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Immunology

Adenovirus E3 14.7-kilodalton protein, an antagonist of tumor necrosis factor cytolysis, increases the virulence of vaccinia virus in severe combined immunodeficient mice

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ABSTRACT **The adenovirus (Ad) 14.7-kDa protein, which is called "14.7K," has been shown to function as a general inhibitor of tumor necrosis factor α (TNF) cytolysis in tissue culture assays, and the effect of this antagonism on viral pathogenesis *in vivo* has recently been explored. In infections of immunocompetent BALB/c mice, we have shown previously that Ad type 2 (Ad2) 14.7K, when cloned into a vaccinia virus (VV) vector in combination with the gene for murine TNF, is able to counteract much of the attenuating effect of TNF on VV virulence. In the present study we utilized VV constructs expressing various combinations of Ad 14.7K and TNF in infections of T- and B-cell-deficient C.B-17 severe combined immunodeficient (SCID) mice to determine whether these cells are directly necessary for 14.7K's reversal of TNF-mediated viral attenuation. The mice were infected by the intranasal route, and mortality, morbidity, histopathology, and virus replication in selected organs were evaluated at various times after infection. We found that, in the SCID murine pneumonia model, neither the attenuation by TNF nor its reversal by Ad 14.7K require the participation of T or B lymphocytes or their secreted products. SCID mice infected with VV expressing both 14.7K and TNF [VV 14.7(+)/TNF] were generally well clinically for the first 7–10 days after infection; however, they developed a subacute or chronic illness, succumbing to disseminated VV infection at least 3 weeks earlier than mice infected with VV expressing TNF alone [VV 14.7(−)/TNF]. Animals infected with VV 14.7(+)/TNF were shown to have higher initial titers of virus and delayed clearance from the lungs as well as more rapid spread of virus to internal organs than animals infected with VV 14.7(−)/TNF. SCID mice infected intranasally with VV without TNF showed a dramatic increase in acute disease and succumbed within the first 1–2 weeks after infection, independent of Ad 14.7K expression.**

The early region 3 (*E3*) genes of adenovirus (Ad) encode a number of proteins with the potential to modulate the host immune response to infection. Among the most well characterized is gp19K, an integral membrane glycoprotein, resident within the endoplasmic reticulum (ER), which associates with the heavy chain of the class I major histocompatibility complex (MHC) (1–3). This interaction results in retention of the heavy chain within the ER, down-regulation of class I MHC expression at the cell surface, and interference with recognition and lysis by $CD8^+$ cytotoxic T cells (4). In addition to evasion of the cell-mediated immune response, Ad has also evolved mechanisms to circumvent the cytokine response to viral infection. An Ad immediate early gene product, Ad E1A, induces sensitization of cells to lysis by tumor necrosis factor α (TNF) (5, 6), while three proteins encoded by the *E3* region function to block this TNF-mediated cytolysis. These include the 10.4- and 14.5-kDa proteins, which are called "10.4K" and "14.5K" and are integral plasma membrane-associated proteins that function in concert to protect cells from the lytic effects of TNF (7). These proteins have an additional function in cell surface receptor modulation. Expressed from a retroviral vector, 10.4K and the 10.4K–14.5K complex produced upon viral infection have been shown to accelerate internalization of the epidermal growth factor receptor (8, 9). Possibly in cooperation with the 14.5K, 10.4K also promotes down-regulation of other type I and type II protein tyrosine kinase receptors (10).

The other TNF antagonist encoded by the *E3* region is the 14.7-kDa protein, which is called "14.7K" and is the focus of the present paper. Ad type 2 (Ad2) 14.7K is a 128-amino acid polypeptide found in the soluble fractions of both nucleus and cytosol (11); 14.7K is capable of preventing cytolysis in cell lines that are spontaneously sensitive to TNF and in a wide variety of cell lines that can be sensitized to TNF lysis by the action of the Ad E1A protein or by treatment with cycloheximide, cytochalasin E, or actinomycin D (11–13). Thus, 14.7K can function in the absence of other Ad2 proteins to protect cells from TNF lysis in tissue culture assays. It has been shown that C3HA mouse embryo fibroblasts stably transfected with the 14.7K gene are resistant to TNF-induced activation of phospholipase A_2 (14), an activation that has been demonstrated to play a role in both the proliferative and cytolytic actions of TNF (15, 16). It is also known that the protective effects of 14.7K do not result from a reduction in TNF receptor number or affinity (13).

The relevance of 14.7K's ability to block TNF lysis for viral pathogenesis *in vivo* has been the subject of recent investigation. Such studies are complicated by the fact that the human Ads do not replicate well in mice, which would provide a convenient model system (17), and the mouse Ad does not appear to encode a homologue of the 14.7K (18). Others have shown that TNF expression is induced upon high-dose Ad type 5 infection of mice (a replication-defective model) (19) and that Ad deleted of the 14.7K gene (as well as genes encoding the other TNF antagonists, 10.4K and 14.5K) induces a primarily neutrophilic infiltrate in the cotton rat lung, in contrast to the largely monocytic inflammatory response to the wild-type virus (20).

We set out to study the effects of 14.7K in isolation from other Ad immunomodulatory proteins in a murine pneumonia model, and toward this end we cloned the 14.7K gene into a vaccinia virus (VV) vector. As a vector, VV provides high-level expression of heterologous proteins, and in these experiments it also served as the pathogen, yielding quantifiable morbidity, mortality, inflammation, and virus replication

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Abbreviations: VV, vaccinia virus; TNF, tumor necrosis factor α; SCID, severe combined immunodeficient; Ad, adenovirus; pfu, plaque forming units.

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upon intranasal infection of mice. For some studies, the gene for mouse TNF was inserted into the same virus expressing 14.7K. It had been demonstrated (21) that virus-directed TNF expression caused significant attenuation of VV for infection of immunocompetent, athymic nude, or gamma-irradiated mice. In the vectors that we constructed, coexpression of TNF and Ad 14.7K provides high local concentrations both of the cytokine, with its attendant antiviral activity, and of the putative cytokine antagonist. We have previously shown, using VV modified to contain various combinations of 14.7K and TNF genes, that 14.7K increases the virulence of VV expressing the TNF gene following intranasal inoculation of BALB/c mice (22). Thus, in immunocompetent mice, Ad 14.7K is able to reverse much of the attenuating effect of TNF on VV virulence, presumably by inhibiting premature lysis of infected cells by TNF. We conducted the experiments in SCID mice described in this manuscript to determine whether T or B cells or their products are necessary for 14.7K's antagonistic effects on TNF-mediated attenuation.

MATERIALS AND METHODS

Mice. C.B-17 severe combined immunodeficient (SCID) mice were purchased from Taconic Farms and infected at 3 weeks of age (mortality experiment) or 4–5 weeks of age (organ titration experiment). Mice were housed in microisolator cages in a specific pathogen-free colony.

Cell Lines/Viruses. The VV recombinants have been described (22). The virus VV 14.7(+)/TNF contains the Ad2 14.7K coding sequence as well as the mouse TNF gene behind the combined early/late VV promoter *P7.5*. VV 14.7(−)/TNF is identical, except that the 14.7K coding sequence was cloned in the reverse, nonexpressing orientation relative to *P7.5*. Two other viruses, VV 14.7(+) and VV 14.7(−), are also double recombinants with the Ad2 14.7K coding sequence [expressing (+) or nonexpressing (−)] but without mouse TNF.

Mouse Infections. SCID mice were infected by intranasal installation of virus as described (22). Infected mice were observed until they succumbed to the infection or were sacrificed at various times after infection for determination of viral titers in organs and histopathology.

PCR Analysis of the Mouse TNF Gene in Individual Plaques. Virus obtained from each SCID organ to be tested was plaqued on 143B cell monolayers under 1% agarose. Individual, well-isolated plaques were picked, and the tip of the pipet was inserted into the reaction tube containing the PCR mix previously described (23). PCR conditions and products were analyzed by electrophoresis as described (23). Primers (purchased from Stratagene) for amplification of the mouse TNF cDNA sequence were nucleotides 157–177 (ATGAGCACAGAAAGCATGATC) of the sense strand and nucleotides 412–432 (TACAGGCTTGTCACTCGAATT) of the antisense strand, yielding a 276-bp product.

RESULTS

SCID Mice Infected Intranasally with VV 14.7(+) or VV 14.7(−) Develop Pulmonary Disease and Succumb Rapidly. SCID mice infected with VV 14.7(+) or VV 14.7(−) died acutely and in a dose-dependent fashion (Fig. 1). The mean time to death and clinical picture were similar to that of BALB/c mice infected with the same viruses, with the rapid onset of weight loss, hunched posture, ruffled fur, and decreased activity followed by death within several days. The mice presumably died of a VV-induced pneumonia, as the pulmonary pathology revealed diffuse congestion, severe edema, and moderate-to-severe perivascular and peribronchial infiltration of inflammatory cells. There was no difference in pulmonary histopathology between mice infected with VV 14.7(+) and VV 14.7(−), and the time to development of illness and death were also very similar but not quite identical for the two viruses. Mice infected with VV 14.7(+) died 1–3 days earlier than those infected with VV 14.7(−) at both doses administered; a similar brief lag period was repeatedly observed in infection of BALB/c mice with these viruses and may be due to antagonism of small amounts of endogenous, host-derived TNF by the 14.7-expressing VV.

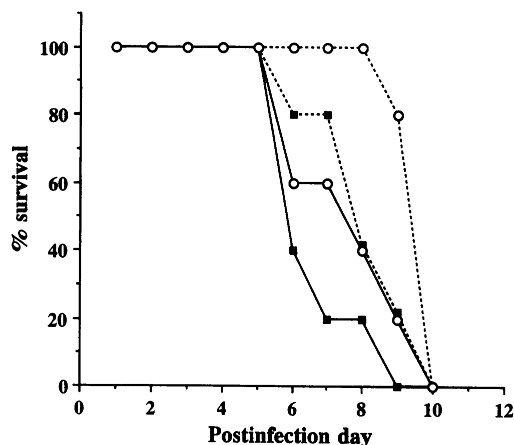

FIG. 1. Survival of SCID mice after infection with VV 14.7(+) or VV 14.7(−). C.B-17 SCID mice (3 weeks old) were infected intranasally with 6.3×10^6 pfu (——) or 6.3×10^5 pfu (- - - -) of VV 14.7(+) (■) or VV 14.7(−) (○) and examined daily for deaths. There were five animals in each of the four groups.

SCID Mice Infected with VV Containing the Mouse TNF Gene Develop Subacute or Chronic Illness, Accelerated in Appearance by the 14.7K Gene. Shown in Fig. 2 is the survival of mice after infection with VV 14.7(+)/TNF or VV 14.7(−)/TNF. These data clearly illustrate the dramatic effect of TNF expression on the course of VV infection in SCID mice. Virus-directed TNF production induces not only a delay in the appearance of the illness but also markedly prolongs its course. The initial illness was milder in SCID mice than in the BALB/c mice reported previously, and although there was severe, generalized pulmonary inflammation (peribronchial, perivascular, and interstitial) during the first week after infection, there was only one death in several experiments performed. Rather than succumbing to a rapidly fatal VV-induced pneumonia, as did SCID mice infected with VV 14.7(+) or VV 14.7(−) (both lacking the TNF gene) or BALB/c mice infected with $>2 \times 10^6$ plaque-forming units (pfu) of VV 14.7(+)/TNF, the SCID mice infected with VV expressing TNF survived the initial infection but eventually developed a subacute or chronic disseminated vaccinial disease. After a period of time, which varied from weeks to months depending on the amount of input virus and the presence of the 14.7K gene, the mice developed cutaneous pox on hairless skin surfaces [along the tail, also involving ears and paws (data not shown)] and evidence of spread to internal organs (discussed below). As shown in Fig. 2, expression of Ad 14.7K in combination with TNF accelerated the time to death from a disseminated VV infection by ≈3 weeks at both input doses. The mean time to death was 30 days for VV 14.7(+)/TNF and 53 days for VV 14.7(−)/TNF at an input dose of 9.7×10^6 pfu (Fig. 2 *Upper*), while the values were 50 days for VV 14.7(+)/TNF and 73 days for VV 14.7(−)/TNF at the 1-order-of-magnitude-lower input dose (Fig. 2 *Lower*). This experiment was repeated twice with essentially similar results. Thus, the enhanced pathogenicity of VV 14.7(+)/TNF as compared with VV 14.7(−)/TNF is apparent in SCID mice as it was in BALB/c mice, although reflected in terms of an accelerated time to death rather than a difference in LD_{50} at the doses administered.

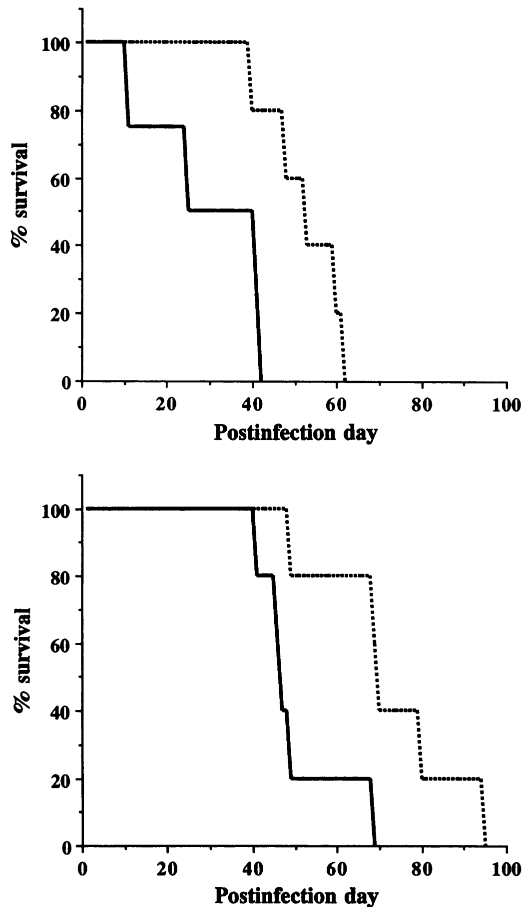

FIG. 2. Survival of SCID mice after infection with VV 14.7(+)/TNF or VV 14.7(−)/TNF. SCID mice were infected intranasally with 9.7×10^6 (*Upper*) or 9.7×10^5 (*Lower*) pfu of VV 14.7(+)/TNF (——) or VV 14.7(−)/TNF (- - - -) and monitored daily for mortality. There were four or five animals in each of the four groups.

VV Containing the TNF and Ad 14.7K Genes Persists in the Lungs After Acute Infection. We also examined growth kinetics of the recombinant vaccinia viruses in selected organs at various times after infection. Mice infected intranasally with VV 14.7(+)/TNF or VV 14.7(−)/TNF were sacrificed during the first week after infection, and the amount of virus in lung tissue was quantified. By postinfection day 4, pulmonary viral titers were generally slightly higher after infection with VV 14.7(+)/TNF than with VV 14.7(−)/TNF. However, the differences were on average smaller than those observed upon infection of BALB/c mice, in which peak lung titers were consistently higher by 2 or more orders of magnitude for VV 14.7(+)/TNF than for VV 14.7(−)/TNF. Therefore, early on (within 2 weeks after infection), the disease severity, mortality, and lung titers indicated an only modestly enhanced pathogenicity of VV 14.7(+)/TNF versus VV 14.7(−)/TNF in infections of SCID mice. However, what may be of greater significance is the observation that VV 14.7(+)/TNF was not cleared from the lungs. For example, at an input dose of 8.4×10^6 pfu, VV 14.7(+)/TNF pulmonary titers were 10^7 pfu at postinfection day 7 as compared with 10^2 pfu for VV 14.7(−)/TNF (Fig. 3 *Left*). At an input dose of 8.4×10^5 pfu, VV 14.7(−)/TNF was reduced to undetectable levels in the lungs by postinfection day 7 while titers of VV 14.7(+)/TNF remained at $\approx 10^4$ pfu (Fig. 3 *Center*). This delayed clearance of VV 14.7(+)/TNF, which was noted even at an input dose of 8.4×10^4 pfu (Fig. 3 *Right*), may contribute to its earlier dissemination. We again examined virus titers at a later time after infection (40 days), when morbidity (disseminated disease) and some mortality (25%) were evident in the VV 14.7(+)/TNF-infected group while all of the VV 14.7(−)/TNF-infected mice remained apparently well. As shown in Fig. 4, the majority of mice infected with VV expressing both 14.7K and TNF had significant titers of virus in all organs examined, while the VV expressing TNF alone was detected only in the lung of two animals [one animal had a low titer (<100 pfu/lung), while one animal infected with the highest dose had a titer of 10^4 pfu per lung]. Thus, these titers represent a window in time, when dissemination had already occurred widely in the VV 14.7(+)/TNF-infected mice but was not yet apparent in the VV 14.7(−)/TNF-infected mice. At even later times after infection, the mice infected with VV 14.7(−)/TNF also developed a disseminated disease apparently identical to that seen weeks or months earlier in the VV 14.7(+)/TNF-infected mice. Interestingly, the precise cause of death in these mice is unknown. Except for the vesicles visible on the skin, pathologic examination of organs from VV 14.7(+)/TNF-infected mice harvested near the time of death revealed no detectable pathology in liver, kidney and brain, while in general only subtle changes (mild interstitial infiltrates) were evident in pulmonary tissue.

Some VV [14.7(+)/TNF and 14.7(−)/TNF] Isolates from Animals at Postinfection Day >40 No Longer Express TNF. Having observed the delayed appearance of disseminated disease in the SCID mice, we determined whether the VV

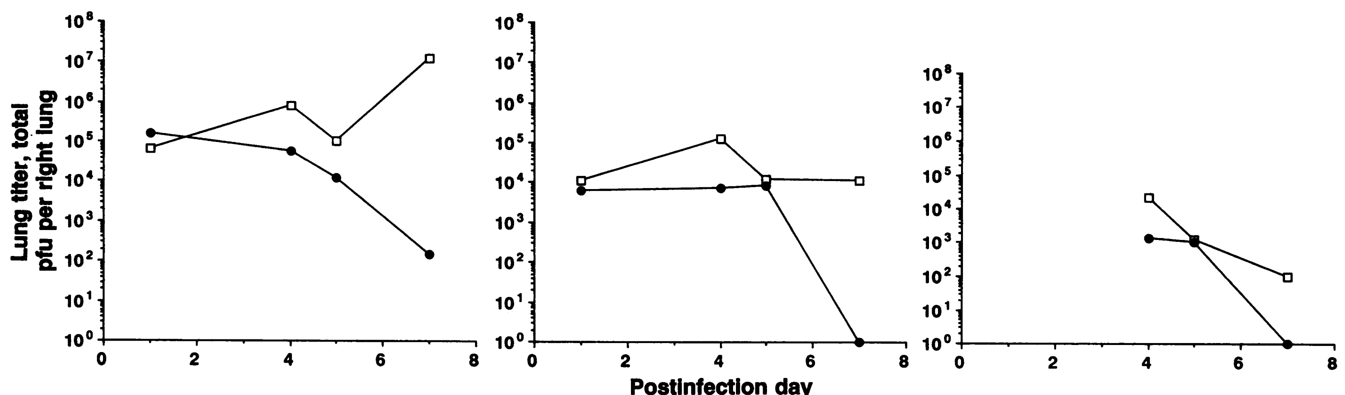

FIG. 3. Kinetics of replication of VV 14.7(+)/TNF and VV 14.7(−)/TNF in lungs of SCID mice during the first week after infection. Intranasally infected SCID mice were sacrificed on the indicated postinfection day, and the right lung was removed, cut into pieces, dispersed by sonication, and plaqued on 143B cell monolayers to determine virus titer (22). The mice were initially infected with 8.4×10^6 (*Left*), 8.4×10^5 (*Center*), or 8.4×10^4 (*Right*) pfu of VV 14.7(+)/TNF (□) or VV 14.7(−)/TNF (●). The lung titers are expressed as total pfu per right lung. Symbols placed at 10^0 indicate that no virus was detected.

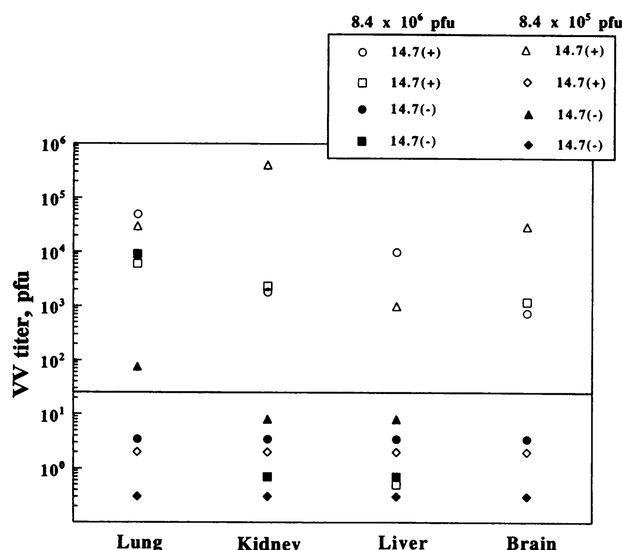

FIG. 4. Presence of VV 14.7(+)/TNF and VV 14.7(−)/TNF in SCID organs at a late time (40 days) after infection. Intranasally infected SCID mice were sacrificed on postinfection day 40, and the amount of VV in various organs was quantified. The VV titers are expressed as (*i*) total pfu per right lung, (*ii*) total pfu per right kidney, (*iii*) pfu per 42 mg of liver protein, and (*iv*) pfu per 8 mg of brain protein. Each unique symbol represents the various organs of one animal. Symbols placed at or below the value of 2.5×10^1 (below the solid line) indicate that no virus was detected in those samples.

that could be recovered from these animals was still capable of producing TNF. Virus from infected organs was amplified by one passage in 143B cells, and the supernatants were assayed for the presence of mouse TNF by ELISA. All organs harvested early (during the first week) after infection contained VV that produced large amounts of immunoreactive TNF, and this was the same whether the samples were from mice initially infected with VV 14.7(+)/TNF or VV 14.7−/TNF (see Table 1, mice nos. 1–4). However, VV from organs harvested late (postinfection day 40 and beyond) was variable in terms of TNF expression (Table 1, mice nos. 5–10). For example, mouse 7, infected with VV 14.7(+)/TNF and sacrificed on postinfection day 40, when disseminated disease was apparent, contained TNF-expressing VV in lung and brain but not in kidney. Because the disease became manifest over such a prolonged period of time, it was possible that TNF expression was compromised in these viruses because of mutation during growth *in vivo* or that the nonexpressing viruses represented outgrowth of a small amount of contaminating parental recombinant VV in the initial inoculum [(i.e., VV 14.7(+) or VV 14.7(−) before insertion of TNF]. Therefore, we carried out PCR amplification of viral DNA from individual plaques using a primer pair specific for the mouse TNF cDNA sequence. We found that four of the five nonexpressing independent virus isolates contained the portion of the TNF gene amplified by our PCR primers (nucleotides 157–432 of the mTNF cDNA sequence) in the four plaques examined for each isolate. These results show that the TNF gene was present initially and suggest that there may be selective pressure favoring loss of TNF expression during chronic carriage of the virus. However, it is not clear that the mechanism of disease and death is dependent on such mutations.

DISCUSSION

TNF can be directly cytotoxic to virally infected cells (25). It has been hypothesized that this effect accounts for the TNF-induced attenuation of VV replication *in vivo* (21) and

Table 1. Loss of expression of TNF gene by VV isolated from SCID mice after 40 days of infection

Virus	VV input, pfu × 10^{-6}	Day p.i.	Organ/ animal no.	TNF (ELISA)	TNF (PCR)	Clinical illness
+	8.4	1	Lung/1	+	+	−
−	8.4	5	Lung/2	+	+	+
−	8.4	1	Lung/3	+	+	−
−	8.4	5	Lung/4	+	+	−
+	8.4	40	Lung/5	−	+	+
			Liver/5	−	ND	
			Brain/5	(low)	+	
+	8.4	40	Lung/6	+	+	+
			Brain/6	+	ND	
+	0.84	40	Lung/7	+	+	+
			Kidney/7	−	+	
			Brain/7	+	ND	
−	8.4	40	Lung/8	−	−	−
−	0.84	40	Lung/9	−	+	−
−	8.4	233	Ear/10	+	ND	+
			Kidney/10	+	ND	
			Brain/10	+	ND	
			Spleen/10	−	ND	
			Lung/10	+	ND	

All animals were infected intranasally with the indicated amount (8.4 or 0.84 × 10^6 pfu) of VV 14.7(+)/TNF, designated (+), or VV 14.7(−)/TNF, designated (−). Animals were sacrificed on the indicated postinfection day (day p.i.), and organs were removed for virus titration. TNF expression was determined by ELISA (22), and the TNF coding sequence was detected by PCR as described in text. ND, not determined.

that interference with premature cytolysis is the mechanism whereby the Ad 14.7K reverses the TNF-mediated attenuation (22). However, TNF is a pleiotropic inducer of a wide array of genes, including many with roles in inflammatory or immune responses, and as such has the potential for numerous indirect antiviral effects on VV pathogenesis. For example, TNF increases adhesion molecule expression on the vasculature as well as class I and II MHC expression, potentially increasing cellular recruitment to the site of infection, promoting more efficient recognition and lysis by $CD8^+$ CTL and enhancing antigen processing and presentation to $CD4^+$ helper T cells. If the above schemes for the indirect action of TNF were of primary importance, then TNF should be less attenuating (or nonattenuating) in SCID mice, which lack mature functional T or B cells. However, we still observed an attenuating effect of TNF in SCID mice, similar to that reported in nude or sublethally irradiated mice (21), lending further support to the direct antiviral effects of TNF as the mechanism of attenuation in our murine pneumonia model. Similarly, the SCID infections demonstrate that Ad 14.7K does not require the presence or cooperation of T or B lymphocytes or their secreted products to reverse the TNF-mediated attenuation. These data lend support to the direct interference of Ad 14.7K with the premature, TNF-mediated lysis of infected cells as the *in vivo* mechanism of action. For infection of SCID mice, virus-directed TNF expression is required to see the effect of the Ad 14.7K on VV pathogenicity. This finding is in agreement with the results of BALB/c infections (22). The importance of TNF as an antiviral agent in infections with wild-type (i.e., non-TNF-expressing) VV is unclear. Flow cytometric analysis has shown that TNF-producing T lymphocytes are present in the spleen following infection with VV (26). However, the effect of this endogenously produced TNF on VV growth may be small compared with the effect of much higher levels of TNF produced by the VV vector.

Our results do not rule out the cooperation of TNF with some other component of the immune response as important

for its antiviral activities. SCID mice have functional natural killer (NK) cells, which can be stimulated by TNF to release γ interferon (IFN-γ) (27–29); 14.7K would then be postulated to block the cytolytic effects of this antiviral cytokine combination (11). The basis for the relatively milder acute disease in SCID mice as compared with BALB/c mice infected with the TNF-expressing VV is unclear especially because both exhibit marked pulmonary inflammation within the first week after infection (data not shown). It may be that the innate immune response of the SCID mouse is actually better able to control the acute VV infection than is the BALB/c mouse. The SCID spleen is enriched for IFN-γ-producing NK cells, and *in vitro*, SCID-derived splenic cells exhibit enhanced IFN-γ secretion in response to a Listeria infection as compared with an immunocompetent C.B-17 control (29).

In the SCID mice described in this report, the VV-producing TNF alone was attenuated but appeared not to be completely eliminated. This observation in SCID mice differs to some extent from the results reported by others regarding infection of other strains of immunodeficient mice with cytokine-encoding VV. Athymic Swiss outbred nude mice (9 weeks old) infected intravenously with 5×10^6 pfu of VV expressing TNF remained as healthy as uninfected mice for >40 days (21). However, our experiments utilized intranasal infections, a route that is more lethal than intravenous infections in BALB/c mice, and we infected younger mice, which may exhibit greater susceptibility to the lethal effects of the virus.

Our results from infections of SCID mice with the TNF-expressing VV may be analogous to findings obtained upon implantation of cytokine-secreting [TNF, interleukins 2 or 4 (IL-2 or IL-4), or γ interferon] tumor cells into immunodeficient mice (24). In the absence of functional T cells (nude or SCID mice), the cytokine-expressing cells gave rise to tumors with approximately a 3-week delay in comparison with the, the nonexpressing parental cell line. Interestingly, all of the originally TNF-, IL-2-, or IL-4-secreting cells that exhibited this delayed tumor outgrowth were found to have absent or dramatically reduced cytokine production, presumably due to preferential destruction of the cytokine-expressing cells and outgrowth of nonproducing variants.

Although the Ad 14.7K gene clearly accelerates the late-appearing VV disease and seems to do this by antagonizing TNF, the mechanisms and target organs to explain the mortality are unclear.

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