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Antiviral Research 45 (2000) 33–45

Antiviral Research

www.elsevier.com/locate/antiviral

The role of natural killer cells in protection of mice against death and corneal scarring following ocular HSV-1 infection

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Received 3 September 1999; accepted 3 November 1999

Abstract

C57BL/6 mice depleted of NK (natural killer) cells with anti-asialo-GM1 antibody were more susceptible to lethal HSV-1 ocular challenge (12% survival) than control C57BL/6 mice (100% survival), $CD4^+$ depleted mice (100% survival), $CD8^+$ depleted mice (80% survival), or macrophage depleted mice (85% survival). NK depletion also resulted in significantly higher levels of HSV-1 induced corneal scarring than was seen with any of the other groups. C57BL/6 mice depleted of NK cells with PK136 (anti-NK1.1 antibody which is more specific for NK cells than is anti-asialo-GM1 antibody) were also more susceptible to HSV-1 ocular challenge than T cell or macrophage depleted mice. Vaccination completely protected NK depleted mice against death and corneal scarring. In contrast to C57BL/6 mice, in BALB/c mice, NK depletion had no effect on survival or corneal scarring following ocular HSV-1 challenge. Experiments with IFN-γ knockout mice (IFN-$\gamma^{o/o}$ mice) suggested that IFN-γ played a minor role in protection of naïve mice against death following HSV-1 challenge. However, IFN-γ did not appear to be an important factor in protection against HSV-1 induced eye disease. Thus, protection against HSV-1 induced corneal scarring in naïve mice appeared to be due to a non-INF-γ NK function. Our results therefore suggest that NK cells were very important in protecting naïve C57BL/6 mice but not vaccinated C57BL/6 mice against corneal scarring and death following ocular HSV-1 challenge.

Keywords: HSV-1; NK depleted mice; Corneal scarring

1. Introduction

Protective immunity induced by a host following infection is dependent on the capacity of the host immune system to elicit the appropriate immune response to either resist, control, or eliminate the pathogen. These protective responses are

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0166-3542/00/$ - see front matter
PII: S0166-3542(99)00075-3

due to innate (i.e. macrophages, NK (natural killer) cells) or adaptive (i.e. neutralizing antibody, CTL) immune responses (Nash et al., 1980; Bonina et al., 1984; Lausch et al., 1987; Kohl et al., 1989; Kohl, 1992; Kunder et al., 1993; Ghiasi et al., 1997a,b). Natural killer cells are an important part of innate immunity (Biron, 1997a,b). Sensitivity to HSV-1 infection is increased in individuals deficient in natural killer cells (Biron, et al., 1989). Similarly, mice are more susceptible to MCMV infection in the absence of NK cells (Bancroft, et al., 1981; Bukowski et al., 1983). In vivo depletion of NK cells in C57BL/6 mice using anti NK1.1 mAb or anti-asialo-GM1 antibody also resulted in increased host susceptibility to infection by HCMV and LCMV (Seaman et al., 1987; Shanley, 1990; Welsh et al., 1990). In contrast, NK cells did not appear to be a major factor in protection of naïve BALB/c or C57BL/6 mice against coccidial infection (Smith et al., 1994).

C57BL/6 mice can be depleted of NK cells in vivo by treatment with anti-asialo-GM1 antibody or anti NK1.1 antibody (Glimcher et al., 1977; Habu et al., 1981; Koo and Peppard, 1984; Trinchieri, 1989). Since asialo-GM1 antigen is found on activated macrophages (Wiltrout et al., 1985) and cytotoxic T cells as well as NK cells (Suttles, et al., 1986), anti-NK1.1 antibody (Bendelac, 1995; Bendelac et al., 1997; Yoshimoto et al., 1995) is more specific for NK cells and may therefore be a preferable antibody for NK depletion studies. However, all experiments investigating the effect of NK depletion on corneal disease following ocular HSV-1 infection, have been done in BALB/c (Staats, et al., 1991; Tamesis et al., 1994) and SCID (Bouley, et al., 1996) mice. Unlike C57BL/6 mice, BALB/c mice do not have the NK1.1 antigen on the surface of their NK cells (Seaman et al., 1987). In this study we have therefore used anti-NK1.1 antibody (PK136) and anti-asialo-GM1 antibody to investigate the role of NK cells in protection of naïve C57BL/6 mice against HSV-1 induced corneal scarring and death following ocular challenge.

NK cells may control viral infection in vivo via the secretion of antiviral cytokines such as IFN-γ (Karupiah, et al., 1990), by lysis of virus infected cells (Shellam et al., 1981; Bukowski et al., 1983), or other factors such as TNF-α (Paya et al., 1988). Therefore, the goals of this study included:

1. investigation of relative contribution of NK cells, T cells, and macrophages in protection against HSV-1 induced corneal scarring and death following ocular infection of naïve mice; and
2. investigate whether protective responses induced by NK cells was associated with their cytolytic activities or IFN-γ production.

We report below that:

1. depletion of NK cells by asialo-GM1 or anti-NK1.1 antibodies resulted in increased corneal scarring and death in C57BL/6 mice but not BALB/c mice following ocular HSV-1 challenge;
2. depletion of $CD4^+$ T cells, $CD8^+$ T cells, or macrophages had less impact on corneal scarring and death than depletion of NK cells; and
3. the absence of IFN-γ had less impact on death and corneal scarring than the absence of NK cells.

2. Materials anf methods

2.1. Virus and cells

Triple plaque purified HSV-1 strains were grown in rabbit skin (RS) cell monolayers in minimal essential medium (MEM) containing 5% fetal calf serum. McKrae, a stromal disease causing, neurovirulent, HSV-1 strain was the ocular challenge virus. KOS, which when given peripherally kills no mice and produces no stromal disease, was used as a live virus vaccine.

2.2. Mice

Inbred BALB/cJ, C57BL/6J, and C57BL/6J-IFN-$\gamma^{O/O}$ mice (5–8 weeks old) were used (The Jackson Laboratory Bar Harbor, ME).

2.3. Immunizations

Mice were vaccinated three times IP at 3 week intervals with 2×10^5 PFU of live KOS in tissue

culture medium. Mock-vaccinated mice were similarly inoculated, but with tissue culture medium (MEM) alone.

2.4. Serum neutralizing antibody titers

Serum neutralizing antibody titers were determined by 50% plaque reduction assays as we described previously (Ghiasi et al., 1994) using serum collected 3 weeks after the final vaccination.

2.5. Ocular challeng

Mice were challenged ocularly with 2×10^5 or 2×10^6 PFU of HSV-1 strain McKrae per eye, in tissue culture medium (Ghiasi et al., 1994).

2.6. Corneal scarring

The severity of corneal scarring in surviving mice was scored in a masked fashion using a one to four scale as we described previously (Ghiasi et al., 1995a).

2.7. Depletion of NK cells with anti-asialo GM1

One microgram of rabbit anti-asialo GM1 immunoglobulin (Wako Chemicals, Dallas, TX) was dissolved in 1 ml of PBS and each mouse received single or multiple IP injections of 100 μg of antibody in 100 μl of PBS as follows:

1. one X depletions were done 3 days before ocular challenge (day −3), 3 days after ocular challenge (day +3), or 6 days after ocular challenge (day +6);
2. two X depletions were done 3 days before ocular challenge (day −3) and 3 days after ocular challenge (day +3) or 3 days after ocular challenge (day +3) and 6 days after ocular challenge (day +6); and
3. three X depletions were done 3 days before ocular challenge (day −3), 3 days after ocular challenge (day +3), and 6 days after ocular challenge (day +6).

Control mice were treated with an equal concentration of freeze-dried normal rabbit serum in PBS.

2.8. Depletion of NK cells with anti-NK1.1 antibody

Depletions were done by IV injection of purified PK136 (anti-NK1.1) antibody (National Cell Culture Center, Minneapolis, MN) as described previously (Seaman et al., 1987). Mice were injected with 100 μg of PK136 monoclonal antibody on days −4, −1, +2, and +5, or with 200 μg of PK136 on days −4, −1, +2, +5 and +8 relative to HSV-1 ocular challenge. In selected mice, the efficiency of NK1.1 depletion efficiency was monitored by FACS analysis of total spleen cells harvested 48 h after the second depletion using PE conjugated PK136 antibody (Pharmingen, San Diego, CA) as described previously (Yanez et al., 1996). Treatment with 100 μg of PK136 completely depleted detectable NK1.1. Control mice were similarly injected with PBS.

2.9. Depletion of T cells

Each mouse received IP injections of 100 μg of purified GK1.5 (anti-$CD4^+$) or 2.43 (anti-$CD8^+$) antibodies (National Cell Culture Center, Minneapolis, MN) in 100 μl of PBS on days −4, −1, +2, and +5 relative to HSV-1 ocular challenge. The efficiency of $CD4^+$ and $CD8^+$ T cell depletion was monitored by FACS analysis. After the second depletion, >95% of $CD4^+$ or $CD8^+$ T cells were depleted from the spleens. Control mice were treated with PBS.

2.10. Depletion of macrophages

Liposome-encapsulation of dichloromethylene diphosphonate (Cl_2MDP) was done as described previously (Berra et al., 1994; Van Rooijen and Sanders, 1994). Briefly, 8-mg cholesterol and 86 mg of phosphatidylcholine (Sigma Co, St. Louis, MO) were dissolved in 10 ml of chloroform in a round-bottomed flask. After low-vacuum rotary evaporation, the inner white film was dispersed in 10 ml PBS alone (mock depletion) or 0.6 M Cl_2MDP in 10 ml PBS (a gift from Boehringer Mannheim, GmbH, Mannheim, Germany). The milky suspension was kept at room temperature for 2 h under nitrogen gas and after gentle shak-

ing the suspension was sonicated for 3 min. After sonication, the suspension was kept under nitrogen gas for another 2 h. Before use, the suspension was centrifuged at $10\,000 \times g$ for 15 min and the pellet was washed twice with PBS (centrifugation at $25\,000 \times g$). Finally, the pellet was suspended in 4 ml of sterilized PBS. To deplete macrophages each mouse received 100 μl of the mixture IV and another 100 μl IP. Depletion was done on days -4, -1, $+2$, and $+5$ relative to HSV-1 ocular challenge.

2.11. Statistical analysis

The Student's *t*-test and Fisher's exact test were performed using the computer program Instat (GraphPad, San Diego) to analyze survival and corneal scarring. Results were considered statistically significant when the $P < 0.05$.

3. Results

Effect of anti-asialo-GM1 antibody depletion of NK cells on survival and corneal scarring. Ganglio-N-tetraosylceramide (asialo GM1) is a neutral glycosphingolipid present at high quantity on the surface of NK cells (Kasai et al., 1980) and antibody to asialo GM1 efficiently depletes NK cells in vivo (Bukowski et al., 1983; Habu et al., 1984). Two experiments were performed to determine the effect of asialo-GM1-antibody depletion of NK cells on survival and corneal scarring.

In experiment 1, mice were depleted either 3 days before ocular challenge (-3), three days after ocular challenge ($+3$), or 6 days after ocular challenge ($+6$) as described in Section 2. One hundred percent (20 of 20) of the control (not depleted) mice survived lethal challenge (Table 1). In contrast, only five of ten mice depleted of NK cells three days before ocular challenge survived the lethal challenge (Table 1). This was significantly less survival than the control ($P = 0.002$, Fisher exact test). Depletion 3 days after infection also resulted in significantly more death than the control (7 of 10 mice survived; $P = 0.03$). Depletion 6 days after ocular challenge did not significantly decrease survival ($P = 0.3$).

Table 1
Effect of time of NK depletion on survival and corneal scarring[a]

Time of depletion (relative to challenge)	Survival (%)	Corneal scarring
3 days before	5/10	0.3 ± 0.1
3 days after	7/10	1.0 ± 0.4
6 days after	9/10	0.3 ± 0.2
Control	20/20	0.2 ± 0.1

[a] C57BL/6 mice were depleted of their NK cells by a single injection of anti-asialo GM1 antibody at the times indicated. Survival and corneal scarring were determined 28 days after ocular challenge.

Corneal scarring was measured in all of the mice that survived until day 28 after ocular challenge (Table 1). Although corneal scarring appeared higher in mice depleted of their NK cells 3 days after ocular challenge, this difference did not reach statistical significance (Table 1, $P > 0.1$, Student's *t*-test).

Experiment 2 was done to determine if multiple NK depletions would further alter survival and corneal scarring. Groups of 10–20 mice were depleted of their NK cells three times (days -3, $+3$, and $+6$, relative to ocular challenge), or two times (days $+3$ and $+6$). As above, all of the control mice (19 of 19) survived ocular challenge (Table 2). In contrast, only 11% (2/19) of the mice that were depleted three times survived ($P < 0.0001$ compared to control, Fisher's exact test).

Table 2
Effect of number of NK depletions on HSV-1 induced corneal scarring[a]

Time of depletion	Survival (%)	Corneal scarring
1st (day $+3$) 2nd (day $+6$)	5/10	2.1 ± 0.5
1st (day -3) 2nd (day $+3$) 3rd (Day $+6$)	2/19	2.7 ± 0.3
Control	19/19	0.1 ± 0.1

[a] C57BL/6 mice were depleted of their NK cells two or three times before and after ocular challenge as described in Section 2. Survival and corneal scarring were determined 28 days after ocular challenge.

Table 3
Protection of NK depleted C57BL/6 mice by vaccination[a]

Treatment[b]	Survival (%)	Corneal scarring
Vaccinated and depleted	10/10 (100)	0
Mock-vaccinated and depleted	1/10 (10)	3.5 ± 0.5

[a] C57BL/6 mice were vaccinated three times with KOS and challenged ocularly with 2×10^6 PFU of McKrae and survival was followed for 4 weeks.
[b] NK depletion of mice was done 3 days before ocular challenge, 3 days after ocular challenge, and 6 days after ocular challenge as described in Section 2

Five of ten mice (50%) depleted two times survived challenge (Table 2; $P = 0.002$ compared to control). Thus, the number and timing of the NK depletions significantly influenced survival.

As above, corneal scarring was measured in all surviving mice 28 days after ocular challenge (Table 2). Mice depleted either two or three times had significantly increased corneal scarring compared to controls ($P < 0.0001$, Student t-test). These results suggest that NK immune mediated responses played an important role in protecting naive mice against both death and corneal scarring.

3.1. Vaccination protects NK depleted mice against death and corneal scarring

To determine if vaccination could provide protection against lethal HSV-1 challenge in NK depleted mice, mice were depleted of NK cells three times as above. Prior to NK depletion, mice were vaccinated with live avirulent HSV-1 (strain KOS) or mock vaccinated as described in Section 2. Only one of ten mock vaccinated, NK depleted mice (control) survived ocular challenge (Table 3). In contrast, vaccination completely protected NK depleted mice against death. All ten (100%) of the vaccinated NK depleted mice survived lethal challenge (Table 3; $P = 0.0001$, Fisher's exact test). Thus, NK cells did not appear to be required for protection against lethal HSV-1 ocular challenge of vaccinated mice.

HSV-1 induced corneal scarring was assessed in the surviving mice. As above, the mock vaccinated, NK depleted mice had high levels of HSV-1 induced corneal scarring (Table 3). In contrast, vaccination completely protected against corneal scarring in NK depleted mice (Table 3, $P < 0.0001$ compared to control). Thus, NK cells were not required for protection against corneal scarring in vaccinated mice.

3.2. Role of IFN-γ in protection against death and corneal scarring

Some studies have suggested that naïve C57BL/6 mice have significant resistance to HSV-1 infection because of high levels of IFN-γ production (Gresser et al., 1976; Kirchner et al., 1983; Bukowski and Welsh, 1986). Since NK cells produce IFN-γ, it was of interest to examine the effect of NK depletion on IFN-$\gamma^{O/O}$ mice. Eighty five percent (Table 4; 17 of 20) of IFN-$\gamma^{O/O}$ mice survived lethal ocular challenge (Table 4). This was not significant compared to the parental C57BL/6 mice in this experiment (20 of 20 survived; $P = 0.2$, Fisher exact). However in numerous experiments with a total of over 200 C57BL/6 mice, none have ever died at the infectious dose used here. Taking this into account ($P < 0.05$), the small change in survival seen in IFN-$\gamma^{O/O}$ mice is probably meaningful, but not as great as the decreased survival seen in NK depleted C57BL/6 mice. NK depletion of IFN-$\gamma^{O/O}$ mice resulted in no survival following HSV-1 challenge (Table 4;

Table 4
Effect of IFN-γ and NK depletion on survival and corneal scarring[a]

Mouse genotype	Survival (%)	Corneal scarring
C57BL/6-IFN-$\gamma^{O/O}$ Control	17/20 (85)	0.3 ± 0.1
C57BL/6-IFN-$\gamma^{O/O}$ NK Depleted	0/20 (0)	ND
C57BL/6 Control	20/20 (100)	0.1 ± 0.1

[a] Mice were depleted twice of their NK cells (3 days before and three days after ocular challenge) and survival and corneal scarring were determined as described in Section 2

zero of 20; $P < 0.0001$ compared to undepleted IFN-$\gamma^{O/O}$ mice). This was also not significant compared to NK depleted C57BL/6 mice (six of 49 NK depleted C57BL/6 mice survived in the above experiments, $P < 0.05$). These results suggest that the majority of the NK cell activity responsible for protecting naïve C57BL/6 mice against ocular HSV-1 challenge was due to an NK function other than IFN-γ production. This was supported by the finding that injection of recombinant IFN-γ into C57BL/6 NK depleted mice did not improve survival following HSV-1 challenge (data not shown).

Following ocular HSV-1 challenge, the average corneal scarring score for the IFN-$\gamma^{O/O}$ mice was similar to that of the control C57BL/6 mice (Table 4, $P = 0.5$). Thus, IFN-γ did not appear to be an important factor in protecting naïve mice against HSV-1 induced corneal scarring. Corneal scarring, which is measured on day 28 post infection, could not be measured in the IFN-$\gamma^{o/o}$-NK depleted mice, since none of these mice survived. Thus, as with survival, these results suggest that the majority of the NK cell activity responsible for protecting naïve C57BL/6 mice against HSV-1 induced corneal scarring was due to an NK function other than IFN-γ production. Again, this was supported by the finding that injection of recombinant IFN-γ into C57BL/6 NK depleted mice did not protect against corneal scarring (data not shown).

3.3. Depletion of NK cells by anti-NK1.1 antibody

To confirm the above anti-asialo GM1 antibody NK depletion experiments, NK depletion of C57BL/6 mice was performed using either 100 or 200 μg injections of anti-NK1.1 monoclonal antibody PK136 as described in Section 2. As expected, all 20 control C57BL/6 mice survived ocular challenge (Fig. 1A). In contrast, only 50% of the mice that received 200 μg injections of PK136 survived ocular challenge (Fig. 1A; $P = 0.002$ compared to control, Fisher's exact). Corneal scarring was also significantly higher in these mice compared to the control mice (Fig. 1B) ($P < 0.001$, Student's t-test). Depletion of NK cells with 100 μg of PK136 monoclonal antibody had less impact. Seven of nine (77%) depleted mice survived ocular challenge and this difference did not quite reach statistical significance (Fig. 1A; $P = 0.09$). As with the mice depleted with 200 μg of PK136, the mice depleted with 100 μg of PK136 had significantly more corneal scarring than the control mice (Fig. 1B; $P = 0.003$). Thus, depletion of NK cells with PK136, confirmed the results obtained with the anti-asialo GM1 antibody.

3.4. Comparison of NK, macrophage, and T cell depleted mice

Groups of 20 C57BL/6 mice were depleted four times of their $CD4^+$ T cells (using GK1.5 mAb), $CD8^+$ T cells (using 2.43 mAb), or macrophages (chemical depletion using Cl_2MDP) as described in Section 2. In addition, 20 mice were depleted three times of NK cells by anti-asialo GM1.

All of the 20 control mice and 20 of 20 mice (100%) depleted for $CD4^+$ T cells survived lethal challenge (Fig. 2A; $P = 1.0$ versus control). In addition, 17 of 20 (85%) macrophage deplete mice and 16 of 20 (80%) $CD8^+$ T cell depleted mice survived lethal ocular challenge (Fig. 2A; $P = 0.2$ and $P = 0.1$, respectively). In contrast only two of 20 (10%) NK-depleted mice survived the lethal challenge ($P < 0.0001$ versus control mice, $CD4^+$ T cell depleted mice, $CD8^+$ T cell depleted mice, or macrophage depleted mice; Fisher's exact). These results suggest that NK played a more important role in protection of naïve mice against lethal ocular HSV-1 challenge than did $CD4^+$ T cells, $CD8^+$ T cells, or macrophages.

Corneal scarring in surviving mice was determined 28 days after ocular challenge. As expected, the amount of corneal scarring was highest in the NK depleted mice (Fig. 2B; $P < 0.0001$ versus control). This was followed by corneal scarring in the $CD4^+$ depleted mice (Fig. 2B; $P = 0.0001$ versus control), the $CD8^+$ depleted mice ($P = 0.02$ versus control) and the macrophage depleted mice ($P = 0.04$ versus control). Thus, as for survival, these results suggested that in naive C57BL/6

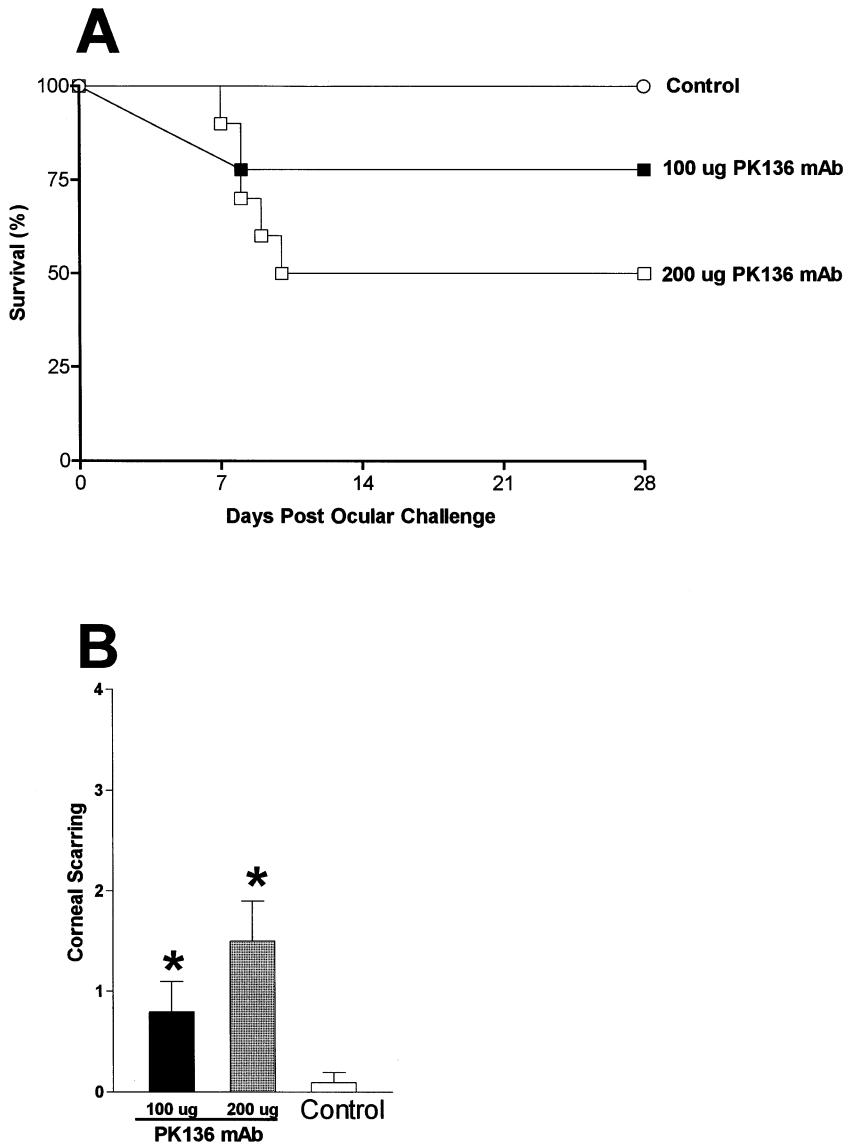

Fig. 1. Survival and corneal scarring of NK1.1 depleted C57BL/6 mice. Mice were depleted of their NK1.1 cells and inoculated ocularly with 2×10^6 pfu of McKrae as described in the Materials and Method. (A) Survival in NK1.1 depleted mice. Survival of NK1.1 depleted mice was measured 28 days after ocular challenge and compared with C57BL/6J control mice. (B) Corneal scarring in NK1.1 depleted mice. Corneal scarring in the above surviving mice was determined 28 days after ocular challenge. For each bar, corneal scarring (y-axis) represents the average of the scarring from 14 eyes (NK1.1, 100 ug PK136), 10 eyes (NK1.1, 200 ug PK136), and 40 eyes (control). '*'; Indicates similar to each other and significantly different from control group ($P < 0.001$, Student's t-test).

mice ocularly challenged with HSV-1, NK cells were more important in protecting against HSV-1 induced corneal scarring than were macrophages, $CD4^+$ T cells, or $CD8^+$ T cells.

3.5. Effect of NK depletion on survival and corneal scarring of BALB/c mice

BALB/c mice were depleted of NK cells with

three injections of anti-asialo GM1 antibody as described above for C57BL/6 mice. Following HSV-1 ocular challenge, only 20% (4 of 20) of the NK depleted BALB/c mice survived lethal ocular challenge (Table 5). This was similar to the undepleted control mice ($P = 1$, Fisher's exact test).

The surviving control and NK depleted BALB/c mice also had similar levels of corneal scarring

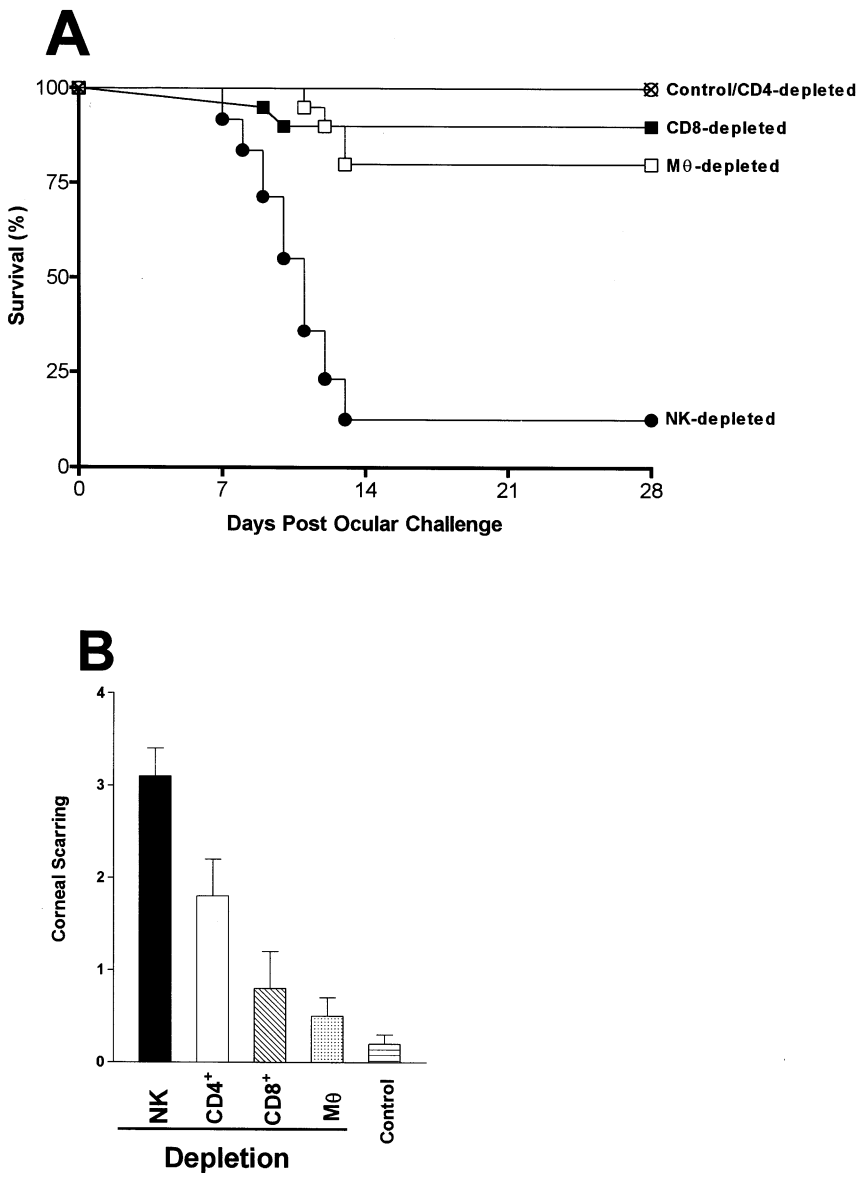

Fig. 2. Comparison of survival and corneal scarring in NK, $CD4^+$, $CD8^+$, or macrophage depleted C57BL/6 mice. (A)Survival of NK, $CD4^+$, $CD8^+$, and macrophage depleted mice following ocular HSV-1 challenge. Depletions were done as described in Section 2. NK depletions were done with anti-asialo GM1 antibody on days -3, 3, and 6. (B)Corneal scarring was measured 28 days after ocular challenge in surviving mice. For each bar, corneal scarring (Y-axis) represents the average of the scarring from four eyes (NK-depleted), 34 eyes (macrophage-depleted), 40 eyes ($CD4^+$-depleted), 32 eyes ($CD8^+$-depleted), and 40 eyes (control). The error bars indicate the SEM.

Table 5
Effect of NK depletion in BALB/c mice[a]

Treatment	Survival (%)	Corneal scarring
Depleted	4/20 (20)	3.5 ± 0.3
No depletion	6/20 (30)	3.1 ± 0.2

[a] Three days after the first NK depletion using anti-asialo-GM1 antibody, BALB/c mice were challenged ocularly with 2×10^5 PFU of McKrae. Second depletion was done three days after ocular challenge. Survival and corneal scarring were determined 28 days after ocular challenge.

(Table 5; $P = 0.7$, student t-test). Thus, in contrast to C57BL/6 mice, in BALB/c mice NK cells did not appear to be important in protection against HSV-1 induced corneal scarring and death.

4. Discussion

Ocular infection with HSV-1 can cause eye disease ranging in severity from blepharitis, conjunctivitis, and dendritic keratitis, to disciform stromal edema and necrotizing stromal keratitis (Dawson and Togni, 1976; Binder, 1984; Dawson, 1984). HSV-1 induced corneal scarring can lead to blindness and as such, HSV-1 is the leading cause of corneal blindness by an infectious agent in developed countries (Dawson, 1984). HSV-1 induced corneal scarring is due to an as yet undefined immune response.

Studies in mice have alternatively suggested that the immune response leading to corneal scarring is associated with $CD4^+$ T cells alone (Newell et al., 1989; Hendricks and Tumpey, 1990; Doymaz and Rouse, 1992; Hendricks, et al., 1992), $CD8^+$ T cells alone (Hendricks and Tumpey, 1990), or both together (Hendricks, et al., 1989; Igietseme et al., 1991; Staats, et al., 1991). NK cells have also been implicated in HSV-1 induced corneal disease (Bouley, et al., 1996). As with T cells, the literature contains conflicting reports. Thus, it has been reported that in BALB/c or SCID mice NK depletion increases corneal disease (Staats, et al., 1991) while other studies in BALB/c mice suggest that NK depletion decreases corneal disease (Tamesis et al., 1994; Bouley, et al., 1996). The role of macrophages in HSV-1 induced corneal disease is also controversial, with some reports suggesting that macrophages increase corneal disease (Berra et al., 1994; Thomas et al., 1997) and others suggesting that macrophages protect against corneal disease (Tumpey et al., 1996). In this study we show that C57BL/6 mice depleted of NK cells were more susceptible to lethal HSV-1 infection and HSV-1 induced corneal scarring than were mice depleted of either $CD4^+$ T cells, $CD8^+$ T cells, or macrophages. This suggests that in naïve C57BL/6J mice, NK cells were more important than T cells or macrophages in providing protection against HSV-1 induced corneal disease.

Murine NK cells are phenotypically characterized by different antigen markers such as NK1.1, Qa-5, and asialo-GM1 (Trinchieri, 1989; Scott and Trinchieri, 1995). In many mice, NK cells can be depleted by antibodies against either asialo-GM1 or against NK1.1, since both of these antigens are found on the surface of their NK cells (Glimcher, et al., 1977; Habu et al., 1981; Koo and Peppard, 1984). In C57BL/6 mice, NK1.1 antigen is expressed on all NK cells (Tutt et al., 1986). In contrast, NK cells in BALB/c mice do not have NK1.1 antigen on their surface (Karlhofer and Yokoyama, 1991; Bendelac, 1995).

All previous NK depletion experiments directed at the role of NK cells on HSV-1 infection employed anti-asialo GM1 to deplete the NK cells. Since anti-asialo-GM1 recognizes other immune cells in addition to NK cells, using this antibody for depletion of NK cells introduces the complication of possible alterations to other immune functions. Therefore, the apparent effects of NK depletion on HSV-1 infection seen in previous studies are equivocal. In the studies reported here, we employed anti-NK1.1 antibody (as well as anti-asialo GM1) to deplete NK cells in C57BL/6 mice. As indicated above, we found that depletion of NK cells resulted in increased susceptibility to lethal HSV-1 infection and HSV-1 induced corneal disease. To our knowledge, this is the first

report showing that depletion of NK cells with an anti-NK1.1 antibody reduced the resistance of naïve mice to either lethal HSV-1 challenge or HSV-1 induced corneal scarring. Since anti-NK 1.1 antibody should deplete NK cells specifically, these results provide the first definitive demonstration that NK cells were involved in innate protection against HSV-1 challenge.

As with HSV-1 induced eye disease, the role of NK cells in protection against HSV-1 induced death remains controversial. Some studies have suggested that NK cells are not involved in protection against lethal HSV-I infection (Chmielarczyk et al., 1985; Bukowski and Welsh, 1986; Tamesis et al., 1994), while other studies have concluded that NK cells are important in protection against HSV-1 induced death (Lopez, et al., 1980; Engler et al., 1981; Habu et al., 1984; Rager-Zisman et al., 1987; Staats, et al., 1991). As with eye disease, these discrepancies could be due to the use of different mouse and virus strains. In general, mouse strains with higher resistance to HSV-1 (e.g. C57BL, FVB/N, and NZB) contain higher NK activity than mouse strains that are more susceptible to HSV-1 (e.g. A, BALB/c, and 129) (Clark et al., 1979). NK cell activity has also been reported to be higher in mouse strains that are resistant to other types of infection such as L. major (Scharton and Scott, 1993). To help resolve the issue as to whether NK cells provide innate protection against lethal HSV-1 challenge and HSV-1 induced corneal scarring, in this report we compared the effect of NK depletion in BALB/c and C57BL/6 mice using either anti-asialo-GM1 or NK1.1 antibody. Our results suggested that in C57BL/6 mice, NK cells play a major role in innate protection against both eye disease and death following HSV-1 challenge. In contrast, in BALB/c mice, NK cells did not appear to be very important. This raises the question of which mouse strain is more like humans. In humans NK cells appear to play a role in resistance to HSV-1 infection (Biron, et al., 1989), suggesting that in some instances C57BL/6 mice may be a better model than BALB/c mice.

Previous studies in BALB/c mice showed that NK cells did not appear in infected cornea until day 15 post infection and then only at low levels (Wang et al., 1989). It is not known when NK cells first appear in the cornea of C57BL/6 mice. If NK cells appear in the cornea of C57BL/6 mice within a few days of ocular HSV-1 infection, it would help explain why NK cells play a significant role in combating ocular HSV-1 infection in C57BL/6 but not BALB/c mice.

All previous NK depletion experiments involving HSV-1 infection of C57BL/6 mice were done using HSV-1 IP challenge. Thus, the role of NK cells in HSV-1 induced corneal disease has not been examined in C57BL/6 mice. Several studies examining the effect of NK depletion on HSV-1 infection have been done in BALB/c mice, and in some of these studies eye disease was examined following ocular challenge. However, BALB/c mice are highly susceptible to ocular HSV-1 infection and routinely develop severe corneal disease. Thus, even if NK cells provide some protection against HSV-1 induced corneal disease, depletion of NK cells in BALB/c mice may not produce an observable effect over the high innate level of eye disease. In contrast, C57BL/6 mice are highly resistant to HSV-1 induced corneal disease. If NK cells are important in this innate resistance, depletion of NK cells should produce a readily observable effect. The results of the NK depletion studies reported here strongly suggest that in C57BL/6 mice NK cells are involved in protection against both lethal HSV-1 challenge and HSV-1 induced corneal disease.

In contrast to our results with C57BL/6 NK depleted mice, beige mice (NK-deficient C57BL/6 mice), were reported to be as resistant to HSV-1 infection as C57BL/6 mice (Tamesis et al., 1994). However, the beige mouse study used 100-fold less input HSV-1 than was used here. This may explain the lack of corneal disease in these mice.

In addition to directly lysing target cells, NK cells produce IFN-γ (Trinchieri, 1989; Arase, et al., 1996), TNF-α (Paya et al., 1988), and other factors. Thus, NK mediated lysis, NK IFN-γ production, and/or NK TNF-α production may be involved in NK mediated protection against HSV-1. IFN-γ is produced by both T

lymphocytes and NK cells (Herberman and Ortaldo, 1981; Mosmann and Coffman, 1989). In this report, we used IFN-γ knockout mice (IFN-$\gamma^{o/o}$ mice) with and without NK depletion to examine the relative contribution of IFN-γ and NK mediated cell lysis in protection against HSV-1. IFN-$\gamma^{O/O}$ mice appeared slightly more susceptible to HSV-1 infection, although the difference was not significant. Depletion of NK cells from IFN-$\gamma^{O/O}$ mice appeared to increase susceptibility to HSV-1 slightly more than depletion of NK cells from normal C57BL/6 mice. Thus, it appeared that NK mediated lysis was more important than IFN-γ, with IFN-γ possibly playing a minor role in protection against HSV-1 induced death. Since NK cells are the major source of TNF-α, it is possible that the absence of TNF-α in NK depleted mice contributed to the increased corneal scarring. This is supported by our previous finding that TNF-α is associated with reduced virus replication in the eyes and protection against corneal scarring (Ghiasi et al., 1995b).

Previously we showed that perforin deficient mice, which lack NK functions, are more susceptible to death following ocular HSV-1 infection than their parental C57BL/6 mice (Ghiasi et al., 1999). This is similar to the results reported here for NK-depleted mice. However, unlike NK-depleted mice, they were not more susceptible to HSV-1 induced corneal scarring. This may be because the perforin-deficient mice have elevated levels of T-cells.

In summary, our findings suggest that:

1. NK cells were important in protection of naïve C57BL/6 mice against HSV-1 challenge, since anti-NK1.1 antibody as well as anti-asialo GM1 depletion of NK cells resulted in increased susceptibility to both death and corneal scarring;
2. in BALB/c mice NK cells were not important in protection of naïve mice against HSV-1 challenge,
3. in naïve C57BL/6 mice NK cells played a more important role in survival and eye disease than T cells or macrophages;
4. NK cells were not a major factor in protection of immunized mice, since vaccination completely protected NK depleted mice against HSV-1 induced death and corneal scarring; and
5. INF-γ had only a minor affect on the ability of C57BL/6 mice to control HSV-1 infection.

Acknowledgements

This work was partially supported by Public Health Service grant EY09224, the Discovery Fund for Eye Research and the Skirball Program in Molecular Ophthalmology.

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