REQUIREMENT OF THYMUS (T) LYMPHOCYTES FOR RESISTANCE TO LISTERIOSIS*

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The resistance to facultative intracellular bacteria such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* involves the interaction of lymphocytes and mononuclear phagocytes (1). In the course of an infection with *Listeria*, lymphoid cells are found in the spleen and thoracic duct lymph with the capacity to transfer protective immunity to normal mice challenged with a lethal dose of the organism (2). The protective immunity involves the participation of highly microbicidal phagocytes at the sites of *Listeria* infection, almost certainly activated by the interaction of the lymphoid cells and the *Listeria* organism or its products. The functional characteristics of these "activated macrophages" have recently been reviewed (1) and are not the subject of this report.

The resistance to *L. monocytogenes* is a form of cell-mediated immunity. Immunity to *Listeria* correlates well with the development of skin sensitivity to *Listeria* extracts and, of more importance, cannot be transferred with antibody, but only with lymphoid cells (2). Other accepted types of cell-mediated immune reactions such as skin sensitization to products of tubercule bacillus (3), allograft rejection (4), tumor rejection (5), and some autoimmune states (6) are transferred to normal recipients by live cells and not by conventional serum antibody. The cells involved in all of these cell-mediated immune reactions are believed to derive from the thymus and to form part of the thymus (T) cell[1] population of peripheral lymphoid organs. (We accept the nomenclature of T lymphocytes as those cells derived from the thymus, and B lymphocytes as those derived from bursa or the mammalian equivalent.) The evidence for T lymphocytes being involved in cell-mediated immunities is in part circumstantial and based upon the observations that such immune states are highly dependent upon an intact thymic function (7). Also, the fact that these immune states are not transferred by antibodies, a B cell product, speaks in favor of T lymphocytes as one of the effector cells. Recently the availability of antibodies to membrane antigens of T or B lympho-

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[1] *Abbreviations used in this paper:* B lymphocytes, bone marrow–derived cells; FCS, fetal calf serum; IV, intravenously; NMS, normal mouse serum; PBS, phosphate-buffered saline; T lymphcytes, thymus-derived cells.

cytes has permitted the direct investigation of the type of cells involved in different immune states (8). It was possible to demonstrate with the use of antibodies to the thymic alloantigen θ that indeed T cells were involved in the killing of tumor cells (9), in helper function (10), and in resistance to some viral infections (11).

If the cellular resistance to facultative intracellular bacteria such as *Listeria monocytogenes* is dependent upon an intact thymic function, then it is expected that such resistance should be transferable to normal hosts only by cell suspensions containing T lymphocytes. We have investigated this point and found such expectation to be true.

Materials and Methods

Animals.—For all experiments involving *Listeria* 8–10-wk-old BC_3F_1 male or female mice obtained from Cumberland Farms (Nashville, Tenn.) were used. AKR and CBA mice were obtained from Jackson Laboratory, Bar Harbor, Maine.

Microorganisms.—Listeria monocytogenes, obtained from Dr. G. B. Mackaness (Trudeau Institute, Saranac Lake, N. Y.), was used in all experiments. The organism was maintained by serial passage in BC_3F_1 mice. For passage approximately 200 organisms were injected intravenously (IV) into each mouse and 2 or 3 days later the spleen was homogenized in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and cultured at 37°C. The organisms were harvested in the log phase of their growth curve, at 16 hr, washed twice in phosphate-buffered saline (PBS), counted, and immediately injected into mice for repassing or experimental studies.

Counting Microorganisms.—The number of organisms was estimated by optical density measurements in a Zeiss spectrophotometer (Carl Zeiss, Inc., New York) at 540 λ. The readings were verified after appropriate dilution and 24 hr of culture in tryptose agar at 37°C in room air. We found that the number of viable organisms correlated reasonably with the number estimated by spectrophotometry.

LD_{50}.—The LD_{50} was determined approximately every 2 months since the virulence of the *Listeria* increased with repassing. At the time of the transfer experiments the LD_{50} was 1×10^5 *Listeria* organisms.

Immunization.—Approximately one-fifth the LD_{50} dose was given IV for maximal primary immunization. Hyperimmunization was achieved by giving approximately five times the LD_{50} dose IV 10 days after the primary immunization.

Assay for Protective Anti-Microbial Immunity.—Anti-microbial immunity was determined by estimating the growth of a challenge dose of *Listeria* in the spleen of actively or passively immunized mice. The rationale for this approach is based upon the clear studies of Mackaness and his group with the same type of organism (1, 2). The challenge dose which was 100% lethal in unimmunized animals was about five times the LD_{50}.

Each experimental group usually contained six mice. The spleens were assayed 2 or 3 days after the IV challenge with *Listeria*. Each spleen was taken with sterile technique, homogenized in tryptic soy broth, and appropriately diluted (10^2, 10^4, and 10^5). Each dilution was cultured in duplicate in tryptose agar at 37°C in room air for 24 hr. The duplicate colony counts were made and averaged to express the individual spleen count. The value for each experimental and control group is represented by the geometric mean of that group and statistical significance is measured by the Student's t test.

Transfer of Immune Lymphocytes.—Normal, immune, and hyperimmune spleen cells were obtained by teasing in Eagle's minimal essential medium (Associated Biomedic Systems, Inc., Buffalo, N.Y.) and 5% fetal calf serum (FCS). The cells were washed two times in the same medium, passed through a nylon filter, and counted. Viability was estimated by trypan blue

dye exclusion. All cell counts were expressed as the number of viable nucleated cells. The cells were transferred to syngeneic recipients IV in 0.4 ml of Eagle's medium containing the challenge dose of approximately 5×10^5 *I. monocytogenes*. The transfer of immunity was assayed in the recipient animals by the quantitative spleen colony count assay described above.

Anti-θ Serum.—Anti-θ serum was produced in AKR mice (6 wk of age at the start of the experiment) immunized with CBA thymus cells. The AKR mice were immunized three times every 2 wk with 10^8 thymus cells injected intraperitoneally. The first injection contained besides the CBA thymocytes, 10^9 dead *Bordetella pertussis* organisms. (The addition of *B. pertussis* serves as an adjuvant and increases the titer of anti-θ antibodies; we thank Dr. J. C. Cerottini, Swiss Institute for Cancer Research, Lausanne, Switzerland, for advising us on this procedure.) The anti-θ serum reacted specifically with T cells. The antisera killed 96–100% of thymus cells and only 30–35% of spleen cells. The spleen cells that were spared by the

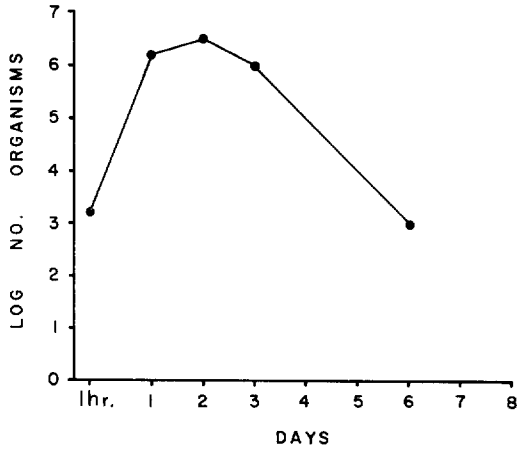

FIG. 1. Growth curve of *L. monocytogenes* in BC_3F_1 mice. 8×10^4 *Listeria* organisms were given IV to BC_3F_1 mice at the beginning of the experiment. Spleens of six mice were harvested at 1 hr, 1, 2, 3, and 6 days. Each point represents the geometric mean of the $\log_{10}$ organisms viable in the spleens.

treatment with anti-θ sera contained surface Ig by immunofluorescence and were considered to be B lymphocytes. The complement source was guinea pig sera obtained lyophilized from Microbiological Associates, Inc., Bethesda, Md. The cytotoxic tests included the trypan blue exclusion test and the ^{51}Cr release from cells.

The cells, 1.5×10^8, were treated with 0.5 ml of sterile anti-θ serum, at 37°C for 30 min in a 2 ml volume. The cells were washed, resuspended in 3 ml of media containing 0.5 ml guinea pig sera and 0.5 mg of DNAse for ½ hr at 37°C. The cells were then washed twice, counted, and tested for viability. Usually 40–50% of the cells were recovered with a viability of 95–98%.

EXPERIMENTS AND RESULTS

Immunity to Listeria in BC_3F_1 mice.—The immune response of BC_3F_1 mice to *Listeria* was studied by measurement of the growth curve of the organism in vivo. 8-wk-old BC_3F_1 mice received 8×10^4 *Listeria* organisms IV. At

1 hr, 1, 2, 3, and 6 days, six mice were sacrificed, and the number of *Listeria* present in the spleen determined by the spleen colony count assay. Each value represented the geometric mean of six animals. Results are depicted in Fig. 1.

The rapid growth of organisms reached a peak at 2 days and by the third day there was already some evidence of protective immunity since the number of organisms was reduced to 20% of the 2 day peak value. For this reason, the second or third days were selected as the time for assay in the experiments of adoptive transfer of immunity. By 6 days the number of organisms was about 1/1000 of that found at 2 days.

The development of protective immunity appeared to be dependent on the

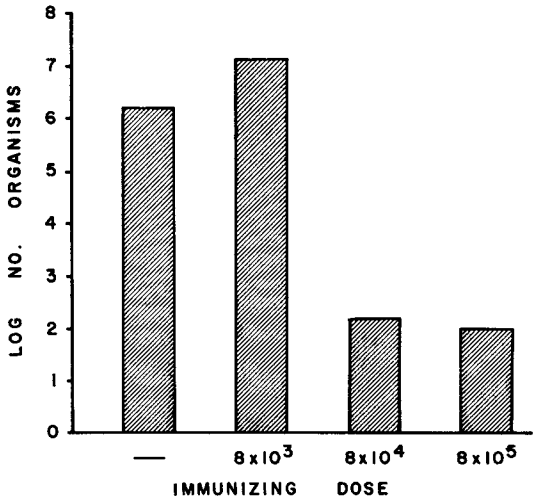

FIG. 2. Development of resistance to lethal challenge of *Listeria*. Mice were given 8×10^3, 8×10^4, or 8×10^5 *Listeria* IV. These mice and a group of normal mice were challenged 7 days later with 3.64×10^6 *Listeria* IV. Spleen culture and colony count assay was done 2 days later and the geometric mean of the six mice in each group is shown in the bar graph above.

strength of the immunizing dose. This was tested in an experiment in which BC_3F_1 mice received no organisms, 8×10^3, 8×10^4, or 8×10^5 *Listeria* organisms IV. 7 days later each group of mice was challenged with 3.64×10^6 organisms and 2 days later a spleen colony count assay was done. The points in Fig. 2 represent the geometric mean values of the six animals in each group. The 8×10^4 and 8×10^5 doses evoked similar degrees of immunity which were approximately 4 $\log_{10}$ differences from control or unimmunized animals challenged with the same number of organisms. The administration of 8×10^3 organisms did not produce any resistance in the test animals.

Transfer of Protective Immunity.—The adoptive transfer of protective immunity to *Listeria* was achieved by the transfer of spleen cells 7 days after the primary immunization (immune cells). Spleen cells were also obtained

from mice which had received a primary immunizing dose of 1/5 LD_{50} *Listeria* and 10 days later a second dose of 5 × LD_{50}. These cells were transferred 4 days after the second injection (hyperimmune cells).

Normal, immune, and hyperimmune BC_3F_1 spleen cells were harvested as described in the Materials and Methods section and transferred into syngeneic BC_3F_1 mice of the same sex, IV, together with a challenge dose of 5 × LD_{50} of *L. monocytogenes*. The transfer of immunity was assayed in the recipient by the quantitative spleen colony count assay described previously.

The results, in Fig. 3, showed that 2×10^8 immune spleen cells adoptively transferred immunity to syngeneic recipient mice challenged with 9×10^5 *L. monocytogenes*. (Some degree of resistance could be achieved by transferring

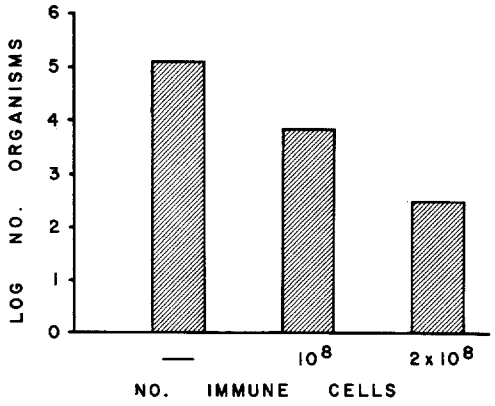

FIG. 3. Adoptive transfer of resistance to lethal challenge of *Listeria*. Either 1×10^8 or 2×10^8 immune spleen cells were transferred into normal syngeneic recipients. At the same time these animals as well as a control group which received no cells were challenged with 9×10^5 *Listeria* IV. Spleen colony count assay was done 2 days later and each group of six mice is represented by the geometric mean.

1×10^8 immune cells but a dose of 4×10^7 cells was ineffective.) As shown in the experiments of Fig. 4, a comparable degree of immunity could be transferred with 4×10^7 hyperimmune spleen cells. This apparent expansion of the specifically committed lymphocyte population in the hyperimmune state was evidently limited: the transfer of 8×10^6 hyperimmune cells failed to confer protective immunity to the recipients.

Immune spleen cells did not transfer resistance into X-irradiated mice. In one experiment all mice that were irradiated with 660 R and received immune spleen cells and *Listeria* organisms died 2–3 days later. Hence, as expected the host mononuclear cells were necessary for the transfer of resistance (1, 2).

Inhibition of Adoptive Transfer with Anti-θ Serum.—Spleen cells from hyperimmunized mice were teased, filtered through nylon, and washed in Eagle's

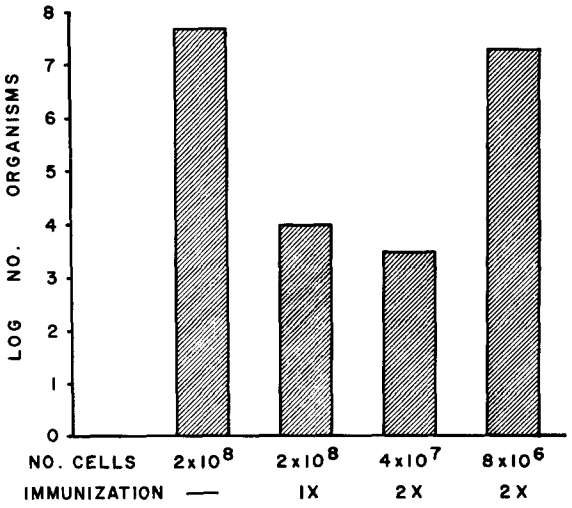

FIG. 4. Enhancement of adoptive transfer by hyperimmunization. 2×10^8 normal spleen cells, 2×10^8 immune spleen cells, 4×10^7 hyperimmune spleen cells, and 8×10^6 hyperimmune cells were transferred IV with 9×10^5 *Listeria* organisms to normal syngeneic recipients. 2 days later spleen colony count assay of the six mice in each group was done and the geometric mean values are represented by the bar graph.

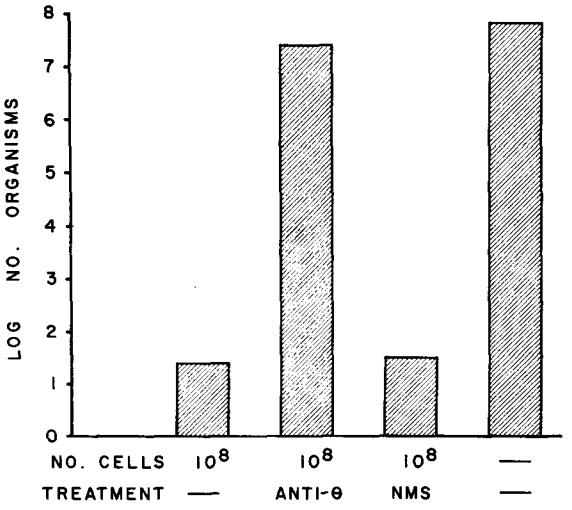

FIG. 5. Inhibition of adoptive transfer of *Listeria* resistance by anti-θ serum. 10^8 hyperimmune spleen cells either untreated, treated with anti-θ serum, or treated with NMS as described in the Materials and Methods section were transferred IV with 4.75×10^5 *Listeria*. A control group received 4.75×10^5 *Listeria* but no cells. Spleen culture and colony count assay of the six mice in each group was performed 3 days later. The bar graph represents the geometric mean of each group.

medium with 5% FCS. The cells were treated with anti-θ sera, normal mouse serum, or not treated at all; 1×10^8 viable cells were injected IV in 0.4 ml together with the challenge dose of 4.75×10^5 *Listeria*. One group of normal mice received a challenge injection of *Listeria* but no cells were transferred. Spleen colony count assay was performed 3 days later.

The results, described in Fig. 5, show that hyperimmune spleen cells transferred 6 $\log_{10}$ degrees of protection against a lethal *Listeria* challenge. Treatment of the hyperimmune cells with anti-θ serum completely abolished this protective effect, but treatment with normal mouse serum (NMS) had no inhibitory effect. Two other experiments of this nature using immune spleen cells gave similar results.

DISCUSSION

The complete abolition of the transfer of cellular immunity by treatment of the lymphocytes with specific anti-θ serum indicates that T cells are of prime importance in the initiation of the immune resistance to listeriosis. The specificity of the anti-θ sera for T cells is reasonably clear. The θ antigen is present on most thymic cells and on most T cells of peripheral lymphoid organs (8). All evidence implies that such θ-bearing cells are T lymphocytes, although the converse has not been clearly established. Our antisera, at the doses and under the technical conditions employed, were reacting mainly, if not completely, with T lymphocytes and spared most, if not all, B lymphocytes. It then was apparent that a cell suspension composed mostly of B lymphocytes as a result of treatment with anti-θ sera was ineffective in conferring resistance to listeriosis.

One function of T lymphocytes, that of killing tumor cells, takes place directly without the apparent intervention of any other type of lymphocyte (9). A second function of T lymphocytes, that of helping in the production of antibody to certain antigens, takes place by regulation of the activity of B lymphocytes (10). Mediating resistance to infection with the intracellular facultative bacteria *L. monocytogenes* is one more function of the T cell. This third function takes place by attracting and stimulating the microbicidal activity of the mononuclear phagocyte system in some way (11). It remains to be shown whether the T lymphocytes are capable of elaborating humoral factors which call for and activate the macrophages and/or whether they do this indirectly by first acting on an intermediate B lymphocyte. The former possibility is most likely; the association of delayed hypersensitivity, or cell-mediated immunity, with mononuclear phagocytes is well established (12). Our results bring one more piece of evidence, this time by a direct approach, in support of the T cell origin of these reactions.

Our studies do not yield information on the characteristics of the T lymphocytes except that they indicate that these cells result from antigen stimulation and that they increase in activity after secondary immunization. Mackaness

and colleagues have shown that the lymphocytes responsible for *Listeria* resistance are sensitive to anti-lymphocyte serum (13), appear in the thoracic duct early after stimulation, do not recirculate from blood to lymph (14), and tend to accumulate in sites of specific or nonspecific inflammation (15). These results may be characteristics of T lymphocytes which may show functional heterogeneity during their life-span. Indeed, T lymphocytes with different properties have been identified (16).

SUMMARY

Spleen cells of mice infected with *Listeria monocytogenes* were adoptively transferred to normal mice. Such lymphocytes conferred resistance to a lethal challenge with *Listeria*. Hyperimmunization of the donor reduces the number of cells necessary to transfer effective immunity. Such spleen cells if treated with anti-θ serum do not transfer resistance to *Listeria*. Hence, thymus (T) lymphocytes are involved in the resistance to infection with the facultative intracellular bacteria *L. monocytogenes*.

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