 hr with rotation, and then immunoprecipitated with 20 μl of Sepharose conjugates (as indicated) for 3 hr with rotation. Sepharose absorbants were washed three times: once with LB; once with LB containing 0.5 M NaCl; and once with LB containing 0.1% sodium dodecyl sulfate (SDS). Absorbed material was eluted at 65°C for 30 min with 20 μl of

sample buffer (0.01 M Tris [pH 8.0] containing 2% SDS, 5% 2-mercapto-ethanol (v/v), 25 μg/ml bromphenol blue, and 10% glycerol (v/v)). Electrophoresis was performed in a 3.3%–20% gradient polyacrylamide gel with a 3% stacking gel (Neville and Glossman, 1974), and autoradiographs were developed with Kodak XAR-5 film.

Virus Inhibition Assay

2×10^5 $T4^+$ JM T cells were exposed to AIDS virus at 0 min. The inhibitors ammonium chloride (20 mM) or amantadine (20 mM) were added at various times during the course of virus infection (0 min, 30 min, and 60 min). After 6 hr, the cells were washed and replated in fresh medium (RPMI/10%FCS). The effect of these agents on AIDS virus infection was determined five days after infection. The fraction of infected cells in the cultures expressing viral antigens was determined by immunofluorescence microscopy as described above (Helenius et al., 1980).

RNA Isolation and Northern Blot Hybridizations

Total RNA was isolated from cells by homogenation in 4M guanidinium thiocyanate, followed by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin et al., 1979). $Poly(A)^+$ selection was achieved by oligo(dT)-cellulose chromatography (Type 3, Collaborative Research) (Aviv and Leder, 1972).

RNA was electrophoresed through a 1% agarose-formaldehyde gel (Scheller et al., 1982) and transferred onto Hybond (Amersham). Northern blot hybridization was performed according to the procedures supplied by the manufacturer. Probes were nick-translated to a specific activity of $0.5–1 \times 10^9$ cpm/μg with α^{32}P-labeled deoxynucleotide triphosphates (Rigby et al., 1977).

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