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Immunology

Local production of tumor necrosis factor encoded by recombinant vaccinia virus is effective in controlling viral replication *in vivo*

(antiviral/immunodeficient mice/attenuation/clearance/cytokines)

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ABSTRACT **Tumor necrosis factor (TNF) has pleiotropic effects on a wide variety of cell types. *In vitro* studies have demonstrated that TNF has antiviral properties and is induced in response to viral infections. However, a role for TNF in the antiviral immune response of the host has yet to be demonstrated. Here we describe the construction of and studies using a recombinant vaccinia virus that encodes the gene for murine TNF-α. By comparing the replication of and immune responses elicited by the TNF-encoding virus to a similarly constructed control virus, we hoped to observe immunobiological effects of TNF in the host. The *in vivo* experiments with this recombinant virus demonstrate that the localized production of TNF-α during a viral infection leads to the rapid and efficient clearance of the virus in normal mice and attenuates the otherwise lethal pathogenicity of the virus in immunodeficient animals. This attenuation occurs early in the infection (by postinfection hour 24) and is not due to the enhancement of cellular or antibody responses by the vaccinia virus-encoded TNF. This evidence suggests that attenuation of the recombinant virus is due to a direct antiviral effect of TNF on cells at the site of infection. Therefore, these results support the suggestion that TNF produced by immune cells may be an important effector mechanism of viral clearance *in vivo*.**

Tumor necrosis factor (TNF)-α was originally described as a tumoricidal factor found in mouse serum after sensitization with viable bacillus Calmette–Guérin and subsequent intravenous injection of bacterial endotoxin (1). Activated macrophages (2) are the major source of TNF-α, although a number of other cell types also produce it. The cDNA cloning of both human (3) and murine (4, 5) TNF-α and hence availability of pure recombinant material has led to the discovery of many biological properties associated with TNF-α (see refs. 6 and 7 for reviews). For instance, it has been demonstrated that TNF-α and TNF-β have antiviral properties *in vitro* (8–10). Furthermore, *in vitro* studies have shown that TNFs are induced in response to viral infection (10–12) and that virally infected cells are selectively killed by TNFs (10, 13, 14). Although these findings suggest a role for TNFs in viral infections, the antiviral properties of TNFs have never been demonstrated convincingly *in vivo*.

Recombinant vaccinia viruses (VVs) have been widely used experimentally as a vector for delivery of biologically relevant antigens and as a mode to study the function of inserted foreign genes (15, 16). The innovative approach of encoding cytokine genes in VV was first described for interleukin 2 (IL-2) (17, 18). Vector-directed IL-2 expression markedly attenuated the pathogenicity of the recombinant VV, enabling athymic nude mice to resolve a normally lethal viral infection. We have since constructed recombinant VVs that encode the gene for murine TNF-α to investigate the *in vivo* properties of this factor. Thus, TNF is transcribed and expressed by VV-infected cells and hence available at the site of infection. Therefore, cells that do not normally express TNF such as fibroblasts and endothelial cells will express this factor when infected with the TNF-encoding virus and may thus resemble TNF-producing effector cells. This experimental approach has enabled us to study the immunoregulatory and antiviral properties of TNF at a concentration and mode of presentation that might mimic the natural physiological condition in the host.

Previous *in vivo* studies (19, 20) in which recombinant TNF was administered i.v. or i.p. to the host have not been able to demonstrate the antiviral effects of TNF. However, the findings reported in this paper support the studies that have demonstrated the potent antiviral effects of TNF *in vitro* (8–10). The recombinant virus encoding TNF, influenza hemagglutinin (HA), and herpes simplex virus thymidine kinase (TK) genes (VV-HA-TNF) is dramatically attenuated *in vivo* compared with its control virus encoding HA and herpes simplex virus TK genes (VV-HA-TK). This system appears to be more effective in demonstrating a role for TNF in antiviral immunity because it allows for the localized production of TNF within the microenvironment of infection and possibly in a membrane-associated form, as previously reported (21), that may be of significance physiologically without the widespread release of TNF in the circulation.

MATERIALS AND METHODS

Mice. CBA/H($H\text{-}2^k$) and athymic Swiss outbred nude mice were bred at the Animal Breeding Establishment, John Curtin School of Medical Research, under specific pathogen-free conditions.

Recombinant Viruses. The recombinant VVs used in this study, VV-HA-TNF and VV-HA-TK, were constructed by using VV vectors and homologous recombination and selection methods as described (17, 22). Briefly, VV-PR8-HA6, a L929 cell line-adapted, wild-type VV strain WR (VV-WR) containing the HA gene of influenza virus strain A/PR/8/34 in the *J* region, was used to construct the recombinant viruses. The recombinant virus VV-HA-TNF contains within the *F* region the whole cDNA coding sequence including the signal peptide for murine TNF-α (5) (cDNA provided by W. Fiers, State University of Ghent, Belgium) under the control of the VV 7.5-kDa promoter, $P_{7.5}$ (from B. Moss, National Institutes of Health, Bethesda, MD), and the TK gene of herpes simplex virus, which was used as a selectable marker. The control virus, VV-HA-TK, similarly contains the TK gene of herpes simplex virus but not TNF-α cDNA in the *F*

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Abbreviations: TNF, tumor necrosis factor; VV, vaccinia virus; IL, interleukin; CTL, cytotoxic T lymphocyte; NK, natural killer; p.i., postinfection; pfu, plaque-forming unit(s); moi, multiplicity of infection; HA, hemagglutinin; TK, thymidine kinase; IFN, interferon.

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region (Fig. 1 *Upper*). VV stocks were grown in CV-1 cells and titrated by using 143B cells.

TNF Bioassay. TNF was assayed on WEHI 164 cells by the method of Espevik and Nissen-Meyer (23) modified as follows. Actinomycin D (Sigma) at 2 μg/ml was added to the cell suspension, which then was seeded into 96-well microtiter plates (Linbro) at a density of 2×10^4 cells per well in 50 μl of growth medium. After the 4-hr incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), the supernatant was not aspirated, but rather 100 μl of 10% sodium dodecyl sulfate in 0.01 M HCl was added to each well, followed by overnight incubation. A standard curve was generated from serially diluted recombinant human TNF-α (supplied by Chiron; specific activity, 5×10^7 units/mg).

TNF ELISA. An indirect ELISA method (24) was used to detect TNF in sera. TN3 19.12 (24), a hamster monoclonal antibody to murine TNF (donated by Celltech, Berkshire, U.K.), was used to coat the plates, and a polyvalent rabbit anti-murine TNF antibody diluted 1:500 (donated by Boehringer Ingelheim) was used as the secondary antibody after addition of the TNF source. Plates were developed by using an alkaline phosphatase-conjugated sheep anti-rabbit immunoglobulin antibody (Sigma) and disodium *p*-nitrophenyl phosphate at 1 mg/ml (Sigma). Standard units were defined from serially diluted murine TNF-α (donated by Boehringer Ingelheim; specific activity, 1.2×10^7 units/mg).

Virus Titrations. Organs were titrated as described by Karupiah *et al.* (25). Cell culture samples for titration were harvested by scraping the adherent cells from the surface of the tissue culture vessel into the supernatant. Samples were then subjected to a freeze/thaw cycle three times before a 100-μl aliquot was removed for titration.

Cells. L929, a continuous fibroblast line from connective tissue of a C3H/An mouse; YAC-1, a line derived from Moloney murine leukemia virus-induced lymphoma from an A/Sn mouse; and CV-1, a cell line derived from African green monkey kidney, were maintained in Eagle's minimal essential medium (GIBCO) supplemented with 5% heat-inactivated fetal calf serum (FCS; Flow Laboratories). 143B, a human osteosarcoma cell line; HeLa, a human epithelioid carcinoma cell line; 293, a human embryonal kidney cell line transformed by adenovirus; and tertiary rat embryo fibroblasts derived from JC rats were maintained in Autopow (Eagle's modified minimal essential medium; Flow Laboratories) supplemented with 5% heat-inactivated FCS. WEHI 164, a mouse fibrosarcoma cell line, was maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) supplemented with 10% heat-inactivated FCS.

Preparation of Spleen Effector Cells and Cytoxicity Assays. Single-cell suspensions from spleens of mice were obtained, and cytotoxicity assays were performed as described (25). L929 targets for cytotoxic T-lymphocyte (CTL) assays were infected with wild-type VV-WR at 20 pfu per cell during ^{51}Cr labeling and were incubated with effector cells for 6 hr. YAC-1 targets for natural killer (NK) cell assays were incubated with effector cells for 4 hr.

Antibody Determination. VV specific antibodies were determined by using a standard ELISA method (26). Titers

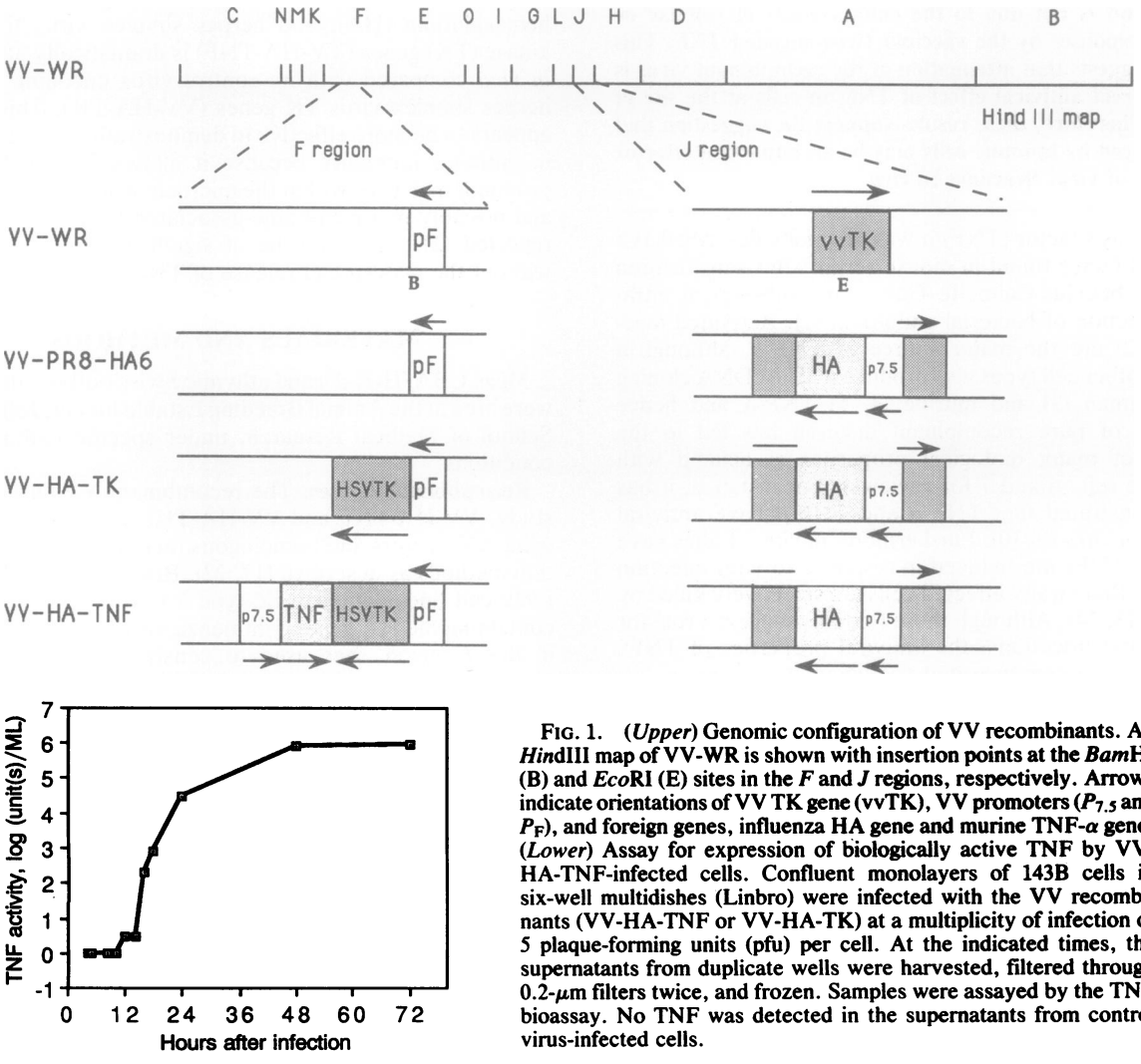

FIG. 1. (*Upper*) Genomic configuration of VV recombinants. An *Hin*dIII map of VV-WR is shown with insertion points at the *Bam*HI (B) and *Eco*RI (E) sites in the *F* and *J* regions, respectively. Arrows indicate orientations of VV TK gene (vvTK), VV promoters ($P_{7.5}$ and P_F), and foreign genes, influenza HA gene and murine TNF-α gene. (*Lower*) Assay for expression of biologically active TNF by VV-HA-TNF-infected cells. Confluent monolayers of 143B cells in six-well multidishes (Linbro) were infected with the VV recombinants (VV-HA-TNF or VV-HA-TK) at a multiplicity of infection of 5 plaque-forming units (pfu) per cell. At the indicated times, the supernatants from duplicate wells were harvested, filtered through 0.2-μm filters twice, and frozen. Samples were assayed by the TNF bioassay. No TNF was detected in the supernatants from control virus-infected cells.

were calculated with an end point that was determined as twice the mean blank value.

RESULTS

To test the effective vector-directed expression of biologically active TNF by cells infected with the TNF-encoding virus, an expression assay on 143B cells was carried out (Fig. 1 *Lower*). High levels of TNF were detected in the culture supernatants of cells infected with the VV-HA-TNF virus, reaching a maximal level between 24 and 48 hr postinfection (p.i.) (Fig. 1 *Lower*). However, after such an infection with a moi of 5 pfu per cell, most cells would be destroyed by 24 hr p.i., and hence this late increase of TNF in the supernatant may be due to the release of the transmembrane form (21). This clearly differs from the expression of VV-encoded IL-2, which reached a maximal level of secreted cytokine by 12 hr p.i. (17).

To compare the *in vitro* replicative efficacy of VV-HA-TNF to that of control virus VV-HA-TK, we performed a single-step growth experiment (moi = 5 pfu per cell). Under these conditions, growth profiles for both recombinant viruses were similar in either CV-1 (simian) or L929 (murine) cell lines (data not shown). This indicates that the ability of VV-HA-TNF to replicate *in vitro* at high multiplicities of infection is not altered by insertion of the TNF gene or by the expression of TNF. To test the sensitivity of VV to the antiviral effects of TNF, the cell lines L929, 143B, 293, and HeLa and tertiary rat embryo fibroblasts were pretreated for 24 hr with 0.1–400 ng of recombinant murine TNF-α (Genentech; specific activity, 1.2×10^7 units/mg, supplied by Boehringer Ingelheim) or of human TNF-α (Asahi, Tokyo; specific activity, 2.2×10^6 units/mg) per ml and then were infected with wild-type VV-WR. When the virus yield was measured 24 hr later, only L929 cells showed reduced virus growth ($\leq$ a 1.5 logarithmic decrease—i.e., a reduction from 7×10^7 to 2.1×10^6 pfu/ml) in the presence of TNF. Some toxicity toward the L929 cells was noted, however, at the concentration of TNF required to inhibit virus replication. Thus, *in vitro* VV is not highly susceptible to the antiviral effects of TNF, which is in agreement with the work of others (27).

In contrast to these *in vitro* results, which suggest that VV is not highly susceptible to the antiviral effects of TNF, expression of TNF markedly attenuated the growth of VV-HA-TNF *in vivo* (Fig. 2). Two models of immunodeficiency were used: athymic Swiss outbred nude mice and euthymic CBA/H mice rendered immunodeficient by a sublethal dose of γ-irradiation (650 R) administered 24 hr prior to infection. Both the nude and irradiated mice infected with control virus VV-HA-TK died from a disseminated vaccinial disease after a mean survival time of $\approx$10 days (Fig. 2 *Left*). In contrast, when infected with VV-HA-TNF, both groups of mice survived and remained as healthy as the uninfected controls, indicating that TNF expression had reduced the pathogenicity of VV in these mice (Fig. 2 *Left*).

The attenuating effect of TNF expression on virus growth was also seen in normal mice (Fig. 2 *Right*). Neither VV-HA-TNF nor its control, VV-HA-TK, produced morbidity or mortality at the doses used. However, VV-HA-TNF was recovered from various organs at significantly reduced titers, and the virus cleared more rapidly as compared with VV-HA-TK (Fig. 2 *Right*). The difference in growth between the two viruses was evident by 24 hr p.i. (Fig. 2 *Right*) and even earlier in some cases (data not shown). Growth in the ovaries provided one of the most prominent indicators of virus attenuation. VV-HA-TK grew to high titers, reaching $10^{8.1}$ pfu per pair of ovaries by day 3, and was cleared only by day 10, whereas VV-HA-TNF reached a mean peak titer of $10^{3.5}$ pfu per pair of ovaries and was cleared 3–5 days p.i. Histological examination of the ovaries from VV-HA-TK-infected mice revealed extensive damage to the stromal tissue and follicles, whereas those from VV-HA-TNF-infected mice appeared normal (data not shown).

The expression of TNF was essential for the reduced pathogenicity of recombinant virus VV-HA-TNF *in vivo* because the similarly constructed control virus containing foreign coding sequences in the *F* and *J* regions of the VV genome but lacking the TNF gene (Fig. 1 *Upper*) was not attenuated. Furthermore, recombinant viruses similarly constructed to express other cytokine genes such as IL-1α, IL-3, IL-4, IL-5, and IL-6 were not attenuated but did produce other biological responses *in vivo* that were associated with

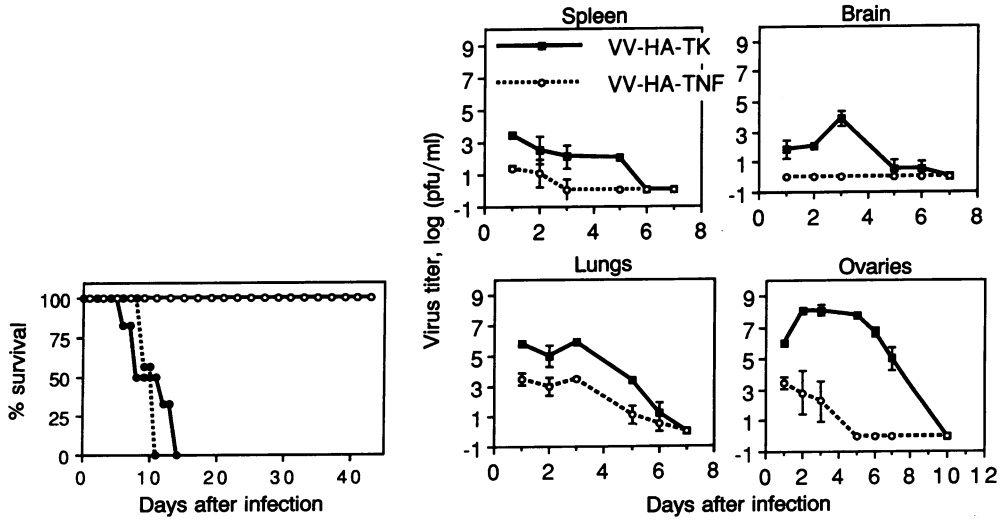

FIG. 2. Attenuation of the VV-HA-TNF *in vivo*. (*Left*) Survival study of VV recombinants in immunodeficient mice. Groups (six mice per group) of 9-week-old Swiss outbred nude and 8-week-old sublethally irradiated CBA/H mice were inoculated i.v. with the recombinant viruses at doses of 5×10^6 pfu and 1×10^7 pfu, respectively. Mortality of VV-HA-TK-infected nude (—●—) and sublethally irradiated CBA/H (--●--) mice and the survival (—○—) of VV-HA-TNF-infected nude, VV-HA-TNF-infected sublethally irradiated, and uninfected control mice are shown. (*Right*) Growth kinetics of the VV recombinants in normal mice. Groups of 9-week-old female CBA/H mice were injected i.v. with a nonlethal dose (10^7 pfu) of either VV-HA-TNF (---) or VV-HA-TK (—). On the indicated days, selected organs were collected for titration of virus on 143B cell monolayers. Mean titers are shown for groups of four mice, with error bars indicating the SEM.

the known properties of the particular cytokine (data not shown).

Coinfection was used to assess the degree of localization of the attenuating effect of VV-directed TNF. Mice were infected with either VV-HA-TNF or VV-HA-TK alone or with a suspension of both viruses to test whether the VV-encoded TNF would affect the replication of another virus when the two were given concomitantly. Briefly, CBA/H mice were injected i.v. with 10^7 pfu of VV-HA-TNF, 10^7 pfu of VV-HA-TK, or a mixture of 10^7 pfu of each VV recombinant (coinfection). Lungs and ovaries were removed on days 1, 3, and 5 and titrated on 143B cells. Titers were calculated as the mean ± SEM for groups of four mice. The titers of the VV-HA-TK infection and coinfection in each group were compared by using Student's *t* test. Early in infection—i.e., on days 1 and 3 p.i., there was no significant difference in the titers of coinfected viruses compared with that of VV-HA-TK when given alone, even though the growth of VV-HA-TNF at this stage was significantly attenuated. On day 5 p.i., by which time VV-HA-TNF had been almost cleared, the virus titer in the ovaries of coinfected mice (log titer, 6.03 ± 0.50 pfu/ml) was lower ($P = 0.046$) than that of mice given VV-HA-TK alone (log titer, 7.10 ± 0.14 pfu/ml), suggesting that the early presence of VV-HA-TNF resulted in some inhibition of growth of the other virus in the ovaries. There was no significant difference, though, on day 5 in the titers of lung tissue of coinfected mice compared with those of mice given VV-HA-TK alone ($P = 0.285$). Overall, these results suggest that the TNF produced by VV-HA-TNF-infected cells effectively controls its own spread but is not as effective in controlling the growth of another coinjected virus that infects cells of the same organ.

A bioassay (23) and ELISA (24) were used to detect TNF in the sera of animals given 10^7 pfu of VV-HA-TNF or VV-HA-TK i.v. (data not shown). The bioassay failed to detect TNF in either case. Although the ELISA measured small amounts of TNF in the sera close to the limit of detection (60 pg/ml), there was no significant difference between levels detected in VV-HA-TNF- or VV-HA-TK-infected mice. This further suggests that the production and distribution of virus-encoded TNF are very localized and are controlled by the self-limiting growth of VV-HA-TNF.

The humoral and cell-mediated immune responses to VV were studied by using euthymic CBA/H mice infected with the recombinant viruses to determine whether they contributed to the rapid clearance of VV-HA-TNF.

Antibody responses were measured in the serum of mice infected with the recombinant VVs (Table 1). VV-specific IgG and IgM levels were observed to be significantly lower in mice immunized with VV-HA-TNF, presumably reflecting the reduced titers and more rapid clearance of the virus. Since levels of antibody were not increased during VV-HA-TNF infection, it seems likely that the observed attenuation of VV-HA-TNF, which occurred rapidly, was not antibody-mediated.

We found no evidence to implicate VV-specific CTL activity as a possible mechanism for the attenuated growth of VV-HA-TNF, even though T cells are thought to be important in the normal clearance of poxviruses *in vivo* (28, 29). CTL levels were not increased in VV-HA-TNF-infected mice compared with levels in mice infected with control virus (Table 2). Furthermore, the earliest VV-specific CTL activity was detected on day 3 p.i. with a peak response between days 5 and 7, whereas inhibition of VV-HA-TNF growth was evident much earlier (Fig. 2 *Right*). This mechanism also could not account for virus clearance in nude mice, which lack mature functional T cells, or in sublethally irradiated mice, which suffer from loss of some immune effector cell functions, including virus-specific CTL activity.

Table 1. Humoral immune response in CBA/H mice infected with VV-HA-TNF and VV-HA-TK

Immunizing virus	Dose i.v., pfu × 10^{-7}	Day p.i.	Humoral immune response* IgM	IgG	Anti-VV
VV-HA-TNF	1	7	250	50	
	1	14	81	250	
	1	21	50	159	250
	0.1	21			50
	10	21			1250
VV-HA-TK	1	7	534	50	
	1	14	138	1113	
	1	21	50	1250	1250
	0.1	21			841
	10	21			6250

*Antibody responses were measured in 7-week-old CBA/H mice immunized with the recombinant VVs at the doses indicated. Serum was collected on days 7, 14, and 21 p.i. as indicated for detection of VV-specific IgG and IgM levels and on day 21 p.i. for detection of anti-VV antibodies. Results are shown as the geometric means of titers for groups of 4 mice.

It has been shown (25) that the rapid clearance of recombinant VV expressing IL-2 (VV-HA-IL2) in both nude and normal mice is mediated by an IL-2-induced augmentation of the NK cell response. Therefore, splenic NK cell activity in mice given VV-HA-TNF or control virus VV-HA-TK was compared on days 1, 2, and 3 p.i. (Table 2). Both the TNF-encoding and control recombinant viruses induced NK cell responses above those of uninfected animals, which reached peak levels on day 3 p.i. However, there was no significant difference between the response in VV-HA-TNF- or control virus-infected mice, suggesting that the attenuation of VV-HA-TNF, unlike that of VV-HA-IL2, is not mediated through enhancement of NK cell activity.

DISCUSSION

The lack of enhanced cell-mediated (CTL, NK) or humoral immune responses in VV-HA-TNF-infected mice suggests that other mechanisms are responsible for the rapid recovery from this infection. We propose that the attenuated growth of VV-HA-TNF *in vivo* is largely due to the antiviral effects of

Table 2. Cell-mediated immune response in CBA/H mice infected with VV-HA-TNF and VV-HA-TK

Immunizing virus	Dose i.v., pfu × 10^{-7}	Cell-mediated response,* lytic units per 10^6 cells CTL†	NK cells‡
VV-HA-TNF	5	9.09	2.99
	0.5	3.33	2.56
	0.05	1.47	1.06
VV-HA-TK	5	11.11	3.08
	0.5	3.85	2.44
	0.05	2.08	1.24
Uninfected controls		Deducted	0.75

*Data are representative of NK cell and CTL responses obtained in the spleens of 6- to 10-week-old CBA/H mice injected i.v. with various doses of the VV recombinants. The % lysis of target cells was calculated as the mean of three mice per group (where SEM < 5%) and converted to lytic units per 10^6 effector cells, where 1 unit represents activity that results in 25% lysis of the target cells. Values (% lysis) for the Unimmunized CTL control group were deducted from the above test values.

†CTL responses were measured on day 5 p.i. using VV-infected L929 cells as targets.

‡NK cell responses were measured on day 3 p.i. using YAC-1 cells as targets.

TNF, possibly acting synergistically with other host-derived factor(s).

In vitro studies have clearly demonstrated that TNF is antiviral for a number of different DNA and RNA viruses, although induction of the antiviral state can be variable between cell types and conditions (8–10). Studies utilizing antibodies to β interferon (IFN-β) have shown that these antiviral effects of TNF are partially or completely mediated through IFN-β (9, 30, 31), while other evidence indicates that they are independent of IFNs (10). The induction of low levels of IFN-β by TNF has been confirmed by using the polymerase chain reaction (32, 33). Cytokines such as IFN-γ (10, 34) and IFN-β (32, 34) also synergize with TNF, dramatically enhancing its antiviral effects. It has also been suggested that TNF eliminates virus by lysing virally infected cells (13, 14).

It is interesting to note that, although the effect of cloning TNF into recombinant VV leads to a dramatic effect on virus growth *in vivo*, the *in vitro* effect of TNF on VV growth is minimal. Whether synergy of virus-encoded TNF with other host-derived cytokines, either induced by virus or TNF itself, or the lysis of infected cells in the host can explain the potent *in vivo* antiviral properties of virus-encoded TNF remains to be determined.

It has been suggested by Old (35) that the real physiological role of TNF may be as a protective agent against infectious diseases, since there is evidence to suggest a role for TNF in fungal, bacterial, and parasitic infections. In this study, we clearly demonstrate the antiviral activity of TNF *in vivo*. Attempts to treat viral infections by the administration of TNF have largely been unsuccessful (19, 20), probably because of the short half-life of free TNF *in vivo* and the need to localize TNF at sites of virus infection, which was somewhat artificially achieved in our VV–cytokine system. As the mice given VV-HA-TNF remained healthy, the antiviral effects of TNF evidently occur at a lower concentration or in a different form than that required to cause pathology.

The role of cytokines, including TNF, in the recovery from viral infections under normal physiological conditions needs to be addressed. Recovery from poxviruses in mice is mediated by $CD8^+$ T lymphocytes, which are thought to recognize and lyse virus-infected cells (28, 29). However, mice deficient in T cells can still resolve infection with recombinant VVs encoding TNF-α, IFN-γ (26), or IL-2 (17, 25) genes. This raises the possibility that the prime function of T cells is to produce and focus lymphokines at sites of virus replication rather than, as previously thought, the direct killing of virus-infected cells.

If cytokines are an important effector mechanism in virus clearance, deficiencies in cytokine production are likely to lead to uncontrolled virus growth. Mononuclear cells from AIDS patients are reported to be deficient in the production of TNF-α (36), IFN-γ (37), and IL-2 (38) in response to mitogen or antigen stimulation. The persistence of human immunodeficiency virus (HIV), despite the presence of HIV-specific CTLs, in seropositive individuals (39) could be attributed to the reduced ability of these and other effector cells to secrete the necessary cytokines to eliminate the virus.

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