INFECTION AND IMMUNITY, Feb. 1974, p. 354-362
Vol. 9, No. 2
Printed in U.S.A.
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Relationship of Antimicrobial Cellular Immunity to Delayed Hypersensitivity in Listeriosis

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Received for publication 1 October 1973

The relationship of antimicrobial cellular immunity to delayed hypersensitivity (DH) was studied in mice antigenically stimulated by living *Listeria monocytogenes* confined to diffusion chambers in peritoneal cavities or by subcutaneous inoculation of sublethal doses of the organism. Mice showed DH reactions when tested 6 days after inoculation, and reactions were positive for at least 90 days in some mice. DH also became established when the mice were stimulated by antigens diffusing from peritoneal chambers containing viable *Listeria*. Mice were categorized as DH positive or DH negative if they developed more or less than a 5% increase in foot volume 24 h after the injection of *Listeria* antigen. Some antigenically stimulated mice did not elicit the DH reaction. Consequently, the animals were arranged as immunized groups (DH positive and DH negative) and *Listeria* chamber implant groups (DH positive and DH negative). When challenged with *L. monocytogenes*, all four groups were significantly resistant as compared with controls. Thus, the in vivo tests for immunity and DH did not show direct correlation. The results suggested that antimicrobial cellular immunity can occur as a phenomenon independent of DH. Evidence for antimicrobial cellular immunity as the principle mechanism of resistance in murine listeriosis is discussed with consideration for possible heterogeneity of function by thymus-derived lymphocytes.

Listeriosis in mice has been a useful model for studying resistance mechanisms to infections by facultative intracellular parasites. The injection of anti-*Listeria* sera into normal mice demonstrated that humoral antibodies alone did not enhance host resistance (20, 27). However, active immunity was achieved after the inoculation of sublethal doses of living *Listeria* cells (14, 27). The appearance of delayed hypersensitivity (DH) was found to correlate with the time when macrophages became highly efficient in the intracellular inactivation of *Listeria monocytogenes* (14). The dependence of increased resistance on sensitized lymphocytes was demonstrated by the injection of heterologous antibodies causing lymphocyte injury. Normal mice were made resistant by the injection of immune spleen cells and the immunity could be abolished by the injection of antilymphocyte globulin (ALG) (19). The requirement for thymus-derived lymphocytes was indicated when spleen cell suspensions lost the ability to passively transfer immunity after treatment with anti-theta serum (12) and T cell-depleted mice failed to generate anti-*Listeria* resistance (2). When antithymocyte serum (ATS) was injected into normal mice at the time of infection, the afferent limb of the immune response was suppressed (29). Injection of ATS in immune mice caused suppression of both delayed hypersensitivity and antimicrobial cellular immunity as measured by lethality of the infectious process (30).

Although the cell-mediated immune (CMI) reactions of delayed hypersensitivity and antimicrobial cellular immunity usually occur concomitantly, the relationship between the two phenomena remains uncertain. The multiple antigenic stimulation of an infectious process was considered useful for studying the possible selection of cell-mediated immune reactions. It was of interest to determine whether the DH state would become established when the antigenic stimuli were presented as live *Listeria* cells confined to diffusion chambers in contrast to organisms inoculated into tissues. The present experiments were under-

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taken to explore these questions and the dependence of antimicrobial cellular immunity on delayed hypersensitivity.

MATERIALS AND METHODS

Bacteria. *L. monocytogenes* strain 3-54 (serotype 4b) was used for immunization and challenge inoculations, for implantation in diffusion chambers, and for the preparation of antigen to detect DH. Methods for cultivating the bacteria and preparing suspensions were reported previously (24, 29). The organism was passaged through mice to maintain virulence and a smooth growth phase.

Diffusion chambers. Diffusion chambers for intraperitoneal implantation were 20 mm in diameter and 5 mm high. Filter membranes (Millipore Corp., Bedford, Mass.) with a 0.22 μm pore size were attached with Millipore cement. Details on the construction, loading, and bacteriological testing of the chambers were described elsewhere (24).

Animal groups for diffusion chamber studies. An outbred line of young adult, female mice of the Swiss-Webster strain (approximately 25 g in weight) was used.

(i) Listeria chamber implant groups. Diffusion chambers containing *Listeria* cells suspended in tryptose broth were implanted in the peritoneal cavities of mice. Methoxyflurane anesthesia was used during the surgery.

(ii) Sham chamber implant group. Diffusion chambers containing tryptose broth were implanted as described for the *Listeria* chamber implant group.

(iii) Immunized groups. Mice in the immunized groups received two subcutaneous inoculations of living *L. monocytogenes* in the region of the back. The immunization procedure began on the same day that diffusion chambers were implanted.

(iv) Normal control groups. Untreated mice were housed under the same conditions as the treated groups and their natural resistance to *L. monocytogenes* was challenged along with the treated animals.

Data analysis. Levels of resistance were measured by the host response to 10-fold graded doses of *L. monocytogenes* expressed as 50% end points (8). Standard error was determined by the procedure of Irwin and Cheeseman (7). The difference between the effects of the challenge on animal groups were evaluated by the formula:

$$Z = \frac{X - Y}{\sqrt{\delta x^2 + \delta y^2}}$$

The Z value represented the difference between two variables (X and Y, the two mean lethal dose [LD_{50}] logs) divided by the standard error of the difference of the two variables. A Z value of 2 would indicate a significant difference in the two groups at the 5% level assuming that the animal response followed a normal distribution. In the study by Irwin and Cheeseman, heterogeneity of response in mice to *Salmonella typhimurium* infection was encountered. They suggested that a value of 3 could more safely be considered a significant difference than a value of 2, when analyzing host responses in animals containing the degree of heterogeneity of those used in their study. In ascribing significance to the data reported here, interpretations were arbitrarily made as follows: Z values of 3.0 and greater were considered significant, 2.5 to 2.9 were possibly significant, and 2.4 and less were not significant.

Antigen for detection of delayed hypersensitivity. Antigen was prepared by inoculating *L. monocytogenes* into Trypticase soy broth which contained only the dialyzable components of the medium (30). The culture was incubated at room temperature for 24 h and then at 37 C on a shaker for an additional 72 h. Bacteria were sedimented by centrifugation at 7,000 $\times$ *g* for 20 min. The supernatant fluid was filtered through a 0.22 μm membrane filter to yield the broth antigen. After testing for bacterial sterility, the antigen was stored at 4 C in sterile vials.

The protein concentration of the antigen used to study DH and for experiment A was 3.1 mg/ml. Another antigen preparation was used in experiment B which had a concentration of 2.6 mg/ml.

The hind footpad of the mouse was injected with 0.02 ml of antigen. The volume of footpad swelling that occurred was determined by liquid displacement (30, 31). The foot volume of each mouse was measured five consecutive times prior to the injection of *Listeria* antigen and again at 24 and 48 h after antigen injection. Increase in the mean volume relative to the volume before antigen injection was recorded as the increase in foot volume. Changes in volume were evaluated by Student's *t*-test. The significance of foot volume changes in mice exposed to *Listeria* compared with unsensitized controls was tested by analysis of variance.

RESULTS

Delayed hypersensitivity reaction. It was necessary to obtain data on the times of DH responses in *Listeria*-sensitized mice and to ascertain the merits of the antigen used for its detection. Groups of five mice each were tested during the period of 6 to 90 days after the intraperitoneal inoculation of live *Listeria* cells. Animals receiving one sensitizing inoculation were given 8.8×10^5 bacteria. Groups receiving two sensitizing inoculations were given 1.6×10^5 *Listeria* cells followed by another 4.4×10^6 cells 14 days later. Measurements of foot volume were made before and after the injection of antigen into the footpad, and a mean foot volume was determined from the five animals in a group. The groups of mice were subjected to testing on two or three occasions with intervals between tests ranging from 12 to 57 days. Results recorded in Table 1 indicate that DH was readily detected 6 days after sensitization with living bacteria. The swelling was usually greater at 24 h, although strong reactions were encountered at the 48-h reading. The sensitization had begun to wane in some individuals by 18 days after the inoculation of live bacteria.

TABLE 1. *Delayed hypersensitivity in mice to Listeria antigen measured by increase in foot volume*

Day after sensitization	One sensitizing inoculation[a]		Two sensitizing inoculations		Unsensitized controls	
	24 h	48 h	24 h	48 h	24 h	48 h
6	13.0 ± 5.1[b] 4/5[c]	4.6 ± 3.2 2/5	15.6 ± 3.4 5/5	12.9 ± 2.2 5/5	−3.4 ± 1.8 0/5	−2.2 ± 2.4 0/5
12	21.0 ± 2.6 5/5	17.2 ± 3.9 5/5	21.0 ± 4.5 5/5	11.4 ± 1.4 5/5	0.7 ± 1.2 0/5	−0.7 ± 1.1 0/5
18	9.2 ± 3.4 3/5	3.5 ± 0.9 2/5	8.1 ± 2.0 3/5	2.9 ± 1.8 2/5	1.7 ± 2.7 1/5	−3.1 ± 4.5 0/5
60						
set 1	5.6 ± 2.8 2/5	8.2 ± 3.2 4/5	ND[d]	ND	3.0 ± 0.5 0/5	2.7 ± 1.5 1/5
set 2	3.8 ± 5.0 2/5	0.2 ± 2.6 1/5	13.4 ± 9.1 2/5	10.9 ± 7.4 3/5	−0.1 ± 1.5 0/5	−1.0 ± 1.8 0/5
75	ND	ND	4.8 ± 2.9 2/5	6.2 ± 1.6 3/5	1.8 ± 1.5 0/5	0.7 ± 1.4 0/5
90	ND	ND	9.5 ± 6.2 2/5	1.6 ± 2.7 1/5	−1.0 ± 1.1 0/5	0.1 ± 1.4 0/5

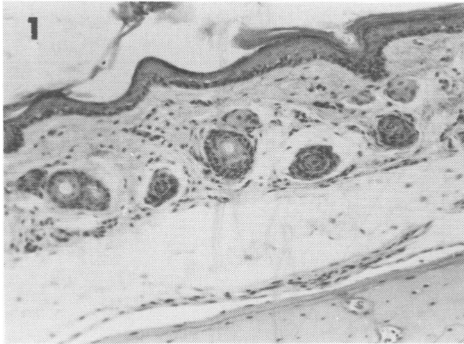
[a] Sensitized by the intraperitoneal inoculation of living *L. monocytogenes*.
[b] Mean percentage of foot volume increase and standard error of the mean. Each value calculated from 25 measurements (five mice per group, five measurements per mouse).
[c] Number of individual mice showing significant swelling per number mice tested ($P < 0.05$).
[d] ND, Not done.

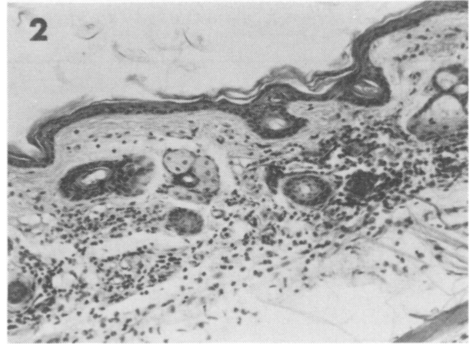
However, significant reactions (5% level of probability) persisted in other animals for at least 90 days. Unsensitized control mice appeared nonreactive to the antigen. Small increases in foot volume sometimes occurred in normal mice which were considered to be the result of trauma to the foot. Among 35 tests on control mice, there were two instances when this swelling reached the significance level (Table 1). In contrast to the usual allergic reactions in sensitized mice, these two swellings did not persist at both the 24- and 48-h readings. Some of the control mice were subjected to three injections of the skin test antigen without showing evidence of sensitization.

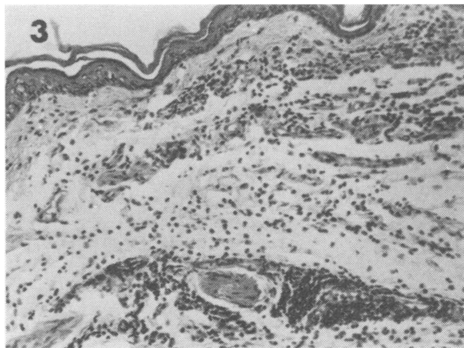
Histological examination of the feet revealed the expected inflammatory changes in sensitized animals, with cellular infiltrations consisting largely of mononuclear cells. Infiltrating cells were frequently found in association with small veins (Fig. 1 to 4).

Diffusion chamber experiment A. An initial experiment using diffusion chambers (experiment A) was undertaken to determine whether the delayed hypersensitive state could be induced when the bacterial cells were confined in diffusion chambers and only soluble substances or possibly subcellular fragments were released as antigens. Diffusion chambers containing approximately 5.8×10^5 *Listeria* cells were implanted intraperitoneally. From previous work it was known that the confined bacteria would replicate to more than 10^8 cells during the first 24 to 48 h (24). Characteristically, the viable population would then decline to about 10^7 cells and persist at that level for more than a year (25). Leukocytes were attracted to the exterior of the chamber membrane as bacterial products were released. Neutrophils were most numerous in a population that also contained large mononuclear cells and some plasma cells (26). The humoral antibody response to the stimulus was feeble, with the development of low titers of mercaptoethanol-sensitive agglutinating antibodies (24).

Fifty-five days after chamber implantation the mice were tested for DH along with normal control mice. Fifty percent of the animals containing diffusion chambers showed allergic reactions of a 5% or greater increase in foot volume (Table 2). The control animals remained negative. Resistance to listeriosis was challenged by the subcutaneous inoculation of 10-fold graded doses of the organism. The challenge inoculation, given 57 days after implanting the chambers, demonstrated a highly significant level of increased resistance in the mice containing diffusion chambers as compared with normal control mice (Z value = 4.19). The mice containing diffusion chambers that did not demonstrate DH were distributed at different dose levels of the challenge, and their resistance appeared to parallel that of DH-positive mice. The experiment was con-







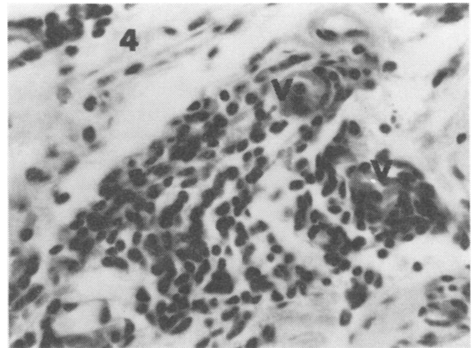


FIG. 1. *Normal mouse skin from the side of the hind foot. The dermal layer contains hair follicles, fibroblasts, and a few leukocytes. Hematoxylin and eosin, ×295.*

FIG. 2. *Cellular infiltration of the dermis and subcutis 24 h after injection of Listeria antigen in the foot pad of a sensitized mouse. Section from the side of hind foot. Hematoxylin and eosin, ×295.*

FIG. 3. *Extensive cellular infiltration of the dermis and subcutis 24 h after injection of Listeria antigen in the foot pad of a sensitized mouse. Section from interdigital region of hind foot. Hematoxylin and eosin, ×295.*

FIG. 4. *Cellular accumulation in the region of small veins (V) 24 h after injection of Listeria antigen in the foot pad of a sensitized mouse. Infiltrating cells are largely mononuclear with some neutrophils. Hematoxylin and eosin, ×944.*

cluded 30 days after the challenge inoculation. The chamber contents from six mice were then transferred to a plating medium to determine the fate of the bacteria in diffusion chambers, and all were shown to contain live *L. monocytogenes*.

It was concluded that the placing of *Listeria*-containing diffusion chambers in the peritoneal cavity was followed by the establishment of DH in many of the mice, and animals with implanted chambers showed increased resistance to listeriosis. Delayed hypersensitivity and the state of antimicrobial cellular immunity coexisted in many of the animals and could lead one to the conclusion that the two states were mutually dependent.

Diffusion chamber experiment B. An expanded diffusion chamber experiment was devised to explore the dependency of antimicrobial cellular immunity on a pre-existing state of delayed hypersensitivity. The intent was to study this in animals that had been sensitized by diffusible antigens from the chambers as well as animals sensitized by the inoculation of living bacteria. It was also necessary to determine the extent of increased resistance afforded by the presence of empty diffusion chambers in the abdominal cavity (26).

Four sets of animals were prepared. The *Listeria* chamber implant set received chambers intraperitoneally containing 10^4 organisms in tryptose broth. Diffusion chambers containing sterile tryptose broth were implanted in the same manner in the sham chamber implant set. The immunized set received two sublethal inoculations of living *L. monocytogenes*. The ini-

TABLE 2. *Delayed hypersensitivity in mice sensitized to L. monocytogenes and mortality after challenge inoculation*

Mouse group	Delayed hypersensitivity reaction[a]	LD_{50}[b]		Treated/ normal[c]
		Log ± SE	Numerical	
Expt A				
Normal	1.24 ± 1.10	6.10 ± 0.23	1.26×10^6	—[d]
Listeria implant				
DH positive	12.78 ± 1.16	7.50 ± 0.24	3.16×10^7	25.1
DH negative	1.71 ± 0.71			
Expt B				
Normal	0.87 ± 0.21	7.47 ± 0.20	2.97×10^7	—
Listeria implant				
DH positive	8.61 ± 0.78	8.95 ± 0.34	8.90×10^8	30.0
Listeria implant				
DH negative	0.62 ± 0.16	9.07 ± 0.30	1.18×10^9	39.7
Immunized				
DH positive	17.01 ± 2.07	8.68 ± 0.23	4.80×10^8	16.2
Immunized				
DH negative	0.99 ± 0.27	8.95 ± 0.35	8.88×10^8	29.9
Sham implant	0.72 ± 0.20	7.83 ± 0.29	6.72×10^7	2.3

[a] Mean percent of foot volume increase and standard error of the mean at 24 h.
[b] Challenged with 10-fold graded doses of *L. monocytogenes* inoculated subcutaneously.
[c] LD_{50}-treated group/LD_{50} normal group.
[d] Not applicable.

tial dose of 10^4 organisms was inoculated subcutaneously on the day that *Listeria* chambers were implanted. Twelve days later, the second dose of 10^5 bacteria was given. A control set of mice was housed with the groups exposed to *Listeria*.

At approximately weekly intervals, a fraction of the mice from each set was tested to determine the accumulation of conversions to the delayed hypersensitive state. Individual animals were scored DH positive if the foot swelling was 5% or more. Forty-five days after chamber implantation, the DH testing had revealed the following positive values: *Listeria* chamber implant mice, 35%; sham chamber implant mice, 0%; immunized mice, 61%; and control mice, 0%. The mice containing *Listeria* chamber implants and those that had been immunized were then segregated according to their delayed hypersensitive status into four groups. This was done to determine whether the positive DH status was necessary to enhance host resistance. The intensity of the DH reactions indicates that the mean foot volume increase was greater in mice immunized with whole bacteria than in the *Listeria* chamber implant mice (Table 2). A small increase in foot volume was encountered in control mice and those containing sham implants. This was considered to be the result of trauma from the injection. A similar level of swelling occurred in the DH-negative animals that had been immunized or that contained *Listeria* chamber implants. The resistance of the mice to ten-fold graded doses of *L. monocytogenes* inoculated subcutaneously was determined 45 days after chamber implantation (33 days after the second immunizing inoculation). The number of mice at each challenge level varied from 6 to 10 in the various groupings. The experiment was terminated 30 days after the challenge inoculation.

Table 2 lists LD_{50} values and indicates increased resistance in both the immunized and *Listeria* chamber implant groups regardless of their DH status. The differences between LD_{50} values for all pertinent groups are compared in Table 3. Invariably, the difference was significant between the normal control group and the four groups receiving antigenic stimulation. As expected, the sham chambers afforded some rise in host resistance, but the LD_{50} for the sham chamber group was not significantly different from that of the normal controls. The LD_{50} was greatest for the DH-negative *Listeria* chamber group. Among the four antigenically stimulated groups, the smallest lethal dose was required for the DH-positive immunized group.

Most deaths occurred during the first 10 days after the challenge inoculation. Dead mice presented the expected gross lesions in livers and spleens indicating involvement of the reticuloendothelial system. Among eight animals dying

TABLE 3. *Significance of differences in mortality between mouse groups after challenge with L. monocytogenes*[a]

Mouse groups compared (expt B)			
Listeria implant (DH positive)[b]		*Listeria* implant (DH negative)	
To normal	3.78[c] (Significant)	To normal	4.42 (Significant)
To *Listeria* implant (DH negative)	0.27 (Not significant)	To *Listeria* implant (DH positive)	0.27 (Not significant)
To immunized (DH positive)	0.66 (Not significant)	To immunized (DH positive)	1.03 (Not significant)
To immunized (DH negative)	0.002 (Not significant)	To immunized (DH negative)	0.27 (Not significant)
To sham implant	2.51 (Possibly significant)	To sham implant	2.96 (Possibly significant)
Immunized (DH positive)		Immunized (DH negative)	
To normal	4.00 (Significant)	To normal	3.69 (Significant)
To immunized (DH negative)	0.64 (Not significant)	To immunized (DH positive)	0.64 (Not significant)
To *Listeria* implant (DH positive)	0.66 (Not significant)	To *Listeria* implant (DH positive)	0.002 (Not significant)
To *Listeria* implant (DH negative)	1.03 (Not significant)	To *Listeria* implant (DH negative)	0.27 (Not significant)
To sham implant	2.30 (Not significant)	To sham implant	2.47 (Not significant)

[a] Sham implant: to normal, 1.01 (not significant).
[b] DH, Delayed hypersensitivity.
[c] Calculated as the value $Z: Z = (X - Y)/\sqrt{\delta X^2 + \delta \upsilon^2}$. Interpreted as: 3.0 and greater, significant; 2.5 to 2.9, possibly significant; 2.4 and less, not significant.

from days 11 through 21, all yielded positive brain cultures. The organism was also recovered from the liver or the spleen of six of the mice.

Interpretation of immunological events in the *Listeria* chamber implant groups depended on maintenance of the barrier between the replicating bacteria in the chamber and the lymphoid cells of the host. In a test to evaluate the structural integrity of the diffusion chambers the spleens, livers, and heart blood of mice were cultured bacteriologically at intervals after implanting chambers containing *Listeria*. Eight mice were sampled during the 4- to 8-day period, and four mice were examined 2.5 to 5 months after implantation. All cultures remained negative for *Listeria*. Early in the study, some animals were examined that had been implanted with chambers containing small leaks in the Millipore membrane. After a few days these intentionally imperfect chambers were invaded by leukocytes, and the bacterial population was greatly diminished. One of the mice died with a generalized *Listeria* infection from the escaping bacteria.

Diffusion chambers taken from dead mice were examined after the challenge inoculation. Gross evidence of breaks in chamber membranes was not encountered among the numerous mice autopsied. The fluid from 15 randomly selected chambers was examined. All of these chambers were considered to be intact because viable *Listeria* counts ranged from 1.4×10^7 to 1.2×10^9 per chamber, and leukocytes were not found on any of the Giemsa-stained smears of chamber contents.

DISCUSSION

Since the pioneering studies by Lurie (13) on tuberculosis, attention has been appropriately focused on understanding the role played by the macrophage in resistance to facultative intracellular parasites. The importance of the macrophage to immunity in listeriosis came into view (14, 22) after it was shown that passive antibodies would not protect the host and that live bacteria could be used to induce resistance (14, 20, 27). Detailed studies demonstrated the increased capacity of activated macrophages for intracellular destruction of both the immunologically specific microorganism that induced the resistant state and a degree of resistance to unrelated disease agents (11, 18, 28, 38). The cellular interactions leading to resistance, and apparently to macrophage activation in listerio-

sis, have been shown to depend upon lymphocytes (19, 29, 30). In mice, the reacting lymphocytes were found to bear the theta antigen of thymus-derived lymphocytes (2, 12). Dumonde et al. (6) used the term "lymphokines" for the multiple biologically active factors released from sensitized lymphocytes after reaction with antigen in cell-mediated immune reactions. Recent work by Klun and Youmans (10) presented in vitro evidence for a lymphocyte product that inhibited the growth of virulent tubercle bacilli within macrophages. Such a newly described lymphokine may represent the lymphocyte mediator affecting the functional role of macrophages. The comments that follow are based on the assumption that antimicrobial cellular immunity will be shown to be a cell-mediated immune reaction involving thymus-derived lymphocytes, lymphokines, and macrophages. Delayed hypersensitivity has been defined as an immunologically specific inflammatory reaction with a characteristic histological appearance (1). To determine DH in vivo one must rely upon development of inflammation at a site of antigen deposition which means, of course, that the reaction depends upon the availability of circulating sensitized lymphocytes (Fig. 1 to 4). Skin-reactive factor needed for the DH response is an activity that has been difficult to distinguish from migration-inhibitory factor (MIF), lymphotoxin, and blastogenic factor (3).

The magnitude of the DH reaction in listeriosis has been reported to correspond directly with the level of resistance (14, 16, 23). Mackaness (15) presented evidence for the mediation of immunity to tuberculosis by tuberculin hypersensitivity. The same author described DH and antimicrobial cellular immunity as different manifestations of the same phenomenon mediated by the same population of committed lymphoid cells (17). Recent studies by others on listeriosis and blastomycosis indicate the temporal relationship of the phenomena or suggest a relationship of DH to resistance (5, 30, 35). The difficulty with proving the interdependence of these phenomena is that the whole array of lymphokines is likely to be released when antigen and cells react. This has given rise to an assumption in studies on cell-mediated immune reactions that each reacting lymphocyte is capable of releasing all lymphokines. The problem of demonstrating this is technically difficult because there is no convenient way of separating cells to determine the validity of the premise. Nevertheless, Simon and Sheagren (34) showed that guinea pig cells cultivated in vitro produced MIF but did not release a factor enhancing the bactericidal function of macrophages. Klun et al. (9) found that supernatant fluids from lymphocytes might contain both MIF and mycobacterial growth inhibitory activity. However, some suspensions of splenic lymphocytes revealed only one of the two activities. The authors noted that "this association has only been observed in vitro and whether one lymphocyte product can be produced in vivo to the exclusion of the other is not known."

The in vivo experiments described in this report were designed to study the relationship of DH to antimicrobial cellular immunity. Mice were exposed to the diverse antigens of living *Listeria* cells in two different ways. After inoculation of bacteria into the subcutaneous tissues, it was presumed that the expected chain of events occurred involving inactivation of the organisms within macrophages, antigen processing (37), stimulation of B and T cells, and macrophage activation. When *Listeria* cells were confined to the diffusion chambers, uncertainty existed concerning the establishment of DH in a situation where macrophages were excluded from direct interaction with the microorganism, and antigen had to diffuse from the chambers in a presumably soluble form. Delayed hypersensitivity developed from both modes of antigen presentation. However, the inoculation of bacteria produced a greater number of responding individuals after 45 days than did the diffusion chamber method (61% as compared to 35%). In addition, the strength of the reactions, as measured by the size of the inflammatory reaction, was greater when bacteria were inoculated (a mean foot volume increase of 17.0% as compared to 8.6% for those sensitized from diffusion chambers; Table 2). The mean foot volume increase for mice scored as DH negative was less than 1%, which compared favorably with the values for nonsensitized control animals (Table 2).

From previous work which showed that immunity to listeriosis in the mouse was dependent upon functioning lymphocytes, the resistance of mice to fatal infection was interpreted as a manifestation of antimicrobial cellular immunity (2, 12, 19, 29, 30). It was considered desirable to test the resistance of the mice by means akin to a natural process. Since natural infections usually go through a stage in which the primary defense barriers are breached, and since immune reactions occur at a peripheral site, it was decided that subcutaneous rather than intravenous inoculation of *Listeria* cells should be used for the challenge. Furthermore, the survival of the host was used as the end point in preference to interruption of the process

at some point to enumerate the parasite population in selected organs.

Before challenging their resistance, the mice were divided into DH-positive and DH-negative groups. The negative skin tests indicated that circulating sensitized lymphocytes were not available to react in a peripheral site to elicit the DH inflammatory reaction. Likewise, it may be presumed that the inoculation of living bacteria into the subcutaneous tissues would not elicit a DH reaction, and the host would then be deprived of the assumed beneficial effects on resistance accruing from DH. If DH is merely a different manifestation of the phenomenon of antimicrobial cellular immunity mediated by the same population of lymphoid cells, one would expect the DH-negative mice to offer no more resistance than would unsensitized control animals to a challenging infection. This was not the case. The DH-negative mice were at least as resistant as the DH-positive animals (Tables 2 and 3). If DH had correlated directly with immunity, the greatest resistance would have occurred in the immunized DH-positive group, and it would have declined in sequence from the *Listeria* implant DH-positive group to the two DH-negative groups. There was no apparent advantage for host resistance afforded by the presence of DH.

Animals exposed to antigens of the bacteria developed antimicrobial cellular immunity quite independently of DH. The presence of a high order of antimicrobial cellular immunity in circumstances where DH was not detected mitigates against the idea that an intense level of CMI reaction equates with the same level of intensity for all CMI phenomena. The results of these experiments suggest that all committed lymphoid cells do not release the same array of lymphokines.

The role of DH in immunity to tuberculosis has long been debated. Some studies support the concept that the processes play independent roles. Neiburger et al. (21) found a high degree of immunity to tuberculosis in mice after the injection of ribonucleic acid prepared from *Mycobacterium tuberculosis*. The immunization did not induce tuberculin sensitivity. Raffel (33) produced tuberculin sensitivity in guinea pigs by injecting wax D and tuberculoprotein, but this did not increase resistance to tuberculous infection. The point might be made that these experiments could be difficult to interpret because only a part of the antigenic composition of the microorganism was used for sensitization. However, support is given to these studies by the present experiments on listeriosis in which all antigens of metabolizing cells were present.

It has been proposed in immunological theory that a high order of cell selection occurs in the processes of antibody formation, and a large body of research supports the theory (4, 36). It seems unlikely that the other sphere of immune responses, namely, the cell-mediated responses, would fail to involve selection. If all committed lymphoid cells do not release the same array of lymphokines, as inferred here, it is further suggested that DH and cellular immunity are mediated by different populations of committed lymphoid cells rather than by the same population. Selection of cells for function might relate to the nature of the antigenic stimulus in terms of the conformation of the antigenic determinant or possibly the physical state in which the antigen is presented. There is some evidence of heterogeneity of T lymphocytes as presented by Raff and Cantor (32). The T_1 cells are thymus dependent and short-lived, while T_2 cells are longer lived, recirculate, and are susceptible to ALS. Selection is implied among T lymphocytes for the performance of specialized functions. Some CMI reactions result in direct lymphocyte-target cell cytotoxicity with a high order of specificity regarding the cells injured. Other T lymphocyte functions result in the release of factors from antigen-stimulated cells that nonspecifically affect other cells in their environment (3). It is in this latter functional category where the present study on listeriosis suggests that some of the T cells release factors producing the DH phenomenon, whereas others are directing the increased antimicrobial function of macrophages.

The constituents of parasitic cells may induce different functional CMI responses. It may be expected that the whole spectrum of CMI reactions can follow from sensitization with viable parasitic cells and thus produce multiple phenomena when antigen is reintroduced. It also seems likely that various components of microbial cells elect and stimulate T lymphocytes for specialized functions.

ACKNOWLEDGMENTS

The technical assistance of Joyce Wu and Pamela Wagner is appreciatively acknowledged.

This investigation was supported in part by Public Health Service training grants GM-762 and GM-1406 from the National Institute of General Medical Sciences.

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