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Role of Natural Killer Cells in Infection with the Mouse Pneumonitis Agent (Murine *Chlamydia trachomatis*)

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Natural killer (NK) activity is increased in both spleen and lung early in pulmonary infection by murine *Chlamydia trachomatis* in both susceptible nude and resistant heterozygous (*nu*/+) mice. Ablation of the rise in NK activity by giving the mice antiasialo GM-1 antibody or stimulation of NK activity by immunomodulators did not affect quantitative tissue counts of the mouse pneumonitis biovar of *C. trachomatis* or significantly affect survival. Studies are needed to further define the role of NK cells in host defense, immunoregulation, and immunopathology during chlamydial infection.

Ample precedent exists for a possible role for natural killer (NK) cells in host defense against nonviral intracellular organisms such as *Toxoplasma gondii* (5, 6) and *Salmonella typhimurium* (11), as well as other nonviral pathogens such as *Cryptococcus neoformans* (10). Further, cytotoxic activity of relatively uncharacterized type has been shown during infection with *Chlamydia psittaci* (2, 9) in mice by use of spleen cells or spleen-derived cytokines as effectors. However, an attempt to find cytotoxic T-cell activity in mice infected with *Chlamydia trachomatis* was unsuccessful (12), as was an attempt with humans (13). We felt this provided ample impetus to further explore the issue of cellular cytotoxicity in *C. trachomatis* infection by monitoring NK activity during infection by use of the mouse pneumonitis biovar of *C. trachomatis* (MoPn). Further, we decided to examine NK cell activity in the actual organ infected (in this case, lung) as well as in the spleen. We also decided to use the standard NK assay with YAC-1 uninfected tumor target cells instead of the macrophage and fibroblast targets used in previous studies (2, 9). We were fortunate that a reliable method for measuring NK function in the murine lung has recently been described (14).

Our model of murine pneumonia caused by MoPn in both nude (*nu/nu*) (susceptible) and heterozygous (*nu*/+) (resistant) mice has been presented in detail in recent publications (15–17).

MATERIALS AND METHODS

MoPn. MoPn was obtained and maintained as previously described (16, 17). The undiluted titer of MoPn was 4×10^9 inclusion-forming units (IFU)/ml.

Inoculation of mice. Groups of 5 to 20 mice were inoculated under pentobarbital anesthesia with 0.05 ml of MoPn agent (16, 17). The infection dose was 5×10^3 IFU per mouse.

Mice. Specific-pathogen-free *nu/nu* and *nu*/+ mice (parent strain BALB/c) were obtained and maintained as previously described (16). They had been made superclean by using germ-free foster mothers and repopulating them with limited bacterial flora of organisms nonpathogenic to mice (16). Mice of both sexes (6 to 8 weeks old) were used in these experiments.

Tumor cell lines. These studies made use of the Moloney virus-induced lymphoma, YAC-1, which was grown as a suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, and glutamine. This cell line was free of mycoplasma and MoPn by culture.

NK assay. The spleen NK assay was the standard 4-h assay (5, 6, 10, 11) with splenic effectors from infected and uninfected *nu*/+ or *nu/nu* mice. The effector/target (E/T) ratios were 100:1, 50:1, and 25:1 with determination of $Na_2{}^{51}CrO_4$ release. Incubation time was 4 h at 37°C and 5% CO_2, at which time a 100-μl sample of the supernatant was counted in a gamma spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). Spontaneous release was determined in media alone, and maximum release was determined with 5% Triton X. All tests were run in triplicate on single mice. Percent cytotoxicity was calculated as follows: % cytotoxicity = [(cpm effectors + targets) − (cpm spontaneous)]/[(maximum releasable cpm) − (cpm spontaneous)]. Spontaneous release was always less than 10% of maximum release.

Isolation of pulmonary NK cells. Isolation of pulmonary NK cells was done according to the method of Talmadge et al. (14). Mice were exsanguinated, the pulmonary vasculature was repeatedly perfused with normal saline to remove residual blood cells, and the lungs were washed by tracheobronchial lavage. The lungs were removed, thymic and bronchial tissues were excised, and the remaining pulmonary tissue was minced and dissociated by two sequential 30-min incubations with 0.14% collagenase and 0.03% DNase. The resulting suspension was twice washed and layered onto a Lympholyte-M Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) gradient with centrifugation at 500 × *g* for 30 min. The effector cells were harvested, washed, and used at effector target ratios of 50:1 and 25:1 in the NK assay as above.

Modulation of NK function. NK function was stimulated by giving 100 μg of poly(I) · poly(C) (Sigma Chemical Co., St. Louis, Mo.) intravenously (i.v.) 18 h before infection with MoPn. NK function was ablated by giving 200 μg of antiasialo GM-1 (Wako Chemicals) i.v. at days 0 and 3 of MoPn infection.

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TABLE 1. Spleen NK function in *nu/+* and *nu/nu* mice

E/T ratio	% Cytotoxicity[a] for:									
	Uninfected mice		Infected mice, days after infection							
			2		5[b]		10		15	
	nu/+	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*[b]	*nu/+*	*nu/nu*
100:1	11.2 ± 3.7	16.1 ± 5.0	12.6 ± 4.0	17.4 ± 4.0	34.6 ± 7.4	45.1 ± 5.7	15.5 ± 4.0	42.5 ± 10.7	12.8 ± 7.8	—[c]
50:1	8.8 ± 3.4	10.8 ± 4.3	9.7 ± 3.7	13.0 ± 6.7	27.7 ± 7.1	37.0 ± 7.8	11.3 ± 3.6	31.11 ± 8.3	8.5 ± 5.9	—
25:1	5.7 ± 2.1	7.1 ± 2.8	6.7 ± 2.5	9.3 ± 3.1	19.2 ± 5.9	27.6 ± 4.2	6.7 ± 2.4	21.5 ± 4.8	6.0 ± 4.5	11.0 ± 0.2

[a] Results are the mean ± standard deviation of data from three mice in each group.
[b] $P < 0.5$ for all values compared with those of uninfected mice.
[c] —, Insufficient cells obtained from very ill mice.

Quantitative culture of lungs for MoPn. Lungs were harvested, minced, and quantitatively cultured for MoPn in McCoy cell culture as in our previous study (17).

Statistics. Statistics were by the Student *t* test with correction for unequal variances.

RESULTS

Table 1 shows the results of NK assays on spleens from *nu/+* and *nu/nu* mice at days 2, 5, 10, and 15 after intranasal infection with MoPn, compared with results of assays on spleens from uninfected *nu/+* and *nu/nu* mice. A statistically significant elevation of NK function occurred in both *nu/+* and *nu/nu* spleen cells at day 5 postinfection with a return to base line by day 10 in *nu/+* spleens. *nu/nu* NK values, however, remained elevated until day 15 (although complete testing was not obtained on that day because of very small spleens in terminally ill *nu/nu* mice).

Of perhaps more biological relevance (Table 2), a similar significant increase occurred in lung NK function (the organ where the infection was occurring) at day 5 postinfection. Thus, significant elevation of NK function occurred in a significant anatomical compartment according to the standard NK assay during infection with a *C. trachomatis* biovar (MoPn).

To determine whether NK function changes had an effect on infection with MoPn (a role in host defense as opposed to immunomodulation alone), NK function was increased by giving poly(I) · poly(C) 18 h before infection or ablated by giving two doses of antiasialo GM-1 during infection with subsequent quantitative culture of MoPn in lungs.

Poly(I) · poly(C) significantly increased NK function in lung 18 h later so that it would be elevated at the time of infection in mice given poly(I) · poly(C) the day before (Table 3). The table also shows that antiasialo GM-1 prevented the normal increase of pulmonary NK function seen by day 5 of infection.

Next, quantitative culture of *nu/+* lungs for MoPn was performed at various times after infection in immunomanipulated and unmanipulated mice. Poly(I) · poly(C) given 18 h before infection to increase NK activity at the time of infection had no effect on MoPn IFU per lung, compared with controls on either day 2 or 3 of infection ($P > 0.30$ compared with controls). Mean IFU per lung at day 2 were 3,390 ± 1,349 in mice given no poly(I) · poly(C) and 3,486 ± 1,159 in mice given poly(I) · poly(C). The values at day 3 were 4,135 ± 1,821 and 4,775 ± 2,070, respectively, in the same two groups. Each value is the mean ± standard deviation of results for five mice. Conversely, antiasialo GM-1 antibody given i.v. to *nu/+* mice to block the normal rise in NK activity by day 5 of infection (Tables 1 to 3) had no effect on MoPn IFU per lung on day 5 postinfection. Values were 13,848 ± 9,784 IFU per lung (unmanipulated *nu/+*), 14,194 ± 4,609 (*nu/+* given antiasialo GM-1), and 13,225 ± 7,509 (unmanipulated *nu/nu*). Thus, no significant effect on MoPn titers was achieved by immunomodulatory techniques designed to increase NK function at the time of infection [poly(I) · poly(C) 18 h earlier] or prevent the normal increase of NK activity with infection (antiasialo GM-1).

Finally, a series of experiments was performed in vivo, giving mice immunomodulators known to increase NK function. We are aware that any immunomodulator is likely to influence other immunologic parameters in addition to NK function over the duration of an experiment, and we recognize that these experiments are likely to be less specific than the quantitative culture data just presented. The first experiment used murine α plus β interferon (Lee Biomolecular, San Diego, Calif.; 1.7×10^4 U) given i.v. every 2 days for a total of four doses. Pulmonary NK activity measured 1 day after the last dose in uninfected *nu/+* mice was significantly increased compared with control mice given excipient control material (at E/T ratio of 50:1, control mice showed a percent cytotoxicity of 0.8 ± 0.3, after interferon 5.1 ± 0.7 [$P < 0.003$]). This admittedly modest increase in NK function had no significant effect on mortality in mice given the same doses in in vivo studies. With *nu/+* mice, mortality at day 20 in mice given 10^5 IFU of MoPn intranasally was 100% in 10 mice given excipient control and 80% in 10 mice given four doses of interferon starting on day −1 ($P > 0.30$ by the

TABLE 2. Lung NK function in *nu/+* and *nu/nu* mice

E/T ratio	% Cytotoxicity for:							
	Uninfected mice		Infected mice, days after infection					
			2		5[a]		10	
	nu/+	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*	*nu/+*	*nu/nu*
50:1	1.7 ± 2.5	3.5 ± 5.0	2.6 ± 1.5	4.7 ± 1.2	44.2 ± 0.8	40.8 ± 8.0	3.7 ± 1.5	4.6 ± 1.4
25:1	1.0 ± 1.1	3.5 ± 4.4	1.9 ± 0.03	3.5 ± 0.5	29.1 ± 1.3	40.1 ± 7.7	3.5 ± 1.0	4.4 ± 1.6

[a] $P < 0.05$ for all values compared with those of uninfected mice.

TABLE 3. Effect of immunomodulation on pulmonary NK function

E/T ratio	% Cytotoxicity with:					
	Poly(I) · poly(C)[a]		No poly(I) · poly(C) (control)		Antiasialo GM-1[b]	
	nu/+	*nu/nu*	*nu/+*	*nu/nu*	Normal *nu/+*, day 5[c]	*nu/+* antiasialo GM-1, day 5
50:1	46.3 ± 14.3	59.9 ± 4.1	0.9 ± 0.4	2.7 ± 0.8	22.9 ± 2.7	1.7 ± 0.4
25:1	44.0 ± 17.1	58.5 ± 5.7	0.1 ± 0.1	0.4 ± 0.1	18.2 ± 3.7	1.3 ± 0.5

[a] 100 μg of poly(I) · poly(C) given i.v. 18 h earlier; mice not infected. $P < 0.05$ compared with control [no poly(I) · poly(C)] for all values.
[b] 200 μg of antiasialo given i.v. on days 0 and 3 of infection with MoPn. NK assay done on day 5 of MoPn infection.
[c] $P < 0.05$ compared with antiasialo for both values.

Wilcoxon two-tail test). With 10^4 IFU of MoPn, mortality was 35% in controls and 50% in interferon-treated mice ($P > 0.30$ by the Wilcoxon two-tail test; 20 mice per group).

Poly(I) · poly(C) given at 100 μg i.v. every 3 days for four doses significantly increased NK function in *nu/+* uninfected mice compared with controls given phosphate-buffered saline (at an E/T ratio of 50:1, control mice showed a percent cytotoxicity of 3.5 ± 1.1, after poly(I) · poly(C) 70.8 ± 7.8 [$P < 0.005$]).

The same regimen with MoPn-infected *nu/+* mice (starting the day before infection) led to no significant mortality difference [mortality: 100% in control; 100% in poly(I) · poly(C)-treated mice at day 20 postinfection; $P > 0.30$ by the Wilcoxon two-tail test; 10 mice per group]. Poly(I) · poly(C) was not toxic to uninfected *nu/+* mice (0% mortality at day 20).

Repeated doses of 200 μg of antiasialo given i.v. were somewhat toxic to the mice in our study and not suitable for mortality studies.

Recognizing that these mortality experiments are not specific studies of NK function and that positive benefits of early NK stimulation could be counterbalanced or outweighed by other effects of these immunomodulators, no significant beneficial effect was observed.

DISCUSSION

These studies demonstrate that pulmonary infection with murine *C. trachomatis* leads to an increase in both spleen and lung NK cell tumoricidal activity, peaking early after infection and declining thereafter. This increase is preventable by treatment of the mice with antiasialo GM-1. The significance of this NK activity is unclear. As discussed in the introduction, NK cells may be directly cytotoxic for some pathogens including nonviral pathogens such as *T. gondii* (5) and *Cryptococcus neoformans* (10) and might play a role in the control of these infections before specific immunity develops. In addition, NK cells also may play an immunoregulatory role. NK cells can suppress the generation of Lyt 2^+ cytotoxic T cells by suppressing or eliminating dendritic cells (4), can suppress B-cell function (1), and can be involved in producing imunomodulators such as interferon (3). Our previous data have shown that T cells are critically important in host defense against pneumonia caused by MoPn in our model (15–17). Nude mice, which frequently have increased NK cell activity (7), were more susceptible to MoPn than were *nu/+* animals, suggesting that specific (T-cell-dependent) immunity is more important to ultimate survival in our model than is early natural or nonspecific immunity. However, our *nu/nu* mice NK activity was not as high endogenously as has been reported previously (7, 8), perhaps because our mice were superclean and had low-level background stimulation. Thus, the fact that our *nu/nu* mice demonstrated no early increased resistance to MoPn with similar lung titers to *nu/+* on day 5 cannot be used to exclude a role for NK cells in host defense against MoPn. However, the facts that ablation of the rise in NK activity by antiasialo GM-1 antibody and stimulation of NK activity by NK inducers had no significant effect on MoPn lung titers and that NK inducers did not delay mortality due to MoPn suggest that the in vivo role of NK cells in directly controlling the infection is at best small. We are in the process of further examining this point by studying the in vitro effects of NK cells on MoPn by using Percoll (Pharmacia) gradient-enriched populations. It appears likely, however, that the in vivo role is one primarily of immunomodulation.

As stated in the introduction, the generation of factors cytotoxic to *Chlamydia* sp.-infected cells during *C. psittaci* infection has been described (2, 9). Currently, the cell type(s) responsible for generation of these factors is unclear, as is its role in host defense or immunopathology. The in vivo role, if any, could be either beneficial or detrimental to the host. Concanavalin A-induced cytotoxic factors can be generated during MoPn infection in our model as well (G. Byrne, D. Williams, and J. Schachter, unpublished data), but optimal generation occurs later than day 5, and the role, if any, of NK cells in the generation of such factors is unclear.

This study demonstrates a significant increase in NK function during chlamydial infection. Further studies are needed to define the role of NK cells in host defense, immunoregulation, and immunopathology during chlamydial infection.

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