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Immunity to Antigenically Related Salmonellae: Effects of Humoral Factors on the Bactericidal Activity of Normal and Immune Peritoneal Exudate Cells

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Immunity against *Salmonella enteritidis* and *Listeria monocytogenes* was studied by measuring in vitro the bactericidal activity of peritoneal exudate cells (PEC) of control (normal PEC) and *S. typhi* Ty2-immune (immune PEC) mice. Specific immune serum, anti-*S. typhi* Ty2, heat inactivated at 56°C for 30 min, significantly inhibited the growth of *S. enteritidis* only with immune PEC. These opsonic factors had no effect upon the activity of normal PEC. That such inhibition could not be demonstrated in *Listeria* experiments, either with immune or normal PEC, suggests that *S. enteritidis* was specifically recognized, in vitro, by the thermostable opsonin anti-*S. typhi* Ty2 and that macrophages from immune PEC were more efficient in inhibiting bacterial growth than those from normal PEC. Thus, the interaction between macrophages and the microoorganism seems to play an essential role in cell-mediated as well as humoral immunity.

Cell-mediated immunity is involved in activating macrophages that acquire an enhanced phagocytosis and a nonspecifically increased bactericidal activity (9). Interactions of lymphokines with macrophages induce in the latter cells a variety of metabolic and functional changes which can be recorded in appropiate test systems. This has been reported in mice for several infections by facultative intracellular bacteria, i.e., tuberculosis (2), listeriosis (11), brucellosis (10), salmonellosis (4, 5), and protozoa such as *Toxoplasma gondii* and *Besnoitia jellisoni* (13).

In salmonella infections, cytophilic antibodies are important for effective function of phagocytic cells (15). According to recent evidence, both humoral and cellular immune responses of the infected host play a role in the expression of specifically acquired immunity (3). Specific antibodies inhibit the multiplication of the bacteria during the first stage of the disease, but the development of cell-mediated immunity is the major mechanism for destroying bacteria (12). Nevertheless, it is as of yet unknown whether specific antibodies (thermostable opsonins) might be involved in the nonspecific bactericidal action of activated macrophages.

This paper describes the results of an in vitro test that measures the bactericidal activity of normal peritoneal exudate cells (normal PEC) and *Salmonella typhi* Ty2 immune PEC against *S. enteritidis*, as antigenically related bacteria, and *Listeria monocytogenes*, as an unrelated microorganism. The effect of specific antibodies against *S. typhi* Ty2 and *S. virginia* is also studied.

MATERIALS AND METHODS

Mice. Five- to six-week-old CF1 male albino mice, outbred, weighing 14 to 17 g, were obtained from the Animal House Section of the Instituto Bacteriologico de Chile.

Bacteria. The strains of *S. typhi* Ty2 (1, 9, 12, Vi:d:), *S. virginia* [8: d: (1, 2)] and *S. enteritidis* (1, 9, 12, g, m:) were obtained from the National Reference Centre for Enterobacteriaceas of the Instituto Bacteriológico de Chile. The strain of *L. monocytogenes* was originally obtained from the Hygiene Institute of Boon, strain 1071/53 (kindly supplied by Pino, Medicine School, Universidad de Chile).

All strains were prepared from overnight cultures in meat peptone agar slants at 37°C. The bacteria were harvested in the log phase of growth, washed twice with 0.01 M phosphate buffered-saline (PBS), pH 7.2, and adjusted to 300×10^6 bacteria per ml. The number of viable microorganisms per milliliter was determined by counting the number of colonies in the agar plates.

Immune sera. Antisera against *S. virginia* and *S. typhi* Ty2 were induced in CF1 mice by one intraperitoneal injection in PBS, without adjuvants of 10^7 bacteria killed by freezing at −70°C for 30 min and thawing at 48°C for 30 min with soft shaking. This treatment was repeated until no viable microorganisms were obtained when control cultures were done. Animals were bled between 7 and 8 days after the antigen injection. Control animals received PBS alone.

The pool of immune sera, anti-*S. typhi* Ty2 and *S. virginia*, was titrated for agglutinating antibody. Both sera were shown to have an anti-d agglutinating anti-

body titer of 1/75. Anti-O9 was undetectable. Normal mouse serum showed neither anti-d nor anti-O9 antibodies. All immune sera were inactivated at 56°C for 30 min before use.

Preparation of PEC. Immune PEC were obtained from a group of 90 CF1 mice immunized with one peritoneal injection of 10^7 *S. typhi* Ty2 killed as described above for immune sera. Twelve days after the injection, PEC were obtained from the peritoneal cavity after the injection of 3 ml of sterile, apyrogenic boiled 3% starch solution in PBS. Cells were collected after 72 h with TC 199 containing 5 U of heparin per ml. PEC from five mice were pooled and adjusted to 5×10^6 cells per ml. No antibiotics and no preservatives were added. About 80 to 85% of the cells were macrophages, 15 to 18% were lymphocytes, and 1 to 2% were polymorphonuclear leukocytes (PMN). Cell viability was 95% by trypan blue exclusion.

Normal PEC were obtained from the peritoneal cavity of five CF1 mice after one peritoneal injection of PBS and 12 days after PEC were obtained after the injection of 3% starch solution in PBS as described above for immune PEC.

Sterility control was performed in both cell suspensions, and no viable organisms were found.

Bactericidal assay. The method of Young and Armstrong (17) was performed by mixing 2.5×10^6 PEC with 2.5×10^6 bacteria in the presence or absence of 12.5% anti-*S. typhi* Ty2, anti-*S. virginia* immune sera, or normal mouse serum. No serum at this concentration (12.5%) caused clumping of the bacteria. The tubes were incubated at 37°C for 90 min with constant shaking.

Viable extracellular and intracellular bacteria were determined after lysis of PEC with distilled water. Appropriate serial dilutions were prepared and plated, in duplicate, on meat peptone agar. Plates were incubated at 37°C overnight, and the colonies were counted. Triplicate analyses were performed.

Statistical evaluation. Difference between mean values were analyzed by Student's *t* test. Values of *P* of < 0.025 were considered to be significant.

RESULTS

Bactericidal activity of heat-inactivated normal and immune mouse sera. No bactericidal activity of heat-inactivated normal serum and immune serum was detected against both *S. enteritidis* and *L. monocytogenes* when 2.5×10^6 bacteria were incubated with 12.5% of either serum at 37°C for 90 min (Table 1).

Bactericidal activity against *S. enteritidis*. No statistical difference between the bactericidal activity was observed when normal or immune PEC were incubated with *S. enteritidis* without the presence of heated sera. The addition of normal anti-*S. typhi* Ty2 or anti-*S. virginia* mouse serum did not modify the survival of the microorganism with normal PEC. Nevertheless, a significant reduction of the survivial of *S. enteritidis* was found when such sera were added to immune PEC. Only the addition of anti-*S. typhi* Ty2 immune mouse serum inhibited significantly the bacterial growth with immune PEC compared with the control done without serum [Fig. 1, G < E ($P < 0.0001$)]. This bacterial growth inhibition due to immune PEC and anti-*S. typhi* Ty2 immune serum was also statistically significant when compared with the effect of normal or anti-*S. virginia* immune serum [Fig. 1, G < F ($P < 0.01$), G < H ($P < 0.01$)].

Bactericidal activity against *L. monocytogenes*. Normal or immune PEC in the presence or absence of normal or immune serum did not modify the survival of *L. monocytogenes* (Fig. 2).

TABLE 1. *Survival of S. enteritidis and L. monocytogenes after 90 min of incubation with 12.5% normal, anti-S. typhi Ty2 and anti-S. virginia immune mouse sera heat inactivated at 56°C for 30 min*[a]

Microorganism	Initial inoculum	Normal mouse serum	Anti-*S. typhi* Ty2 mouse serum	Anti-*S. virginia* mouse serum
S. enteritidis	2.5×10^6	1.8×10^7	2.8×10^7	1.6×10^7
L. monocytogenes	2.5×10^6	4.4×10^7	3.6×10^7	3.2×10^7

[a] Survival expressed as mean values of the number of colony-forming units.

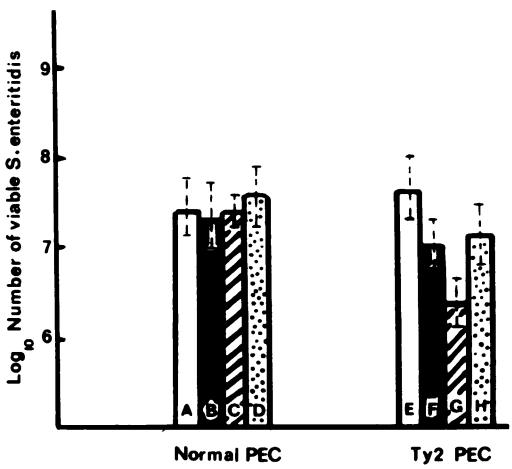

FIG. 1. *Inhibitory activity of PEC of normal and S. typhi Ty2 primary immune mice against S. enteritidis in the absence or presence of normal and immune mouse sera against S. typhi Ty2 and S. virginia. Ty2 PEC, PEC of S. typhi Ty2 primary immune mice; Key: □, Bacteria + PEC; ■, bacteria + PEC + NMS (normal mouse serum); ▨, bacteria + PEC + a. Ty2 (S. typhi Ty2 immune mouse serum); ▦, bacteria + PEC + a. virg. (S. virginia immune mouse serum). Bars represent mean value of nine experiments ± 1 standard deviation. G < E ($P < 0.0001$), G < F ($P < 0.01$), G < H ($P < 0.01$)*

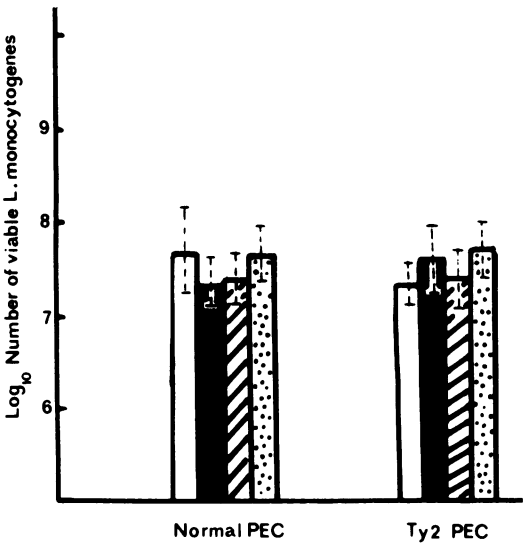

FIG. 2. *Lack of inhibitory activity of PEC of normal and S. typhi Ty2 primary immune mice against L. monocytogenes in the absence or presence of normal mouse serum and immune mouse serum against S. typhi Ty2 and S. virginia. Ty2 PEC, Peritoneal exudate cells of S. typhi Ty2 primary immune mice. Key: □, Bacteria + PEC; ■, bacteria + PEC + NMS (normal mouse serum); ▨, bacteria + PEC + a. Ty2 (S. typhi Ty2 immune mouse serum); ▩, bacteria + PEC + a. virg. (S. virginia immune mouse serum). Bars represent mean value of nine experiments ± 1 standard deviation.*

DISCUSSION

Mononuclear phagocytes play an important role in resistance to samonella infections (16). Zinkernagel (18) has suggested that immunity to *S. typhimurium* needs specific humoral factors besides macrophage activation.

In our study the in vitro interaction between immune PEC with *S. enteritidis* is strongly influenced by the presence of anti-*S. typhi* Ty2 serum. This enhanced activity was exerted only upon immune PEC and not upon normal PEC. Both normal and immune PEC had a similar cell distribution. Besides, both normal and immune PEC were obtained in identical experimental conditions, after starch stimulation, which is known to produce a certain degree of macrophage activation. Thus, we can conclude that macrophages from immune PEC were more efficient in inhibiting bacterial growth than were macrophages from normal PEC, leading to destruction of *S. enteritidis* in the presence of opsonic antibodies.

That only macrophages from immune PEC were able to inhibit growth of bacteria opsonized with such antibodies could mean that in the expression of acquired cellular resistance, specific humoral factors are important not only in the adherence and ingestion of bacteria, but also in their digestion by macrophages. Similar findings have also been found by Jones et al. (8), who have shown that heated anti-toxoplasma serum enhanced ingestion and intracellular killing of toxoplasma by immune macrophages. The influence of the opsonic factors is also evident in our results through the null bactericidal activity of the immune PEC against *S. enteritidis* when the in vitro test was performed in the absence of mouse serum (Fig. 1).

Normal PEC were unable to inhibit growth of *S. enteritidis* in spite of the presence of opsonic antibodies, although *S. enteritidis* shares some antigenic structures with *S. typhi* Ty2. This result opposes those previously reported, in which specific opsonins were pointed out to limit the intracellular multiplication of the microorganism by normal or immune macrophages (7). However, our results are similar to those obtained by Anderson et al. (1), who have shown that pretreatment of toxoplasma with heat-inactivated mouse serum, anti-toxoplasma, enabled normal macrophages to kill the organism while pretreatment with heat-inactivated mouse serum, anti-*Besnoitia*, did not confer this capibility on normal macrophages, in spite of the fact that these two protozoa share some antigenic structures.

Both normal and immune PEC had no bactericidal effect upon *L. monocytogenes*. The same results have been previously described for *S. typhimurium* (6) with normal macrophages. This experimental finding suggests that macrophages from immune PEC are unable to inhibit the growth in vitro of the unrelated bacteria. North and Spitalny (14) have shown in vivo that the transference of anti-θ and complement-treated PEC from an immune mouse to a normal mouse failed to control a lethal challenge with the microorgainsm in spite of the presence of functional immune macrophages.

On the bases of the above data, some interesting facts can be concluded: (i) *S. typhi* Ty2 killed by repeated freezings at −70°C and thawings at 48°C that is injected intraperitoneally in PBS is able to elicit the production of macrophages (immune PEC) that inhibit the growth of *S. enteritidis*, a microorganism that shares an antigenic structure with *S. typhi* Ty2. (ii) Heat-inactivated anti-*S. typhi* Ty2 mouse serum contains opsonic factors cross-reactive with an antigenic determinant specific for *S. enteritidis*. These factors are necessary in the intracellular digestion process of *S. enteritidis*. (iii) The action of these thermostable opsonins is specific because immune PEC, in the presence of heat-inactivated anti-*S. typhi* Ty2 mouse serum, has

no effect upon *L. monocytogenes*. (iv) Our results suggest that growth inhibition of the microorganism plays an important role in the cellular (macrophages) as well as in the humoral (opsonic antibodies) immune response.

Studies are in progress to try to bring about better understanding of the quality, amount, and influence of both humoral specific factors and the antigens shared by the microorganism in the induction of the immune response.

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