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Early Gamma Interferon Production by Natural Killer Cells Is Important in Defense against Murine Listeriosis

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A spot enzyme-linked immunosorbent assay was used to show that subcutaneous inoculation of a sublethal number of *Listeria monocytogenes* resulted in the early appearance of gamma interferon (IFN-γ)-producing cells in the draining lymph nodes. In contrast, inoculation of UV-killed *L. monocytogenes* failed to cause the appearance of IFN-γ-producing cells. The appearance of IFN-γ-secreting cells in response to the living organisms peaked at 24 h of infection and then declined. The draining lymph node cells responsible for secreting IFN-γ belonged to a cell population that was positive for the NK1.1, asialo-GM_1, and Thy-1 markers but negative for the CD4 and CD8 T cell subset markers. Early elimination of natural killer (NK) cells by treatment with anti-NK cell antibodies resulted in severe exacerbation of infection, as did early neutralization of endogenous IFN-γ by treatment with a rat anti-murine IFN-γ monoclonal antibody. In contrast, depletion of $CD4^+$ and $CD8^+$ T cells failed to exacerbate infection. The results serve to show that the early production of IFN-γ by NK cells, rather than by T cells, is an essential event in resistance to listeriosis.

It is well documented (26) that gamma interferon (IFN-γ) plays an important role in resistance to infections with intracellular pathogens, presumably by virtue of its ability to activate the microbicidal activity of host macrophages (5, 32, 33). It has been shown (4, 30) in the case of murine listeriosis that the neutralization of endogenous IFN-γ by a specific anti-IFN-γ monoclonal antibody (MAb) can result in a severe, and in some cases lethal, infection. Moreover, according to one study, to cause exacerbation of infection, anti-IFN-γ MAb needs to be given just before, or up to 1 day after, mice are inoculated with *Listeria monocytogenes* (30). Additional evidence in support of a role for IFN-γ in immunity to *L. monocytogenes* comes from a publication (19) showing that the administration of recombinant IFN-γ results in the increased resistance of mice to a subsequent challenge infection with this organism.

Because it is well known that T cells are major producers of IFN-γ (45), it might be expected that these cells are responsible for IFN-γ production in mice infected with *L. monocytogenes*. Indeed, it has been shown that splenic T cells from 6-day-infected mice produce IFN-γ when cultured in the presence of killed (14, 28) or live (3) *L. monocytogenes* in vitro. That T cells are important in resistance to infection with this bacterium is suggested by data from several laboratories (1, 23, 36) showing that T cells from 6-day-infected mice can, on passive transfer, protect naive mice against a lethal *L. monocytogenes* inoculum. It is generally believed, as was first suggested by Mackaness (21), that T cells operate in adoptively immunized recipients to activate the listericidal function of macrophages. Given that IFN-γ has been implicated as having a role in macrophage activation (5, 32, 33), this proposed in vivo function of T cells would seem to be in keeping with their ability to secrete IFN-γ after antigen exposure in vitro.

However, an essential role for T cells in resistance to infection with *L. monocytogenes* can be questioned, given the finding that athymic mice (34, 35) and euthymic BALB/c mice depleted both of $CD4^+$ and $CD8^+$ T cells by treatment with appropriate MAbs (40) display a surprising ability to resist infection. Therefore, if IFN-γ is essential for resistance to listeriosis, it is likely that it is produced by cells other than T cells. Of relevance to this possibility is a recent publication (31) showing that maximum endogenous IFN-γ production in response to listeriosis occurs on day 2 of infection, before T cell-mediated immunity is generated (37). Natural killer (NK) cells must be considered possible candidates for the production of this IFN-γ, because there are a number of published reports (reviewed in references 41 and 46) showing that NK cells secrete IFN-γ in response to microbial pathogens.

The purpose of the study presented here was to determine whether T cells or NK cells are responsible for the IFN-γ production that is essential for the early control and resolution of murine listeriosis. We show that the early production of IFN-γ is carried out predominantly by cells that are identified as NK cells by their morphology and surface markers. We also show that peak numbers of IFN-γ-secreting cells are present at 24 h of infection and that their elimination by anti-NK cell reagents results in severe exacerbation of infection.

MATERIALS AND METHODS

Animals. $CB6F_1$ male mice were supplied by the Trudeau Institute Animal Breeding Facility (Saranac Lake, N.Y.) and were used for experiments at 8 to 10 weeks of age.

Bacteria. *L. monocytogenes* EGD (serotype 3b) contained in a spleen homogenate from infected $CB6F_1$ mice was used to seed a Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) culture, which was harvested during the log phase, dispensed in 1-ml aliquots, and frozen at −70°C until needed. An inoculum of bacteria was prepared for injection by thawing an aliquot and diluting it appropriately in 0.9% NaCl solution. Infections were initiated by injecting 10^6 *L. monocytogenes* in 0.05 ml of saline subcutaneously (s.c.) into the right hind footpad. The 50% lethal doses for $CB6F_1$ mice were about 2×10^4 for mice inoculated intravenously (i.v.) and more than 10^7 for mice inoculated s.c. in the footpad. Bacterial growth was monitored by plating

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serial 10-fold dilutions of homogenates of footpads, draining lymph nodes (DLN), or spleens on Trypticase soy agar and enumerating colonies after 24 h of incubation at 37°C. UV-killed *L. monocytogenes* was used as an antigen to stimulate popliteal lymph node cells in the spot enzyme-linked immunosorbent assay (ELISA) (see below). UV exposure was performed by a published procedure (11).

Antibodies and antisera. Rat anti-murine IFN-γ MAb was produced by the R46A2 hybridoma (12) growing as ascites fluid in the peritoneal cavity of $CB6F_1$ mice primed with pristane. The MAb was precipitated with sodium sulfate and purified by DEAE anion-exchange chromatography (12). A rabbit anti-murine IFN-γ polyclonal antiserum (13) was also used in the spot ELISA. The neutralizing titers (units per milliliter) for the monoclonal and polyclonal anti-IFN-γ preparations were determined in a biological assay (13) and are defined as the reciprocal of the dilution of antibody which neutralizes 50% of the antiviral activity of 20 U of recombinant murine IFN-γ.

Ascites fluids containing anti-NK cell MAbs from hybridomas PK136 (20), SW5EC (42), and SW10A7 (generated in Michael Bennett's laboratory at the University of Texas Southwestern Medical Centre, Dallas) were kindly provided by Henry Winn (Massachusetts General Hospital, Boston). MAb from hybridoma PK136 binds to the NK1.1 marker on NK cells (20), whereas MAbs from SW5EC and SW10A7 bind to overlapping subsets of $NK1.1^+$ cells (42). The anti-NK1.1 antibody was purified from whole ascites fluid by immunoaffinity chromatography and was conjugated directly to fluorescein isothiocyanate (FITC). It was used for staining cells for flow cytometric analyses (see below). Polyclonal rabbit anti-asialo-GM_1 antiserum was obtained from WAKO Pharmaceuticals, Dallas, Tex., and polyclonal FITC-conjugated goat $F(ab')_2$ anti-rabbit immunoglobulin (Ig) was obtained from Sigma Chemical Co., St. Louis, Mo.

The anti-T cell MAbs used were anti-CD4 (clone GK1.5; provided by Frank Fitch, University of Chicago, Chicago, Ill.), anti-CD8 (clone TIB 210; American Type Culture Collection, Rockville, Md.), and anti-Thy-1.2 (clone 30.H.12; American Type Culture Collection). The production of MAb-containing ascites fluids, purification of MAbs, and conjugation of $F(ab')_2$ fragments to FITC were done as described in detail elsewhere (7).

In vivo cell depletion. In vivo depletion of NK cells was achieved 1 day before infection by intraperitoneal injection of 0.2 ml of phosphate-buffered saline (PBS) containing 40 μl of rabbit anti-asialo-GM_1 antiserum or 0.1 ml each of SW5EC and SW10A7 whole ascites fluids. An equivalent amount of normal rabbit or mouse IgG (Sigma) was used to control for the nonspecific effects of injecting foreign Ig. T cell depletion was achieved by injecting mice i.v. with 0.2 ml of PBS containing 2.5 mg of anti-CD4 or anti-CD8 MAb per ml 2 days before infection. The extent of T cell depletion was determined by flow cytometry (see below).

In vivo neutralization of IFN-γ. Endogenously produced IFN-γ was neutralized by injecting mice intraperitoneally with 10^5 neutralizing units of anti-IFN-γ MAb (specific activity, 1.8×10^5 neutralizing units per mg of IgG) 3 h before infection. Control mice received the same quantity of purified rat IgG (Sigma).

Spot ELISA. The procedure used to identify IFN-γ-producing cells was based on a spot ELISA described by others (43). It uses a 96-well nitrocellulose membrane filtration plate (Millititre HA; Millipore Corp., Bedford, Mass.). To obtain lymph node cells, we carefully cut DLN or contralateral lymph nodes (CLN) of mice infected with *L. monocytogenes* into pieces and pressed them through a 200-mesh stainless steel screen with a rubber plunger. The resulting cell suspension was centrifuged and resuspended in culture medium containing 10% heat-inactivated fetal calf serum (RPMI–10% FCS). One lymph node equivalent of the cell suspension in 100 μl of culture medium was added to the first well of the 96-well flat-bottomed ELISA plate, which previously had been treated overnight at 4°C with a 1-μg/ml solution of purified rat anti-murine IFN-γ MAb, blocked for 2 h at 37°C with PBS containing 2% bovine serum albumin, and washed. The cells contained in 100 μl of culture medium were serially titrated in twofold dilutions across 12 wells of the plate. Each well then received an additional 100 μl of culture medium or 100 μl of culture medium containing either 10^6 UV-killed *L. monocytogenes* or 5 μg of purified phytohemagglutinin (PHA; purchased from Wellcome Diagnostics, Research Triangle Park, N.C.) per ml. The plate was incubated undisturbed overnight in a humidified atmosphere of 5% CO_2 at 37°C. Each cell population was assayed in triplicate. Positive controls consisted of wells treated with 100 μl of a crude murine IFN-γ preparation (approximately 500 U/ml) in place of cells. Negative controls consisted of wells to which 100 μl of diluent was added. After overnight incubation, the plate was vigorously washed to remove the cells and the IFN-γ was detected by the addition of rabbit anti-IFN-γ polyclonal antiserum (diluted 1/5,000 in PBS containing 1% bovine serum albumin) for 1 h at room temperature, by washing, and by the addition of alkaline phosphatase-conjugated goat anti-rabbit Ig (diluted 1/1,500 in PBS containing 1% bovine serum albumin and 50 μl of normal goat serum per ml). After the plate was allowed to stand for 1 h at room temperature, the wells were washed and the enzyme substrate 5-bromo 4-chloro-3-indolyl phosphate-*p*-toluidine salt (BRL Life Technologies, Gaithersburg, Md.) was added, as was the catalyst nitroblue tetrazolium chloride (BRL Life Technologies). Fifteen minutes later, when blue dots had developed on the membranes, the reaction was terminated by rinsing with distilled water. The membranes were dried, and the dots were counted at a magnification of ×4 under an inverted microscope. The mean number of IFN-γ-secreting cells counted in replicate samples at a single dilution was used to calculate the total number of IFN-γ-secreting cells per lymph node. Samples in which less than 200 dots were present in the first well were considered unreliable estimates and were not included. The results are reported as the mean ± standard deviation of the number of IFN-γ-producing cells per lymph node.

Flow cytometric analysis. Cells from the DLN or CLN of infected mice were examined after reaction with the appropriate fluorescent reagents by use of a FACScan (Becton Dickinson, Sunnyvale, Calif.) for the presence of cells with T cell or NK cell surface antigens. T cell staining was generally carried out with FITC-conjugated $F(ab')_2$ anti-CD4 and anti-CD8 MAbs (final dilution, 1/500). In one experiment, however, a three-label analysis was carried out by treating the cells first with biotinylated $F(ab')_2$ anti-Thy-1.2 MAb and then, after washing, with FITC-$F(ab')_2$ anti-CD4, FITC-$F(ab')_2$ anti-CD8, and streptavidin-phycoerythrin. Cells bearing the NK cell surface antigen, NK1.1, were detected by direct staining with FITC-conjugated anti-NK1.1 MAb (final dilution, 1/500). To determine the percentage of cells bearing the asialo-GM_1 surface marker, we incubated the cells for 1 h at 4°C with polyclonal rabbit anti-asialo-GM_1 antiserum (final dilution, 1/200), washed them, and incubated them with polyclonal FITC-conjugated goat anti-rabbit Ig for 1 h at 4°C. After being stained, they

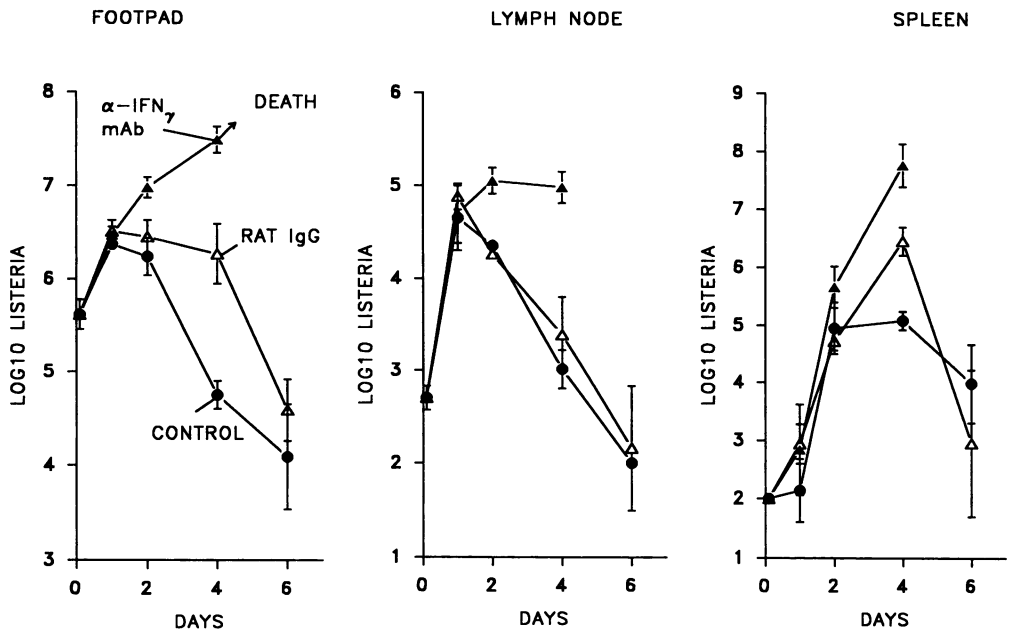

FIG. 1. Growth curves of *L. monocytogenes* in the footpads, lymph nodes, and spleens of anti (α)-IFN-γ MAb-treated, IgG-treated, or untreated mice. Mice were injected with 10^6 bacteria s.c. in the right hind footpad, and viable organisms were enumerated at progressive times. Anti-IFN-γ treatment caused progressive bacterial growth that resulted in death of the mice, whereas untreated mice and those treated with rat IgG controlled their infection and survived. Each point represents the mean ± standard deviation for five mice per group.

were washed and resuspended in sheath buffer before analysis. In every case, propidium iodide (2 μg/ml) was added to stain dead cells that were excluded from the analysis.

Density gradient centrifugation. Popliteal DLN or CLN from mice inoculated 1 day earlier with *L. monocytogenes* were harvested, and cell suspensions were prepared as described above. Adherent cells were removed from the cell suspensions by plating on plastic petri dishes for 2 h at 37°C. Nonadherent cells (2×10^7 to 5×10^7 contained in 1 ml of RPMI–10% FCS) were removed from the dishes, washed, loaded onto a five-step discontinuous Percoll gradient (27), and centrifuged for 40 min at 300 × *g*. The fractions obtained were numbered from 0 to 5, with 0 being the cells that failed to enter the gradient and 1 being the fraction with the lowest density. Cells in each fraction were washed three times in RPMI–10% FCS to remove excess Percoll before being subjected to the spot ELISA or flow cytometric analysis.

RESULTS

Treatment with a rat anti-IFN-γ MAb converts a sublethal *L. monocytogenes* infection to a lethal one. Neutralization of IFN-γ in mice infected with *L. monocytogenes* by treatment with a hamster anti-murine IFN-γ MAb has been shown to cause lethal exacerbation of a sublethal infection (4). To determine whether the rat anti-murine IFN-γ MAb available in this laboratory is also capable of converting a sublethal infection to a lethal one, we treated mice with either 10^5 neutralizing units (0.5 mg of specific IgG) of this rat MAb or 0.5 mg of control rat Ig 1 day before inoculating them s.c in the footpad with 10^6 *L. monocytogenes*.

Figure 1 shows that treatment with the rat anti-IFN-γ MAb resulted in severe exacerbation of infection. In untreated mice, the multiplication of *L. monocytogenes* was controlled in the footpad and DLN from day 1 of infection onwards and in the spleen from day 2 onwards. By contrast, in mice treated with the rat anti-IFN-γ MAb, progressive growth of *L. monocytogenes* occurred in all organs after day 1, and all of these mice died by day 6. Moreover, it was apparent that anti-IFN-γ MAb treatment caused the organism to reach the spleens at an earlier time, thereby indicating that the organism was disseminated more rapidly from the site of inoculation in treated mice. However, there was also some enhancement of bacterial multiplication in the footpads and spleens of mice given control Ig, although in all cases mice so treated were able to almost completely resolve the infection by 6 days. This exacerbating action of control Ig indicates that the exacerbation caused by anti-IFN-γ MAb treatment was partly the result of a depressive effect on host resistance of foreign Ig. This result serves to show the importance of including controls for the effect of foreign Ig in experiments that involve studying the effect of MAbs in vivo.

IFN-γ is produced by non-T cells during the first 2 days of infection. The foregoing results showing that the neutralization of IFN-γ results in an increase in the multiplication and dissemination of *L. monocytogenes* by 24 to 48 h of infection imply that IFN-γ is essential for early resistance to infection. It was anticipated, therefore, that cells making this lymphokine would be found in responding lymphoid tissue during the first 24 h of infection. Thus, a spot ELISA was used to enumerate IFN-γ-secreting cells in the popliteal nodes of mice inoculated in a hind footpad with 10^6 *L. monocytogenes*. In this experiment, T cell-depleted, as well as T cell-intact, mice were examined to determine whether T cells are directly or indirectly involved in the production of IFN-γ. Individuals of an additional group of mice were inoculated s.c. in the hind footpad with 4×10^6 UV-killed *L. monocytogenes* to determine whether nonreplicating bacteria are capable of inducing the appearance of IFN-γ-secreting cells.

TABLE 1. Numbers of IFN-γ-secreting cells in the DLN and CLN of mice infected for 1 day with *L. monocytogenes*

MAb treatment[a]	*L. monocytogenes*[b]	Node	In vitro stimulation[c]	IFN-γ-secreting cells[d]		% Stained cells[e]		
				Total/node (10^2)	Frequency	Thy-1$^+$	CD4$^+$	CD8$^+$
None	Live	DLN	None	123.0 ± 27.0	1/325	39.3	20.9	19.8
			L. monocytogenes	106.0 ± 30.0	1/377			
			PHA	160.0 ± 12.0	1/250			
		CLN	None	BDL[f]		63.2	36.4	25.2
			L. monocytogenes	BDL				
			PHA	8.4 ± 3.1	1/1,290			
	Dead	DLN	None	17.0 ± 11.4	1/2,611	42.0	19.9	18.2
Anti-CD4 plus anti-CD8	Live	DLN	None	132.0 ± 12.0	1/395	6.90	0.14	0.08
			L. monocytogenes	137.0 ± 44.0	1/438			
			PHA	124.0 ± 26.0	1/484			
	Dead	DLN	None	10.5 ± 9.2	1/1,743	5.0	0.00	0.10

[a] MAbs (500 μg of each) were given i.v. 2 days before infection.
[b] 1×10^6 live or 4×10^6 UV-killed *L. monocytogenes* organisms were inoculated in the right hind footpad.
[c] Lymph node cells were incubated overnight in the presence of killed *L. monocytogenes* or PHA.
[d] Popliteal DLN and CLN were harvested on day 1 of infection, and the number of IFN-γ-secreting cells was enumerated in a spot ELISA. The results represent the mean ± standard deviation for five replicates obtained with pooled lymph node cells from five mice per group.
[e] As determined by flow cytometry analysis.
[f] BDL, below detectable level ($<2 \times 10^2$).

At 24 h of infection, the DLN of T cell-intact mice contained large numbers of cells (1.2×10^4 per DLN), at a frequency of about 1 of 300, which secreted IFN-γ spontaneously when placed in culture alone and that the frequency was no higher in the presence of UV-killed *L. monocytogenes* or PHA (Table 1). Depleting the host of T cells before initiating infection had little effect on the total number of IFN-γ-secreting cells produced (Table 1). Therefore, the majority of IFN-γ-secreting cells were not T cells. Additional experiments (data not shown) showed that as early as 6 h of infection there were already about 6.6×10^3 IFN-γ-secreting cells in the DLN and that on day 3 their number had dropped to less than 2×10^3.

In mice given an intrafootpad inoculum of 4×10^6 UV-killed *L. monocytogenes* (Table 1), the number of IFN-γ-producing cells in the DLN was only 14% of the number in the DLN of mice inoculated with 1×10^6 live organisms. However, UV-killed *L. monocytogenes* caused the cellularity of the DLN to increase to the same extent as did live organisms, causing a similar reduction in the percentage of T cells in the total cell population. No increase in cellularity or in the number of IFN-γ-secreting cells was seen in the CLN of mice given either live or UV-killed organisms, although a small number of cells that were capable of secreting IFN-γ in response to stimulation with PHA were present. These cells, however, were not present in the CLN of T cell-depleted mice (data not shown).

NK cells are responsible for the early secretion of IFN-γ. Since it was shown above that the cells in the DLN that secrete IFN-γ at 24 h of infection are not T cells, experiments were performed to determine whether NK cells are responsible for secreting this lymphokine. To investigate this possibility, we removed adherent cells from the DLN or CLN cell populations of mice infected 1 day earlier with *L. monocytogenes* and subjected the nonadherent cells to density gradient fractionation, which separated most large granular lymphocytes (LGL) from other cell types. The morphology, surface phenotype, and IFN-γ-producing ability of cells in the different fractions were examined.

Greater than 80% of the recovered IFN-γ-secreting cells were found in the lowest-density fractions (1 and 2) of DLN cells, whereas none were found in the higher-density fractions (3 to 5) (Table 2). Stained cytospin preparations (data not shown) of the cells in various fractions showed that fractions 1 and 2, but not fractions 3 and 4, contained a high proportion of LGL, in contrast to the original unfractionated population, which contained a very small proportion of LGL.

Flow cytometric analysis (Fig. 2) revealed that the fraction of DLN cells that contained most of the IFN-γ-secreting cells (fraction 2) also contained a large percentage of cells positive for the asialo-GM_1 and NK1.1 markers. Fraction 3, on the other hand, contained few of these cells, and they were essentially absent in the remaining higher-density fractions, i.e., fractions 4 and 5 (data are shown only for fraction 4). Although fraction 1 was found to contain some IFN-γ-secreting cells, the FACScan data for this fraction are not

TABLE 2. Number of IFN-γ-secreting cells in density gradient fractions of DLN cells of mice infected for 1 day with *L. monocytogenes*

Fraction[a]	IFN-γ-secreting cells/node equivalent[b]	
	No. recovered (10^2)[c]	% of total cells recovered
0	7.67 ± 2.3	4.78
1	41.80 ± 4.4	26.10
2	93.93 ± 7.5	58.64
3	14.07 ± 3.3	8.78
4	2.62 ± 2.9	1.64
5	BDL[d]	0.0

[a] Pooled cells of 10 DLN separated into six separate fractions (bands) when subjected to centrifugation through a discontinuous Percoll gradient as described in Materials and Methods.
[b] As determined by the spot ELISA.
[c] A total of 1.34×10^4 IFN-γ-secreting cells per DLN were present in the unfractionated starting population.
[d] BDL, below detectable level ($<2 \times 10^2$).

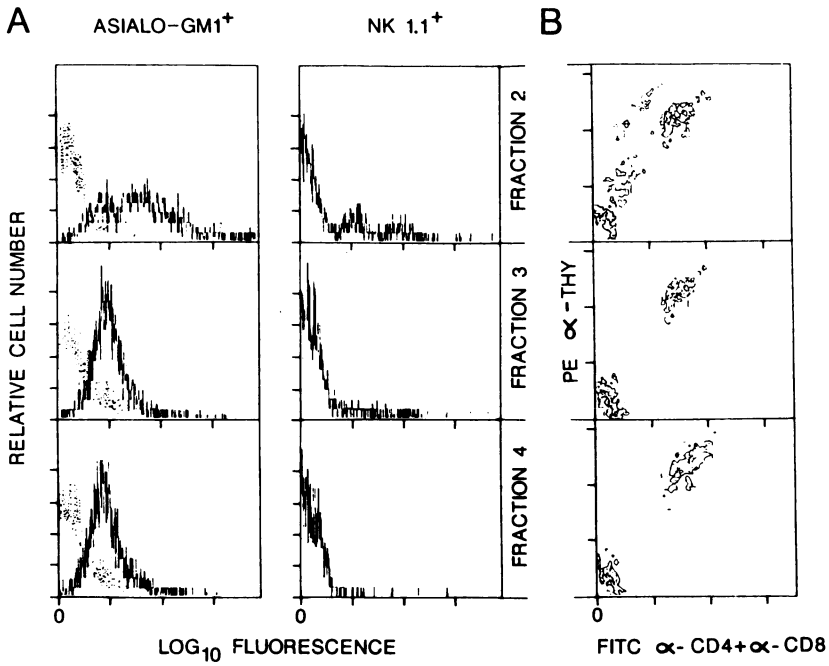

FIG. 2. Evidence that cells with NK cell markers in the responding lymph node of mice infected 1 day earlier can be assigned to a low-density fraction. Nonadherent lymph node cells fractionated on a discontinuous Percoll gradient were analyzed by flow cytometry by the procedures outlined in Materials and Methods. (A) Fraction 2 of the lymph node suspension contained an appreciable percentage of cells that stained with FITC–anti-asialo-GM_1 antiserum (left) or with FITC–anti-NK1.1 MAb (right). (B) Results of a dual-color fluorescence analysis of cells stained with biotinylated $F(ab')_2$ anti-Thy-1.2 MAb (α-THY), FITC-$F(ab')_2$ anti-CD4 (α-CD4), FITC-$F(ab')_2$ anti-CD8 (α-CD8), and streptavidin-phycoerythrin. A proportion of the cells in fraction 2 were $CD4^-$ $CD8^-$ $Thy\text{-}1^+$ (low).

presented because the presence of a relatively high percentage of residual macrophages not deleted in the adherence step made the detection of specifically stained asialo-GM_1^+ and $NK1.1^+$ cells difficult because of the ability of such macrophages to bind the FITC reagents nonspecifically. However, the number of positively stained cells observed in this fraction was still far lower (<8% of total cells) than that observed in fraction 2 (about 17%), which contained almost 60% of the total IFN-γ-secreting cells. According to the three-label flow cytometric analysis with FITC-$F(ab')_2$ anti-T cell reagents (Fig. 2), two different $Thy\text{-}1^+$ $CD4^-$ $CD8^-$ cell populations were present in fraction 2. One of these expressed a low level of the Thy-1 antigen, whereas the other expressed a higher level, similar to that expressed by $CD4^+$ and $CD8^+$ T cells. It is well documented, in this connection, that NK cells can express a low level of the Thy-1 antigen (15, 22). At this time, however, it is not possible to state with certainty that the $Thy\text{-}1^+$ (low) $CD4^-$ $CD8^-$ cells are identical to those that express the asialo-GM_1 and NK1.1 markers.

The same analytical procedures failed to show the presence of IFN-γ-secreting cells in CLN, even though a cytospin preparation of the few cells that made up fraction 2 showed the presence of a small percentage of LGL. Overall, therefore, the results are consistent with the interpretation that NK cells are responsible for secreting IFN-γ in response to an intrafootpad inoculum of *L. monocytogenes*.

In vivo depletion of T cells fails to interfere with resistance to infection. Given the foregoing evidence that IFN-γ is necessary for resistance to listeriosis and that IFN-γ is secreted by NK cells rather than T cells, it was predicted that T cell depletion would have little effect on the course of a primary infection initiated in the footpad. This prediction proved to be correct, in that the depletion of both $CD4^+$ and $CD8^+$ T cells (Fig. 3) had no significant effect on the growth of *L. monocytogenes* in the footpad, lymph nodes, and spleen. In fact, T cell-depleted mice were capable of inactivating *L. monocytogenes* at a faster rate. Depletion in mice of either T cell subset alone (data not shown) also had no significant effect on bacterial growth. Flow cytometric analysis showed that MAb treatment resulted in the depletion of over 98% of the targeted T cells (data not shown).

In vivo depletion of NK cells results in a loss of IFN-γ-secreting cells and in exacerbation of infection. Mice were next depleted of NK cells alone or of NK cells plus T cells to determine whether such depletion would result in a loss of cells that produce IFN-γ and in increased bacterial growth. Depletion of NK cells was carried out with two anti-NK cell MAbs or polyclonal rabbit anti-asialo-GM_1 antiserum as described in Materials and Methods. About 90% of the cells capable of producing IFN-γ in the DLN of 1-day-infected mice were eliminated by treatment with antibodies against NK cell markers, regardless of whether T cells were present (Table 3). T cell-depleted mice treated with control Ig instead of anti-NK cell antibodies had a number of IFN-γ-secreting cells in their lymph nodes, equivalent to the number in control mice.

It needs to be mentioned, however, that although Table 3 shows that treatment with anti-NK cell reagents removed most IFN-γ-secreting cells, it does not shown that this was the result of the removal of NK cells. It seems safe to assume that this was the case, however, in view of the results of an additional experiment that was designed to determine the number of asialo-GM_1^+ cells in low-density

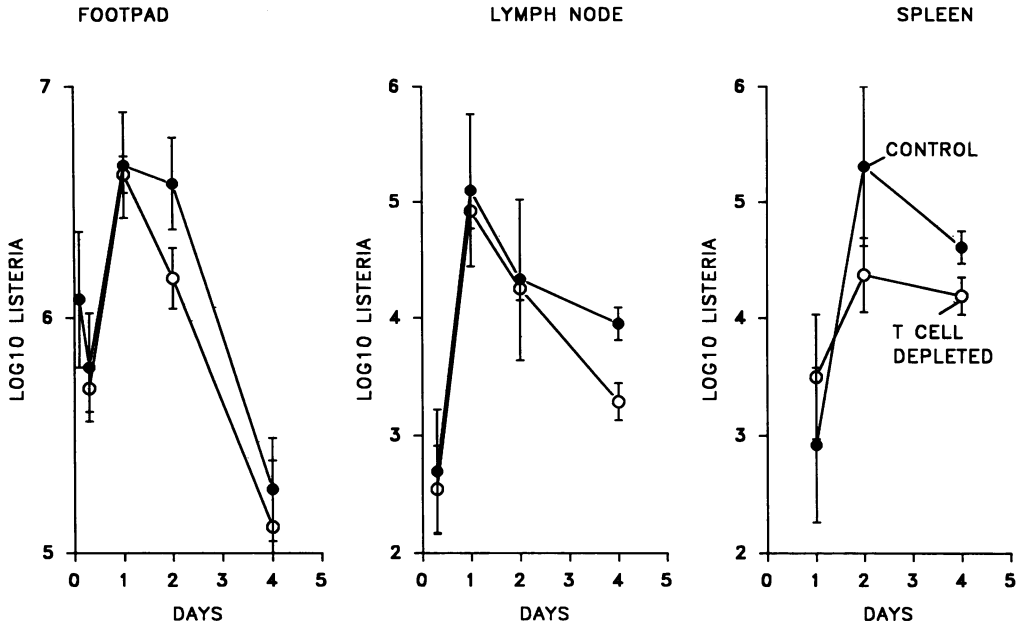

FIG. 3. Effect of depletion of $CD4^+$ and $CD8^+$ T cells on the multiplication of *L. monocytogenes* (10^6) inoculated in a hind footpad. Depletion of T cells did not prevent mice from resolving infection in the footpad, lymph node, or spleen during the time course of the experiment. If anything, T cell-depleted mice displayed more resistance. In this experiment, MAb treatment was given 2 days before infection. Each point represents the mean ± standard deviation for five mice per group.

fractions of DLN cells from mice depleted with anti-T cell reagents alone or in combination with anti-asialo-GM_1 antiserum. According to these results, the percentage of asialo-GM_1^+ cells in the NK cell-rich, pooled low-density fractions (1 and 2) was reduced from 24 to 4% by treatment with anti-asialo-GM_1 antisera.

Figure 4 shows that the loss of IFN-γ-producing cells was associated with the exacerbation of infection, in that by day 3 postinoculation, a larger number of *L. monocytogenes* organisms were present in the footpads of mice treated with anti-NK cell reagents. This was also true for the DLN. However, in the case of mice treated with the anti-asialo-GM_1 antiserum, exacerbation of infection was less pronounced. The number of mice used in this experiment was limited by the availability of the anti-NK cell reagents, which needed to be given in relatively large quantities for in vivo depletion. Consequently, growth curves were not obtained. However, five additional mice per group were monitored for survival, and of these, two in the group treated with the anti-NK cell reagents died by day 5 of infection, whereas none in the remaining groups died. All anti-asialo-GM_1 antiserum-treated mice were visibly sick.

DISCUSSION

This paper deals with a mechanism of resistance against listeriosis which operates during the first 48 h of infection and which is highly dependent on the production of IFN-γ by cells other than T cells. We show, in agreement with the published results of others (4, 30), that treatment with a neutralizing anti-IFN-γ MAb renders mice incapable of controlling an otherwise sublethal infection. Likewise, treating mice with anti-NK cell antibodies, but not anti-T cell antibodies, severely exacerbates infection, thereby indicating that NK cells, rather than T cells, are of importance in early resistance to infection with *L. monocytogenes*. In addition, the results of a spot ELISA showed that NK cells were responsible for producing the IFN-γ secreted soon

TABLE 3. Evidence that the cells that secrete IFN-γ are NK cells

Antibody treatment[a]	No. of IFN-γ-secreting cells/ responding node (10^3)[b]	% Stained cells[c]		
		Thy-1^+	$CD4^+$	$CD8^+$
None	54.8 ± 1.0	26.1	12.3	13.3
Anti-CD4 + anti-CD8	61.0 ± 8.9	3.8	0.1	0.0
Anti-CD4 + anti-CD8 + anti-NK cell MAbs	4.2 ± 1.7	6.0	0.2	0.1
Anti-CD4 + anti-CD8 + anti-asialo-GM_1	1.1 ± 0.9	2.3	0.2	0.1
Anti-CD4 + anti-CD8 + control IgG	49.2 ± 8.0	ND[d]	NT	NT
Anti-asialo-GM_1	3.9 ± 1.7	31.9	15.8	14.3

[a] Mice were injected before infection with the indicated antibodies.
[b] As determined by the spot ELISA. The results represent the mean ± standard deviation for five replicates obtained with pooled lymph node cells from five mice per group.
[c] As determined by flow cytometric analysis.
[d] NT, not tested.

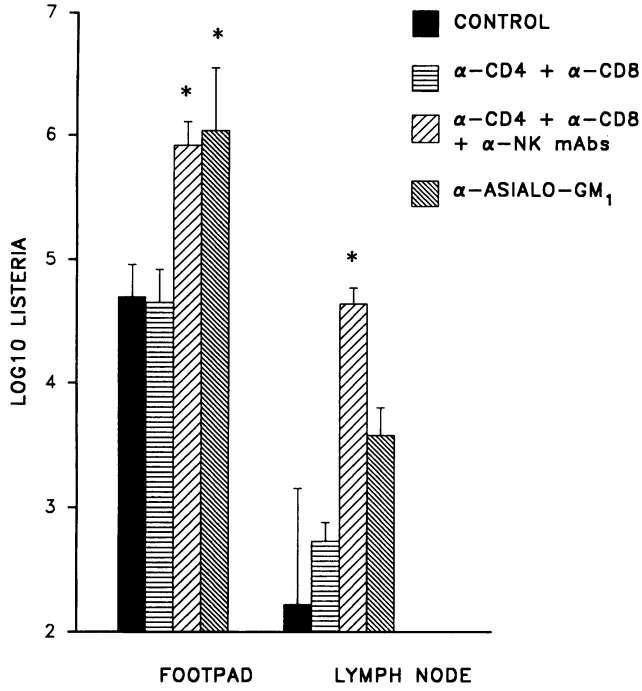

FIG. 4. Effect of treating mice with anti-NK cell antibodies on resistance to *L. monocytogenes* infection initiated in the footpad. Mice were treated with anti-CD4 and anti-CD8 MAbs to deplete them of T cells, with these same MAbs plus anti-NK cell MAbs to deplete them of T cells plus NK cells, or with anti-asialo-GM_1 antiserum to preferentially remove NK cells. The antibody treatments were given before the mice were inoculated with 10^6 *L. monocytogenes* in the footpad as described in Materials and Methods. Depletion of $CD4^+$ or $CD8^+$ T cells had no significant effect on bacterial growth in the footpad and lymph node, whereas depletion of NK cells in addition to T cells by treatment with anti-T cell plus anti-NK cell MAbs or depletion of NK cells alone by treatment with anti-asialo-GM_1 antiserum caused increased bacterial growth. The results represent the mean ± standard deviation for five mice per group. Asterisks represent groups that were significantly different from controls ($P < 0.05$), as determined by Student's *t* test. α, anti.

after inoculation of *L. monocytogenes*, in that there was a marked drop in the number of cells secreting IFN-γ in the DLN of NK cell-depleted, but not T cell-depleted, mice. This finding was supported by the additional finding that enrichment for the IFN-γ-secreting lymph node cells resulted in enrichment for cells which stained positively for the asialo-GM_1 and NK1.1 antigens and for cells with LGL morphology. Taken as a whole, therefore, these results show that NK cells are predominantly responsible for secreting the IFN-γ needed for resistance to infection with *L. monocytogenes*.

The evidence presented here showing that NK cells are essential participants in resistance to murine listeriosis gives meaning to interesting earlier findings published by others indicating that NK cells are involved in the response to infection. For example, it was shown recently (17) that nonadherent cells that lyse NK cell-susceptible tumor targets and bear NK cell-associated markers accumulate in the peritoneal cavity of mice infected at this site with *L. monocytogenes*. Again, spleen cells of SCID mice that are known to contain NK cells (6), but not functional T cells, have been shown to secrete IFN-γ when stimulated in vitro with heat-killed *L. monocytogenes* (2). NK cells have also been shown to participate in defense against other microorganisms, including viruses, bacteria, and fungi (reviewed in reference 9). However, whether NK cells are protective against all infectious agents because of their ability to secrete IFN-γ is not known. It remains possible that resistance depends, instead, on NK cell-mediated lytic mechanisms (16) or antibody-dependent cellular cytotoxicity (25).

The way in which IFN-γ functions in defense against listeriosis is not shown by this study, but it is likely that it serves to activate the listericidal ability of macrophages (39), the cells that are thought to be ultimately responsible for destroying *L. monocytogenes*. The ability of IFN-γ to activate the microbicidal function of macrophages has been revealed with other pathogens (5, 10, 44). However, an alternative role of NK cell-secreted IFN-γ in resistance to listeriosis may be that of promoting the expression of class II antigen on the surface of infected macrophages (24), thereby facilitating increased antigen presentation by these cells for the induction of T cell-mediated immunity. This would seem unlikely, given the findings here and elsewhere (34) that T cells are not essential for the resolution of primary listeriosis. It should also be pointed out that the T cells that adoptively immunize against *L. monocytogenes* are $CD8^+$ (1, 23), meaning that their production does not depend on antigen presentation by class II-antigen-positive macrophages. Further evidence against IFN-γ being needed to facilitate increased T cell production is provided by the results of additional experiments performed in our laboratory (unpublished data) showing that the neutralization of endogenous IFN-γ with anti-IFN-γ MAb fails to cause a reduction in the number of *L. monocytogenes*-specific T cells produced by the host during the first 6 days of infection, as measured by adoptive immunization.

It seems highly likely from the results of this study that the presence of IFN-γ-secreting NK cells in the responding lymph nodes of infected mice was dependent on the recruitment of these cells form blood, because the 100-fold increase in their number over a 24-h period could not have resulted from the replication of local cells. In keeping with this interpretation is the knowledge (18) that lymph nodes contain relatively few NK cells, compared with the spleen and blood. It is also significant that the NK cells in the DLN of infected mice were already secreting IFN-γ at the time of harvest. The reason why dead organisms failed to cause the appearance of IFN-γ-producing cells in the DLN, despite increased cellularity, is not known. It is possible that dead organisms caused the recruitment of NK cells to the DLN but that these cells were not induced to secrete IFN-γ. This possibility could be determined by examining the ability of DLN cells to produce IFN-γ in response to dead *L. monocytogenes* in vitro. It has been shown by others, in this connection, that in vitro stimulation with dead *L. monocytogenes* can induce IFN-γ secretion by spleen cells of infected immunocompetent mice (14, 28) and uninfected SCID mice (2).

The results presented here showing that $CD4^+$ and $CD8^+$ T cells play little role in early resistance to infection and are not involved in early secretion of IFN-γ should not be surprising, given the knowledge that protective *L. monocytogenes*-specific T cells are not acquired by the host until after day 2 of infection (37). Indeed, even after day 2 of infection, the contribution of T cells may be limited, in view of publications showing that mice depleted of T cells by thymectomy and irradiation (34) or by treatment with MAb (40) are able to express considerable resistance to a sublethal *L. monocytogenes* infection. This is not to say that T cells do

not produce IFN-γ later in infection. On the contrary, it has been demonstrated by others (11, 28) that T cells obtained from the spleens of 6-day-infected mice can secrete large quantities of IFN-γ in response to stimulation with killed *L. monocytogenes* or mitogen in vitro and that mice challenged on day 6 of infection with live *L. monocytogenes* or an *L. monocytogenes* cell wall preparation produce T cell-dependent IFN-γ (29). In support of these particular published findings are recent experimental results from our laboratory (unpublished data) showing that a significant number (approximately 5×10^4) of T cells in the spleens of 6-day-infected mice secrete IFN-γ spontaneously when placed in culture.

On the other hand, although the present study clearly shows an important role for NK cells in resistance to infection with *L. monocytogenes*, it is unlikely that these cells are the only nonphagocytic cells that are involved during the initial phase of the host response. Indeed, it is probable that they may be one of several essential cellular components necessary for early defense. A likely additional component could be Thy-1$^+$ CD4$^-$ CD8$^-$ T cells, possibly γδ T cells, which have been shown to accumulate in the peritoneal cavity of mice infected at this site with *L. monocytogenes* (38). This possibility seems likely in view of the demonstration in this paper that Thy-1$^+$ CD4$^-$ CD8$^-$ cells are present in the DLN (Fig. 2) and of the results of a recent study in our laboratory (8) showing that treatment of mice already depleted of CD4$^+$ and CD8$^+$ T cells with anti-Thy-1.2 MAb causes a significant and often lethal exacerbation of infection.

Lastly, the reason why this study dealt with infection initiated s.c. instead of i.v. is that the infection-enhancing action of the rat anti-IFN-γ MAb used is more pronounced with infections initiated extravascularly. This topic will be the subject of a forthcoming publication from our laboratory. It may be the reason why the use of this same anti-IFN-γ reagent failed to cause lethal exacerbation in i.v.-infected mice in the experiments of others (30).

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