

Interferon γ Is Involved in the Recovery of Athymic Nude Mice from Recombinant Vaccinia Virus/Interleukin 2 Infection

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Summary

Athymic nude mice recover from an infection with recombinant vaccinia virus (VV) encoding murine interleukin 2 (IL-2), but treatment with a mAb to IL-2 accentuated infection. Administration of a mAb against interferon γ (IFN- γ) to mice infected with the IL-2-encoding virus completely prevented the IL-2-induced mechanisms of recovery. Both asialo-GM₁⁺ (NK) and asialo-GM₁⁻ (non-NK) cells were participants in the IFN- γ -mediated recovery of nude mice from infection with the IL-2-encoding VV recombinant. Depletion of asialo-GM₁⁺ cells exacerbated infection, though not as much as anti-IFN- γ mAb. In vitro, both asialo-GM₁⁺ and asialo-GM₁⁻ nude mouse splenocytes produced IFN- γ in response to IL-2.

Antiviral cytotoxic T (Tc)¹ cells play a crucial role in recovery from primary virus infections (1-5), including vaccinia virus (VV) (6). T cell-deficient nude mice are therefore unable to resolve a VV infection and usually die of a disseminated vaccinal disease. However, nude mice can successfully overcome infection with recombinant VV that encode murine or human IL-2 (7, 8). Both nude and normal mice infected with the recombinant VV-encoding murine IL-2, VV-HA-IL2, have elevated NK cell responses coinciding with the rapid clearance of this virus (9). In addition, our studies (9) using beige mice also suggested that both NK cell-dependent and -independent mechanisms may be involved in the rapid clearance of VV-HA-IL2 compared with a control virus, VV-HA-TK. Virus-encoded IL-2 could have induced a cascade of immunological events, including NK cell activation, which contributed to rapid clearance of VV-HA-IL2 and recovery of nude mice. In this report, we present evidence that the recovery of nude mice from infection with VV-HA-IL2 is mediated through the release of IFN- γ by both asialo-GM₁⁺ (asGM₁⁺) (NK) and as-GM₁⁻ (non-NK) cells. The antiviral activity of the IL-2-triggered IFN- γ was abolished by treatment with a mAb to IFN- γ , which prevented clearance of VV-HA-IL2 and resulted in 100% mortality in an otherwise sublethal infection.

Materials and Methods

Mice. 6-9-wk-old female athymic Swiss outbred nude mice, raised under specific pathogen-free conditions, were obtained from

¹ Abbreviations used in this paper: MTD, mean time to death; Tc, cytotoxic T cells; TK, thymidine kinase; VV, vaccinia virus.

the Animal Breeding Establishment of The John Curtin School of Medical Research (JCSMR).

Viruses. Construction of VV recombinants from the wild-type virus, VV-WR, has been described in detail elsewhere (7). Briefly, the IL-2-encoding recombinant VV-HA-IL2 was constructed by the insertion of HSV thymidine kinase (TK) gene and a chimeric promoter-IL-2 fragment into the HindIII F region of a VV recombinant, VV-HA-PR8 (10), which had been engineered to express the influenza A/PR/8/34 hemagglutinin (HA). The control virus VV-HA-TK encoding HSV TK and A/PR/8/34 HA but not IL-2 was similarly constructed. Murine IL-2 was detectable in culture supernatants of murine L929 or human 143B cells within 4-6 h of infection with VV-HA-IL-2. Virus stocks, prepared from infected CV-1 cells, were diluted in 2 ml of gelatin saline to contain the desired PFU/ml and dispersed by sonication twice for 5 s at 50 W before use.

Cell Lines. YAC-1 (H-2^a), a line derived from Moloney leukemia virus-induced lymphoma in A/Sn mouse (11), CV-1, a cell line derived from African green monkey kidney (12) and 143B, a human osteosarcoma cell line (13) were maintained in DMEM (Gibco Laboratories, Grand Island, NY) supplemented with antibiotics and 5% heat-inactivated FCS (Flow Laboratories, North Ryde, Australia). YAC-1 cells were used as targets in NK cytotoxicity assays, CV-1 cells were used for the preparation of virus stocks, and 143B cells were used for virus titrations as described elsewhere (9).

Cytotoxicity Assays. The standard ⁵¹Cr-release assays were carried out in triplicate for each E/T ratio as described in detail elsewhere (9).

Antisera/Monoclonal Antibody. Rabbit anti-asGM₁, a polyclonal antibody (Wako Pure Chemicals Industries, Osaka, Japan) was used to deplete as-GM₁⁺ (NK) cells in vivo. Anti-as-GM₁ was administered intravenously, followed by the intranasal route after light anesthesia with ether. The procedure involving antibody ad-

ministration via two routes is based on the findings of Stein-Streilein and Guffee (14), where treatment with anti-as-GM₁ by the two routes was superior to the intravenous route alone and was particularly essential for the depletion of NK cells in the lung. Treatment was continued every day after the day of infection, since preliminary depletion experiments showed the re-emergence of as-GM₁⁺ cells (assessed by flow cytometry and lysis of YAC-1 targets) in spleens of mice infected with VV-HA-IL2. Anti-as-GM₁ antiserum treatment does not deplete as-GM₁⁻ NK cell precursors and IL-2 is known to give rise to as-GM₁⁺ cells from precursors that lack the marker (15).

Purified rat anti-mouse IFN- γ , a neutralizing mAb with specific activity of 13,000 U/ml (5,700 U/mg) was obtained from Lee Biomolecular Research Inc., San Diego, CA. A rat anti-mouse hybridoma line, S4B6.31, which is a subclone of the originally described clone, S4B6 (16), was obtained from DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, CA). The clone secretes mAb (IgG₂ subclass) to murine IL-2. The mAb was obtained as ascites, grown in pristane-primed outbred nude mice, and used after partial purification by ammonium sulphate precipitation. The precipitate was dissolved in PBS and dialyzed three times against PBS at 4°C, filter sterilized, and stored at -20°C.

The following mAbs and antiserum were used at the dilutions indicated for in vitro cell depletion with C: anti-Thy-1.2, clone F7D5 (Serotec Ltd., Blackthorn Bicester, England) at a dilution of 1/1,000; anti-CD4, clone RL174 (17), and anti-CD8, clone 31M (18), kindly provided by Dr. R. Ceredig (John Curtin School of Medical Research) at dilutions of 1/5; and anti-as-GM₁ at a dilution of 1:50 (1:5 for some experiments). Low toxicity rabbit C (Cedarlane Laboratories, Ltd., Canada) was used at a final dilution of 1:10.

Assay for IL-2. The presence of IL-2 in supernatants of VV-HA-IL2-infected cells, sera, or peritoneal fluid of mice was assessed by CTL2 cell proliferation and uptake of [³H]TdR as described elsewhere (19).

Administration of Murine rIL2 and rIFN- γ to Nude Mice Infected with VV-HA-TK. Affinity column purified murine rIL-2, with a specific activity of 64,000 U/ml, was kindly provided by Dr. P. Hodgkin, DNAX Research Institute of Molecular and Cellular Biology. Murine IFN- γ (300,000 IU/ml) was a kind gift of

Boehringer Ingelheim, Vienna, Austria. Nude mice that had been infected 1 h earlier with VV-HA-TK were given 600 U i.p. of murine rIL-2 or rIFN- γ and dosed thereafter every 8 h for a period of 5 d. Uninfected controls were treated similarly to assess the toxicity of the cytokines.

Assay IFN- γ in Supernatants of Nude Mouse Splenocyte Cultures Stimulated with IL-2. Splenocytes from outbred nude mice were cultured in the presence of rIL-2 to determine whether as-GM₁⁺ and as-GM₁⁻ cells produced IFN- γ . Whole splenocytes (5×10^6 cells treated with C only) were cultured in 1 ml of complete DMEM containing 5×10^{-4} M 2-ME and 100 U rIL-2 for 3d at 37°C in a humidified atmosphere containing 5% CO₂ in air. Similarly, splenocytes depleted of as-GM₁⁺ cells (5×10^6 cells treated with anti-as-GM₁ + C) were cultured under the same conditions. Controls were not exposed to IL-2. Presence of IFN- γ in the culture supernatants was determined using a commercial sandwich ELISA (Holland Biotechnology bv, Leiden, Netherlands). The ELISA was performed according to the manufacturers' instructions. IFN- γ activity (IU/ml) was calculated from optical density values using a standard curve.

Results

VV-HA-IL2 Is Cleared Rapidly from Organs of Nude Mice. The kinetics of virus replication in organs of VV-HA-TK- or VV-HA-IL2-infected mice is shown in Fig. 1. Both the viruses successfully seeded the ovary and lung, but whereas VV-HA-TK titers rose sharply 3 d after infection, VV-HA-IL2 was rapidly cleared and virus could no longer be recovered beyond 6 d after infection. VV-HA-TK persisted for at least 13 d after infection in these organs, by which time mice were beginning to die. The replication of these recombinant viruses in spleens and brains of nude mice showed a somewhat different pattern. VV-HA-IL2 was detected in spleens 12 h after infection but could not otherwise be recovered from spleens or brains. VV-HA-TK persisted in spleens over the entire course of infection and was recovered from brains after

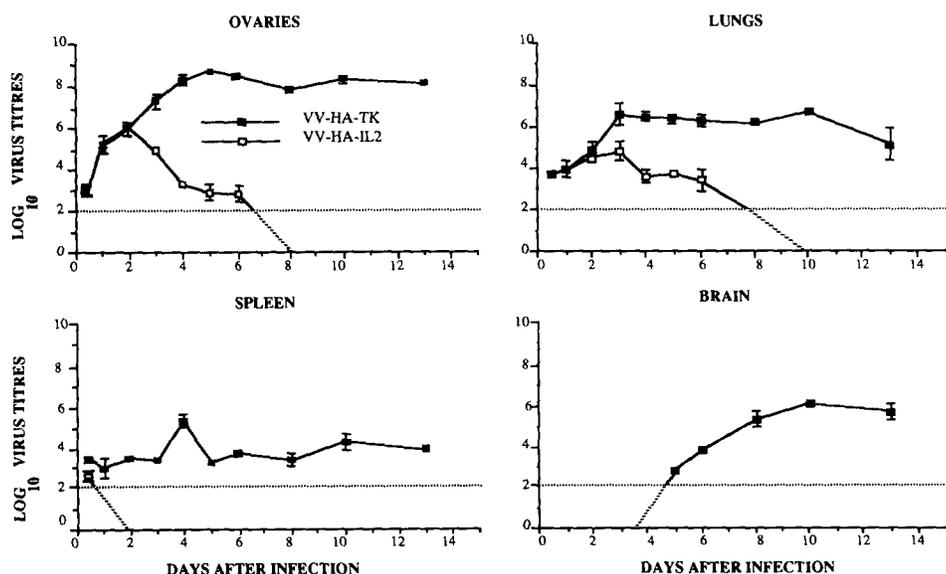


Figure 1. Kinetics of recombinant vaccinia virus replication in organs of outbred nude mice. Female nude mice between the ages of 6 and 9 wk were infected intravenously with 10^7 PFU of VV-HA-IL2 (□) or VV-HA-TK (■) and on the days indicated organs were removed to determine virus titers. Data shown are the geometric means of three individual organs per day for each group \pm SEM. The sensitivity of virus detection in the assay system used is >100 PFU, indicated by the horizontal dotted line.

Table 1. *The Effect of Anti-IL-2 mAb Treatment on the Survival of Nude Mice Infected with VV-HA-IL2 or VV-HA-TK*

No. of mice	Virus	Anti-IL-2 mAb treatment	Percent survival	MTD* (death on days p.i.)†
7	VV-HA-IL2	-	100	-
7	VV-HA-IL2	+	0	6.9 (5, 5, 6, 7, 8, 8, 9)
7	VV-HA-TK	-	0	7.6 (6, 7, 7, 7, 8, 8, 10)
7	VV-HA-TK	+	0	7.3 (6, 6, 7, 7, 7, 9, 9)

4-5-wk-old outbred nude mice were injected i.v. with 10^7 PFU VV-HA-IL2 or VV-HA-TK. On days 0 (day of infection), 1, 2, 3, and 4, mice were given $\sim 500 \mu\text{g}$ i.p. of anti-IL-2 mAb (+) and controls were left untreated (-). Mortality was monitored in all groups. Nude mice treated with anti-IL-2 mAb only all survived (data not shown).

* Mean time to death.

† Numbers in parentheses indicate the days on which individual mice died.

the 4th day of infection and persisted at close to the peak level attained after the 10th day of infection.

The kinetics of VV-HA-IL2 clearance suggested that the mechanisms induced by virus-encoded IL-2 operated as early as the 3rd day of infection, and as reported before (9), coincided with an elevated splenic NK cell response (data not shown).

The Effect of Anti-IL-2 mAb Treatment on Viral Titers and Survival of Nude Mice. In order to establish that IL-2 was indeed produced in vivo during an infection with VV-HA-IL2 and that the highly attenuated phenotype of this virus was due to the antiviral mechanism(s) induced by virus-encoded IL-2, groups of outbred nude mice infected intravenously with 10^7 PFU of VV-HA-IL2 or VV-HA-TK were simultaneously treated with a mAb to murine IL-2. Treatment with the mAb to IL-2 should reduce the activity of IL-2 produced in vivo during infection with VV-HA-IL2 and increase the severity of infection. As expected, all seven mice infected with VV-HA-IL2 and treated with the mAb to IL-2 died, whereas mice given virus alone survived (Table 1). Antibody-treated nude mice infected with VV-HA-TK all died with a mean time to death (MTD) of 7.3 d, which was not significantly different ($p > 0.05$) from those given VV-HA-TK only.

5 d after infection, mean titers of VV-HA-IL2 in ovaries and lungs of mice treated with the mAb were significantly higher ($p < 0.001$) than untreated controls (Table 2). Only marginal increases were apparent in VV-HA-TK titers in ovaries and lungs of mice treated with the antibody compared with untreated controls.

NK Cell Depletion In Vivo Results in Enhanced VV-HA-IL2 Titers. We investigated the possible causal relationship between IL-2-activated NK cells and their antiviral effects in vivo. Outbred nude mice were depleted of as-GM₁⁺ cells by repeated injections with a polyclonal antibody to as-GM₁. The antibody treatment resulted in the elimination of >98% of splenic as-GM₁⁺ cells, as assessed by flow cytometry, and completely abolished the cytolytic activity on YAC-1 targets (data not shown).

Virtually no cytolytic activity on YAC-1 or L939 targets could be demonstrated in spleens of recombinant virus-infected mice that had been treated with anti-as-GM₁ (data not shown).

Significant increases in the mean VV-HA-IL2 titers in lungs and ovaries of NK cell-depleted nude mice were demonstrable 3 and 5 d after infection (Table 3). NK cell depletion in nude mice infected with VV-HA-TK resulted only in marginal and insignificant increases in the mean viral titers in these organs compared with control, untreated nude mice. Despite a satisfactory depletion of NK cells, ovarian VV-HA-IL2 titers were never as high as levels of VV-HA-TK. This may have been a reflection of our earlier finding (Karupiah, G., and R. V. Blanden, manuscript submitted for publication) that anti-as-GM₁ partially inhibited VV infection of murine ovaries.

Separate groups of nude mice that were treated with anti-as-GM₁ and infected with either recombinant virus, were

Table 2. *The Effect of Anti-IL-2 mAb Treatment on VV-HA-IL2 and VV-HA-TK Titers in Nude Mice*

Virus infection and treatment*	Log ₁₀ virus titers \pm SEM†	
	Ovaries	Lungs
VV-HA-TK	8.3 \pm 0.1	6.3 \pm 0.1
VV-HA-TK + anti-IL-2 mAb	8.8 \pm 0.2	6.7 \pm 0.3
VV-HA-IL2	3.0 \pm 0.1	3.3 \pm 0.1
VV-HA-IL2 + anti-IL-2 mAb	7.8 \pm 0.4§	5.6 \pm 0.2§

* 4-5-wk-old mice were infected with virus and treated with anti-IL-2 mAb as described for Table 1. On day 5 after infection, mice were killed and virus titers in lungs and ovaries were determined.

† Data shown are the geometric means of four individual organs per group \pm SEM.

§ Increase in titer highly significant ($p < 0.001$; Student's *t*-test) compared with untreated group infected with VV-HA-IL2 only.

Table 3. The Effect of as-GM₁ Antiserum Treatment in Nude Mice on Recombinant VV Replication

Exp.	Days after infection	Virus and treatment*	Log ₁₀ virus titers ± SEM [†]	
			Ovaries	Lungs
1	3	VV-HA-TK + PBS	7.5 ± 0.3	6.3 ± 0.2
		VV-HA-TK + anti-as-GM ₁	7.8 ± 0.1	6.7 ± 0.4
		VV-HA-IL2 + PBS	5.0 ± 0.2	5.2 ± 0.1
		VV-HA-IL2 + anti-as-GM ₁	5.8 ± 0.1	6.4 ± 0.4
2	5	VV-HA-TK + NRS	8.3 ± 0.3	6.2 ± 0.2
		VV-HA-TK + anti-as-GM ₁	8.6 ± 0.1	6.5 ± 0.1
		VV-HA-IL2 + NRS	4.3 ± 0.1	4.2 ± 0.1
		VV-HA-IL2 + anti-as-GM ₁	5.3 ± 0.2	5.8 ± 0.1

* 6–9-wk-old female outbred nude mice were given antiserum to as-GM₁ (stock diluted to 1/5 in PBS) in a 150 µl volume i.v. and 50 µl i.n. on days –5, –3, –1, 0, 1, and 2 for Exp. 1 and similarly for Exp. 2 with additional treatments on days 3 and 4. Control mice were given only PBS (Exp. 1) or normal rabbit serum (NRS) (Exp. 2). On day 0, mice were injected i.v. with 10⁷ PFU virus, and on the days indicated for each experiment, organs were removed for determination of viral titers.

† Data shown are the geometric means of titers of organs from four individual mice ± the SEM.

monitored for signs of morbidity and mortality. All mice that were infected with VV-HA-TK died within 14 d after infection, and NK cell depletion did not accelerate the MTD (data not shown). Despite the enhanced virus titers (Table 3), no mortality was recorded in NK cell-depleted nude mice that had been infected with VV-HA-IL2. These results indicate that as-GM₁⁺ NK cells contribute to control of VV-HA-IL2 infection in nude mice, but that other mechanisms may be involved.

Enhanced VV-HA-IL2 Replication In Vivo after Treatment with IFN-γ Antibody. The IL-2-induced antiviral mechanism(s) could have been mediated in vivo either through direct cytolysis of virus-infected cells and/or through secretion of antiviral factors. There was a possibility that virus-encoded IL-2, apart from augmenting NK cytolytic activity, also triggered the secretion of antiviral factors such as IFN-γ and TNF-α by as-GM₁⁺ (NK) and as-GM₁[–] (non-NK) cells. To determine the role of host-derived IFN-γ in recovery from VV-HA-IL2 infection, nude mice were treated with a neutralizing

Table 4. The Effect of Treatment with mAb to IFN-γ in Nude Mice on the Replication of VV-HA-TK or VV-HA-IL2

Exp.	Days after infection	Virus infection and treatment	Log ₁₀ virus titers ± SEM*	
			Ovaries	Lungs
1	3	VV-HA-TK	7.5 ± 0.2	6.3 ± 0.2
		VV-HA-TK + IFN-γ mAb	8.3 ± 0.3	7.2 ± 0.2
		VV-HA-IL2	5.0 ± 0.2	5.1 ± 0.3
		VV-HA-IL2 + IFN-γ mAb	7.5 ± 0.3	6.9 ± 0.3
2	5	VV-HA-TK	8.6 ± 0.2	6.5 ± 0.2
		VV-HA-TK + IFN-γ mAb	9.0 ± 0.1	6.9 ± 0.2
		VV-HA-IL2	4.0 ± 0.1	4.2 ± 0.1
		VV-HA-IL2 + IFN-γ mAb	8.8 ± 0.1	6.5 ± 0.2

6-wk-old female nude mice were given 500 µl of mAb to IFN-γ i.p. on days –1, 0, 1, and 2 for Exp. 1 and an additional day (day 3) for Exp. 2. Mice were infected i.v. with 10⁷ PFU virus on day 0, and 3 (Exp. 1) or 5 (Exp. 2) d later, mice were killed and virus titers in organs were determined. 1 U of mAb neutralizes 10 U of murine IFN-γ in vitro.

* Data shown are the geometric means of log₁₀ virus titers ± SEM of four individual lungs or pairs of ovaries.

mAb to IFN-γ and virus titers were determined in organs, 3 and 5 d after infection.

Titers of VV-HA-TK in ovaries and lungs of mice treated with mAb to IFN-γ increased by 0.8–0.9 log₁₀ PFU over untreated controls 3 d after infection (Table 4). In marked contrast, mAb treatment in VV-HA-IL2-infected mice resulted in increases of 2.5 log₁₀ PFU in ovaries and 1.8 log₁₀ PFU in lungs. By day 5 after infection, VV-HA-TK titers in mAb-treated mice were only 0.4 log₁₀ PFU above control levels in these organs, but VV-HA-IL2 titers in ovaries and lungs of mAb-treated mice increased by 4.8 and 2.3 log₁₀ PFU, respectively, above untreated controls. These results indicate that IFN-γ plays a crucial role in clearance of VV-HA-IL2 in both ovaries and lungs of nude mice.

mAb to IFN-γ Inhibits the Ability of Nude Mice to Survive Infection with VV-HA-IL2. The above data suggested that IFN-γ, triggered by virus-encoded IL-2, played an important role in virus clearance. The effect of anti-IFN-γ mAb treatment on survival of nude mice infected with VV-HA-IL2 was next determined. None of the nude mice given the mAb against IFN-γ survived infection with VV-HA-IL2, which was in sharp contrast to the group of mice given VV-HA-IL2 only in which all the animals survived (Table 5). Moreover, the concentration of mAb administered affected the MTD. For instance, nude mice infected with VV-HA-

Table 5. Mortality in Nude Mice Treated with Anti-IFN- γ mAb and Infected with VV-HA-TK or VV-HA-IL2

Virus and/or mAb treatment	No. of mice	Percent mortality	MTD* (death on days p.i.)
mAb only	3	nil	—
VV-HA-TK	5	100	15.4 (12, 14, 15, 18, 18)
VV-HA-TK + mAb	5	100	14.6 (13, 13, 14, 16, 17)
VV-HA-IL2	5	nil	—
VV-HA-IL2 + mAb	5	100	9.6 (5, 6, 7, 13, 17)

10-wk-old female nude mice were given 500 U i.p. of mAb to IFN- γ on days -1, 0, 1, 2, and 3. Mice were infected i.v. with 10^7 PFU of virus on day 0. Control mice were left uninfected.

* Mean time to death.

IL2 and treated with 100 U of mAb for four successive days had a MTD of 11.0 d whereas a similar treatment protocol with 500 U of the mAb resulted in an MTD of 6.0 d (Table 6). mAb-treated mice that died between 5 and 8 d after infection with VV-HA-IL2 had no obvious signs of a vaccinal disease but appeared to suffer from the toxic effects of IL-2, manifested in the form of vascular leak syndrome (erythroderma, ascites, and massive accumulation of mononuclear cells in the peritoneal cavity). High levels of IL-2 could be detected in sera and ascites of VV-HA-IL2-infected mAb-treated mice but not in mice given virus alone (data not shown). However, no significant levels of TNF- α could be detected in sera and ascites of these mice.

Treatment with Anti-IFN- γ mAb in VV-HA-IL2-infected Mice Results in Enhanced NK Cell Activity. We investigated whether treatment with the mAb to IFN- γ in VV-HA-IL2-infected mice prevented the enhanced NK cell response previously observed (9). The splenic NK activity in mice infected with VV-HA-IL2 and treated with the mAb was more than three-fold higher than activity induced by infection with VV-HA-IL2 only (Fig. 2). Using a panel of antiserum and mAb and C, the splenocytes from VV-HA-TK-infected mice mediating lysis of YAC-1 targets were phenotyped as as-GM $_1^+$, Thy-1.2 $^+$, CD4 $^-$, and CD8 $^-$. Similarly, lysis of YAC-1 targets by splenocytes from VV-HA-IL2-infected mice was mediated mainly by as-GM $_1^+$, Thy-1.2 $^+$, CD4 $^-$, and CD8 $^-$ effectors, although some lysis was mediated by effectors that lacked these markers. On the other hand, there were at least two distinct populations of splenocyte effectors from VV-HA-IL2-infected mice that had been treated with the mAb to IFN- γ . One was as-GM $_1^+$, Thy-1.2 $^+$, CD4 $^-$, and CD8 $^-$ and the other was as-GM $_1^-$, Thy-1.2 $^+$, CD4 $^-$, and CD8 $^-$. The phenotype of peritoneal cells obtained from the mAb-treated and VV-HA-IL2-infected mice and that lysed YAC-1 targets had a similar phenotype to splenocytes. Both splenocyte and peritoneal effectors obtained from these mice were

Table 6. The Effect of Increasing IFN- γ mAb Concentration on Mortality of Nude Mice Infected with VV-HA-IL2

No. of mice	mAb concentration	Infected/uninfected	MTD (days p.i. of death)
4	100 U	VV-HA-IL2	11.0 (9, 11, 11, 13)
2	100 U	—	—
4	200 U	VV-HA-IL2	9.8 (7, 9, 10, 13)
2	200 U	—	—
4	300 U	VV-HA-IL2	8.5 (7, 8, 9, 10)
2	300 U	—	—
4	400 U	VV-HA-IL2	7.8 (6, 7, 8, 10)
2	400 U	—	—
4	500 U	VV-HA-IL2	6.0 (5, 5, 6, 8)
2	500 U	—	—

6-wk-old female nude mice were given i.p. varying concentrations of mAb to IFN- γ on days -1, 0, 1, 2, and 3. Mice were infected i.v. on day 0 with 10^7 PFU virus and controls were left uninfected.

quite resistant to treatment with anti-as-GM $_1$ + C, despite the use of 10-fold higher concentration of the antiserum.

Effect of Administration of rIL-2 or rIFN- γ on Mortality and Survival Time of Nude Mice Infected with VV-HA-TK. The above data clearly established that IFN- γ is involved in the rapid clearance of VV-HA-IL2 and recovery of nude mice. It was therefore of interest to determine whether the consequences of local IL-2 production in foci of VV-HA-IL2 infection and subsequent IFN- γ secretion and viral clearance could be mimicked by the administration of exogenous rIL-2 or rIFN- γ during infection with VV-HA-TK.

Groups of three to five outbred nude mice infected intravenously with 10^7 PFU VV-HA-TK were given 600 U of rIL-2 or rIFN- γ intraperitoneally every 8 h for a period of 5 d. No morbidity, mortality or signs of toxicity were recorded in control groups of mice given only rIL-2 or rIFN- γ (Table 7). All mice given VV-HA-IL2 alone survived with no overt disease. Nude mice infected with VV-HA-TK alone showed signs of disease by 6 d after infection and all mice died with an MTD of 12.2 d (Table 7). Treatment with exogenous rIL-2 or rIFN- γ delayed the onset of disease signs that appeared between 11–16 d after infection, and significantly ($p < 0.001$) prolonged survival of nude mice. Nevertheless, all mice that had been infected with VV-HA-TK and treated with either rIL-2 or rIFN- γ succumbed to disseminated disease and died with MTD of 23.4 and 25.8 d, respectively.

These results suggested that systemic IL-2 or IFN- γ inhibited VV-HA-TK replication during the 5-d course of treatment, but that virus persisted and then grew to lethal levels after treatment was withdrawn.

Both as-GM $_1^+$ and as-GM $_1^-$ Nude Mouse Splenocytes Produce IFN- γ in Response to Stimulation with IL-2 In Vitro. To determine whether as-GM $_1^+$ and especially as-GM $_1^-$ lym-

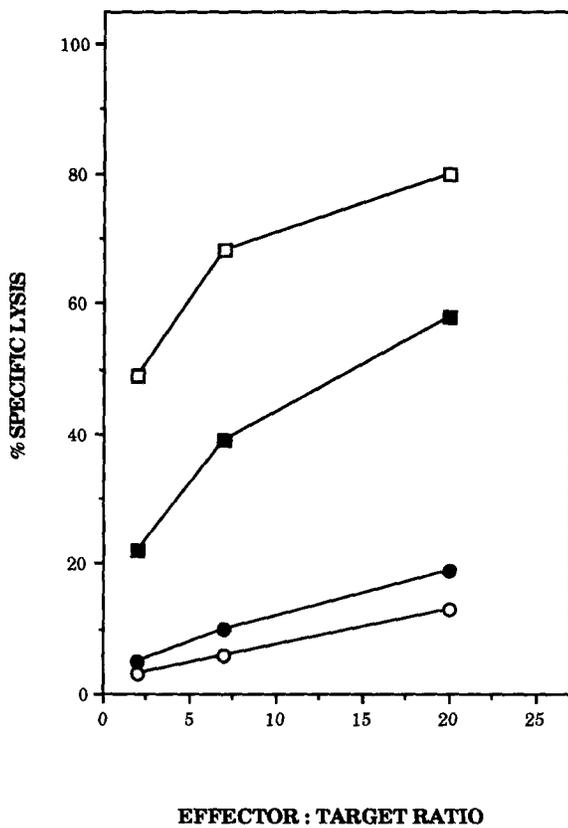


Figure 2. The effect of IFN- γ mAb treatment on splenic NK cell activity. 6–8-wk-old female nude mice were given 500 U i.p. of anti-IFN- γ mAb on days –1, 0, 1, 2, and 3. Mice were infected intraperitoneally with 10^7 PFU virus on day 0, and 5 d later the splenic cytolytic activity (percent specific lysis) on ^{51}Cr -labeled YAC-1 targets was determined using pooled splenocytes from VV-HA-TK-infected (●), VV-HA-TK-infected mAb-treated (○), VV-HA-IL2-infected (■), or VV-HA-IL2-infected mAb-treated (□) mice. The assay was carried out in triplicate for each E/T ratio and SEM were <5%.

phoid cells from nude mice produced IFN- γ after stimulation with IL-2, splenocytes, either whole or depleted of as-GM $_1^+$ cells, were cultured in media containing 100 U/ml of rIL-2 for 3 d, after which the presence of IFN- γ in cultures was assayed using an ELISA. More than 40 U/ml of IFN- γ was detected in whole splenocyte culture supernatants, whereas between 26 and 30 U/ml of the cytokine was detected in supernatants of cultures depleted of as-GM $_1^+$ cells. IFN- γ was not detected in supernatants of control cultures that had been maintained in the absence of IL-2.

Discussion

Athymic, nude mice lack mature T cells and are therefore unable to mount T-dependent immune responses. They usually succumb to VV because of a deficiency in functional CD8 $^+$ Tc cells which are essential for recovery from primary poxvirus infections (1, 2, 7, 8). T cells are the principal producers of IL-2, which is pivotal for the generation and regulation of an immune response (20). Apart from its direct stimulatory effects on lymphoid (21, 22) and myeloid (23, 24) cells, IL-2 also regulates the production of other cytokines like IFN- γ (25–28). The nude mouse model provided an opportunity to study the function of VV-encoded IL-2 in vivo by analysis of the immune mechanisms induced by IL-2 that allowed recovery from VV infection.

The highly “attenuated” phenotype of VV-HA-IL2 in mice was apparently due to antiviral mechanisms induced by virus-encoded IL-2, because experiments in vivo using mAbs to IL-2 to IFN- γ clearly established that in these circumstances VV-HA-IL2 could replicate to high titers in mouse tissues, sometimes as high as the control VV-HA-TK. Therefore, the reduced virulence of VV-HA-IL2 did not result simply from the insertion of a second foreign gene into the VV genome. Furthermore, the consequences of local IL-2 production in foci of VV-HA-IL2 infection and subsequent activation of antiviral mechanisms could not be mimicked by the administration of exogenous rIL-2 or rIFN- γ at 8-h intervals over the first 5 d of infection with VV-HA-TK. All of these mice

Table 7. The Effect of Exogenous rIL-2 or rIFN- γ Administration on Survival of Nude Mice Infected with VV-HA-TK

No. of mice	Virus*	Treatment	Percent survival	MTD (death on days p.i.)
3	nil	rIL-2	100	—
3	nil	rIFN- γ	100	—
5	VV-HA-IL2	—	100	—
5	VV-HA-TK	—	0	12.2 (9, 11, 12, 14, 15)
5	VV-HA-TK	rIL-2	0	23.4 (19, 21, 24, 25, 28)†
5	VV-HA-TK	rIFN- γ	0	25.8 (18, 23, 27, 28, 33)†

* 8-wk-old male nude mice were given 10^7 PFU virus i.v. 1 h after infection, mice were given 600 U i.p. of rIL-2 or rIFN- γ and thereafter every 8 h for a period of 5 d. Controls were given PBS only (—).

† Significant, $p < 0.001$, Student's t -test, compared with group given VV-HA-TK only.

ultimately died, although death was delayed beyond the time of death of untreated VV-HA-TK-infected controls.

The results presented here demonstrate that in the complete absence of classical CD8⁺ Tc cells, as-GM₁⁺ (NK) and as-GM₁⁻ cells (non-NK) can contribute to recovery from infection with recombinant VV encoding murine IL-2. Depletion of as-GM₁⁺ cells *in vivo* exacerbated infection, though not as much as anti-IFN- γ mAb, thus indicating that NK cells activated by virus-encoded IL-2 contributed to antiviral activity and also suggesting the involvement of non-NK cells. In the absence of IL-2 producing functional T cells, virus-encoded IL-2 would be necessary for nude mouse lymphoid cell activation and IFN- γ secretion. Although IFN- γ is known to be produced by NK cells after stimulation with IL-2 (27, 28), our data suggest that in nude mice, as-GM₁⁻ cells may also produce this cytokine. This could explain why NK cell-depleted nude mice survived infection with VV-HA-IL2 despite enhanced viral replication. The identity of these as-GM₁⁻ cells is not known, but there is a possibility that they are precursors of NK or T cells. Furthermore, young adult, athymic, nude mice have been shown to possess T cells that express the TCR- γ/δ (29, 30) and that could produce cytokines with antiviral activity, but further investigations are necessary to confirm the role of this class of cells. Additionally, since IL-2 induces the differentiation of as-GM₁⁻ progenitors to as-GM₁⁺ cells (15), it is possible that in nude mice depleted of as-GM₁⁺ cells and infected with VV-HA-IL2, a regeneration of as-GM₁⁺ cells from precursors may explain why such mice survive.

NK cell responses were not inhibited by the mAb to IFN- γ . Splenic NK cell cytolytic activity, measured *in vitro*, was enhanced further in mAb-treated VV-HA-IL2-infected mice. This could have been due to enhanced viral replication and hence higher IL-2 production, resulting in further NK cell activation.

The finding that a proportion of VV-HA-IL2-infected mice that had been treated with the mAb against IFN- γ died from IL-2 toxicity is also consistent with increased IL-2 production resulting from increased titers of VV-HA-IL2. The high levels of IL-2 may have either directly, or through a cascade, induced the secretion of other cytokines that are thought to mediate, in part, the toxic effects of IL-2 (31–33). However, we could not demonstrate the presence of one such cytokine,

TNF- α , in mice that showed signs of IL-2 toxicity and in which IL-2 was detectable in sera and ascitic fluid.

IFN- γ may have contributed in a number of possible ways to the limitation of viral replication and mediation of recovery of nude mice. First, the induction of an antiviral state in uninfected cells surrounding infected foci could be one direct effect of IFN- γ in preventing spread of infection. Second, IFN- γ could have mediated antiviral activity *in vivo* through activation of mononuclear phagocytes. Both monocytes and macrophages have a well-defined antiviral role *in vivo* (2, 34) and anti-IFN- γ mAb can abrogate the antiviral and antitumour activities of macrophages (35). Third, IFN- γ upregulates the expression of β_2 -microglobulin, which may be a ligand for NK cell recognition and lysis of target cells (36). Therefore, treatment with the mAb to IFN- γ may have interfered with target cell recognition and elimination of virally infected cells by NK cells. Finally, IFN- γ could have mediated antiviral function *in vivo* in synergy with other host-derived antiviral factors like TNF- α/β (37) and IFN- α/β (38). However, our preliminary results from experiments using antibodies to TNF- α and IFN- β suggests that both these cytokines may not be crucial for the recovery of nude mice from infection with VV-HA-IL2, assuming that the available antibodies were efficient in blocking the activity of these factors *in vivo*.

The currently held view with regard to the role of Tc cells in the host defense to viral infection is based on the capacity of these effectors to recognize viral peptide in association with the MHC class I molecule (39) on the surface of infected cells followed by cytolysis (2, 40, 41) and local release of IFN- γ (2, 42, 43). The IFN- γ , released in an antigen-specific and H-2-restricted fashion, could inhibit further spread of virus either through antiviral effects on surrounding cells, or by upregulation of class I MHC antigen expression, thus increasing the efficiency of the cytotoxic function of Tc cells (44, 45). However, the mechanisms of recovery described in this report involve "nonspecific" components of the immune system that can clearly be activated to mediate efficiency antiviral functions to compensate for the absence of T cell-mediated immune mechanisms in nude mice. These results further support recent reports (46–48) that IFN- γ is an essential component of antiviral immune mechanisms and that IL-2 is important for the induction and regulation of IFN- γ production *in vivo*, consistent with previous *in vitro* findings (25–28).

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