Brief Definitive Report

ACQUIRED RESISTANCE TO *LISTERIA MONOCYTOGENES* IS MEDIATED BY Lyt-2+ T CELLS INDEPENDENTLY OF THE INFLUX OF MONOCYTES INTO GRANULOMATOUS LESIONS

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Infections by facultative intracellular bacteria result in inflammatory tissue responses, such as delayed-type hypersensitivity (DTH), and the focal accumulation of mononuclear cells within granulomatous lesions (1, 2). Both DTH and granuloma formation depend on an intact T cell response and have so far been accepted to be indispensable for protection against facultative intracellular bacteria, including *Listeria monocytogenes*. As recently shown (3), however, DTH and protective immunity can be dissociated from each other in secondarily infected mice. Thus, whereas DTH critically depended on the presence of L3T4+ T lymphocytes, Lyt-2+ T cells proved necessary and sufficient for the rapid elimination of *Listeria*.

Although the protective capacity of Lyt-2+ T cells in murine listeriosis has been demonstrated also at the clonal level (4), the mode of action of these cells is still unclear. Two different mechanisms were proposed (5): (*a*) the direct activation of infected macrophages by lymphokines; and (*b*) the lysis of infected, but insufficiently microbicidal/host cells followed by the attraction and activation of blood-borne monocytes.

This report summarizes data from an immunohistochemical study of T cell subset–depleted mice suffering from a secondary *Listeria* infection. The data show that protection against a lethal challenge with *L. monocytogenes* in immune mice can be achieved by Lyt-2+ T cells in the absence of invading blood-borne monocytes. The results, therefore, support the hypothesis that CD8+ T cells mediate protection by directly activating resident macrophages.

Materials and Methods

Mice. Female C57BL/6 mice raised in our own breeding facilities were used at the age of 10–16 wk.

Bacteria. *L. monocytogenes*, strain EGD, was kept virulent by continuous mouse passages. Cultures were obtained by growing samples of spleen homogenates from infected mice overnight in tryptic soy broth (Oxoid, Wesel, FRG), dispensed in vials of 0.2-ml lots, and stored at −70°C until use. For each experiment, the appropriate number of vials from suspension was thawed and diluted in PBS for intravenous inoculation.

mAbs. Four rat mAbs, obtained from ascitic fluid from pristane-primed nude mice intraperitoneally injected with the relevant hybridoma line, were used in vivo. The hybridoma

lines 30-H12 (anti-Thy-1.2, rat IgG2b [IgG2b] [6]), 2.43 (anti-Lyt-2, rat IgG2b [7]), and GK 1.5 (anti-L3T4, rat IgG2b [8]) were purchased from the American Type Culture Collection, Rockville, MD (Tumor Immunology Bank [TIB] 107, 210, and 207). The mAb designated 23-7 (unrelated specificity, rat IgG) was kindly provided by T. Diamantstein, Department of Immunology, Freie Universität Berlin, FRG, and served as a control for nonspecific effects of ascitic fluid. Ascitic fluid was cleared and delipified by centrifugation. The concentration of antibodies was determined by an ELISA with specificity for rat IgG and calculated with a commercially available standard of rat IgG2b (no. 1330; Becton Dickinson & Co., Mountain View, CA) as a reference. Dilutions containing 1 mg/ml of mAb in PBS were stored at $-70°C$ until use. 1 mg of mAbs was injected into the peritoneal cavity on day 3 before reinfection. Two additional mAbs were used for immunohistological examination. Culture supernatants of the hybridomas M1/70 (rat-anti-Mac-1, IgG2b [9]) and F4/80 (rat-anti-mononuclear phagocyte, IgG2b [10]) were generous gifts of R. Steinman, The Rockefeller University, New York.

Infection with Listeria Monocytogenes.   Primary infection with *L. monocytogenes* was performed by an intravenous injection of 3×10^4 viable bacteria in a volume of 0.2 ml of PBS. For secondary infection, mice were injected with 10^6 *Listeria* organisms on day 28 after primary infection. Bacterial growth in spleens and livers was determined by plating 10-fold serial dilutions of organ homogenates on tryptic soy agar. The detection limit of this procedure was 10^2 *Listeria* organisms per spleen. Colonies were counted after 24 h of incubation at 37°C. Numbers of bacteria are given as $\log_{10}$ figures.

Immunohistology.   Liver specimens were snap frozen in liquid nitrogen immediately after killing of the animals. The immunoalkaline phosphatase antialkaline phosphatase (APAAP) procedure was used as described (11, 12). After fixation in acetone and chloroform, frozen sections were incubated with the primary antibody in appropriately diluted ascitic fluid (anti-Thy-1.2; anti-Lyt-2; anti-L3T4) or culture supernatant (anti-Mac-1; anti-F4/80) washed in Tris-buffered saline (TBS; 0.05 M Tris/0.15 M NaCl, pH 7,6), incubated with a rabbit anti-rat Ig antiserum (1:20; Dianova, Hamburg, FRG), supplemented with a 1:10 dilution of normal mouse serum (Dianova), washed in TBS, and incubated with a rat APAAP complex, kindly provided by R. Schwarting, Department of Pathology, Klinikum Steglitz, Berlin, FRG. All incubations were performed for 30 min at room temperature. The incubations with the secondary reagents were repeated once for 10 min each. Alkaline phosphatase was developed with new fuchsin and naphthol-AS-BI-phosphate (Sigma Chemical Co., Deisenhofen, FRG) for 30 min at room temperature. Sections were then counterstained with hematoxyline and mounted in Kaiser's glycerol gelatine (Merck, Darmstadt, FRG).

Analysis of T Cell Subsets in Spleens of mAb-treated Mice.   Spleen cells from reinfected mice treated with mAbs in vivo were incubated with anti-Thy-1.2, anti-L3T4, anti-Lyt-2 , and 23-7 (control antibody) mAbs (25 µg/ml) in 100 µl of PBS supplemented with 5% FCS and 0.02% NaN$_3$ on ice for 30 min. After washing, cells were treated with FITC-labeled goat anti-rat IgG (no. 6270; Medac, Hamburg, FRG) for 30 min on ice. Afterward, cells were washed three times, fixed in 0.1% paraformaldehyde, and subsequently analyzed on a cytofluorograph (Coulter Electronics Inc., Hialeah, FL). The in vivo treatment with the different mAbs resulted in a reduction of the respective T cell subset to background levels as shown previously (3).

Results and Discussion

Normal, immunocompetent mice immunized with *L. monocytogenes* 4 wk previously were depleted of T cell subsets by intraperitoneal injections of mAbs (13) with specificities to Thy-1.2, L3T4, and Lyt-2. 3 d later, mice were charged with a lethal reinfecting dose of *L. monocytogenes* and the course of secondary infections was followed. While acquired resistance was markedly decreased in mice treated with anti-Thy-1.2 or anti-Lyt-2 mAbs, there was no significant difference in the numbers of bacteria recovered from the livers and spleens of either control or L3T4$^+$ T cell-depleted mice (Fig. 1); in fact, both spleens and livers of anti-L3T4 mAb-treated mice lacked bacteria 6 d after reinfection (data not shown). The anti-Thy-1.2-treated
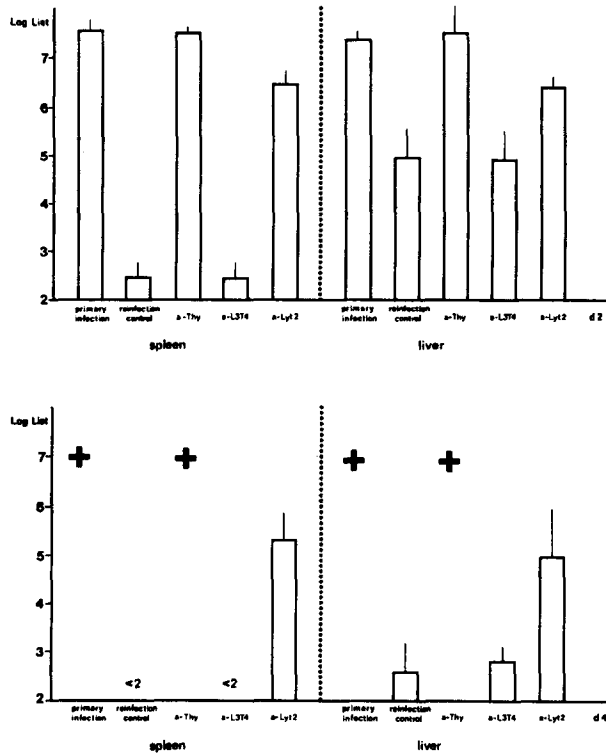
FIGURE 1. Effects of T cell subset depletion in vivo by mAbs to Thy-1.2, L3T4,, and Lyt-2 on the eradication of bacteria from spleens and livers of secondarily infected animals. Mice were immunized with 3 × 10⁴ viable *Listeria* organisms and reinfected with a lethal dose of bacteria 4 wk later. 3 d before secondary infection, mice were treated with an intraperitoneal injection of 1,000 μg of the appropriate mAb. Untreated primarily infected mice were investigated in parallel to demonstrate the degree of acquired immunity. Results are expressed as the mean numbers of *Listeria* (log₁₀ CFU) recovered from the spleens and livers of five mice per group 2 and 4 d after reinfection. (+) Lethal outcome.

group completely lost immunologic memory and all animals died on the third or fourth day after infection. Anti-Lyt-2-treated animals also showed a markedly reduced capacity to eradicate bacteria, especially in the spleen; however, these mice did not succumb to lethal infection.

Immunohistochemical examination carried out simultaneously on days 2 and 4 after reinfection demonstrated that livers of control mice contained numerous inflammatory foci consisting of F4/80⁺/Mac-1⁺ macrophages and L3T4⁺ T lymphocytes (Fig. 2, a–c). A similar pattern was seen in livers of Lyt-2⁺ T cell–depleted mice, although most granulomas were smaller and less densely packed than in control animals (Fig. 2, i–l). Livers from L3T4⁺ T cell–depleted mice, on the other hand, despite established immunity, were devoid of both focally organized accumulations of Mac 1⁺ macrophages and L3T4⁺ T cells (Fig. 2, f and g).

Kinetic studies carried out in order to examine the dynamics of hepatic granuloma formation in secondarily infected, Listeria-primed mice revealed that L3T4⁺ T cells markedly increased in numbers between days 2 and 3 of reinfection, whereas the number of Lyt-2⁺ cells increased only slightly after reinfection. Thus, L3T4⁺ T cells constituted the predominant lymphocyte population in the inflammatory foci (see also Fig. 2 c). In early stages of granuloma formation (on day 2), Lyt-2⁺ T cells were preferentially located at the periphery of the lesions (data not shown). A similar pattern of inflammatory cell influx has been shown to prevail in an adoptive transfer system (14).
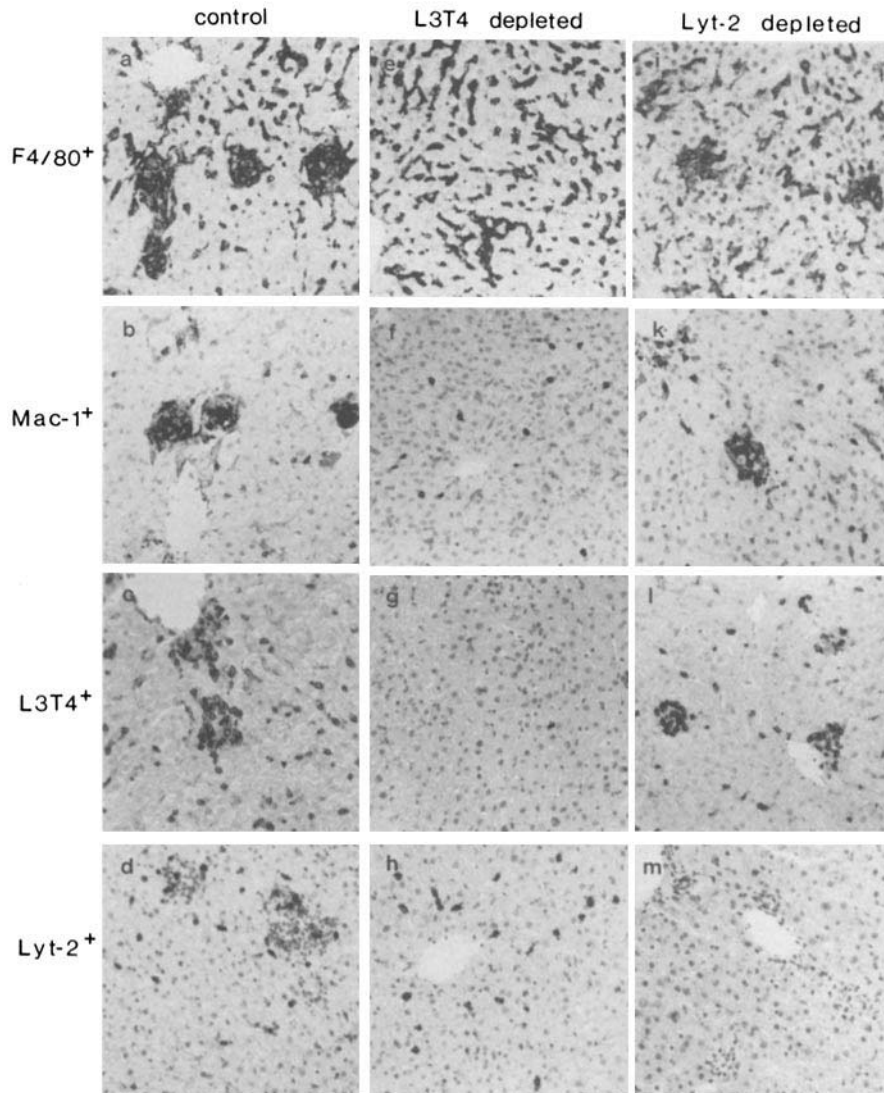
FIGURE 2.   Histomorphology of the immune response to *Listeria* in livers of normal control, or T cell subset-depleted secondarily infected mice. Cryostat sections from livers taken 4 d after reinfection were stained with mAbs with specificity to the surface antigens F4/80, Mac-1, L3T4, and Lyt-2 using the APAAP method.

The data presented indicate that granulomatous lesions in murine listeriosis, besides Mac-1$^+$ macrophages, mainly contain L3T4$^+$ T cells. The latter presumably mediate the attraction and accumulation of blood-borne monocytes into granulomatous lesions, which therefore appear to represent parenchymal expressions of delayed hypersensitivity. Yet, after treatment with anti-L3T4 mAb, immune mice are able to eradicate a manifold lethal bacterial burden in the absence of any L3T4$^+$ T cells, invading monocytes, and granulomatous inflammation. Instead, evenly distributed

Lyt-2$^+$ T lymphocytes (Fig. 2 h) and F4/80$^+$/Mac-1$^-$ (Fig. 2 e), i.e., resident macrophages, appear to be active in the rapid elimination of $L.$ $monocytogenes$.

In conclusion, the results of this study show that L3T4$^+$ T cells represent the predominant lymphocyte population in granulomatous lesions and can contribute to protection via the attraction and subsequent activation of invading mononuclear phagocytes, as postulated in the classical concept. However, as most clearly demonstrated in the absence of L3T4$^+$ T cells, another and more rapidly expressed protective mechanism exists. The latter most likely depends on an interaction of $Listeria$-primed Lyt-2$^+$ T cells and resident macrophages that results in listericidal activity.

It should be emphasized that our data do not preclude a more conspicuous role of granulomas in protection against other, especially more chronic, infections by facultative intracellular bacteria.

Summary

Facultative intracellular bacteria induce specific T cell responses of both the CD4$^+$ and the CD8$^+$ subsets. The immunohistological study of the tissue responses to $Listeria$ $monocytogenes$ in T cell subset–depleted, $Listeria$-primed mice revealed that CD4$^+$ cells not only represent the predominant lymphocyte population in granulomatous lesions but mediate the attraction and accumulation of blood-borne monocytes into inflammatory foci. On the other hand, CD8$^+$ T cells are able to mediate protection in the absence of CD4$^+$ T cells, invading monocytes, and granulomatous inflammation, and therefore appear to activate resident macrophages for listericidal activity.

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