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Gamma Interferon-Mediated Increase in the Number of Ia-Bearing Macrophages during Infection with *Listeria monocytogenes*

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The role of gamma interferon (IFN-γ) in an increase in Ia-bearing macrophages during *Listeria monocytogenes* infection was studied. The peritoneal macrophages from *L. monocytogenes*-infected mice contained a high proportion of Ia. Intraperitoneal injection of the supernatant from a culture of spleen cells from *L. monocytogenes*-infected mice induced Ia-rich exudates in normal mice. The Ia-inducing activity in the culture supernatant was abrogated by the pretreatment of spleen cells with anti-Thy-1.2 antibody plus complement. Immunoadsorption of the culture supernatant with anti-recombinant IFN-γ antibody and protein A-Sepharose CL-4B completely abrogated its Ia-inducing activity. These results suggested that an increase in Ia-bearing macrophages during *L. monocytogenes* infection was attributable to T-cell-derived IFN-γ.

Ia molecules are essential for the antigen-presenting function of macrophages, and their expression is regulated positively and negatively (28). An increase in Ia-bearing macrophages has been observed under most of the conditions resulting in T-cell activation (27). The most notable are infections with intracellular pathogens such as *Listeria monocytogenes* (4), *Mycobacterium tuberculosis* (7), or *Trypanosoma cruzi* (3). This increase in Ia-bearing macrophage expression is ascribed to T-cell-derived lymphokines (21, 25, 26). Activated T cells thus play a crucial role in increases in Ia-bearing macrophages during infection, and such increases seem to result in the up-regulation of the immune response (17).

It has been reported that the factor responsible for the induction of macrophage Ia expression has the same biochemical characteristics as gamma interferon (IFN-γ) (24, 29), and recombinant IFN-γ can induce macrophage Ia expression in vitro (2, 16) and in vivo (19, 23). Although recombinant IFN-γ can induce macrophage Ia expression, it has not been clearly shown yet that the lymphokine IFN-γ is the sole factor capable of inducing Ia expression. At the present level of knowledge, the possibility that some lymphokines other than IFN-γ cause Ia induction cannot be excluded. In this study, we investigated whether in *L. monocytogenes*-infected mice the regulation of macrophage Ia expression is mediated by IFN-γ or whether some other lymphokine is involved.

BALB/c mice were infected with 10^3 viable *L. monocytogenes* EGD. At various times after infection, peritoneal exudate cells (PEC) were recovered. Adherent cells were obtained by the removal of glass-nonadherent cells after 1 h of incubation of PEC suspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% NU-SERUM (Collaborative Research, Inc., Lexington, Mass.), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 5×10^{-2} mM 2-mercaptoethanol at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells adherent to glass cover slips were examined for the expression of Ia antigen as described previously (30). Briefly, 10^6 cells in medium were incubated with 2.5 μg of monoclonal anti-IA^d antibody (Becton Dickinson and Co., Sunnyvale, Calif.) per ml for 30 min on ice. The cells were washed twice with medium and exposed to an optimal concentration of fluorescein-conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin G (1:80; Cooper Biomedical, Inc., West Chester, Pa.) for 30 min. After being washed again, the cells were examined for their Ia expression by fluorescence microscopy. The specificity of the reaction was confirmed by the following findings: (i) cells incubated with fluorescein-conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin G alone never showed fluorescence; (ii) no positive reaction was seen when an unrelated antibody, monoclonal anti-Ia^k antibody (Becton Dickinson), was used as a control.

Spleen cells (5×10^6/ml) from mice infected with *L. monocytogenes* 14 days before were cultured for 24 h with killed listeriae (10^7/ml). After incubation, the culture fluid was centrifuged at 1,600 × *g* for 15 min, and the supernatant was centrifuged at 12,000 × *g* for 20 min. The supernatant was passed through a 0.45-μm-pore membrane filter and injected into mice immediately or was kept frozen at −70°C until use. A control culture supernatant was prepared similarly from a culture of spleen cells from noninfected mice. In some experiments, spleen cells were treated with monoclonal anti-Thy-1.2 antibody plus complement to remove T cells as follows; spleen cells were incubated with a 1:3,000 dilution of monoclonal anti-Thy-1.2 antibody (F7D5; Serotec Ltd., Oxon, United Kingdom) for 30 min at 4°C, washed, and then exposed to a 1:10 dilution of Low-Tox rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) for 45 min at 37°C.

Macrophage Ia-inducing activity in the culture supernatant was assayed in vivo. Undiluted culture supernatant (1 ml) was injected intraperitoneally (i.p.) twice with an interval of 24 h into naive recipient mice. The percentage and absolute number of Ia-bearing macrophages in the PEC were determined 48 h after the last injection.

Macrophage-activating factor (MAF) activity in the culture supernatant was assessed, by the method of Pace and Russell (20) with some modifications, as the ability of P815 mastocytoma cells to induce cytotoxic activity in PEC. The

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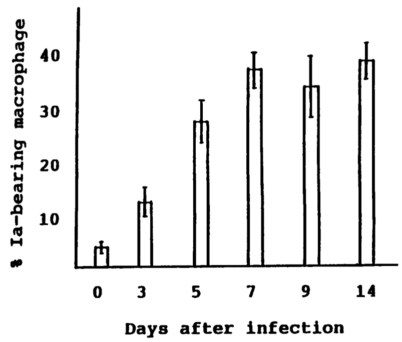

FIG. 1. Kinetics of the increase in Ia-bearing macrophages in peritoneal exudates after infection with *L. monocytogenes*. Mice were infected i.p. with 10^3 *L. monocytogenes* on day zero. PEC were collected for examination for Ia expression on adherent cells at various days after infection.

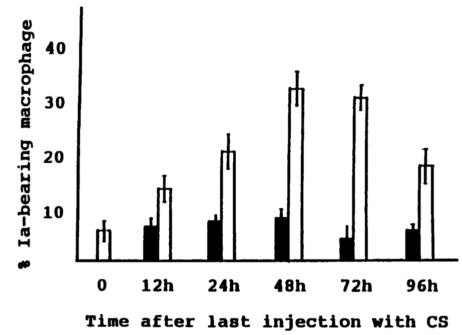

FIG. 2. Kinetics of induction of Ia-bearing macrophages in peritoneal exudates after injection with culture supernatant (CS). Mice were injected i.p. twice, with a 24-h interval, with 1 ml of undiluted CS of spleen cells from *L. monocytogenes*-infected mice (□) or of spleen cells from noninfected mice (■). PEC were collected for examination for Ia expression on adherent cells at various days after the last injection.

culture supernatant diluted 1:1 with medium was added to proteose peptone-induced PEC adhering to 96-well tissue culture plates. After incubation for 12 h at 37°C, the wells were washed, and ^{51}Cr-labeled P815 cells in medium containing 25 ng of lipopolysaccharide (*Escherichia coli* O111:B4; Difco Laboratories, Detroit, Mich.) per ml were added to yield an effector cell/target cell ratio of 20:1. After further incubation for 18 h, the tissue culture plates were centrifuged for 5 min, and 100 μl of supernatant was taken from each well. The released radioactivity in each supernatant was measured in an automatic gamma counter, and the specific ^{51}Cr release was calculated by the following formula: percent specific release = (experimental release − spontaneous release)/(total release − spontaneous release) × 100.

Rabbit antiserum raised against mouse recombinant IFN-γ, MI3, was a kind gift from H. Naruse and A. Inoue, Daiichi Seiyaku Co. Ltd., Tokyo, Japan. The IFN-γ-neutralizing titer of MI3 was 200 U/ml at a final dilution of 1:800. MI3 (10 μl) was added to 8 ml of culture supernatant. After incubation for 2 h at 4°C, protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) was added to the mixture (pH 7.4) to produce a 10% packed volume, and the tube was kept rotating at 4°C for 2 h. After centrifugation at 1,600 × *g* for 10 min, the supernatant was filtered through a 0.45-μm-pore membrane filter prior to use in experiments.

The percentage of Ia-bearing peritoneal macrophages from normal mice was usually less than 10%. Infection of mice with viable *L. monocytogenes* resulted in a significant increase in the percentage of Ia-bearing peritoneal macrophages. This change was not apparent at 3 days after infection but became pronounced by 5 days and persisted up to 14 days (Fig. 1). The absolute number of Ia-bearing peritoneal macrophages from mice infected with *L. monocytogenes* also increased accordingly (data not shown).

Culture supernatants of spleen cells derived from mice infected with *L. monocytogenes* 14 days before were injected twice i.p. into naive recipient mice, because a single injection did not cause any significant change in the percentage and absolute number of Ia-bearing macrophages in peritoneal exudates, a result which was consistent with the observations of Scher et al. (21). We also examined the kinetics of appearance of Ia-bearing macrophages in peritoneal exudates. An increase in the percentage of Ia-bearing macrophages was found as early as 12 h after the last injection, and the maximal level of increase was observed 48 to 72 h after the last injection (Fig. 2). Therefore, we used the protocol of injecting 1 ml of undiluted culture supernatant twice with a 24-h interval and harvesting PEC 48 h after the last injection as the optimal schedule for inducing a high percentage of Ia-bearing macrophages. With this protocol, culture supernatants of spleen cells from *L. monocytogenes*-infected mice induced about 20 to 40% of Ia-bearing macrophages in peritoneal exudates in naive recipient mice, while culture supernatants of normal spleen cells had no effect.

When spleen cells were treated with anti-Thy-1.2 antibody plus complement, the Ia-inducing activity found in culture supernatants was completely abrogated (Fig. 3). The Ia-inducing activity was T-cell dependent, probably attributable to T-cell-derived lymphokines.

Immunoadsorption was carried out to determine whether IFN-γ present in the culture supernatant was the sole mediator required for the induction of macrophage Ia expression. Interferon activity in the culture supernatant of spleen cells from *L. monocytogenes*-infected mice was identified by the ability to protect L-929 cells from infection by vesicular stomatitis virus in the cytopathic effect assay. The culture supernatant contained 8 U of IFN-γ per ml in this assay (Y. Watanabe, M. Mitsuyama, T. Koga, and K. Nomoto, submitted for publication). The culture supernatant was treated with anti-recombinant IFN-γ antiserum at a final dilution of 1:800. This amount of antibody was sufficient to adsorb 8 U of IFN-γ per ml. After the treatment, Ia-inducing activity and MAF activity in the culture supernatant were

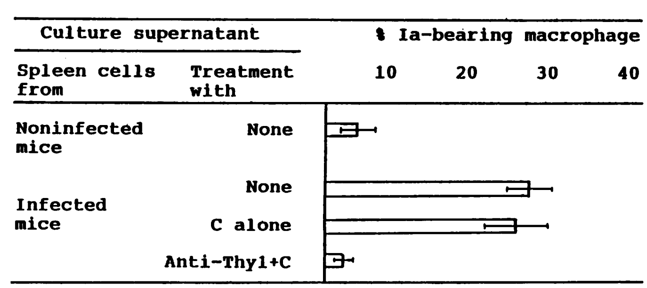

FIG. 3. Effect of treatment with anti-Thy-1.2 antibody plus complement (C) on the generation of Ia-inducing activity by spleen cells. Spleen cells from *L. monocytogenes*-infected mice were treated with anti-Thy-1.2 antibody plus complement or complement alone before removal of the culture supernatant. Mice were injected i.p. with 1 ml of undiluted culture supernatant 72 and 48 h before PEC were collected.

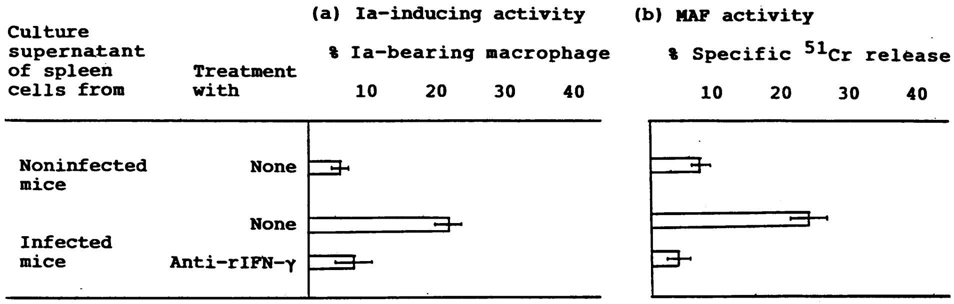

FIG. 4. Ia-inducing activity (a) and MAF activity (b) in a culture supernatant treated with anti-recombinant IFN-γ (Anti-rIFN-γ). The culture supernatant was treated or not treated with anti-recombinant IFN-γ diluted to 1:800; each culture supernatant was then incubated with protein A-Sepharose CL-4B. Ia-inducing activity was determined in vivo, and MAF activity was assessed as the ability of P815 mastocytoma cells to induce cytotoxic activity in PEC.

completely abolished (Fig. 4). This result suggested that the Ia-inducing activity in the culture supernatant which was detectable in our assay system was attributable to IFN-γ.

In the present study, infection with *L. monocytogenes* resulted in a marked increase in Ia-bearing peritoneal macrophages as early as 5 days after infection, and this increase was ascribed to T-cell-derived IFN-γ.

The major lymphokine responsible for the induction of macrophage Ia expression is thought to be IFN-γ, as initially reported by Steeg et al. (24). They showed that biochemical manipulations that enriched for or depleted IFN-γ correlated with the enrichment or loss of Ia-inducing activity, respectively, and that antiserum prepared against semipurified murine IFN-γ abolished Ia-inducing activity. Furthermore, Schreiber et al. reported that monoclonal antibodies to recombinant murine IFN-γ inhibited Ia-inducing activity in lymphokine preparations (22). The above-mentioned studies utilized the culture supernatant of concanavalin A-stimulated spleen cells as the source of Ia-inducing lymphokines. Purified cDNA-derived IFN-γ possessed the capacity to induce Ia expression (2, 16, 19, 23). These facts suggest that IFN-γ is the only lymphokine capable of inducing macrophage Ia expression. However, Groenewegen et al. reported that a mediator other than IFN-γ in the supernatants of human leukocytes induced Ia expression (8). Bancroft et al. reported that infection of lymphocyte-deficient CB-17 SCID mice with *L. monocytogenes* led to an unexpected increase in Ia-bearing peritoneal macrophages (1). Ziegler et al. found that i.p. injection of lipopolysaccharide into mice caused a substantial increase in Ia-bearing peritoneal macrophages (31). Chang and Lee reported that tumor necrosis factor-α induced Ia antigen and that stimulation by the combination of tumor necrosis factor-α and IFN-γ was more than additive relative to the effects of each cytokine alone (6). These studies raised the possibility that some soluble factor other than IFN-γ or some other pathway which is independent of T cells may induce macrophage Ia expression. In view of the above-described findings, it was important to investigate whether the regulation of macrophage Ia expression is mediated by only IFN-γ in *L. monocytogenes* infections. The present study demonstrated that IFN-γ, one of the most important T-cell-derived lymphokines, is solely responsible for the Ia-inducing activity found in the culture supernatant of *L. monocytogenes*-activated spleen cells (Fig. 3 and 4).

We previously showed that *L. monocytogenes* infections resulted in an increase in Ia-bearing macrophages in neonatally thymectomized mice but not in athymic nude mice (18). A T-cell subset generating the mediator was thymus dependent but seemed to require the presence of the thymus only for a short period in its development, while the production of interleukin 2 was not found in this T-cell subset (14).

It was shown that both Ia-inducing activity and MAF activity in the culture supernatant of spleen cells from *L. monocytogenes*-infected mice were attributable to the same molecule, IFN-γ (Fig. 4). IFN-γ is produced by T cells from *L. monocytogenes*-infected mice (5, 9, 13) and is involved in antilisterial protection via the activation of macrophages (5, 15). A question emerged from these observations. We wanted to find out whether macrophage listericidal-enhancing activity in culture supernatants could also be attributed only to IFN-γ. Schreiber et al. showed that anti-recombinant IFN-γ inhibits the activity in culture supernatants of concanavalin A-activated spleen cells to enhance the killing of *L. monocytogenes* (22). Hoover et al. reported the activation of human monocytes to kill *Leishmania donovani* by a lymphokine physicochemically and antigenically distinct from IFN-γ (10). It would be interesting to know whether a non-IFN-γ mediator of this activity exists in culture supernatants.

The rapid increase in Ia-bearing macrophages during *L. monocytogenes* infection and its persistence (Fig. 1) may potentially augment the immunoregulatory capacity of macrophages. To what extent this increase is responsible for the immune response noted in *L. monocytogenes* infections may be important. Jerrells reported that Ia-bearing macrophage influx is associated with the genetic resistance of mice to infection with *Rickettsia tsutsugamushi* (11). Johnson and Zwilling reported that the continuous expression of Ia in peritoneal macrophages correlates with the genetic resistance of mice to *Mycobacterium bovis* BCG (12). In our previous study (17), we reported that the secondary immune response to sheep erythrocytes was augmented as a result of an increase in Ia-bearing macrophages through the administration of a lymphokine-containing culture supernatant prepared in the same way as described in the present study. It needs to be clarified whether the IFN-γ-mediated increase in Ia-bearing macrophages is directly related to the antibacterial resistance of mice to *L. monocytogenes*.

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