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Myxoma Virus M-T7, a Secreted Homolog of the Interferon-γ Receptor, Is a Critical Virulence Factor for the Development of Myxomatosis in European Rabbits

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Myxoma virus is a leporipoxvirus of New World rabbits (*Sylvilagus* sp.) that induces a rapidly lethal infection known as myxomatosis in the European rabbit (*Oryctolagus cuniculus*). Like all poxviruses, myxoma virus encodes a plethora of proteins to circumvent or inhibit a variety of host antiviral immune mechanisms. M-T7, the most abundantly secreted protein of myxoma virus-infected cells, was originally identified as an interferon-γ receptor homolog (Upton, Mossman, and McFadden, *Science* 258, 1369–1372, 1992). Here, we demonstrate that M-T7 is dispensable for virus replication in cultured cells but is a critical virulence factor for virus pathogenesis in European rabbits. Disruption of both copies of the M-T7 gene in myxoma virus was achieved by the deletion of 372 bp of M-T7 coding sequences, replacement with a selectable marker, p7.5Ecogpt, and selection of a recombinant virus (vMyxlac-T7gpt) resistant to mycophenolic acid. vMyxlac-T7gpt expressed no detectable M-T7 protein and infected cell supernatants were devoid of any detectable interferon-γ binding activities. Immunohistochemical staining with anti-β-galactosidase and anti-CD43 antibodies demonstrated that in vMyxlac-T7gpt-infected rabbits the loss of M-T7 not only caused a dramatic reduction in disease symptoms and viral dissemination to secondary sites, but also dramatically influenced host leukocyte behavior. Notably, primary lesions in wild-type virus infections were generally underlayed by large masses of inflammatory cells that did not effectively migrate into the dermal sites of viral replication, whereas in vMyxlac-T7gpt infections this apparent block to leukocyte influx was relieved. A second major phenotypic distinction noted for the M-T7 knockout virus was the extensive activation of lymphocytes in secondary immune organs, particularly the spleen and lymph nodes, by Day 4 of the infection. This is in stark contrast to infection by wild-type myxoma virus, which results in relatively little, if any, cellular activation of germinal centers of spleen and lymph node by Day 4. We conclude that M-T7 functions early in infection to (1) retard inflammatory cell migration into infected tissues and (2) disrupt the communication between sentinel immune cells at the site of primary virus infection in the subdermis and lymphocytes in the secondary lymphoid organs, thereby disabling the host from mounting an effective cellular immune response. To summarize, in addition to neutralizing host interferon-γ at infected sites, we propose that M-T7 protein also modifies leukocyte traffic in the vicinity of virus lesions, thus effectively severing the link between antigen presenting cells of the infected tissue and the effector lymphocytes of the peripheral immune organs.

INTRODUCTION

Virus survival within immunocompetent hosts requires defensive strategies to circumvent a wide array of potent antiviral mechanisms (Gooding, 1992; Smith, 1994; McFadden and Kane, 1994; Rinaldo, 1994; Marrack and Kappler, 1994; McFadden, 1994a, 1995; Barry and McFadden, 1995). Poxviruses, the largest of all animal viruses, have evolved a wide range of immune evasion strategies in order to effectively propagate within their host, including the reduction of inflammation, repression of major histocompatibility class I proteins, inhibition of the complement cascade, and interference with cytokines and growth factors (Turner and Moyer, 1990; Buller and Palumbo, 1991; Smith, 1993; Spriggs, 1994; McFadden and Graham, 1994; McFadden *et al.,* 1995). While the central regions of most poxvirus genomes are well conserved and encode for proteins essential for viral replication and structure, the genomic termini are enriched for genes which express proteins that influence virus–host interactions and pathogenesis (Turner and Moyer, 1990; Traktman, 1990; Buller and Palumbo, 1991).

In order to begin to decipher the complex interplay between host immune defense mechanisms and viral immune evasion strategies, it is useful to consider the unique biological origins of individual poxviruses. Myxoma virus, a leporipoxvirus, is one of the few poxviruses whose natural host, the South American tapeti rabbit (*Sylvilagus brasiliensis*), has been well established (Fenner and Ratcliffe, 1965; McFadden, 1988, 1994b; Fenner and Ross, 1994). In its natural host, myxoma virus causes benign lesions that persist for many months but is relatively apathogenic, while in the European rabbit (*Oryctolagus cuniculus*), myxoma virus induces a rapidly lethal

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infection known as myxomatosis (Fenner and Ratcliffe, 1965; Fenner and Myers, 1978). Hallmarks of myxomatosis include the following: fulminating lesions at the primary site of inoculation, characterized by tissue degeneration, necrosis, edema, and hemorrhage; rapid viral dissemination through the lymphoreticular system via infected leukocytes to secondary internal organs and peripheral external sites such as nose, ears, lips, and eyelids; and supervening Gram-negative bacterial infection in the respiratory tract and conjunctiva. The rapidly disseminating nature of the infection coupled with the extensive immunosuppression that is associated with both cellular immune defects and cytokine dysregulations leads to death within 2 weeks (Strayer, 1989; McFadden, 1994b).

To date, a variety of myxoma proteins have been shown to play a role in myxoma virus pathogenesis in the European rabbit (reviewed in McFadden and Graham, 1994; McFadden *et al.,* 1995). In this communication, we examine the biological role of M-T7, which was originally characterized as a functional homolog of the mammalian cellular interferon-γ (IFN-γ) receptor (Upton *et al.,* 1992). M-T7 is expressed from an early viral promoter, processed in the Golgi/endoplasmic reticulum, and secreted from myxoma virus-infected cells at levels which exceed 10^7 molecules per cell per hour at early times of infection (Mossman *et al.,* 1995c). Furthermore, M-T7 was shown to bind rabbit IFN-γ with high affinity (K_d = 1.2 n*M*) and efficiently abrogate the antiviral state induced by rabbit IFN-γ on rabbit cells (Upton *et al.,* 1992; Mossman *et al.,* 1995c). Unlike the case of IFN-γ receptor homologs encoded by members of the orthopoxvirus genus, myxoma M-T7 protein is specific for the rabbit ligand (Mossman *et al.,* 1995d; Alcamí and Smith, 1995). The open reading frame encoding for M-T7 is present as two copies, one within each of the terminal inverted repeat regions of the myxoma virus genome, where genes important in the determination of virulence are particularly enriched (Upton *et al.,* 1991; Opgenorth *et al.,* 1992; Macen *et al.,* 1993).

IFN-γ is a potent antiviral and immunoregulatory cytokine produced primarily by NK cells and T cells (Epstein, 1984; Vilcek *et al.,* 1985; Farrar and Schreiber, 1993). Given the importance of IFN-γ in the immune response to poxvirus infection (Buller and Palumbo, 1991; Mossman *et al.,* 1995a), we initiated a study to determine the role of M-T7 during myxoma virus pathogenesis in the European rabbit. Thus, both copies of the M-T7 gene were inactivated with a dominant selectable marker in order to construct a recombinant virus which is deficient for the expression of the M-T7 protein. Here, we compare the disease profile and immune response characteristics in rabbits infected with recombinant M-T7 knockout virus with those of wild-type myxomatosis and speculate that M-T7 may possess biological activities in addition to its previously described biochemical property of interferon-γ inhibition.

MATERIALS AND METHODS

Cells and viruses

vMyxlac, the myxoma virus (strain Lausanne) derivative containing a β-galactosidase cassette in an intergenic location near the left terminal inverted repeat, has been previously described (Opgenorth *et al.,* 1992). The construction of vMyxlac-T7gpt is described below. BGMK cells, a primate cell line (obtained from the American Type Culture Collection, ATCC), were maintained in Dulbecco's minimal essential medium (DMEM) with 10% newborn calf serum. RK13 cells, a rabbit kidney cell line (ATCC), were maintained in DMEM with 10% fetal bovine serum.

Construction of myxoma T7$^-$ mutant virus

A 1.9-kb *Bam*HI–*Sst*II segment of the myxoma virus *Bam*HI K fragment (Russell and Robbins, 1989), containing the complete M-T7 open reading frame (792 bp) flanked by the partial open reading frames of M-T8 and M-T6, was blunt-end cloned into the *Sma*I site of pBluescript (KS$^+$), creating the plasmid pKMBD-8. A 372-bp *Sma*I–*Nru*I fragment within the M-T7 gene was deleted and, following the addition of *Bgl*II linkers, the deleted M-T7 coding sequence was replaced with a *Bgl*II cassette containing a dominant selectable marker, the *Escherichia coli* guanosine phosphoribosyl transferase (Ecogpt) gene driven by the vaccinia 7.5K promoter (Falkner and Moss, 1988). The resulting plasmid, pMT7G-4, thus contained the p7.5Ecogpt cassette inserted into the *Sma*I–*Nru*I deletion of the M-T7 open reading frame such that the selectable marker would be transcribed in the same direction as the M-T7 gene. PCR amplification using primers directed toward the 5′ (5′-AGGTCGACATGGACGGGAGACTGGTGTTTC-3′) and 3′ (5′-AGGAATTCTTATTCGAAGACATCTTCCAC-3′) ends of the M-T7 open reading frame was used to amplify the recombinant M-T7/p7.5Ecogpt fragment, designated M-T7gpt, for transfection into myxoma virus-infected cells.

To construct the M-T7$^-$ mutant virus (vMyxlac-T7gpt), BGMK cells infected with vMyxlac at a multiplicity of infection of 0.1 were transfected with M-T7gpt using Lipofectin (GIBCO BRL). After 24 hr infectious virus was isolated and subjected to three passages under mycophenolic acid selection (25 μg/ml mycophenolic acid, 20 μg/ml xanthine, 15 μg/ml hypoxanthine), as described (Falkner and Moss, 1988). Individual foci were then subjected to three rounds of plaque purification, and isolates of vMyxlac-T7gpt were grown up for analysis and stored at −70° for further use. Polymerase chain reaction (PCR) analysis with M-T7 primers and Western blotting analysis with affinity-purified anti-M-T7 (E266) antibody were used

to confirm both the absence of an intact M-T7 open reading frame and the lack of secreted M-T7 protein, respectively. To obtain viral DNA suitable for PCR analysis, virally infected cells from a single well of a six-well multidish were harvested, resuspended in 5 m*M* Tris, pH 8, 5% NP-40, 5% Tween-20, 0.5 mg/ml proteinase K, and incubated at 55° for a minimum of 2 hr. Following inactivation of proteinase K by boiling, the mixture was subjected to phenol:chloroform extraction and the DNA stored at 4° for further use.

Western blotting analysis with M-T7 antibody

The glutathione S-transferase (GST) gene fusion system (Pharmacia) was used to express M-T7 for polyclonal antibody production. An M-T7 fragment amplified by PCR was cloned in frame into the expression vector pGEX-2T, creating the plasmid pGST7. pGST7 was transformed into *E. coli* BL21(DE3)pLysS (Studier and Moffatt, 1986), induced with 0.1 m*M* IPTG, and the inclusion bodies containing the bulk of the M-T7 fusion protein were isolated, as previously described (Mossman *et al.,* 1995b). For the immunization of two rabbits, lyophilized acrylamide gel slices containing denatured M-T7-GST fusion protein were prepared for use as antigen, as described elsewhere (Harlow and Lane, 1988). Polyclonal antiserum from one rabbit (E266) was further subjected to affinity purification against immobilized denatured M-T7-GST fusion protein as outlined elsewhere (Harlow and Lane, 1988). Detection of M-T7 protein in supernatants prepared from virus-infected cells was performed by Western blotting analysis, as described (Schreiber and McFadden, 1994), with a primary antibody dilution of 1:1000. For the detection of M-T7/interferon-γ complexes, chemical crosslinking of supernatants harvested from mock or virally infected cells with unlabeled rabbit interferon-γ (gift from Genentech) was performed as previously described (Upton *et al.,* 1992; Mossman *et al.,* 1995c), with the exception that Western blotting analysis was used to detect M-T7 protein.

Single-step growth analysis in tissue culture

Cultures of RK13 cells in six-well multidishes were infected with either vMyxlac or vMyxlac-T7gpt at a multiplicity of infection of 5 for 1 hr at 37° in 0.4 ml serum-free DMEM. The inoculum was removed, the cells were washed with serum-free DMEM three times and replaced with DMEM supplemented with serum, and the cultures were harvested at various times postinoculation. Virus titers were determined by a plaque assay on RK13 cells followed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of fixed monolayers, as outlined (Opgenorth *et al.,* 1992). Both vMyxlac and vMyxlac-T7gpt growth analyses were performed in triplicate.

Infection of rabbits with vMyxlac and vMyxlac-T7gpt

Female New Zealand White rabbits (*O. cuniculus*) (3 kg) were obtained from a local supplier and housed in C-level containment facilities under the guidelines of Canadian Council on Animal Care. Injections were performed intradermally in each thigh with 1000 PFU virus per site. Rabbits were monitored daily for symptoms of myxomatosis, including appearance of primary and secondary tumors, conjunctivitis, and rhinitis, and responsiveness to stimuli (for review of myxomatosis, see Fenner and Ratcliffe, 1965; McFadden, 1994b). Rabbits which became moribund were sacrificed with euthanyl administered intravenously after anesthesia. A total of 13 rabbits were inoculated with vMyxlac-T7gpt and 3 with the vMyxlac parental virus. In previously published studies (Upton *et al.,* 1991; Opgenorth *et al.,* 1992; Macen *et al.,* 1993), in which a minimum of 18 rabbits have been systematically tested with vMyxlac, we have observed a mortality rate of approximately 95% with this virus. Since this overall lethality is slightly less than the $>$99% ascribed to the original Lausanne isolate of myxoma, we have classified vMyxlac as possessing grade II virulence, using the original definition of Fenner (Fenner and Ratcliffe, 1965).

Histological analysis and immunohistological staining

For histological studies, six rabbits each were inoculated with vMyxlac or vMyxlac-T7gpt as described above. Two rabbits from each group were sacrificed at Days 4, 7, and 10 postinfection, a complete postmortem examination was performed, and tissue sections were removed and placed in neutral buffered formalin. Samples were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin for viewing by light microscopy. For immunohistological studies, the ExtrAvidin Peroxidase Staining kit (Sigma Immunochemicals) was utilized, as per the manufacturer's specifications. Briefly, paraffin was removed with xylene, and samples were rehydrated before being counterstained with Harris Hematoxylin. Samples were then subjected to treatment with 3% hydrogen peroxide, followed by staining with mouse monoclonal anti-rabbit CD43 or anti-β-galactosidase antibodies (Spring Valley and Promega, respectively), at a dilution of 1:500, or normal mouse serum as a control at a dilution of 1:100. The CD43 antibody recognizes rabbit T cells, monocytes, and macrophages, while the β-galactosidase antibody detects the protein product of the *E. coli* β-galactosidase cassette expressed from the vMyxlac and vMyxlac-T7gpt viruses. Following application of affinity-purified biotinylated goat anti-mouse immunoglobulin and ExtrAvidin–Peroxidase, samples were subjected to the substrate 3,3′-diaminobenzidine tetrahydrochloride (Sigma). Finally, samples were dehydrated, mounted in Permount, and photographed using Kodak Ektachrome 64T color reversal film.

RESULTS

Construction and characterization of M-T7$^-$ mutant virus

The myxoma virus M-T7 open reading frame is present as two copies, one within each of the terminal inverted repeats of the 160-kb myxoma virus genome (Fig. 1A). In order to inactivate both copies of the M-T7 gene, a dominant selection method was adopted, using the *E. coli* guanine phosphoribosyltransferase (Ecogpt) marker, shown to be useful for the construction of other poxviral recombinants (Falkner and Moss, 1988; Boyle and Coupar, 1988). A 372-bp *Sma*I–*Nru*I fragment within the M-T7 coding sequence was removed and replaced with a 950-bp Ecogpt cassette driven by the vaccinia virus 7.5K promoter, creating the recombinant M-T7gpt cassette in pMT7G-4 (outlined in Fig. 1B). PCR amplification, using primers directed toward the terminal regions of the M-T7 gene, allowed for amplification of M-T7gpt, which was subsequently used to construct the M-T7$^-$ mutant virus, vMyxlac-T7gpt. As outlined under Materials and Methods, a recombinant viral stock was prepared by sequential plaque purification under selection pressure, in the presence of mycophenolic acid.

To ensure disruption of both copies of the M-T7 open reading frame with M-T7gpt, PCR analysis was performed on DNA isolated from plaque purified virus. As shown in Fig. 1B, both M-T7 and M-T7gpt contain identical sequences at their terminal ends, to which the M-T7 5′ and 3′ primers hybridize. Based on size, the parental M-T7 sequence (792 bp) is readily distinguished by agarose gel electrophoresis from the recombinant M-T7gpt (1370 bp) (Fig. 2A, lanes 1 and 2, respectively). Thus, viral DNA derived from any contaminating wild-type vMyxlac or constructs with only one M-T7 allele disrupted can be distinguished from viral DNA derived from pure recombinant vMyxlac-T7gpt by comparing the size of amplified products of the M-T7 open reading frame using M-T7 primers. After three initial blind passages under mycophenolic acid selection, both wild-type and recombinant virus M-T7 gene sequences were clearly present in the uncloned virus stocks, since both species of viral DNA were detected in comparable amounts (Fig. 2A, lane 3), whereas following three rounds of plaque purification, only recombinant M-T7gpt DNA derived from vMyxlac-T7gpt was detected (Fig. 2A, lane 4).

M-T7 was originally characterized as the major secreted protein from myxoma virus-infected cells and was shown to be efficiently expressed at levels capable of neutralizing 2000–3000 units of rabbit IFN-γ per infected cell at early times during infection (Upton *et al.,* 1992; Mossman *et al.,* 1995c). Disruption of both copies of the M-T7 open reading frame, however, completely abrogated expression of detectable M-T7 protein by the recombinant virus vMyxlac-T7gpt, as determined by Western blotting with affinity-purified anti-M-T7 antibody (Fig. 2B, compare lanes 1 and 3). Previously, M-T7 protein was shown to specifically bind and inhibit rabbit IFN-γ (Upton *et al.,* 1992; Mossman *et al.,* 1995c). Since vMyxlac-T7gpt does not secrete detectable M-T7 protein, no M-T7–IFNγ complex can be detected either (Fig. 2B, compare lanes 2 and 4). Furthermore, while supernatants from cells infected with parental myxoma virus efficiently abrogate the antiviral state induced by rabbit IFN-γ on a rabbit cell line (Upton *et al.,* 1992; Mossman *et al.,* 1995c), supernatants from cells infected with vMyxlac-T7gpt recombinant virus had no effect on the induction of the antiviral state by rabbit IFN-γ (data not shown). Thus, unlike wild-type myxoma virus which efficiently secretes M-T7 protein that forms inhibitory complexes with rabbit IFN-γ, the recombinant myxoma virus, vMyxlac-T7gpt, does not express any detectable M-T7 protein.

Effects of M-T7 disruption on virus virulence

No defects were noted in the replication of the M-T7 knockout virus, vMyxlac-T7gpt, compared with wild-type vMyxlac, in cultured rabbit RK-13 fibroblasts *in vitro* in a single-step growth curve analysis (Fig. 3). Similar growth curves were also measured in primate BGMK cells (data not shown). However, as outlined in Table 1, vMyxlac-T7gpt was significantly attenuated following inoculation of European rabbits, compared to the parental virus, vMyxlac. Dramatic differences were noted in the size and progression of skin lesions, the onset and severity of secondary Gram-negative bacterial infections, and the ability of the animal to clear the viral infection. In fact, the majority (12/13) of vMyxlac-T7gpt-infected animals exhibited virtually none of the most severe symptoms of myxomatosis that are associated with the wild-type virus, but instead recovered completely following a moderated disease course characterized by relatively mild symptoms. Specifically, at 4 days postinfection, the primary lesions of rabbits infected with vMyxlac-T7gpt were smaller than those characteristic of infection with vMyxlac and displayed less hemorrhage and necrosis. By 7 days postinfection, rabbits infected with the M-T7 knockout virus displayed fewer and smaller secondary lesions (if at all) compared with the numerous, large secondary skin lesions on the ears, eyelids, and noses of rabbits following a typical wild-type myxoma infection. Whereas vMyxlac infection typically induces extensive secondary Gram-negative bacterial infections (rhinitis and conjunctivitis), only about half of the rabbits infected with vMyxlac-T7gpt showed any symptoms at all of bacterial colonization of the respiratory tract or conjunctiva. Finally, by 10 days postinfection when rabbits infected with vMyxlac possess symptoms of myxomatosis severe enough to require euthanasia, only 1 of 13 rabbits infected with vMyxlac-T7gpt became moribund. The remaining 12 rabbits ranged in their severity of disease symptoms from extremely mild to moderate and completely recovered

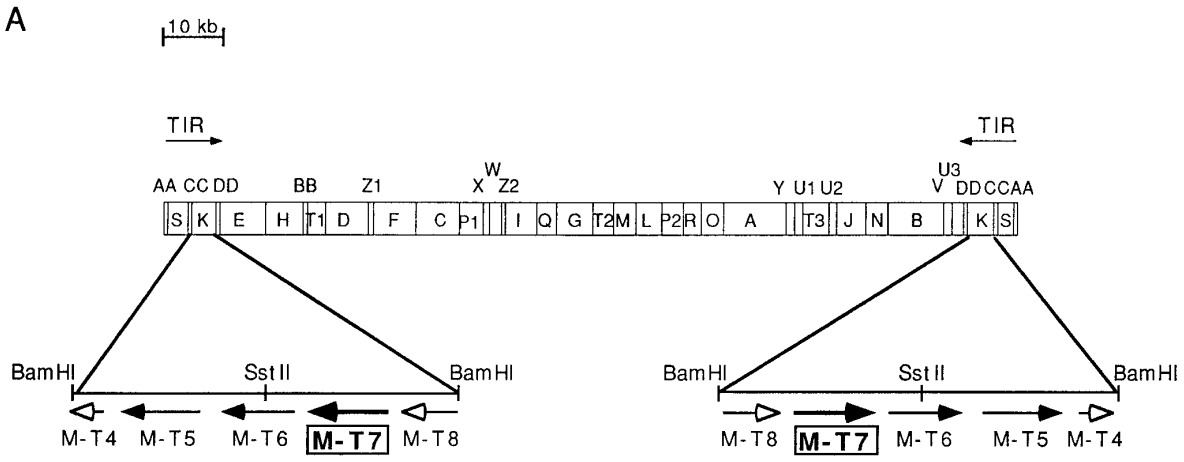


FIG. 1. Construction of a recombinant myxoma virus containing a disrupted M-T7 gene. (A) Location of the M-T7 open reading frames within the 160-kb myxoma virus genome. The *Bam*HI map of the myxoma virus DNA genome (Russell and Robbins, 1989) is illustrated. The M-T7 open reading frame exists in two copies within the *Bam*HI K fragment, located within the terminal inverted repeat region of the myxoma virus genome. The location of the single *Sst*II site within the *Bam*HI K fragment is illustrated. Solid arrows denote complete open reading frames within the *Bam*HI K fragment, whereas open arrows indicate flanking open reading frames that span the *Bam*HI sites within this fragment. (B) Construction of vMyxlac-T7gpt disruption virus. An *Sst*II–*Bam*HI fragment, containing the entire M-T7 open reading frame, was cloned into pBluescript, creating the plasmid pKMBD-8. A 372-bp internal *Sma*I/*Nru*I fragment of the M-T7 coding sequence was removed and, upon addition of *Bgl*II linkers, replaced with a 950-bp p7.5Ecogpt *Bgl*II cassette, creating the plasmid pMT7G-4. PCR amplification using primers directed toward the 5′ and 3′ ends of the M-T7 gene was used to obtain 1370 bp of M-T7gpt DNA. M-T7gpt DNA was subsequently transfected into vMyxlac-infected cells to create the recombinant virus vMyxlac-T7gpt. The size of the M-T7 gene of wild-type vMyxlac, as amplified with the primer pairs used to create M-T7gpt, is illustrated for comparison.

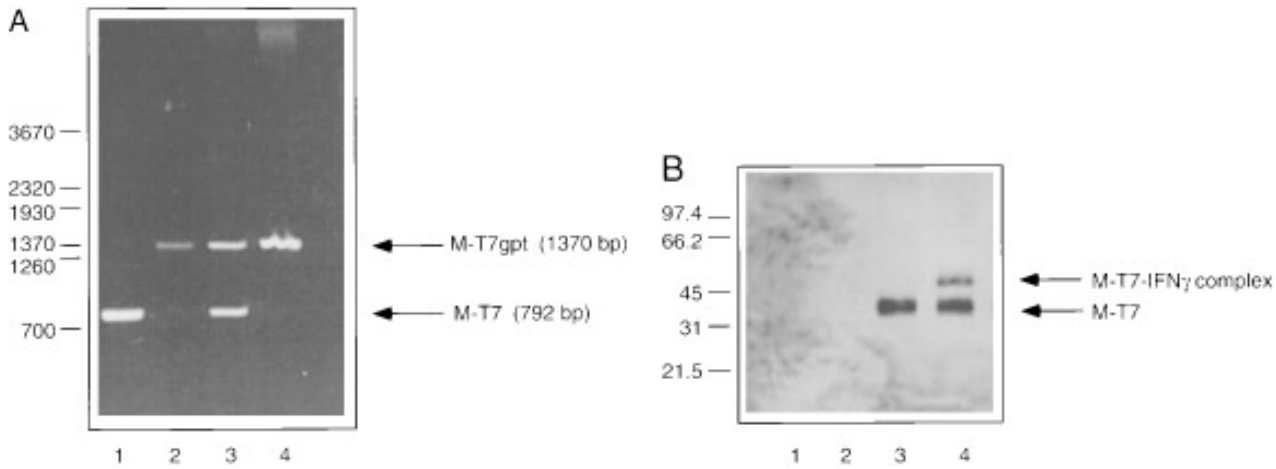


FIG. 2. Characterization of M-T7 from wild-type vMyxlac and recombinant vMyxlac-T7gpt viruses. (A) Agarose gel electrophoresis of PCR products amplified by M-T7 5′ and 3′ primers (see Fig. 1B). Migration of wild-type M-T7 (lane 1) and recombinant M-T7gpt (lane 2) amplified from pKMBD-8 and pMT7G-4 plasmid DNA, respectively. PCR analysis of viral DNA following three blind passages under mycophenolic acid selection pressure (refer to Materials and Methods) illustrates the presence of both M-T7 (792 bp) and M-T7gpt (1370 bp) DNA (lane 3). Following three rounds of plaque purification from a single viral focus, a purified stock of vMyxlac-T7gpt virus was analyzed (lane 4). Location of *Bst*EII-cut λ markers is shown to the left. (B) Detection of M-T7 protein secreted from virus-infected cells by Western blotting analysis. Supernatants from vMyxlac-T7gpt-infected cells (lane 1) and vMyxlac-infected cells (lane 3), subjected to Western blotting analysis using affinity-purified anti-M-T7 (E266) antibody (refer to Materials and Methods). The M-T7–IFNγ complex is measured following chemical crosslinking of rabbit IFNγ to supernatants from vMyxlac-T7gpt-infected cells (lane 2) and vMyxlac-infected cells (lane 4). The location of low-molecular-weight markers is shown to the left.

by 4 weeks. Furthermore, these survivor rabbits were immune to subsequent challenge by wild-type vMyxlac. Thus, we conclude that the M-T7 protein plays a significant role in myxoma virus pathogenesis and can be defined as a classical virus virulence factor.

In the study of other documented virulence factors expressed by the myxoma virus genome, the disease characteristics of vMyxlac have been well characterized (Upton *et al.,* 1991; Opgenorth *et al.,* 1992; Macen *et al.,* 1993). In this particular study, 3 rabbits were injected with vMyxlac for comparative pathological analysis, and 6 rabbits for histological analysis (described below). Thus, since the construction of vMyxlac in 1991, a minimum of 24 rabbits have now been studied following infection with vMyxlac. While most rabbits injected with vMyxlac became moribund 10 days postinfection, typical of the myxomatosis disease progression within this strain, 1 rabbit was capable of mounting an immune response sufficient for recovery. As described under Materials and Methods, we have now tentatively assigned a grade II virulence to vMyxlac, using the original nomenclature proposed by Frank Fenner (Fenner and Ratcliffe, 1965).

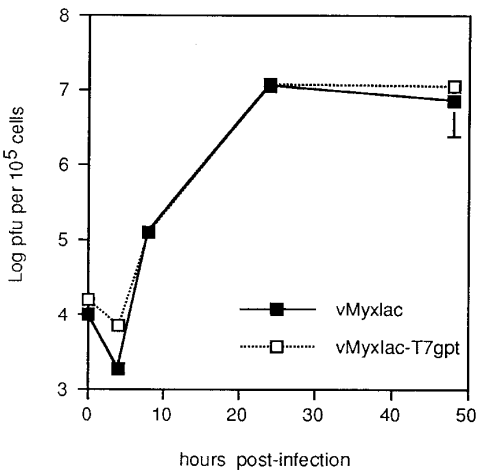


FIG. 3. Single-step growth analysis of the replication of vMyxlac and vMyxlac-T7gpt in cultured rabbit kidney fibroblasts. RK13 cells were infected with vMyxlac (solid squares) or vMyxlac-T7gpt (open squares) with a multiplicity of infection of 5. Cultures were harvested at various times postinoculation and infectious virus was titered on RK13 cells. Each viral growth assay was performed in triplicate.

vMyxlac-T7gpt exhibits reduced viral dissemination in infected rabbits

A common feature of myxomatosis is the rapid and efficient dissemination of virus via lymphatic channels from primary inoculation sites to numerous secondary sites, most prominently the nose, ears, eyelids, respiratory tract, and gonads (Fenner and Ratcliffe, 1965). Our initial observations demonstrated that, while vMyxlac-T7gpt is attenuated in rabbits, the virus is still capable of disseminating to some degree from the primary site of inoculation to the normal spectrum of secondary sites. External examination of rabbits infected with vMyxlac-T7gpt, however, demonstrated a clear reduction in the onset, number, and size of lesions at peripheral locations (refer to Table 1). It is possible that the $T7^-$ recombinant virus still possesses near normal capacity to disseminate to secondary sites by the infection of macrophages and lymphocytes, but that the cellular immune system has

TABLE 1

Pathogenicity of vMyxlac-T7gpt in European Rabbits

Day	vMyxlac (wild type)	vMyxlac-T7gpt (T7 mutant)
0	Inoculation of rabbits with vMyxlac[a]	13 rabbits inoculated intradermally at two sites with 1000 PFU vMyxlac-T7gpt per site
4	Primary lesions at inoculation sites, approx 2 cm, soft, flat, turning necrotic in center	Primary lesions at inoculation sites, approx 1 cm, raised, hard; slight necrosis detected in lesions of 2 rabbits only
7	Gram-negative bacterial infections of nasal and conjunctival mucosa; multiple secondary skin lesions on face and ears	Minor Gram-negative infections of nasal and/or conjunctival mucosa in 6 of 13 rabbits; secondary skin lesions on face and ears smaller than observed with vMyxlac (wild type)
10	Severe Gram-negative bacterial infections in conjunctiva and respiratory tract; secondary skin lesions turning necrotic; rabbits sacrificed due to increasing severity of symptoms	Gram-negative infections undetected in 5 of 13 rabbits, mild in 4 of 13 rabbits, severe in 4 of 13 rabbits; secondary lesions numerous but small; 12 of 13 rabbits highly responsive to stimuli; 1 rabbit sacrificed due to severity of symptoms
14	—	Gram-negative infections mild to absent; primary and secondary lesions regressing
28	—	Absence of Gram-negative infections; primary lesions consist of small, dry scabs; small scars remain from secondary lesions
		Surviving rabbits immune to subsequent challenge with vMyxlac (wild type) infection

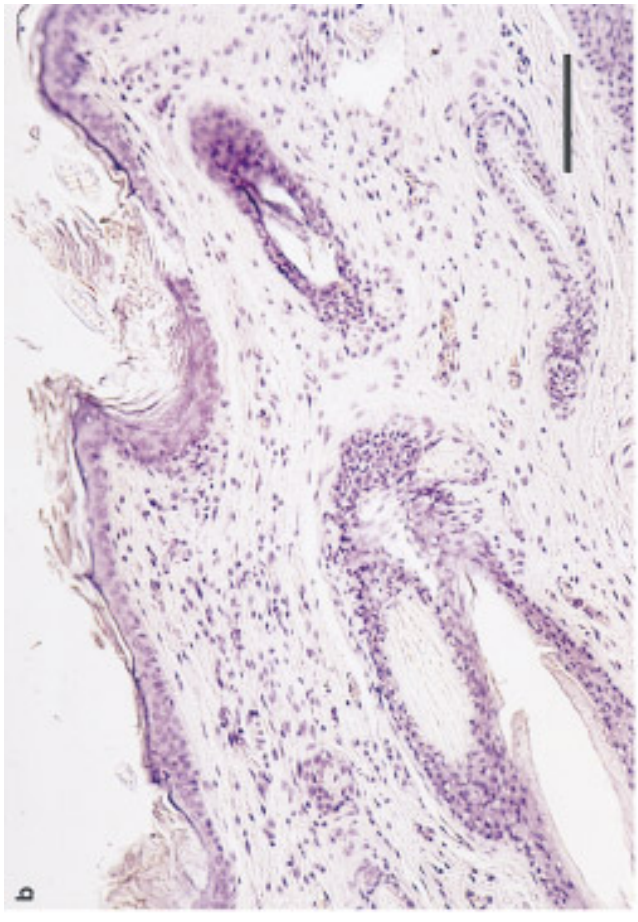
[a] As described under Materials and Methods, the pathogenic profile is a summation of multiple studies carried out with vMyxlac, which carries a lacZ cassette at an intergenic location in myxoma, strain Lausanne.

an increased potential to mount a more vigorous and effective response to the infection. Since both the parental virus strain, vMyxlac, and the M-T7 knockout virus each express an *E. coli* lacZ transgene (Opgenorth *et al.,* 1992), localization of either vMyxlac or vMyxlac-T7gpt virus in infected rabbit tissues is facilitated by staining tissue sections with an antibody against β-galactosidase. Such virus localization studies are important, since a direct correlation between the extent of viral replication and lesion size cannot be assumed. Thus, a time course study was initiated in which six additional rabbits each were infected with either vMyxlac or vMyxlac-T7gpt, and at 4, 7, and 10 days postinoculation, two rabbits from each group were sacrificed and subjected to a full postmortem necropsy. Primary and secondary tissue samples were then prepared and immunostained for β-galactosidase as described under Materials and Methods.

No significant difference in the level of β-galactosidase staining, which corresponds approximately to the level of expression of other myxoma virus late genes, was observed in primary lesions at the inoculation site from vMyxlac- or vMyxlac-T7gpt-infected rabbits at early stages of infection (data not shown). By 7 days postinoculation, peripheral tissues from rabbits infected with vMyxlac demonstrated high levels of β-galactosidase, consistent with efficient virus propagation at secondary sites. Figure 4a demonstrates the typical extent of β-galactosidase expressed at Day 7 within the secondary ear lesion of a rabbit infected with wild-type virus. While viral propagation was not limited to just this particular section, it does appear to be focused at the epidermis and underlying dermis, although some staining of β-galactosidase is observed throughout the entire dermis. In contrast, a typical section of an ear lesion harvested from a rabbit infected with vMyxlac-T7gpt does not contain any comparable intensely staining areas that would be indicative of unrestrained viral propagation (Fig. 4b). Similar observations were obtained when secondary site tissue sections were harvested 10 days postinoculation (not shown). Thus, we conclude that viral load at secondary sites following dissemination was significantly and systematically reduced in rabbits inoculated with the recombinant M-T7$^-$ mutant virus.

vMyxlac and vMyxlac-T7gpt elicit distinct cellular immune responses

Since the pathogenic profile of vMyxlac-T7gpt appears to differ significantly from the parent, vMyxlac, with respect to both virulence and ability to establish secondary infection sites, a more detailed histological analysis was performed on the tissues harvested in the time course experiment described above (summarized in Table 2). Four days postinoculation, the primary sites of both vMyxlac and vMyxlac-T7gpt inoculation manifested essentially similar characteristics of disease progression, including edema of the deeper dermis with an increase in the number of mononuclear inflammatory cells diffusely present throughout both the superficial and deeper der-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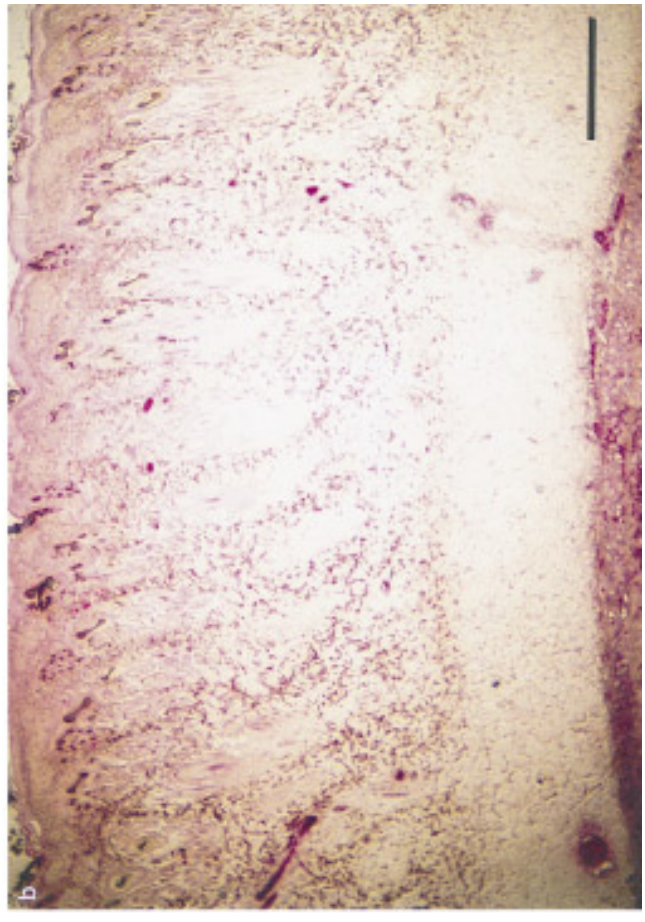
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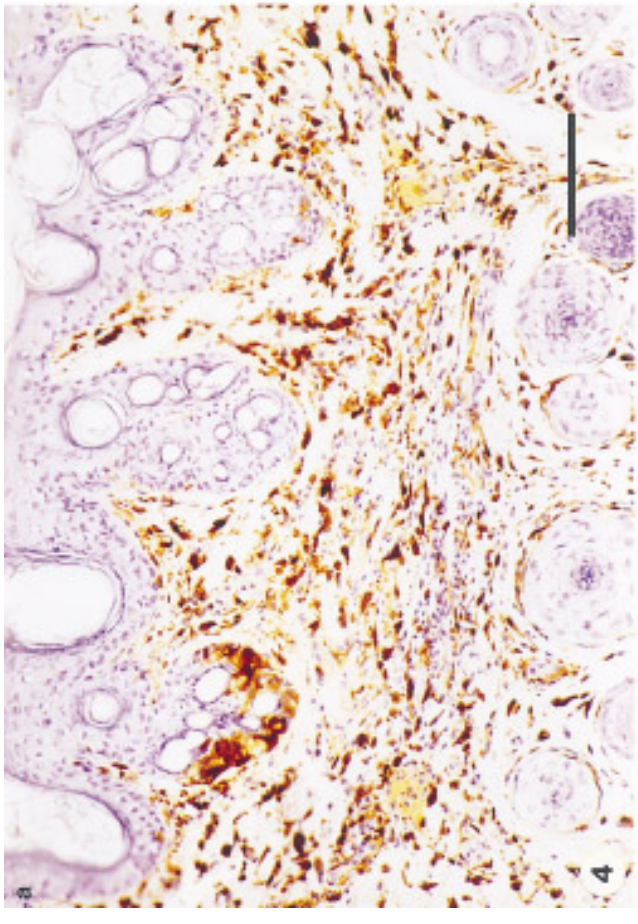
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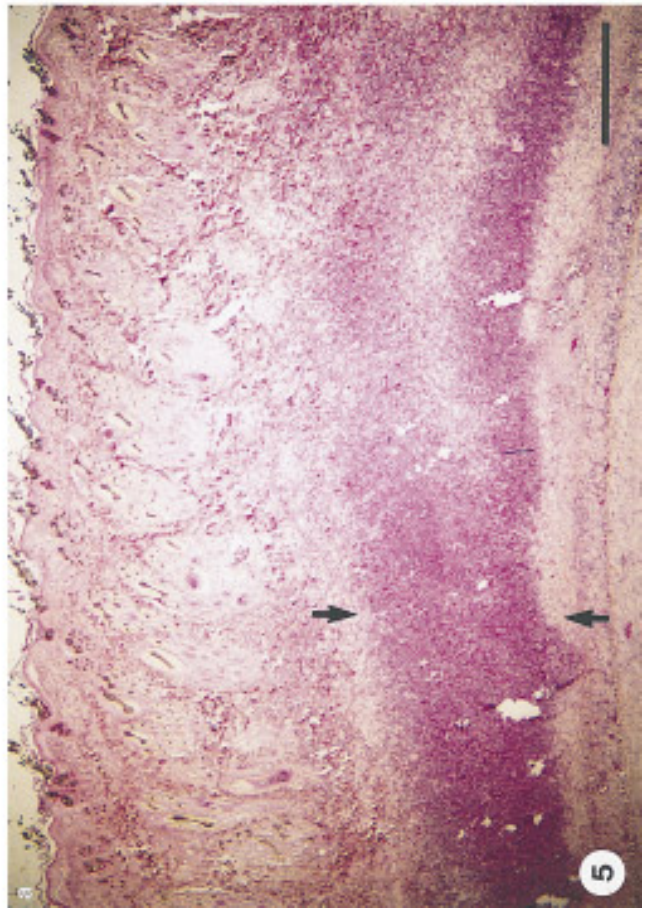
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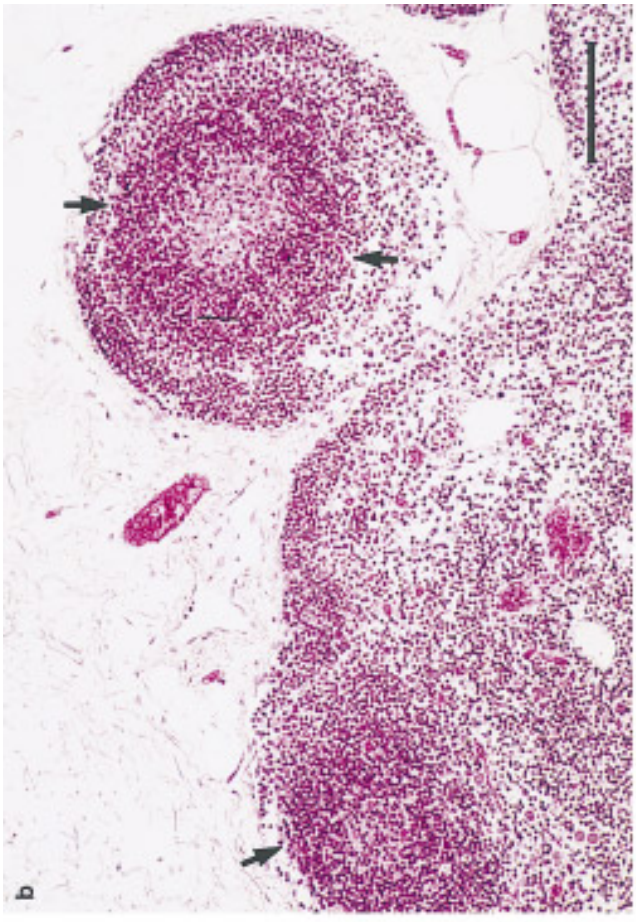
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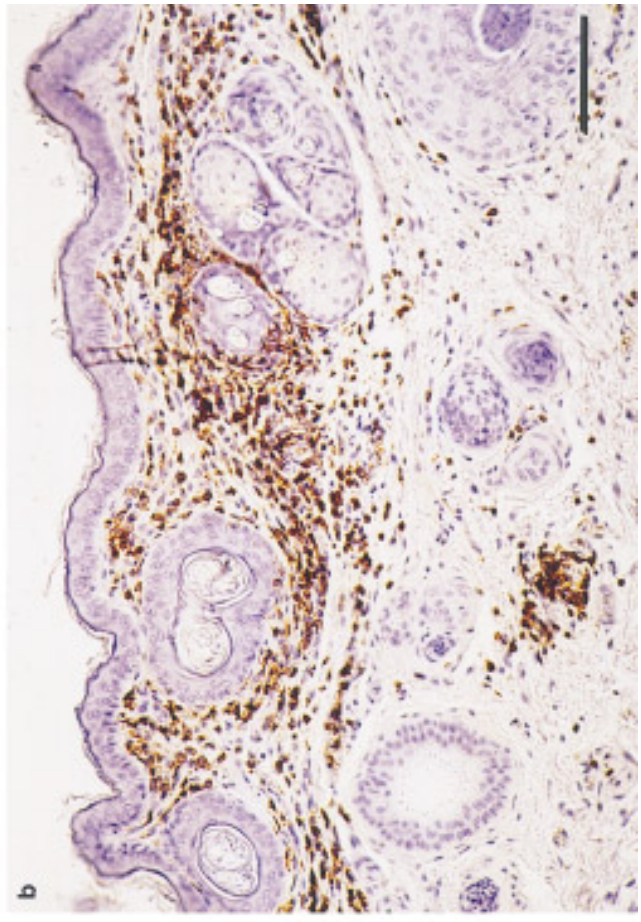
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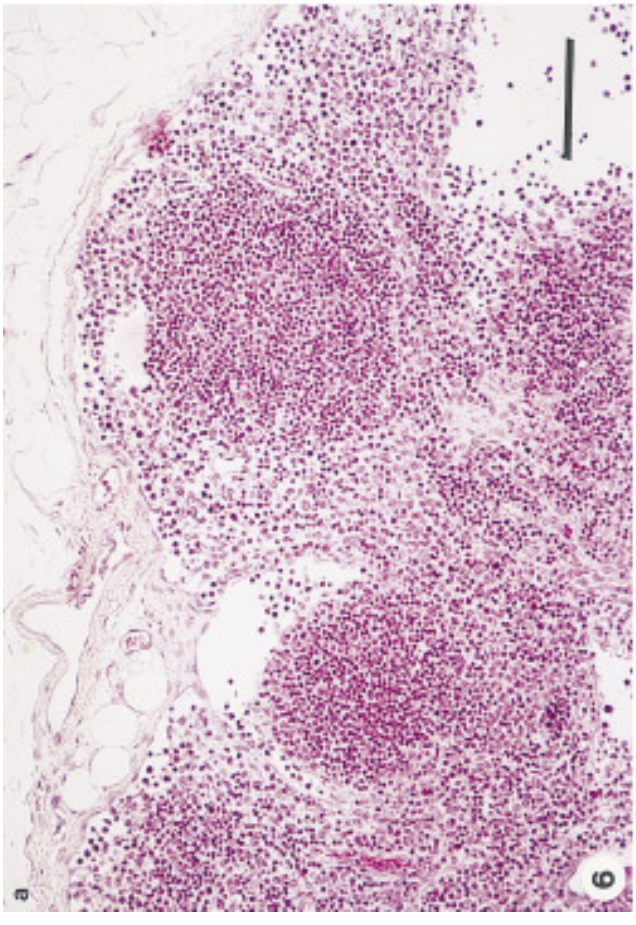
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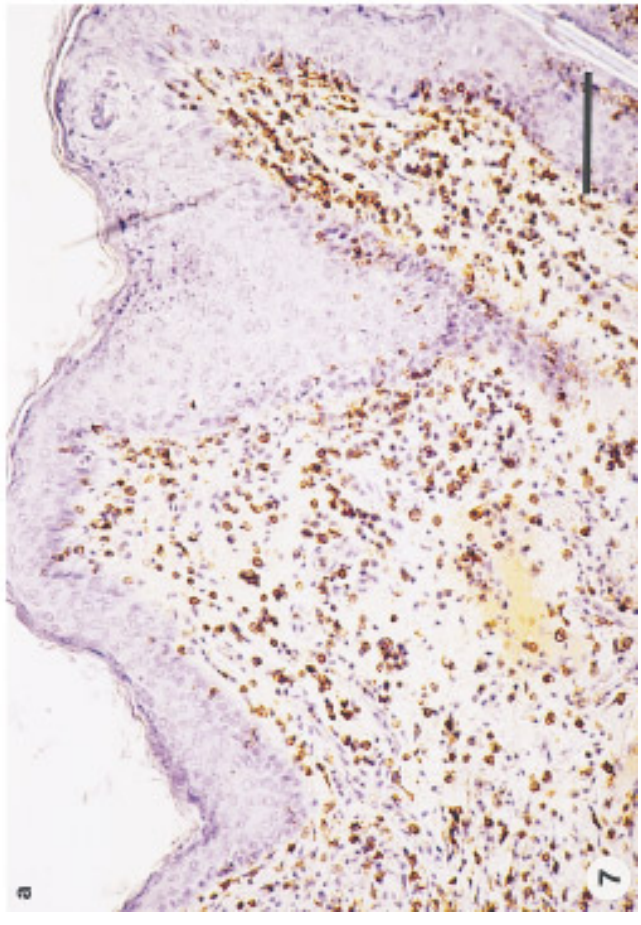
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TABLE 2

Major Histological Differences between Wild-Type Myxoma and vMyxlac-T7 gpt Infection in European Rabbits

	vMyxlac (wild type)	vMyxlac-T7gpt (M-T7 mutant)
Day 4	Primary sites[a]	Primary sites[a]
	Acute inflammatory reaction, primarily heterophils and macrophages, mostly focalized in subdermal tissues below areas of viral replication	Less inflammatory reaction, primarily heterophils and macrophages, dispersed throughout lesion
	Edema	Enhanced edema
	Secondary sites[b]	Secondary sites[b]
	No detectable reaction	No detectable reaction
	Lymph nodes — very mild activation	Lymph nodes — highly reactive lymphocytes
	Spleen — normal	Spleen — reactive lymphocytes
Day 7	Primary sites[a]	Primary sites[a]
	Acute coagulation necrosis of epidermis and dermis	Acute coagulation necrosis of epidermis and dermis
	Acute inflammation with edema	Acute inflammation with edema
	Hemorrhage	Hemorrhage
	Secondary sites[b]	Secondary sites[b]
	Thickened, disorganized epidermis	Normal epidermis
	Acute inflammatory reaction in dermis and subcutis	Mild inflammatory reaction in dermis, some edema in subcutis
	Heterophils and macrophages predominate	Infiltrating lymphocytes predominate
Day 10	Primary sites[a]	Primary sites[a]
	Full thickness necrosis of dermis	Full thickness necrosis of dermis
	Intense inflammatory reaction in deep dermis	No inflammatory reaction in deep dermis
	Heterophils predominate	Infiltrating lymphocytes predominate
	Degeneration of epithelium	Vacuolization of epithelium
	Secondary sites[b]	Secondary sites[b]
	Thickened, disorganized epidermis	Normal epidermis
	Intense inflammation with edema	Mild inflammation with edema
	Heterophils and macrophages predominate	Infiltrating lymphocytes predominate

[a] Primary site samples taken from site of inoculation in the thigh.
[b] Secondary site samples taken from ear, lip, nose, and eyelid.

mis. While areas which underlay the primary site of vMyxlac inoculation showed an accumulation of inflammatory cells (Fig. 5a), the primary site of vMyxlac-T7gpt inoculation appeared to contain a greater level of edema and fewer mononuclear cells in the deeper dermis (Fig. 5b). No significant differences were observed at any of the external peripheral tissues that normally signal myxomatosis at this time point. A very striking difference was observed, however, between internal inguinal lymph nodes harvested from rabbits infected with each virus. At 4 days postinoculation with vMyxlac, lymph nodes demonstrated either an absence of or only a very mild degree of lymphocyte reactivity in the germinal centers and edema of the sinusoids (Fig. 6a). By Day 4, vMyxlac-T7gpt infection, however, induced a dramatic level of lymph node cellular reactivity, characterized by edema of both the subcapsular and medullary sinusoids, an increase in the number of heterophils within the sinusoidal

FIG. 4. Secondary ear lesions harvested at Day 7 postinoculation and stained with an anti-β-galactosidase antibody. (a) Ear lesion from a rabbit infected with wild-type vMyxlac virus. High levels of β-galactosidase staining correlate with the extent of virus late gene expression. (b) Ear lesion from a rabbit infected with recombinant vMyxlac-T7gpt virus. Tissue sections were harvested and stained as outlined under Materials and Methods. 100-μm scale is shown.

FIG. 5. Primary site of infection tissue harvested at Day 4 postinoculation. (a) Primary site of vMyxlac inoculation possesses an enhancement of accumulated inflammatory cells within the deeper dermis (indicated by arrows). (b) Primary site of vMyxlac-T7gpt inoculation demonstrates enhanced edema and lack of inflammatory cell accumulation within the deeper dermis, compared to the wild-type lesion in a. 1-mm scale is shown.

FIG. 6. Lymph node tissue harvested at Day 4 postinoculation. (a) Lymph node (internal inguinal) tissue from a rabbit infected with vMyxlac possesses a low level of cellular activity within the germinal centers. (b) Highly reactive lymph node harvested from a rabbit infected with vMyxlac-T7gpt. Note the more ordered structure and greater cellularity of germinal centers. Arrows indicate zone of increased mitotic activity within the lymphoid germinal centers. Tissue sections were harvested and stained as outlined under Materials and Methods. 100-μm scale is shown.

FIG. 7. Secondary eyelid lesions harvested at Day 10 postinoculation and stained with an anti-CD43 antibody to identify rabbit T cells, monocytes, and macrophages. (a) Tissue section from a rabbit lesion infected with vMyxlac demonstrates extensive diffuse staining of CD43-positive cells throughout the dermis. (b) CD43-positive cells are highly focalized within the superficial dermis in lesions from a rabbit infected with vMyxlac-T7gpt. Tissue sections were harvested and stained as outlined under Materials and Methods. 100-μm scale is shown.

areas, and a reduced population in the central areas of the lymphoid germinal centers with increased mitotic activity (Fig. 6b). Differences were also observed within another lymphoid organ, namely, the spleen. Spleens harvested from animals infected with vMyxlac were essentially indistinguishable from normal uninfected controls, while animals infected with vMyxlac-T7gpt possessed highly reactive and edematous spleens, although not to the levels observed within the peripheral lymph nodes (data not shown). We conclude that by 4 days postinoculation animals infected with the wild-type myxoma virus had mounted only a poor cellular immune reaction, if at all, to the infection while in vMyxlac-T7gpt infected rabbits, the cellular reactivity in secondary immune organs was particularly vigorous.

At 7 days postinoculation, secondary sites of infection, such as the ear and eyelid, were dramatically different between the two viruses (Table 2). Animals infected with vMyxlac demonstrated secondary lesions characterized by a thickened and disorganized epidermis with focal erosion and ulceration. Further, a widespread acute inflammatory reaction within the dermis consisted primarily of heterophils and macrophages, and an acute inflammatory reaction with considerable edema was observed within the subcutis. Secondary lesions from animals infected with vMyxlac-T7gpt, on the other hand, contained a normal epidermis, a focal, mainly mononuclear cell inflammatory reaction with infiltrating lymphocytes predominating within the dermis, and no inflammation but some edema within the subcutis. Thus, in general, while secondary sites of infection with vMyxlac-T7gpt demonstrated what appeared to be a primary inflammatory immune reaction, this reaction was more focalized and did not extend as deeply into the dermis and connective tissues. These results are consistent with our observations at the gross level and suggest that in the absence of T7 expression, reactive lymphocytes now contribute to the clearance of vMyxlac-T7gpt at secondary sites.

Behavior of reactive leukocytes at late times (Day 10) of infection

By 10 days postinoculation, the primary site of infection with vMyxlac revealed extensive necrosis of the skin down to the deeper dermis, with an intense cellular inflammatory reaction localized in the deeper dermis immediately superficial to the cutaneous muscle (Table 2). The cellular infiltration and accumulation of large numbers of heterophils with smaller numbers of mononuclear type cells occupied approximately one-half of the depth of the deep dermal layer, suggesting a block to the free cellular migration into the primary site of viral replication. Diffuse interstitial inflammation with vascular congestion and an infiltrate of a mixed population of heterophils and lymphocytes accumulated in large masses at the edges of the zone of necrosis. As well, opportunistic coccoid bacteria were colonizing the necrotic epidermal surface. In contrast, while the primary site of infection with vMyxlac-T7gpt also demonstrated full thickness necrosis from the epidermis to the subcutis, a comparable mass of reactive inflammatory cells within the deeper dermis was not observed. The epidermis was characterized by vacuolation of the epithelial cells with lifting of the necrotic epidermis off the basement membrane with the intervening space being filled with protein-rich fluid. As well, the subcutaneous muscle exhibited a diffuse infiltrate of lymphoid cells, with a distinctively lymphoid population developing in the dermis at the edges of the necrotic area. Clear demarcation of the primary site of inoculation was seen, which corresponds to the resolution of these primary lesions observed by Day 14 upon gross analysis.

The clustering of inflammatory cells was also more extensive at secondary sites of infection with vMyxlac as opposed to vMyxlac-T7gpt. For example, secondary lesions from the eyelids of rabbits infected with vMyxlac revealed a diffuse inflammatory reaction throughout the full thickness of the connective tissue from the epidermal surface to the conjunctival mucosal surface. Diffuse edema and accumulation of heterophils, mononuclear, and lymphoid cells predominated, while the epidermal surface was reactive and appeared somewhat disorganized. In contrast, secondary lesions from the eyelids of rabbits infected with vMyxlac-T7gpt displayed a relatively normal epidermis with an inflammatory reaction, consisting mainly of mononuclear cells with lymphocytes predominating, being restricted to the underlying dermis. Eyelid tissue sections harvested from rabbits infected with vMyxlac and stained with an anti-rabbit CD43 antibody, which recognizes rabbit T lymphocytes, monocytes, and macrophages, showed diffuse cellular staining throughout the dermis (Fig. 7a). However, similar tissue sections taken from rabbits infected with vMyxlac-T7gpt demonstrated a very focal staining pattern of the dermis directly beneath the epidermis (Fig. 7b). Differences in the organization of the epidermis and dermis can also be seen in these sections.

Thus, in rabbits infected with vMyxlac, an intense, deep, and diffuse inflammatory reaction was established, at both the primary site of inoculation and particularly prominent beneath sites where secondary lesions form. Importantly, many of the reactive inflammatory cells cluster in large arrays in areas that border the infected site, as if migration of early response inflammatory cells to the infected sites is impeded. In rabbits infected with vMyxlac-T7gpt, however, a less intense yet more focalized cellular immune reaction was observed, primarily at the superficial dermis, as if the reactive leukocytes were now able to more effectively penetrate into the viral lesion to gain increased access to the infected cells. Furthermore, an intense primary cellular immune response was established at early times after infection by vMyxlac-T7gpt within the regional lymph nodes and the spleen,

which is not observed to any significant extent upon infection with wild-type myxoma virus.

We conclude that expression of the M-T7 protein in the wild-type myxoma infection establishes what appears to be a partial blockade on the effective migration of reactive inflammatory leukocytes into the infected sites and reduces substantially the extent of communication between sentinel immune cells at the primary site, probably including resident professional antigen presenting cells, and resting lymphocytes in the secondary immune organs. Thus, the loss of M-T7 expression in vMyxlac-T7gpt induces viral attenuation in European rabbits by allowing the development of a more effective cellular immune recognition and subsequent recruitment of activated immune effector lymphocytes from the spleen and lymph nodes to the tissues harboring active virus replication.

DISCUSSION

The importance of IFN-γ in the resolution of poxvirus infections has been well documented (reviewed in Buller and Palumbo, 1991; Mossman *et al.,* 1995a). For example, recombinant vaccinia virus capable of expressing encoded mouse IFN-γ demonstrates a dramatic attenuation in pathogenicity following infection of immunodeficient mice (Kohonen-Corish *et al.,* 1990). Similarly, mice treated with a monoclonal antibody directed against mouse IFN-γ during the course of vaccinia virus infection succumb to a lethal infection (Ruby and Ramshaw, 1991). Using knockout mice that lack the IFN-γ receptor, it was found that IFN-γ increases the susceptibility of mice to vaccinia virus infection by severely diminishing the early defense systems against such an infection, allowing for high viral titers and a lethal pathology (Huang *et al.,* 1993). Subsequently, IFN-γ was shown to inhibit the replication of vaccinia virus, ectromelia virus, and herpes simplex-1 virus in murine macrophages via the induction of nitric oxide synthase, which enhances macrophage cytotoxicity through the production of nitric oxide (Karupiah *et al.,* 1993b; Melkova and Esteban, 1994). Finally, anti-IFN-γ treatment of mice infected with ectromelia virus resulted in enhanced viral spread and replication and demonstrated that IFN-γ is crucial in all aspects of viral clearance except at the primary site of inoculation (Karupiah *et al.,* 1993a).

Since IFN-γ, produced prominently by activated T cells and NK cells, is now recognized as one of the most effective host defense mechanisms against viral infection, it is logical to speculate that inhibition of this potent immunoregulatory cytokine would be a critical factor in myxoma virus virulence. Numerous observations lend support to this speculation. First, the most abundantly secreted protein by far from myxoma virus-infected cells is the M-T7 protein, which specifically binds rabbit IFN-γ with high affinity (K_d = 1.2 n*M*) and can completely block induction of the antiviral state by rabbit IFN-γ (Upton *et al.,* 1992; Mossman *et al.,* 1995c). Second, by comparing the pathogenicity of wild-type vMyxlac with an M-T7 knockout virus, vMyxlac-T7gpt, we show here that some of the features predicted to be the consequence of IFN-γ inhibition do indeed appear to play a role in the progression of a disease state in rabbits infected with myxoma virus. Not only is vMyxlac-T7gpt significantly attenuated in rabbits, but decreased viral dissemination and lower viral titers are observed at secondary sites of infection, while no significant differences in viral replication at primary sites of inoculation are evident. Furthermore, no differences were observed in tissue culture growth curves of vMyxlac and vMyxlac-T7gpt, indicating that M-T7 does not influence the growth of myxoma virus in cultured fibroblasts.

Myxoma virus possesses an extensive and complex arsenal of immune evasion strategies in order to circumvent the host immune defense system and cause a lethal myxomatosis infection (McFadden and Graham, 1994; McFadden *et al.,* 1995). M-T7 is thus only one of many proteins shown to date to play a role in myxoma pathogenesis in the European rabbit host. M-T2, a secreted protein with homology to the tumor necrosis factor receptor (Smith *et al.,* 1990, 1991), specifically binds and inhibits rabbit tumor necrosis factor (Schreiber and McFadden, 1994) and was the first "viroceptor" shown to contribute to virus virulence by sequestration of an important host antiviral cytokine (Upton *et al.,* 1991). The myxoma virus growth factor, a ligand of the epidermal growth factor receptor, as well as the protein product of the M11L open reading frame, whose function remains to be determined, also both play key roles in determining the pathogenicity of myxoma virus in infected rabbits (Opgenorth *et al.,* 1992). Finally, SERP1, a serine proteinase inhibitor, was found to contribute to virus virulence by interfering with the early inflammation response to virus infection (Macen *et al.,* 1993). While the removal of each virulence factor individually attenuates the otherwise lethal myxomatosis disease state, myxoma virus likely requires the entire array of its diverse virulence factors to function in concert in order to completely circumvent the intricate and complex host immune system.

As such, each virulence factor may exercise a distinct activity in modulating different aspects of the potential immune repertoire activated by myxoma virus infection. The data provided here suggest that M-T7 plays a critical role in two distinct, yet likely interlinked, phenotypic responses: (1) blockade of the migration of early response inflammatory cells to the site of viral replication in the infected rabbit dermis, and (2) interruption of the flow of information by resident immune cells which traffic between the site of inoculation and the lymphoid organs where the initiation of acquired immune responses normally develop. The two most dramatic differences in immune responsiveness observed between rabbits infected with vMyxlac and vMyxlac-T7gpt are the accumu-

lation of inflammatory cells at sites bordering virus replication when T7 protein is expressed, but not when it is deleted, and the high state of lymphocyte reactivity at the lymph nodes and spleen at early times of infection when T7 expression is absent. While only a very mild degree of what we speculate to be antigen-specific lymphocyte reactivity is seen within the germinal centers of lymph nodes harvested from vMyxlac-infected rabbits, a dramatically elevated level of lymphocyte activation is observed in both lymph nodes and spleen harvested from vMyxlac-T7gpt-infected rabbits.

The high degree of reactivity observed within the lymph nodes of vMyxlac-T7gpt-infected rabbits substantiates the fact that nonself antigens which enter a host via the skin and mucosa are generally processed and transported by antigen presenting cells (such as macrophages and resident dendritic cells) through the lymphatic system to regional draining lymph nodes, where major histocompatibility complex-restricted activation of both T and B lymphocytes occurs. While anti-virus antibodies may help to decrease viral spread, particularly in previously challenged animals, it is believed that virus neutralization through antibody-mediated mechanisms is incapable of protecting an immunologically naive host from infection with a rapidly developing virulent poxvirus, such as myxoma. Instead a cell-mediated response is believed crucial in determining whether an infection becomes resolved or develops into a lethal syndrome (reviewed in Buller and Palumbo, 1991). Since the lymph nodes are critical for the initiation of T cell responses to lymphborne antigens, we propose that myxoma virus M-T7 protein suppresses the antigenic activation of T lymphocytes within these lymphoid tissues, by interrupting some aspect of the communication between antigen presenting cells resident in the infected dermal tissues at the site of inoculation and resting lymphocytes at peripheral nodes. Additionally, in rabbits infected with vMyxlac-T7gpt, not only was virus propagation at peripheral tissue sites severely inhibited, localization of $CD43^+$ immune cells at both primary and secondary sites was much more highly organized in distinct focal areas beneath the epidermis compared to the wild-type infection. Thus, myxoma virus M-T7 protein may not only circumvent the activation of reactive lymphocytes, but histological analysis suggests that it interferes with leukocyte trafficking in general near infected tissues.

Thus, we propose that the differences in viral virulence, viral dissemination, and immune response seen with vMyxlac-T7gpt and the wild-type virus involve more than the localized inhibition of host IFN-γ. M-T7 was originally characterized as a viral homolog of the mammalian IFN-γ receptor, but since IFN-γ has yet to be shown to influence leukocyte migration, our results indicate that M-T7 may be a multifunctional virulence factor. Indeed, there are precedents for viral proteins which are capable of performing more than one antiimmune activity. Tanapox virus, for example, secretes a glycoprotein at early times of infection which is capable of binding and inhibiting three completely distinct cytokines, namely, human IFN-γ, interleukin-2, and interleukin-5 (Essani *et al.,* 1994). Recently, we have observed that M-T7, in addition to its ability to bind and inhibit rabbit IFN-γ, is capable of binding a wide array of chemotactic cytokines collectively referred to as chemokines (A. Lalani, K. Graham, K. Mossman, K. Rajarathnam, H. Kung, I. C. Lewis, and G. McFadden, manuscript in preparation). Although it is not yet clear if the chemokine binding activity of M-T7 contributes to the apparently aberrant migration of leukocytes in the vicinity of myxoma lesions, mutational analysis of M-T7 may provide clues as to the functional domains of this protein which are important to virulence. Interestingly, sequence alignment of the soluble poxviral IFN-γ receptor homologs with the known cellular mammalian IFN-γ receptors (Mossman *et al.,* 1995a,c) demonstrated that all of the known poxviral proteins contain approximately 60 amino acids at their carboxy-terminal ends which are unrelated to the mammalian receptors, but are well conserved among each other. We are currently investigating the role that this carboxy-terminal region plays in the structure and function of the M-T7 protein.

In order to circumvent host antiviral immune mechanisms and establish a lethal myxomatosis disease state, myxoma virus elicits a diverse array of virulence factors, each of which plays a define yet coordinated role within the viral infection. M-T7 is a critical component of this immune evasion strategy and the dissection of the apparently multiple roles played by this protein, which may not be limited to just IFN-γ inhibition, becomes exceedingly relevant to the identification of new host molecules which orchestrate the host immune response to virus infections.

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