INFECTION AND IMMUNITY, June 1993, p. 2703–2707
0019-9567/93/062703-05$02.00/0

Vol. 61, No. 6

Leukocyte-Mediated Lysis of Infected Hepatocytes during Listeriosis Occurs in Mice Depleted of NK Cells or CD4$^+$ CD8$^+$ Thy1.2$^+$ T Cells

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Received 21 December 1992/Accepted 11 March 1993

An important early component of defense against listeriosis in mice is the lysis of infected hepatocytes by leukocytes that accumulate at foci of infection in the liver. This serves to release *Listeria monocytogenes* from permissive parenchymal cells for ingestion and inactivation by phagocytic cells. It is shown here that lysis of infected hepatocytes is as extensive in mice depleted of T cells or NK cells as it is in control mice. This supports the original interpretation that hepatocyte lysis is mediated mainly by neutrophils.

Listeria monocytogenes is a facultative intracellular bacterial pathogen capable of invading and multiplying in nonprofessional phagocytes in vitro (7, 9, 18) and in vivo (19, 21). Furthermore, *L. monocytogenes* can spread directly from cell to cell within a tissue via points of plasma membrane contact (19, 23). In this way, the organism can avoid being exposed to phagocytes capable of ingesting and destroying it. In murine listeriosis, hepatocytes are the major sites of intracellular bacterial multiplication in the liver (2–4, 21). According to the results of recent studies (2–4), the host deals with this situation by directing leukocytes to sites of infection to lyse infected hepatocytes, thereby releasing *L. monocytogenes* into the extracellular environment for ingestion and destruction by phagocytes. If leukocyte accumulation at foci of infection in the liver is prevented by treating mice with monoclonal antibodies (MAbs) directed against the type 3 complement receptor (CR3) of myelomonocytic cells (2–4, 21), hepatocytes are not lysed and progressive cell-to-cell transmission of infection proceeds unchecked. Because during the first 24 h of infection neutrophils far outnumber other cells at foci of infection and can be seen to be in physical contact with hepatocytes undergoing lysis, it was suggested (2) that neutrophils are mainly responsible for hepatocyte dissolution during this early phase of the infection process. However, it remained possible that other cells better known for their cytolytic functions might be responsible for lysing infected hepatocytes. For example, NK cells (10, 24) and T cells (16) reportedly can express CR3 and might therefore be functionally impaired in mice treated with anti-CR3 MAbs. Although T cells are generally considered not to participate in anti-*Listeria* defense during the first 24 h of infection, a significant influx of CD4$^+$ and CD8$^+$ T cells into the liver parenchyma reportedly (8) does occur during this time. NK cells are also recruited into the liver in large numbers during the first day of listeriosis (8). It was necessary to determine, therefore, whether lysis of infected hepatocytes takes place in mice depleted of only NK cells or T cells, both of which are known to be capable of lysing infected target cells in vitro (11–13, 17) and of participating in host defense against listeriosis in vivo (1, 5, 6, 15). The results show that lysis of infected hepatocytes proceeds normally in mice depleted of NK cells or T cells and that depletion of either cell type does not result in an increase in bacterial numbers during the first 12 h of infection.

B6D2F$_1$ (C57BL6 × DBA/2) male mice, 9 to 12 weeks old, were obtained from the Trudeau Institute Animal Breeding Facility (Saranac Lake, N.Y.) and used in all experiments. Mice were infected by intravenous inoculation with 10^4 or 10^8 CFU of a streptomycin-resistant strain (22) of *L. monocytogenes* (strain 10403SR, serotype 1). Mice were killed by cervical dislocation 12 h after inoculation, and bacteria in the livers were enumerated by plating 10-fold serial dilutions of homogenates of the liver on Trypticase-soy agar. Colonies were counted after incubation for 24 h at 37°C.

MAb 5C6 (20), directed against the CD11b polypeptide chain of the CD11b-CD18 heterodimeric type 3 complement receptor of myelomonocytic cells, was a gift from S. Gordon (University of Oxford, Oxford, United Kingdom). Anti-CD4 (clone GK 1.5), anti-CD8 (clone TIB.210), anti-Thy1.2 (clone 30.H.12), anti-CD3 (clone 145-2C11), and anti-NK1.1 (clone PK136) MAbs were obtained from the American Type Culture Collection, Rockville, Md. Polyclonal rabbit anti-asialo GM1 antiserum was purchased from WAKO Pharmaceuticals, Dallas, Tex., and normal rabbit immunoglobulin (Ig) was purchased from Sigma Chemical Company, St. Louis, Mo. Fluorescein isothiocyanate (FITC)-conjugated F(ab')$_2$ goat anti-rat Ig used for flow cytometric analyses was obtained from Tago Immunochemicals, Burlingame, Calif. FITC-conjugated F(ab')$_2$ fragments of anti-CD4, anti-CD8, anti-Thy1.2, and anti-CD3 MAbs and FITC-conjugated anti-NK1.1 MAb (whole antibody) were prepared in our laboratory by methods described previously (6). Mice were injected intravenously with 0.5 mg of purified MAb 5C6 1 h before infection to prevent recruitment of myelomonocytic cells to foci of liver infection. In vivo depletion of cells bearing CD4, CD8, or Thy1.2 surface markers was achieved by treating mice with appropriate MAbs as described previously (6). To deplete NK cells, mice were given 40 μl of anti-asialo GM1 antiserum intravenously 1 day before infection. Control mice received an equivalent amount (0.9 mg) of normal rabbit Ig.

Flow cytometric analysis was carried out as previously described (5, 6) with pooled spleen cells obtained from two antibody-treated or two untreated mice inoculated with 10^8 CFU of *L. monocytogenes* 12 h earlier. For analysis of T-cell-depleted spleens, unfractionated spleen cells were used, whereas for NK-cell-depleted spleens, the spleen cells

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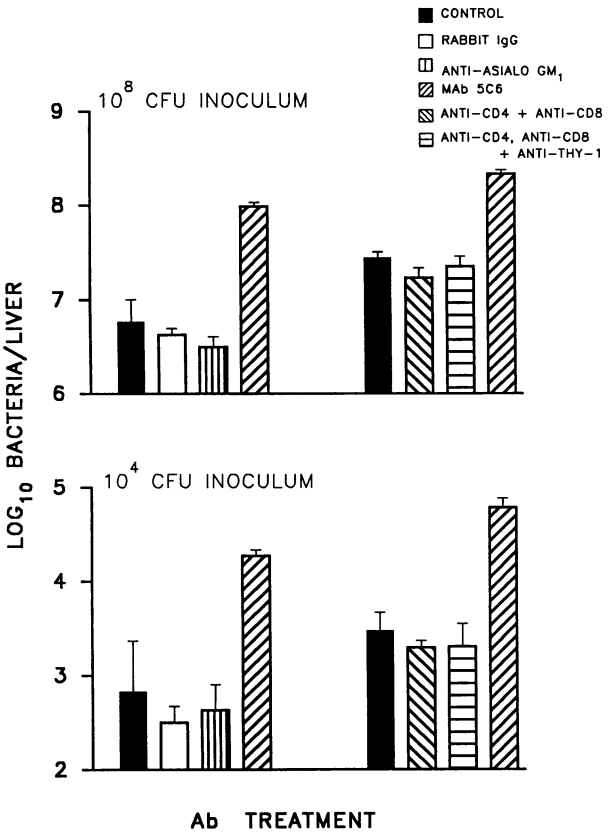

FIG. 1. Recovery of *L. monocytogenes* bacteria from the livers of mice treated with antibodies directed against T cells, NK cells, or cells bearing CR3. Mice were treated with anti-T-cell or anti-NK-cell antibodies and inoculated with either 10^8 or 10^4 CFU of *L. monocytogenes* as indicated. Depletion of T cells or NK cells caused no increase, compared with controls, in the number of bacteria present in the liver at 12 h of infection. By contrast, treatment with anti-CR3 MAb 5C6 caused an increase of 1 $\log_{10}$ unit or more in bacterial numbers by 12 h of infection. The results represent the means ± standard deviations for three mice per group. Ab, antibody.

were depleted first of adherent cells by incubation in plastic petri dishes for 2 h at 37°C and then of B cells by using goat anti-mouse Ig-coated magnetic microspheres (Advanced Magnetic, Inc., Cambridge, Mass.). Magnetic-microsphere-coated cells were then removed by magnetic separation, and the residual cells were separated by density gradient centrifugation into low-density (NK-enriched) and high-density (NK-depleted) fractions as previously described (5). Direct fluorescence labelling of the cell suspensions was performed by using FITC-conjugated $F(ab')_2$ fragments of anti-T-cell MAbs (anti-CD4, anti-CD8, anti-Thy1.2, and anti-CD3) or anti-B-cell antibody (goat anti-mouse Ig). Antibody-coated, intact T cells remaining after MAb treatment were detected by staining with FITC-conjugated $F(ab')_2$ goat anti-rat IgG. NK cells were labelled with FITC-conjugated anti-NK1.1 MAb. Stained cells were analyzed by using a FACScan cytofluorograph (Becton Dickinson, Sunnyvale, Calif.) equipped with Lysis II software. Functional NK-cell activity was determined for low- and high-density spleen cell fractions (prepared as described above) obtained from infected mice treated with normal rabbit Ig or depleted of NK cells by anti-asialo GM1 treatment. Spleen cell fractions were assayed for cytotoxicity against NK-cell-sensitive YAC-1 target cells in a standard 4-h chromium release assay. The percent specific lysis was calculated according to the following equation: % specific lysis = 100 × (experimental ^{51}Cr release − spontaneous release)/(maximum release − spontaneous release). Spontaneous release of ^{51}Cr never exceeded 7% of the maximum release. For histology, mice were killed by cervical dislocation at 12 h of infection and their livers were removed en bloc into 10% buffered formalin, cut into small pieces, and left to fix for 24 h. The pieces were then washed in water, dehydrated in ethanol, and embedded in glycol methacrylate (JB-4 embedding kit; Polysciences, Inc., Warrington, Pa.). Thin sections (1 to 2 μm thick) were cut with glass knives and stained with McNeal's stain.

According to previous studies (2–4), preventing leukocytes from accumulating at sites of *L. monocytogenes* infection in the liver by treating mice with anti-CR3 MAbs causes substantial exacerbation of infection within 12 h. To determine whether the effects of anti-CR3 treatment were due, in part, to inhibition of NK-cell or T-cell function, the growth of *L. monocytogenes* in mice depleted of these cells was compared with its growth in mice treated with anti-CR3 MAb 5C6. If NK cells or T cells are required to control the growth of *L. monocytogenes* during the first 12 h of infection, then increased bacterial multiplication should occur in mice selectively depleted of these cells. As shown in Fig. 1, this was not the case. By 12 h after inoculation with 10^4 or 10^8 CFU of *L. monocytogenes*, the livers of mice depleted of NK cells or T cells contained the same numbers of bacteria as the livers of control mice. By contrast, there were up to 20-fold (range, 8- to 20-fold) more bacteria in the livers of 5C6-treated mice.

A key function of inflammatory leukocytes that accumulate at foci of *L. monocytogenes* infection in the liver is to lyse infected hepatocytes and thereby release the organism for ingestion by phagocytes (2). If dissolution of infected hepatocytes is dependent on NK cells or T cells, then hepatocyte lysis should not take place or should be greatly reduced in the livers of mice depleted of these cells. To investigate this possibility, a histological study of the livers of control mice and mice depleted of NK cells or T cells, both infected for 12 h, was performed. This study showed that in the control mice (Fig. 2a), by 12 h of infection, leukocytes, mainly neutrophils, had migrated to foci of liver infection to occupy space created by lysed hepatocytes. Moreover, neutrophils were close to infected hepatocytes

FIG. 2. Appearance of foci of *L. monocytogenes* infection in the livers of control mice (a) and mice treated with anti-CR3 MAb 5C6 (b). In control mice, by 12 h of infection, large numbers of neutrophils had accumulated at foci of infection. Hepatocytes that once occupied the central area of the lesion have been destroyed, creating holes in the parenchyma that are now occupied by inflammatory cells. Infected hepatocytes (bacteria are indicated with arrows) at the periphery of the lesion are in the process of undergoing dissolution. Similar lesions were observed in the livers of mice depleted of T cells or NK cells. By contrast, neutrophils failed to accumulate at foci of infection in 5C6-treated mice. Under these circumstances, infected hepatocytes were not lysed and *L. monocytogenes* bacteria were found throughout their cytoplasm. Bar = 10 μm.

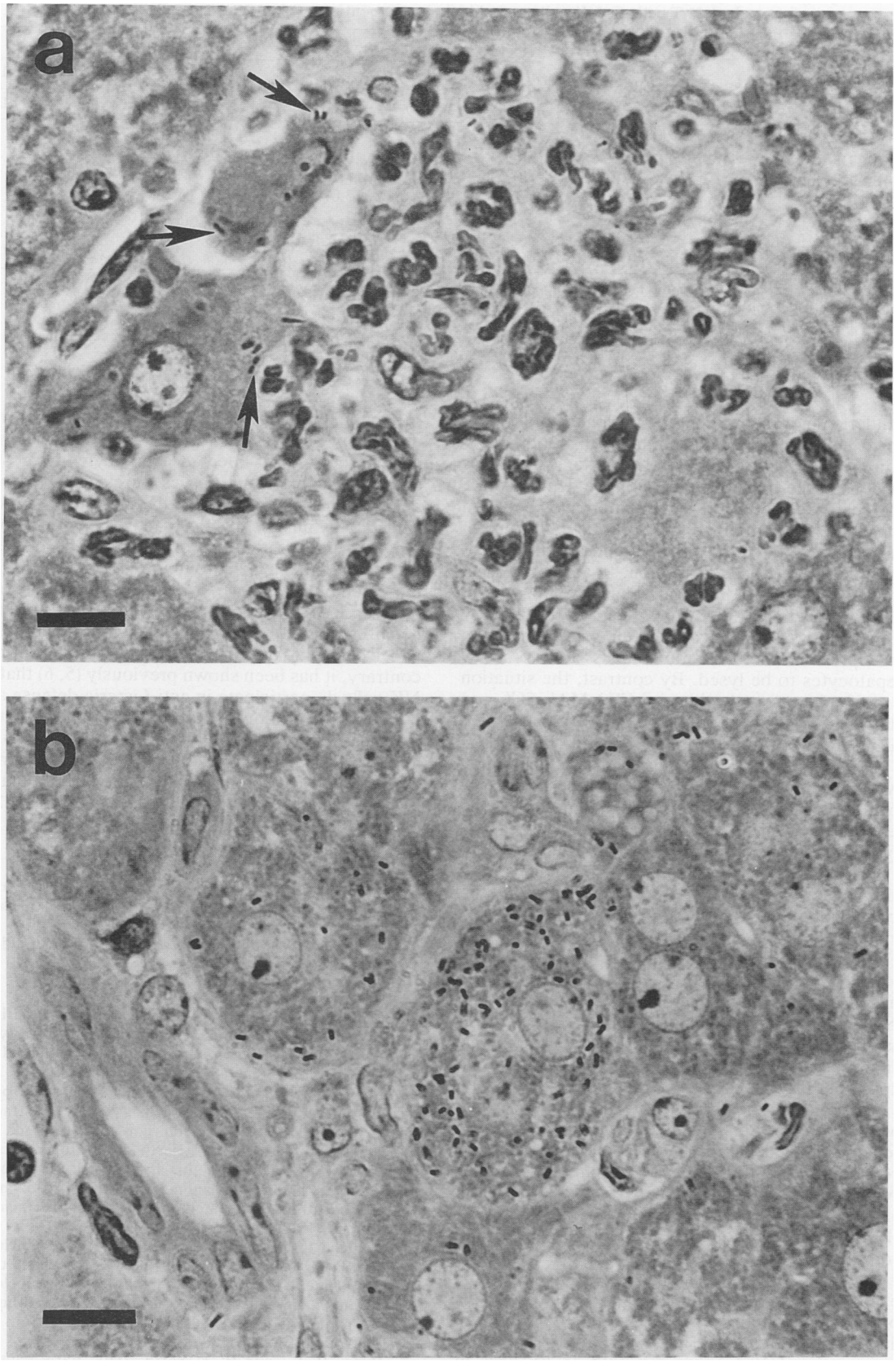
a
b

TABLE 1. Flow cytometric analysis of cell surface markers on spleen cells taken from control mice and mice treated with antibodies to deplete T cells or NK cells or to block recruitment of myelomonocytic cells

MAb treatment[a]	Total cells (10^8)[b]	% of stained cells[c]							
		CD4+	CD8+	Thy+	CD3+	Rat Ig+	NK1.1+[d]		
							ASC	LDF	HDF
None	2.84	10.63	6.63	16.40	17.20	0.00			
Anti-CD4, anti-CD8	2.63	0.03	0.03	2.60	1.90	3.23			
Anti-CD4, anti-CD8, anti-Thy1.2	2.71	0.00	0.03	0.20	0.07	1.70			
Anti-CR3	2.93	11.83	6.57	17.07	17.53	2.33			
Rabbit IgG	2.81						3.43	20.18	0.34
Anti-asialo GM1	2.69						0.06	0.58	0.42

[a] Before infection, mice were injected with the indicated antibodies as outlined in the text.
[b] Number of viable cells per spleen.
[c] Determined by flow cytometric analysis.
[d] ASC, all spleen cells; LDF, low-density fraction of spleen cells from Percoll gradient; HDF, high-density fraction of spleen cells from Percoll gradient.

that were undergoing dissolution at the periphery of lesions. In addition to neutrophils, small numbers of mononuclear cells were present at foci of infection. An extensive examination of lesions in the livers of infected mice depleted of T cells or NK cells revealed no obvious differences between these lesions and those in infected controls in terms of extensiveness of hepatocyte destruction and the accumulation of neutrophils at sites of destruction. In all three cases, it was noteworthy that although individual hepatocytes at foci of infection contained only a few bacteria at this early time, this low level of intracellular infection was sufficient to attract neutrophils and other mobile cells and to allow infected hepatocytes to be lysed. By contrast, the situation in the livers of mice treated with anti-CR3 MAb 5C6 was markedly different (Fig. 2b) in that inflammatory cells failed to accumulate at foci of infection and infected hepatocytes were not lysed. Instead, neutrophils were found almost exclusively within liver sinusoids. Under these circumstances, foci of infection consisted mainly of lightly infected hepatocytes that were morphologically intact.

To verify that in vivo treatment with appropriate antibodies caused depletion of T cells or NK cells, spleen cells from treated mice were harvested at 12 h postinfection and analyzed by cytofluorometry for the presence of cells bearing T-cell or NK-cell surface markers. According to the results in Table 1, treating mice with a combination of all three anti-T-cell MAbs resulted in a >98% reduction in the number of spleen cells expressing CD4, CD8, and Thy1.2 markers. Staining with anti-CD3 MAb confirmed this result by showing that the reduction in the percentage of cells bearing this T-cell marker was comparable to the reduction in cells expressing CD4, CD8, and Thy1.2. Very few of the cells that remained in the spleens of MAb-treated mice stained positively for rat Ig, showing that the majority of the targeted cells were lysed. Similarly, in mice depleted of NK cells by treatment with anti-asialo GM1, a phenotypic analysis of low-density (NK-cell-enriched) fractions of spleen cells revealed a substantial depletion (>95%) of NK1.1+ cells (Table 1). As an additional measure of NK-cell depletion, low-density-fraction, gradient-enriched cells were assayed for cytolytic activity against ^{51}Cr-labelled YAC-1 cells. The results showed that anti-asialo GM1 treatment, but not control Ig treatment, essentially ablated anti-YAC-1 cytotoxicity, resulting in only 1.1% specific lysis at an effector/target ratio of 10:1 compared with 48.4% lysis in controls (data not shown).

In conclusion, previous publications (2–4, 21) have shown that a key early event in defense against listeriosis in the liver is the lysis of infected hepatocytes by host inflammatory leukocytes, which serves to release *L. monocytogenes* from permissive cells into the external environment for ingestion by phagocytes that can destroy it. According to the present study, neither NK cells nor T cells are necessary for this defense mechanism to function. The absence of either of these cell types made no difference in the number of bacteria in liver lesions at 12 h of infection or in the extent of hepatocyte lysis at lesions. This is not to say that NK cells and T cells play no role in defense against listeriosis. On the contrary, it has been shown previously (5, 6) that T cells and NK cells do participate in anti-*Listeria* defense after 24 h of infection. As for control mice, neutrophils were the predominant cells present in early lesions in the livers of NK-cell- and T-cell-depleted mice. The finding that neutrophils were in close physical contact with infected hepatocytes undergoing lysis reinforces the original interpretation (2) that neutrophils are mainly responsible for this aspect of host defense.

We thank Ron LaCourse, Debra Duso, Lynn Ryan, and Linda Schaefer for their excellent technical assistance and Mary Durett for typing.

This study was supported by a grant from the Trudeau Institute.

REFERENCES

1. **Baldridge, J. R., R. A. Barry, and D. J. Hinrichs.** 1990. Expression of systemic protection and delayed-type hypersensitivity to *Listeria monocytogenes* is mediated by different T-cell subsets. Infect. Immun. **58**:654–658.
2. **Conlan, J. W., and R. J. North.** 1991. Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. J. Exp. Med. **174**:741–744.
3. **Conlan, J. W., and R. J. North.** 1992. Roles of *Listeria monocytogenes* virulence factors in survival: virulence factors distinct from listeriolysin are needed for the organism to survive an early neutrophil-mediated host defense mechanism. Infect. Immun. **60**:951–957.
4. **Conlan, J. W., and R. J. North.** 1992. Monoclonal antibody NIMP-R10 directed against the CD11b chain of the type 3 complement receptor can substitute for monoclonal antibody 5C6 to exacerbate murine listeriosis by preventing the focusing of myelomonocytic cells at infectious foci in the liver. J. Leukocyte Biol. **52**:130–132.
5. **Dunn, P. L., and R. J. North.** 1991. Early gamma interferon production by natural killer cells is important in defense against

murine listeriosis. Infect. Immun. **59:**2892–2900.
6. **Dunn, P. L., and R. J. North.** 1991. Resolution of primary murine listeriosis and acquired resistance to lethal secondary infection can be mediated predominantly by Thy-1$^+$ CD4$^-$ CD8$^-$ cells. J. Infect. Dis. **164:**869–877.
7. **Gaillard, J.-L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55:**2822–2829.
8. **Goossens, P. L., H. Jovin, and G. Milon.** 1991. Dynamics of lymphocytes and inflammatory cells recruited in liver during murine listeriosis. J. Immunol. **147:**3514–3520.
9. **Havell, E. A.** 1986. Synthesis and secretion of interferon by murine fibroblasts in response to intracellular *Listeria monocytogenes*. Infect. Immun. **54:**787–792.
10. **Holmberg, L. A., and K. A. Ault.** 1984. Characterization of natural killer cells induced in the peritoneal exudates of mice infected with *Listeria monocytogenes*: a study of their tumor target specificity and their expression of murine differentiation antigens and human NK-associated antigens. Cell. Immunol. **89:**151–168.
11. **Katz, P., H. Yeager, G. Whalen, M. Evans, R. P. Swartz, and J. Roeklin.** 1990. Natural-killer cell-mediated lysis of *Mycobacterium avium* complex infected monocytes. J. Clin. Immunol. **10:**71–77.
12. **Kaufmann, S. H. E., E. Hug, and G. DeLibero.** 1986. *Listeria monocytogenes* reactive T lymphocyte clones with cytolytic activity against infected target cells. J. Exp. Med. **164:**363–368.
13. **Klimpel, G. R., D. W. Nielsen, and K. D. Klimpel.** 1986. Natural cytotoxic effector cell activity against *Shigella flexneri*-infected HeLa cells. J. Immunol. **136:**1081–1086.
14. **Koo, G. C., F. J. Dumont, M. Tutt, J. Hackett, and V. Kumar.** 1986. The NK1.1(−) mouse: a model to study differentiation of murine NK cells. J. Immunol. **137:**3742–3747.
15. **Mackaness, G. B., and W. C. Hill.** 1969. The effect of anti-lymphocyte globulin on cell-mediated resistance to infection. J. Exp. Med. **129:**993–1012.
16. **McFarland, H. I., S. R. Nahill, J. W. Maciaszek, and R. M. Welsh.** 1992. CD11b (Mac-1): a marker for CD8$^+$ cytotoxic T cell activation and memory in virus infection. J. Immunol. **149:**1326–1333.
17. **Pamer, E. G., J. T. Harty, and M. J. Bevan.** 1991. Precise prediction of a dominant class 1 MHC-restricted epitope of *Listeria monocytogenes*. Nature (London) **353:**852–855.
18. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.
19. **Racz, P., K. Tenner, and E. Mero.** 1972. Experimental *Listeria enteritis*. I. An electron microscope study of the epithelial phase in experimental listeria infection. Lab. Invest. **26:**694–700.
20. **Rosen, H., and S. Gordon.** 1987. Monoclonal antibody to murine type 3 complement receptor inhibits adhesion of myelomonocytic cells *in vitro* and inflammatory cell recruitment *in vivo*. J. Exp. Med. **166:**1685–1701.
21. **Rosen, H., S. Gordon, and R. J. North.** 1989. Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. J. Exp. Med. **170:**27–37.
22. **Sun, A. N., A. Camilli, and D. A. Portnoy.** 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect. Immun. **58:** 3770–3778.
23. **Tilney, L. G., and D. A. Portnoy.** 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell Biol. **109:**1597–1608.
24. **Timonen, T., M. Patarroyo, and C. G. Gahmberg.** 1988. CD11a-c/CD18 and GP84 (LB-2) adhesion molecules on human large granular lymphocytes and their participation in natural killing. J. Immunol. **141:**1041–1046.