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Delayed Hypersensitivity in Relation to Suppression of Growth of *Listeria monocytogenes* by Guinea Pig Macrophages

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Prototypes of delayed hypersensitivity (tuberculin allergy, graft rejection immunity, and contact dermatitis) were established in guinea pigs. The macrophages from peritoneal exudates of such animals were examined for their capacities to suppress the growth of *Listeria monocytogenes* in vitro. Only the macrophages from animals sensitized to BCG clearly exhibited this property.

The peritoneal macrophages of animals immunized with any of several bacteria (*Listeria monocytogenes, Brucella abortus, Mycobacterium tuberculosis, Salmonella typhimurium*) are reported to have increased capacity to kill or suppress any one of them (2, 21-26). This ability is apparently independent of antigenic relationships among the microorganisms; it seems rather to coincide with the development of delayed hypersensitivity to the sensitizing bacterium which in turn can lead to "activation" of macrophages under appropriate circumstances (24).

The question of relationship between delayed hypersensitivity and acquired immunity to infectious agents is an old one, particularly in respect to tuberculosis. It occurred to us that it would be interesting to find evidence on this general question by looking into delayed hypersensitive reactivities of other categories, not involving infectious agents, to find whether these would lead to an increase in bactericidal or static properties of peritoneal macrophages. We have explored the question in guinea pigs where prototypes of delayed hypersensitivity, including infectious allergy, graft rejection, and allergic contact dermatitis, can be readily established.

We found that only macrophages from animals sensitized to BCG showed a clearly increased capacity to suppress *L. monocytogenes*.

MATERIALS AND METHODS

Animals. Random-bred albino guinea pigs were purchased locally. Inbred strain 13 animals were from our own colony.

Bacteria. BCG cultured on solid medium (Lowenstein-Jensen or Trudeau) at 37 C was washed twice in 0.05% Tween-80 water, heated to 80 C for 1 hr, again washed twice, and harvested by filtration through membrane filters (Millipore Corp., Bedford, Mass.). Wet weights were determined and suspensions were prepared in phosphate-buffered saline (*p*H 7.4) containing 0.5% phenol and 0.05% Tween-80. Bacteria were dispersed by grinding in a Ten-Broeck glass apparatus. Stock suspensions were kept frozen until used. Cultures were made to assure the presence of killed mycobacteria only.

L. monocytogenes was kindly provided in lyophilized form by George B. Mackaness. This strain showed the expected cultural reactions and had only moderate virulence for the guinea pig; a dose of 5×10^9 given intraperitoneally was lethal for 600-g guinea pigs in 3 to 4 days. Stock cultures on Tryptose Agar slants (Difco) were stored frozen. Bacteria were grown at 37 C for 24 hr in Tryptose Broth. The bacteria in the stationary phase were washed three times in Pomerat solution (sodium chloride, 8 g; potassium chloride, 0.2 g; sodium phosphate, monobasic, 0.05 g; sodium bicarbonate, 1 g; dextrose, 2 g; and double-distilled water to 1 liter) before use.

Contact allergen. 1-Chloro-2,4-dinitrobenzene (DNCB, Eastman) was recrystallized once from absolute ethyl alcohol. A 50% solution in acetone was painted onto a 1-cm^2 area of skin repeatedly for epicutaneous sensitization (12). A 1% solution in olive oil was used for testing (8). The final re-stimulation of sensitivity was applied either epicutaneously or intraperitoneally. For the former, 10% DNCB in acetone was painted over the entire two sides of the animals. For the latter, a suspension of dinitrophenylated guinea pig skin was used, prepared as follows. The shaven flanks of a normal animal were painted with 10% DNCB in acetone; 24 hr later the skin was removed, trimmed of fat, weighed, washed with distilled water, cut with scissors, and homogenized in distilled water in an Omnimixer (Sorvall) and a motor-driven Duall conic glass tissue grinder (Kontes). The suspension was treated with 2,4-dinitrobenzene sodium sulfonate by the method of Eisen et al. (14). Unconjugated hapten was eliminated

by dialysis. The product was freed of fat by centrifugation in the cold and filtered through a 110-mesh stainless-steel sieve.

The protein content of the suspension was estimated by precipitating a sample with an equal volume of 20% cold trichloroacetic acid, hydrolyzing the precipitate with 6 N HCl at 110 C in vacuo for 24 hr (17), and employing the method of Satake et al. (28) to determine amino acid concentration with a DL-leucine standard. The 2,4-dinitrophenyl (DNP) concentration was determined by reading the optical density at 360 nm of the neutralized hydrolysate, with dilutions of 6-(2,4-dinitrophenyl) aminocaproic acid used as a standard. The suspension was heavily labeled with approximately 31 μmoles of amino groups and 2 μmoles of DNP per ml. Normal and DNCB-sensitized animals were tested intradermally with dilutions of the suspension and of its centrifuged supernatant fluid. Reactions occurred only in sensitized animals tested with the undiluted suspension.

Tissue grafts were made with 2-cm^2 pieces of skin taken from the trunk of a strain 13 guinea pig. The grafts were cleaned of subcutaneous tissue, fitted into beds prepared in the flanks of outbred albino recipients, and anchored with elastic bandage.

Spleen cells were obtained by slicing the organ of a strain 13 donor and gently grinding the fragments in a loose-fitting Ten-Broeck glass grinder. The suspending fluid was cold Medium 199 with Hanks basal salt solution (Gibco) containing 2% normal inactivated guinea pig serum (Hyland) and 10 units of preservative-free heparin (Calbiochem) per ml. The suspension was put through a 110-mesh stainless-steel filter, and the number of cells was determined by hemocytometer. Viability (usually about 60%) was estimated by the erythrosin B method (27).

Cell-bacteria preparations. The method used for assaying the activity of macrophages upon *Listeria* was as follows. Guinea pigs were sensitized and restimulated as described. Five days later, guinea pigs were sacrificed without anesthetic, and the abdominal cavity was opened aseptically and washed out with 20 ml of the cold suspending medium used for spleen cells. The cells were washed once and suspended in tissue culture medium [Medium 199, Earle basal salt solution (Gibco), with 15% inactivated guinea pig serum and 1% additional glutamine]. Samples were counted and suspensions were adjusted to 4×10^6 nucleated cells per ml. It was necessary to pool cells from two guinea pigs for an experiment. Smears were stained with Wright-Giemsa for examination of cell morphology and differential count.

A 1-ml amount of suspension containing 4×10^6 nucleated cells was put into each of a series of Leighton tubes containing slides. These were incubated at 37 C in a 95% air, 5% CO_2 atmosphere for 3 to 4 hr. The fluid was decanted to eliminate unattached cells, and 10^5 washed *Listeria* cells suspended in 1 ml of the cell culture medium were added. After 2 hr at 37 C, the medium was decanted, the tubes were washed twice with Pomerat solution, and fresh medium containing 2 μg of streptomycin per ml was added. The cell-bacterial mixtures were then incubated at 37 C for 22 hr. The cells remaining attached to the slides were counted through the walls of the tubes by use of an ocular grid. The nutrient fluid was decanted, and the slides were washed twice with Pomerat solution and sonically treated for 4 min in a DF 101 oscillator (Raytheon). Bacterial counts were made by pour-plate dilutions in Tryptose Agar. Results were evaluated by the Wilcoxon rank-two sample-two tail test.

RESULTS

Of the 16 experiments carried out with the peritoneal cells of normal guinea pigs, 14 were untreated. The remaining two were injected with incomplete Freund adjuvant in the rear foot pads and reinjected intravenously 3 weeks later with 1 ml of an emulsion of oil, saline, and Tween-80 (1:2:1) 5 days before harvesting cells. This conformed to the general scheduling of events in the experimental groups. Table 1 shows the results in terms of bacteria per macrophage recovered after 22 hr of incubation, as determined by quantitative cultures of cells remaining attached to slides in the Leighton tubes. Additional information was obtained by lightly sedimenting cells from the supernatant fluids and by washing, sonically treating, and culturing these. The results correlated well with those shown for the slide preparations in these and the other experimental groups to be described.

An average of 56 multiples of the initial inoculum of bacteria per macrophage was found after the 22-hr incubation period (assuming that all added bacteria had been phagocytosed).

BCG sensitization. Animals were injected with 0.5 mg (wet weight) of heat-killed bacilli in Freund emulsion, divided between the rear footpads. Three weeks later, they were reinjected intravenously with 3.2 mg of bacilli in Medium 199 (Hanks base) with 10 μg/ml of heparin. Five days later, peritoneal cells were obtained for testing.

These results (Table 2) indicate a significant suppression of bacterial growth compared to the findings with cells of normal animals. Although there was some increase in numbers of intracellular bacteria, this was restricted to an average of about 7.5 multiples of the original inoculum.

Hypersensitivity to DNCB. Hypersensitivity to DNCB was induced by painting 50% DNCB in acetone onto various sites in rotation (ears, flanks, areolae and nipples, and inguinal areas). The lengths of sensitization periods were not uniform, since animals were repeatedly tested and retreated until they reacted to 1% DNCB in olive oil with responses graded as 4+ or better by the criteria of Chase (8). As the final stimulus, animals received either 1 ml of DNP-treated skin suspension mixed with 15 mg of Phenergan intraperitoneally, or they were repainted with 10%

TABLE 1. *Normal animals*

Expt	Macrophages in inoculum	Bacteria/ macrophage initially	Macrophages on slide[a] (per cent of inoculum)	Bacteria/ macrophage on slide	Multiples of bacteria/ macrophage
1	0.96×10^6	0.1	4.4	0.7	7
2	2.16×10^6	0.05	2.5	1.4	28
3	1.60×10^6	0.06	14.5	0.8	13
4	2.28×10^6	0.04	4.2	8.1	202
5	1.92×10^6	0.05	7.0	3.5	70
6	1.96×10^6	0.05	8.3	5.9	118
7	1.96×10^6	0.05	7.2	4.3	86
8	1.12×10^6	0.09	16.6	1.5	16
9	1.48×10^6	0.07	11.7	1.2	17
10	1.52×10^6	0.07	12.8	0.6	9
11	2.04×10^6	0.05	10.8	0.7	14
12	2.68×10^6	0.04	6.1	0.6	15
13	1.04×10^6	0.09	16.1	4.9	54
14	2.32×10^6	0.04	9.3	1.9	47
15[b]	1.84×10^6	0.05	7.6	6.2	124
16[b]	1.08×10^6	0.09	11.9	6.8	75
Avg	1.75×10^6	0.06	9.44	3.2	56

[a] In Leighton tube.
[b] Received incomplete Freund adjuvant in footpads and intravenously.

TABLE 2. *BCG sensitization*

Expt	Macrophages in inoculum	Bacteria/ macrophage initially	Macrophages on slide[a] (per cent of inoculum)	Bacteria/ macrophage on slide	Multiples of bacteria/ macrophage
1	2.20×10^6	0.05	5.6	0.1	2.0
2	1.76×10^6	0.06	2.9	0.008	0.1
3	1.12×10^6	0.09	10.0	0.04	0.4
4	2.56×10^6	0.04	7.5	0.4	10.0
5	1.40×10^6	0.07	12.0	0.8	11.0
6	1.76×10^6	0.06	7.5	0.8	13.0
7	1.88×10^6	0.05	12.2	0.8	16.0
Avg	1.81×10^6	0.06	8.3	0.4	7.5

[a] In Leighton tube.

DNCB in acetone over both sides. Again, peritoneal cells were harvested 5 days later for tests with bacteria.

At the time of harvest of cells from the animals restimulated intraperitoneally, there were indications of earlier hemorrhage in the peritoneal cavity; in some cases ecchymoses were seen in the intestinal walls. These hemorrhages apparently resulted from hypersensitive reactions, since they were not seen in normal animals injected with the same material. The numbers of cells harvested were greater than obtained from other groups, although differential counts showed the customary patterns. The data in Table 3 indicate that the macrophages of these animals behaved, in relation to *Listeria*, as had those of controls.

Hypersensitivity induced by cells and tissue grafts. Guinea pigs were treated in several ways to induce anti-tissue reactivity. Group A (Table 4) included two animals (1 and 2) which were injected intravenously with 250×10^6 spleen cells and 2 weeks later received a skin graft, 12 days before harvest of peritoneal cells. Three other animals (3–5) were given two skin grafts on two occasions from inbred (strain 13) donors. Group B included two guinea pigs (6 and 7) grafted with skin and retreated 3 weeks later, after their grafts had been rejected, with 250×10^6 spleen cells intravenously, 5 days prior to collecting their peritoneal cells. Four other animals (8–11) received two skin grafts in each flank; this was repeated 12 days later from an isologous donor, and 5 days later 400×10^6 spleen cells were injected intravenously. Again, peritoneal cells were harvested 5 days later. Thus, the subjects in this experiment were sensitized by skin grafting

TABLE 3. *DNCB sensitization*

Group	Expt	Macrophages in inoculum	Bacteria/ macrophage initially	Macrophages on slide[a] (per cent of inoculum)	Bacteria/ macrophage on slide	Multiples of bacteria/ macrophage
A[b]	1	1.68×10^6	0.06	6.6	4.6	76
	2	2.20×10^6	0.05	8.5	2.7	54
	3	1.36×10^6	0.07	11.9	1.4	20
	4	1.16×10^6	0.09	12.1	1.3	14
	5	1.32×10^6	0.08	6.8	2.8	35
	6	1.64×10^6	0.06	11.2	6.8	108
Avg		1.51×10^6	0.068	9.5	3.2	51
B[c]	7	1.84×10^6	0.05	5.6	2.7	54
	8	1.40×10^6	0.07	7.3	3.2	46
	9	2.32×10^6	0.04	6.4	2.9	72
	10	2.16×10^6	0.05	7.8	0.9	18
	11	2.00×10^6	0.05	3.8	2.4	48
	12	2.16×10^6	0.05	5.2	1.3	26
Avg		1.98×10^6	0.061	6.0	2.2	44

[a] In Leighton tube.
[b] Epicutaneous restimulation.
[c] DNP-skin, intraperitoneally.

TABLE 4. *Homologous tissue sensitization*

Group	Expt	Macrophages in inoculum	Bacteria/ macrophage initially	Macrophages on slide[a] (per cent of inoculum)	Bacteria/ macrophage on slide	Multiples of bacteria/ macrophage
A[b]	1	1.44×10^6	0.07	11.2	1.1	16
	2	2.04×10^6	0.05	8.0	3.2	64
	3	1.16×10^6	0.09	16.8	0.1	1
	4	2.64×10^6	0.04	5.4	9.1	227
	5	2.04×10^6	0.05	6.6	0.1	2
Avg		1.86×10^6	0.06	9.6	2.7	62
B[c]	6	2.36×10^6	0.04	4.3	7.6	190
	7	2.20×10^6	0.05	7.0	2.7	54
	8	1.24×10^6	0.08	15.8	0.9	11
	9	2.0×10^6	0.05	8.1	8.1	162
	10	0.88×10^6	0.11	13.2	9.8	89
	11	0.76×10^6	0.13	15.9	6.5	50
Avg		1.57×10^6	0.075	10.7	5.9	93

[a] In Leighton tube.
[b] Skin grafts as final stimulus.
[c] Skin grafts, with spleen cells intravenously as final stimulus.

twice or by injection with spleen cells followed by grafts (group A) or by grafts followed by spleen cells (group B). Variations in the results of individual experiments were wide in group A in which, in fact, the mode of sensitization was least analogous to that used in sensitizing animals to mycobacteria. The overall results of all 11 experiments (Table 4) failed to show evidence of activity of macrophages as seen in the BCG-sensitized group.

Statistical analysis showed the following for results with cells of the variously treated groups: BCG versus normal, $P < 0.05$; BCG versus DNCB, $P < 0.01$; BCG versus grafted, $P < 0.05$. Comparisons of other pairs in various combinations showed no significant differences.

DISCUSSION

The experiments reported were undertaken to explore the proposition that the delayed hypersensitive response to immunogens of various bacteria can give rise to an "activation" of macrophages which endows them with the ability to suppress the growth of various intracellular bacteria. It was thought that if the underlying immunological response involved is in fact delayed hypersensitivity, the same end result might be seen in a variety of circumstances involv-

ing this reactive state, including contact sensitivity and antigraft reactivity.

The results presented confirm previous reports that peritoneal macrophages from animals appropriately sensitized to BCG have a significant capacity to suppress the growth of an intracellular test bacterium, *L. monocytogenes*. This conclusion is based on numbers of bacteria cultured from macrophages of BCG-sensitized animals in contrast to those recovered from cells of normal ones. Among the experimental vagaries in such a comparison, we can think of none which would spuriously favor the results seen. Thus, the macrophages of animals immunized to *M. tuberculosis* are described as being more actively phagocytic than are cells from normal subjects (19). In this case, more of the initial inoculum of *Listeria* should have been engulfed by these cells, and the fact that fewer are found in them at the conclusion of the experiment suggests a suppressive activity beyond that indicated by the actual values found. Again, it may be that multiplication of *Listeria* in macrophages injures or kills cells, causing them to detach from the slides and perhaps to rupture and release bacteria into the streptomycin-containing medium; such bacteria would have been missed in the final census. There is no reason to suppose that this contingency would lead to a wrong inference in respect to the activity of cells of BCG-reactivated animals, and, in any case, the count of macrophages present at the conclusion of experiments in the various experimental groups did not differ significantly.

In contrast to the results seen with cells from BCG-sensitized animals, those found with cells from guinea pigs in which contact hypersensitivity to dinitrochlorobenzene or antihomograft reactivity to skin and spleen cells had been induced did not show increased capacities to suppress bacteria. The results of individual experiments in all groups varied widely. Cells in the 7 BCG experiments manifested a degree of suppression of bacterial growth in the range which was found in 7 of 16 experiments with normal cells, in 1 or 2 of 12 experiments with cells from DNCB-sensitized animals, and in 4 of 11 experiments with grafted animals. Thus, all experiments with cells from BCG animals were in a range of activity seen in 12 of 39 experiments with cells from non-BCG-sensitized animals.

The inhomogeneity of individual results might be accounted for by the thesis (18, 20) that populations of macrophages differ in the proportions of "activated" members. Some of the cells obtained from "normal" subjects may have this characteristic, perhaps because of spontaneously occurring low-level sensitizations, whereas more of the cells from a deliberately sensitized subject would be activated.

The fact that only animals with induced "bacterial allergy" gave rise to the effect seen is difficult to explain, particularly in view of the many uncertainties that surround the definition of what constitutes delayed hypersensitivity and what the effector of this may be. There are a number of "phenomena" presumably related to delayed hypersensitivity, generally by reason of chronological association rather than direct evidence, which has so far been difficult to obtain.

One of these phenomena is the macrophage inhibition reaction (16) which appears to depend upon a "factor" released by lymphoid cells of sensitive animals upon exposure to antigen (6); this in turn affects macrophages to hobble their movement. It would be satisfying to incorporate this factor into a viewpoint encompassing the enhanced suppressive effect of such macrophages upon engulfed bacteria, for it is known that macrophages can be stimulated to an increased synthesis of lysosomes and to an exacerbation of metabolic activities in a variety of circumstances (7, 9, 10, 13). However, inhibition of migration of macrophages occurs when the lymphoid cells of homografted animals are exposed to donor cells in vitro (1); presumably the macrophage-inhibiting factor is concerned here also, but an antibacterial activity of the macrophages was not found. Similarly, the macrophage inhibition reaction is demonstrable with cells from animals with delayed reactivity to DNP (11), the hapten used here, but again the antibacterial effect was not found. In experiments in vivo carried out by Forbes (15), mice sensitized to ovalbumin in complete Freund adjuvant and restimulated with the protein alone showed no increased clearance or destruction of injected *Listeria*.

Quantitative factors may account for the difference in results found among the three experimental groups described. For example, fewer lymphoid cells may respond to a chemical inducer, such as DNCB, or to homologous tissue antigens than to immunogens of a bacterium which may share antigenic qualities with many other antigens in nature to which guinea pigs can respond. Consequently, a larger number of responding cells may be induced by the bacterial stimulus. If this were so, one would think that reactivity to tuberculin, for example, should be transferred by fewer peritoneal cells than are required for a hapten such as DNCB. It is common experience, in fact, that about 10^8 cells are required in both cases.

In relation to the quantitative aspects of sensitization required for the "activation" of peritoneal macrophages to become apparent, Blanden et al. (4) recently reported that mice need to be stimulated with a large dose of BCG, or restimulated with a second dose, for this to occur.

In our various experiments secondary stimulation was routinely employed.

In the case of the grafted animals, a different consideration may enter. Most of the reactive lymphoid cells may be localized in lymph nodes rather than in circulating lymph and blood, and would not find their way to the peritoneal cavity (3).

In a current report, Blanden (5) showed that the graft-versus-host response induced in hybrid mice by injection of parental cells increased resistance of peritoneal macrophages to *Salmonella*. However, the pretreatment of mice with allogeneic cells did not have this effect, i.e., the host-versus-graft reactions, as reported in our experiments also, failed to induce resistance to infectious agents.

Finally, it may be that delayed cellular reactivity is not concerned in the activation of macrophages that leads to the antibacterial effects described by Mackaness and observed here. Macrophages may be activated by components of bacterial cells in a manner that is not related to specific immunological reactivity, although various experiments (Mackaness) strongly indicate that antigenic specificity is centrally involved in the reactive state; e.g., when the antibacterial power of macrophages has waned, it can be revived by injection of the bacterium to which primary sensitivity has been established, but not by an unrelated organism.

We conclude by restating the observation that, on the basis of the experiments reported here, only macrophages from animals sensitized to a bacterium showed a capacity to suppress bacterial growth beyond that seen with cells from normal subjects. Those sensitized to a chemical hapten or to homologous tissues failed to show this effect.

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