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# Granzyme A is critical for recovery of mice from infection with the natural cytopathic viral pathogen, ectromelia

(poxvirus/cytolytic leukocytes/proteases)

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ABSTRACT **Cytolytic lymphocytes are of cardinal importance in the recovery from primary viral infections. Both natural killer cells and cytolytic T cells mediate at least part of their effector function by target cell lysis and DNA fragmentation. Two proteins, perforin and granzyme B, contained within the cytoplasmic granules of these cytolytic effector cells have been shown to be directly involved in these processes. A third protein contained within these granules, granzyme A, has so far not been attributed with any biological relevance. Using mice deficient for granzyme A, we show here that granzyme A plays a crucial role in recovery from the natural mouse pathogen, ectromelia, by mechanisms other than cytolytic activity.**

$CD8^+$ T cells together with natural killer (NK) cells are the main cytolytic leukocytes that are involved in recovery from infection by intracellular pathogens (1–3) and that *in vitro* and *in vivo* induce cell lysis as measured by $^{51}Cr$ release and DNA fragmentation or apoptosis (4). Three proteins, namely perforin and the two serine proteinases, granzyme A (gzm A) and granzyme B (gzm B), are exclusively associated with cytolytic lymphocytes (4). Gene-targeted mice have been used to show that perforin and gzm B are directly involved in these processes (5–9). A lack of perforin reduces lysis of target cells by NK and cytolytic T (Tc) cells (5, 7–9). More importantly, perforin knock-out mice fail to clear lymphocytic choriomeningitis virus infection (5, 9). On the other hand, lack of gzm B in cytolytic lymphocytes results in a profound defect in their ability to induce rapid DNA fragmentation and apoptosis (6).

We have recently been able to show that lack of gzm A does not affect the cytolytic potential of either NK or Tc cells as measured by $^{51}Cr$ release or nuclear DNA fragmentation (10). These data not only emphasize the unique role of gzm B in NK/Tc cell-mediated cytotoxicity but also indicate that the biological activities of gzm A and gzm B are not redundant and interchangeable. In addition, we were unable to consistently observe an effect on virus elimination after infection of gzm A mutant mice with the noncytopathic arenavirus, lymphocytic choriomeningitis virus (10). We thus turned to investigate the role of gzm A in recovery from the cytopathic orthopoxvirus, ectromelia. Classic studies have implicated $CD8^+$ T cells and NK cells in recovery from poxvirus infection (1–3), and detailed information on the etiology of mousepox exists (11).

## MATERIALS AND METHODS

**Animals.** The gzm A-deficient mouse mutant (gzm $A^{-/-}$) was generated by homologous recombination, using BL/6-III ES cells, as described recently (10). Female gzm $A^{-/-}$ and normal littermates between 6 and 12 weeks of age were used throughout the experiments.

**Viruses.** The virulent Moscow strain ectromelia virus was grown in mouse spleen, the intracellular naked ectromelia virus (INV), and the extracellular enveloped ectromelia virus (EEV) in tissue culture, as has been described previously (12, 13). Viruses were purified and titrated on BS-C-1 monkey cell monolayers as has been described (14). The predominance of either EEV or INV, in the purified virus fractions, was verified by electron microscopy.

**Generation of Cytolytic Effector Cells.** Mice were immunized with $10^6$ plaque forming units (pfu) of Moscow strain ectromelia, unless otherwise stated, into the hind footpads. Splenocytes were tested for cytolytic activity 2–8 days p.i.

**Cytotoxicity Assays.** The NK cell-sensitive YAC-1 cell line, the NK-insensitive mastocytoma cell line P815 (H-$2^d$), and the H-$2^b$ matched MC57 methylcholantrene-induced fibrosarcoma cell line were used as target cells. MC57 target cells were infected with 2 pfu of ectromelia virus per cell for 1 h or mock infected. The methods used for cell lines has been described in detail elsewhere (15). The duration of the assays was 6 h. The percent specific lysis was calculated using the following formula: % specific lysis = [(experimental release − medium release)/(maximum release − medium release)) × 100. Data given are the means of triplicates. SEM were always < 5%.

**Virus Titration of Organs.** Organs were frozen and thawed, homogenized and diluted in PBS, and titrated in duplicate on BS-C-1 monkey cell monolayers as has been described (2). Values given are calculated for total organs and represent the mean of three individual animals, unless stated otherwise. Significance was evaluated by Student's *t*-test.

**Histological Evaluation.** Mice were immunized with $10^6$ pfu of virulent Moscow ectromelia via the footpad. At the indicated days after injection, spleen and liver (from three mice) were removed and fixed in 5% formaldehyde in PBS for embedding in paraffin. Sections of organs were stained with haematoxylin/eosin and embedded in Entellan (Merck). Samples were properly coded and examined under double-blind conditions.

**Liver Enzyme Levels in Serum.** Mice were bled by cardiac puncture. Activities of aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.1.2) were measured by standard laboratory methods (16).

**Proteolytic Inactivation of Purified Ectromelia Virus.** gzm A was purified from an aged $CD8^+$ Tc cell line as described

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*Abbreviations*: NK, natural killer; pfu, plaque forming units; gmz A, granzyme A; gzm B, granzyme B; INV, intracellular naked ectromelia virus; EEV, extracellular enveloped ectromelia virus; Tc, cytotoxic T; fp, footpad; p.i., postinfection.

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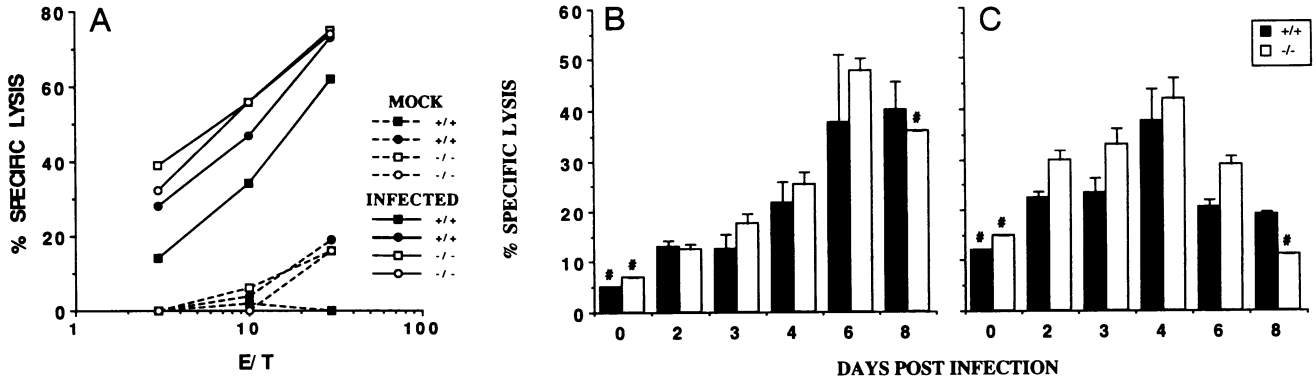


FIG. 1. Cytolytic activity of ectromelia-immune splenocytes and kinetics of induction by gzm $A^{-/-}$ and gzm $A^{+/+}$ mice. (*A*) The lytic activity of 6-day immune splenocytes from two individual gmz $A^{-/-}$ (open symbols) and two gzm $A^{+/+}$ littermates (closed symbols) on H-2-matched, MC57, mock (- - -) or ectromelia virus-infected (——) target cells. (*B*) Mean lytic activity of splenocytes from three individual mice immunized with $10^6$ pfu virulent Moscow ectromelia via the footpad on ectromelia-infected MC57 target cells. All values are from a 4-fold titration curve and were resolved by log regression analysis at an effector to target ratio of 10:1. Closed bars represent splenocytes from gzm $A^{+/+}$ mice; open bars of gzm $A^{-/-}$ mice. Bars marked # represent the values of a single animal. Lysis of mock-infected MC57 targets was not significant and is not shown for clarity. (*C*) As for *B*, but splenocytes were tested on NK cell-sensitive YAC-1 target cells. Lysis of control P815 targets was not significant and are obmitted for clarity.

(17). Plasmin was obtained from Chromogenic (Haemochron Diagnostica GmbH; Essen, Germany). Purified INV and EEV were diluted in reaction buffer (100 mM Tris·HCl, pH 8.5, plus 10 μg/ml heparin) to give $4 \times 10^3$ pfu/ml. 500 μl of virus suspension was incubated with 25 μl of purified gzm A (280 units; specific enzymatic activity, 11,800 units/mg), 25 μl of plasmin ($1 \times 10^6$ units/mg), or buffer only, at 37°C overnight. Samples were diluted with cell culture medium to give a final volume of 1 ml and titrated in duplicates on BS-1 monkey cell monolayers.

Table 1. Histopathological findings in ectromelia-infected gzm $A^{-/-}$ and gzm $A^{+/+}$ mice between days 2 and 8 p.i.

| Time post infection (day) | Pathological alterations in Liver | | Spleen | |
|---|---|---|---|---|
| | gzm $A^{-/-}$ | gzm $A^{+/+}$ | gzm $A^{-/-}$ | gzm $A^{+/+}$ |
| 2 | − | − | − | − |
| | − | − | − | − |
| | − | − | − | − |
| 3 | + | − | + | − |
| | ± | − | − | − |
| | + | − | + | − |
| 4 | ++ | − | ± | − |
| | ++ | − | + | − |
| | + | − | + | − |
| 6 | + | ± | + | ± |
| | + | − | ++ | − |
| | + | − | ++ | − |
| 8 | ± | − | ± | − |
| | * | − | * | − |
| | * | − | * | − |

Histological changes in spleen and liver of gzm $A^{-/-}$ mice infected with ectronelia virus ($1 \times 10^6$ pfu Moscow strain) were already apparent on day 3 p.i. and increased with time after infection. No regeneration of the organs occured. On day 8 p.i., one gzm $A^{-/-}$ mouse was moribund, two were dead. No or only marginal changes were seen in organs of gzm $A^{+/+}$ mice at any time point tested. Evaluation, spleen: focal necrosis, in particular in the red pulp with fragmentation of the lymphoid follicles; ++, severe; +, partial; ±, few; −, none. liver: scattered necrotic foci throughout the parenchyma, infiltration with mononuclear cells in portal tracts; ++, severe; +, partial; ±, few; −, marginal/none.
*Dead.

## RESULTS AND DISCUSSION

Initial experiments indicated that Tc cells from spleens of 6 day ectromelia-immune, gzm $A^{-/-}$ and gzm $A^{+/+}$ mice did not show a significant difference in lysis of H-2-matched, ectromelia-infected target cells (Fig. 1*A*). To obtain information regarding the kinetics of induction and function of NK and Tc cells from the two mouse strains, a time course with three animals per group was performed by inoculation of virulent Moscow strain of ectromelia virus via the footpad ($1 \times 10^6$ pfu). This mode of infection of susceptible inbred strains of mice leads to a disease state indistinguishable from naturally acquired mousepox except for the localization of the primary skin lesion (11).

Fig. 1 *B* and *C* shows the mean lysis of ectromelia-infected H-2-matched MC57 (Fig. 1*B*) and the NK cell-sensitive YAC-1 (Fig. 1*C*) targets by virus-immune splenocytes. No significant difference in lytic activity was observed between the gzm $A^{-/-}$ and gzm $A^{+/+}$ effector cell populations at any time point between days 2 and 8. The kinetic profiles of the two cytotoxic lymphocyte populations are similar to those reported previously for ectromelia (18) and other pox viruses (2). However, the most telling observation was the mortality on day 8 (Table 1). Two out of three of the gzm $A^{-/-}$ mice had died, and the third was moribund, whereas the gzm $A^{+/+}$ mice did not show any noticeable signs of illness. This was confirmed in more comprehensive experiments (also see Table 3). This is the more intriguing since among inbred mouse strains C57BL/6 mice show high resistance to ectromelia (19). Despite this,

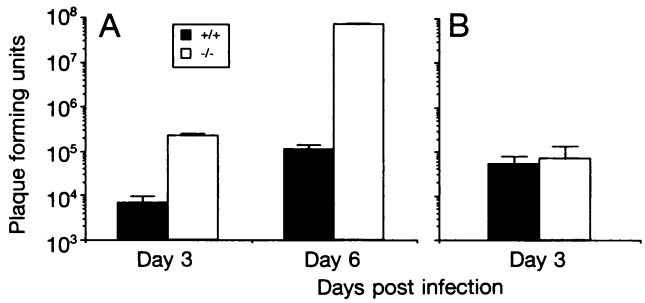


FIG. 2. Virus titers of spleen and liver of gzm $A^{-/-}$ and gzm $A^{+/+}$ mice after virulent ectromelia infection. Mice were infected via the footpad with $1 \times 10^6$ pfu of ectromelia virus (Moscow strain). At days 3 and 6 p.i., ectromelia virus titers were determined in spleen (*A*) and liver (*B*) of three individual animals with standard deviation shown. Significance tested by Student's *t*-test.

Table 2. Kinetics of virus titers in the liver of gzm $A^{-/-}$ and gzm $A^{+/+}$ mice infected with ectromelia.

| Time post infection (day) | gzm $A^{-/-}$ | gzm $A^{+/+}$ |
|---|---|---|
| 6 | $6.6 \times 10^8$ | $4.4 \times 10^7$ |
| | $8 \times 10^7$ | $1.6 \times 10^5$ |
| | $1.8 \times 10^8$ | $1.0 \times 10^5$ |
| 8 | $1.2 \times 10^8$ | $<10^2$ |
| | $2 \times 10^7$ | $<10^2$ |
| | $2 \times 10^5$ | $<10^2$ |
| 10 | $1.2 \times 10^5$ | $<10^2$ |
| | * | $<10^2$ |
| | * | $<10^2$ |

Mice were infected via foot pad with $5 \times 10^5$ pfu of ectromelia virus (Moscow strain). At the indicated time p.i., livers of individual mice were removed and virus titers were determined as described.
*Dead.

splenocytes of the moribund mouse were normal with regard to lytic activity on H-2-matched ectromelia-infected targets (Fig. 1*B*).

We next analyzed virus titers in spleen and liver, the main visceral target organs infected by ectromelia (11), 3 and 6 days p.i.via the footpad. Spleen titers were significantly higher in gzm $A^{-/-}$ mice than in gzm $A^{+/+}$ controls on both day 3 ($P < 0.0001$) and day 6 ($P < 0.0177$) (Fig. 2*A*) and reached almost $10^8$ pfu on day 6 in gzm $A^{-/-}$ mice, but only around $10^5$ pfu in gzm $A^{+/+}$ littermates. Liver titers were not significantly different on day 3 (Fig. 2*B*). However, in separate experiments using a lower virus inoculum ($5 \times 10^5$ pfu), significantly higher virus titers were observed in the liver of gzm $A^{-/-}$ mice than in those of their wild-type littermates 6–10 days p.i. (Table 2).

The difference seen in virus titers in spleen and liver was also reflected by histopathology (Tables 1 and 3). Spleens of gzm $A^{-/-}$ mice showed histological changes similar to those of genetically susceptible mice—i.e, DBA/1, A/J, and C3H (11): focal necrosis, particularly in the red pulp, and fragmentation of the lymphoid follicles were observed from day 3 postinfection on. The necrotic foci extended in size and numbers with time after infection (days 3–6), and spleen regeneration did not occur. As expected (11), only marginal changes, if at all, were seen in spleen tissue of gzm $A^{+/+}$ mice at any time point (days 2–8). Furthermore, scattered foci of necrosis were apparent throughout the liver parenchyma of gzm $A^{-/-}$ but not gzm $A^{+/+}$ mice; they extended with time p.i. with small lymphoid cell accumulations in the portal tracts (Table 1). Spleen and liver tissues of one of the gzm $A^{-/-}$ mice that was still surviving on day 8 p.i. only showed mild histological alterations indicating recovery from infection. Similar results were obtained in a repeat experiment in which the dose of virus

Table 3. Histopathological findings in gzm $A^{-/-}$ and gzm $A^{+/+}$ mice infected with increasing doses of ectromelia virus on days 6, 7/8, and 10.

| | | Liver | | | | Spleen | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | gzm $A^{-/-}$ | | gzm $A^{+/+}$ | | gzm $A^{-/-}$ | | gzm $A^{+/+}$ | |
| Days p.i. | Virus dose | Necrosis | Infiltration | Necrosis | Infiltration | Necrosis | Infiltration | Necrosis | Infiltration |
| 6 | $5 \times 10^5$ | ++ | * | ± | ++ | +++ | * | − | ± |
| | | +++ | * | − | + | ++ | + | − | ± |
| | | ++ | ++ | − | + | + | ++ | − | ± |
| | $1 \times 10^6$ | ++ | ++ | − | − | + | ++ | ++ | + |
| | | ± | ++ | − | ± | ± | ++ | − | ± |
| | | † | | − | ± | † | | − | ± |
| | $2 \times 10^6$ | ++ | ± | − | − | +++ | * | +++ | − |
| | | ++ | + | − | − | +++ | * | − | + |
| | | † | | − | − | † | | − | ± |
| 7/8 | $5 \times 10^5$ | +++ | * | − | − | +++ | * | − | − |
| | | +++ | * | − | − | ++ | ++ | − | − |
| | | − | ± | − | − | − | ± | − | − |
| | $1 \times 10^6$ | − | − | − | − | ± | ± | − | ± |
| | | − | + | − | − | ++ | + | − | ± |
| | | − | − | − | − | − | ++ | − | ± |
| | $2 \times 10^6$ | + | + | − | − | − | ++ | ± | ± |
| | | + | + | − | − | − | ++ | − | − |
| | | − | ± | − | − | − | ± | − | ± |
| 10 | $5 \times 10^5$ | − | ++ | − | ± | ± | ± | − | ± |
| | | † | | − | ± | † | | − | ± |
| | | † | | − | ± | † | | − | ± |
| | $1 \times 10^6$ | − | − | − | ± | − | ++ | − | ± |
| | | − | ± | − | − | + | + | − | + |
| | | − | ± | t.f. | t.f. | − | ± | t.f. | t.f. |
| | $2 \times 10^6$ | − | − | − | − | + | + | − | + |
| | | − | − | t.f. | t.f. | ± | + | t.f. | t.f. |
| | | † | | t.f. | t.f. | † | | t.f. | t.f. |

On day 6 p.i. (ectromelia virus Moscow strain), liver and spleen tissues of all gzm $A^{-/-}$ mice showed considerable histological changes characterized by prominent necrosis and cell infiltrations, independent of the virus dose; at later stages of infection (days 7/8, 10) the microscopical alterations in both organs were less pronounced. Two gzm $A^{-/-}$ mice had died on day 6 p.i., three gzm $A^{-/-}$ mice had died on day 10 p.i. With two exceptions (day 6; $1 \times 10^6$ pfu, $2 \times 10^6$ pfu), none of the infected gzm $A^{+/+}$ mice showed significant histological alterations in the respective organs at any of the time points tested. Evaluation of spleen/liver tissues, necrosis: +++, complete; ++, large areas/multiple; +, partial; ±, some; −, none; infiltration: ++, massive/general; +, multiple; ±, few; −, none. t.f., technical failure.
*Not present in preparation.
†, Dead.

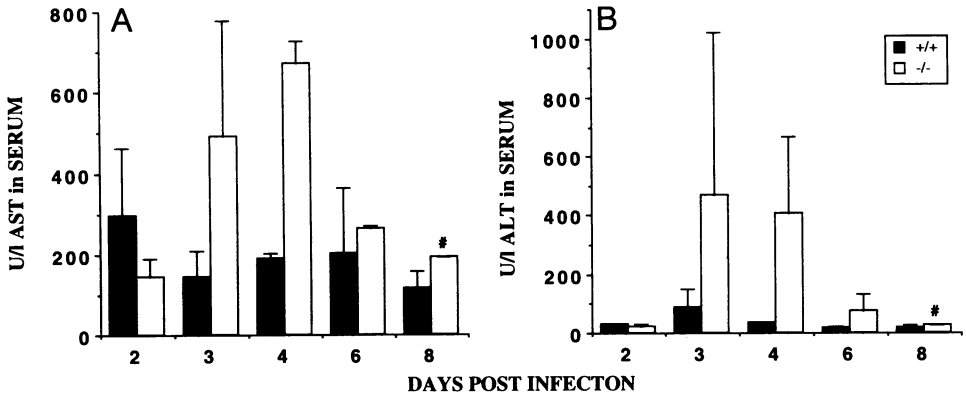


FIG. 3. Liver enzyme levels in serum of ectromelia-infected gzm $A^{-/-}$ (□) and gmz $A^{+/+}$ (■) mice. Means of aspartate aminotransferase (*A*) and alanine aminotransferase (*B*) levels in units per liter of serum (± SD) from three individual animals immunized (footpad) with $1 \times 10^6$ pfu ectromelia virus (Moscow strain). #, Enzyme levels from a single surviving animal.

was varied from $5 \times 10^5$ to $2 \times 10^6$ pfu and in which the observation time was extended to 10 days (Table 3). Again, 2/9 and 3/9 gzm $A^{-/-}$ mice had died on day 6 ($1 \times 10^6$ pfu, $2 \times 10^6$ pfu) and 10 p.i. ($5 \times 10^5$ pfu, $2 \times 10^6$ pfu), respectively, whereas all gzm $A^{+/+}$ mice survived the infection independent of the virus dose. The most prominent histological changes in spleen and liver tissues of gzm $A^{-/-}$ mice, as characterized by necrosis and cell infiltration, were seen on day 6 p.i. in all three experimental groups. At later stages (days 7, 8, and 10), histological changes were much less pronounced, particularly in mice previously infected with $1 \times 10^6$ or $2 \times 10^6$ pfu. With the exception of two animals, none of the infected gzm $A^{+/+}$ showed important histopathological alterations in spleen and liver at any time point tested. The finding that only a fraction of gzm $A^{-/-}$ mice die during ectromelia infection emphasizes the critical role of gzm A for optimal protection against ectromelia-induced pathology but also clearly indicates the involvement of additional essential factors for the control of virus infection.

To further characterize ectromelia infection in gzm $A^{-/-}$ mice, levels of liver enzymes in serum were determined. This method has been shown previously to correlate with viral or immunopathological damage (16, 20). Fig. 3 *A* and *B* shows the levels of aspartate aminotransferase and alanine aminotransferase in serum from the animals tested above for cytolytic activity (Fig. 1 *B* and *C*) and histopathology (Fig. 3). As reported by others (16), we observed great variation between individual animals within a group, but liver enzyme levels on days 3 and 4 were far higher in gzm $A^{-/-}$ mice than in gzm $A^{+/+}$ mice. Since both types of mice express similar cytolytic potential in their cytolytic effector cells after ectromelia virus infection (Fig. 1), the pronounced hepatic destruction in gzm $A^{-/-}$ but not gzm $A^{+/+}$ mice must be due to a higher infection rate of liver cells in the mutant strain, resulting in increased cytopathic and/or Tc cell-mediated lytic processes.

Table 4. Enzymatic inactivation of EEV and INV preparations of ectromelia virus

| | | Ectromelia (pfu) | | | |
|---|---|---|---|---|---|
| | | Extracellular (EEV) | | Intracellular (INV) | |
| Treatment* | Virus dilution: | $10^{-1}$† | $10^{-2}$† | $10^{-1}$† | $10^{-2}$† |
| Buffer | | >100 | 20 | >100 | 22 |
| gzm A | | >100 | 26 | >100 | 27 |
| Plasmin | | 6 | <1 | 87 | 7 |

*Virus suspensions were treated with enzymes and subsequently plaqued as described in Material and Methods.
†Virus dilution.

Together with previous studies (10), these results clearly demonstrate that gzm A is not directly involved in cytolytic activity but that the enzyme is critical in the control of ectromelia virus infection. To this end we investigated *in vitro* the possibility that gzm A or plasmin inactivates mature viral particles by proteolytic digestion. We included plasmin as it is known that gzm A is able to activate pro-urokinase, thereby initiating a proteolytic cascade that leads to the generation of plasmin (21). Two morphological variants of ectromelia virus were used, the enveloped virus, EEV, which is released from cells by budding, and the nonenveloped or naked virus, INV, which is released from disrupted cells (13). The two virus preparations were incubated with either purified gzm A or plasmin or in control buffer for 16 h. Residual virus titers were then determined (Table 4). The number of plaques of gzm A-treated virus was not reduced over virus incubated with buffer alone. However, incubation with plasmin had a differential effect on both virus preparations, by reducing the virus titer of EEV by 1.5 logs but that of INV marginally. This may be due to the presence of a small percentage of EEV particles in the INV preparation. Although there is no ready explanation for this finding at present, one could speculate that plasmin is generated in the microenvironment of NK and Tc cells within foci of ectromelia virus infection and that it degrades the receptors on EEV required for infectivity. However a more detailed study with a wider range of enzyme concentrations will be necessary to conclusively establish the precise role of gzm A. The possibility that gzm A can act directly under pathophysiological conditions cannot be excluded.

It is also possible that other molecules and/or processes that are not present/operative in *in vitro* culture systems are involved synergistically with gzm A in the clearance of ectromelia virus. It is worth mentioning that it was shown that gzm A is also able to process interleukin 1β precursor molecules to yield the bioreactive cytokine (22). The activation of plasmin from plasminogen and the generation of active interleukin 1 are thought to be involved in inflammatory processes (21, 22) and thus may contribute, directly or indirectly, to the clearance of ectromelia virus.

Furthermore, it is reasonable to imply that Tc/NK cells employ both, gzm A and gzm B, in addition to perforin (5) for optimal control of viral infections: gzm B to induce DNA fragmentation in infected target cells (4, 6) and gzm A to initiate an as yet unknown mechanism(s) (10). However, in ectromelia infection, gzm B in Tc/NK cells may not be active because of its inhibition by a viral cysteine protease inhibitor, crmA, recently described for cow pox virus (23). CrmA is probably also encoded by other pox viruses, including ectromelia. This assumption is supported by previous findings showing that crmA inhibits the IL-1β-converting enzyme (23), a pro-

tease with similar specificity to gzm B. Together with these observations, the present study indicates that in the absence of gzm B activity due to viral-mediated inhibition, gzm A is critical for the elimination of ectromelia virus from the host.

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