Characteristics of Cells Present in Peritoneal Fluids of Mice Injected Intraperitoneally with *Bordetella pertussis*

C. W. FISHEL,* D. G. HALKIAS, T. W. KLEIN, AND A. SZENTIVANYI

Departments of Medical Microbiology* and Pharmacology, College of Medicine, University of South Florida, Tampa, Florida 33620

Received for publication 2 September 1975

Peritoneal fluids obtained from mice after the intraperitoneal administration of *Bordetella pertussis* vaccine, heated vaccine, an extract of the organisms, killed *Escherichia coli*, or thioglycolate medium were examined in terms of total cells and percentage that adhered to glass cover slips during a 2-h incubation period. All these substances were found to increase the number of leukocytes in peritoneal fluid within 1 to 2 days after the injection. This increase appeared to be due to an influx of macrophages and polymorphonuclear leukocytes with relative proportions at a given time dependent upon the material involved in the induction of the response. The initial increase after pertussis vaccine seemed to be due mainly to an influx of mononuclear cells, whereas with *E. coli* neutrophils constituted the major portion of the cell population. The percentage of peritoneal cells that attached to glass was also found to be markedly reduced in preparations obtained from mice after the injection of *B. pertussis* or *E. coli*. There appeared to be differences in persistence of this phenomenon, with preparations containing the histamine-sensitizing factor being the most active in affecting adherence properties. Thus these data would suggest that the action of *B. pertussis* on macrophages (or precursors) and neutrophils is not expressed in terms of suppression of emigration properties, as has been reported by others for lymphocytes, but is manifested in the alteration of glass-adherence characteristics. Within experimental limitations, it is believed that macrophages are possibly more involved in terms of altered function than are the polymorphonuclear cells.

In the years since Parfentjvo and Goodline (23) reported the increased histamine sensitivity of *Bordetella pertussis*-injected mice, numerous other biological properties of the organism have been described. Thus mice and rats become very susceptible to the lethal effects of serotonin, endotoxin, acetylcholine, bradykinin, and nonspecific stimuli such as cold stress (20). The injected animals exhibit a depressed responsiveness to substances such as epinephrine (8, 11), and the general immune response including both humoral and cellular events is also influenced by the microorganism (6, 19, 21, 27). Although these phenomena are well recognized, the underlying mechanism(s) in most if not all has not been completely identified.

The development of a marked leukocytosis is also a prominent reaction that follows the injection of pertussis (or soluble products thereof) in man and experimental animals (17, 18). The response is characterized by an increase in numbers of both blood granulocytes and lymphocytes and, in the experimental animal, follows either the intravenous or intraperitoneal administration of the organism (7, 18). The leukocytosis is believed to be due to alterations in the distribution of cells rather than increased production; i.e., there is initially a mobilization of cells from lymphoid organs followed by a suppression of the recirculation from blood to lymph nodes (2, 25). The failure to "home" seems to be due to an alteration of the lymphocyte surface by a pertussis component(s). In support of this idea, it was shown that spleen, lymph node, and thoracic duct cells reversibly combined and removed the leukocytosis-promoting factor from preparations of the pertussis organism (33). It would appear, therefore, that a pertussis component does have the potential to attach to at least certain cells of the mobilizable lymphoid pool and the characteristics of these cells are altered to some extent, at least, as a result of the combination.

By contrast to the information available concerning the influence of *B. pertussis* on cells in the circulation, little evidence has been obtained relative to the effect of the organism on
cells in the peritoneal fluid. An increase in the number of neutrophils in the peritoneal cavity of mice 5 days after the intraperitoneal administration of *B. pertussis* has been observed (9). The present studies were designed to investigate the influence of the organism on cells of the peritoneal fluid, with emphasis upon those that adhere to glass. It was found that the intraperitoneal administration of *B. pertussis* vaccine (PV), heated pertussis vaccine, an extract of the organism (PE), killed *Escherichia coli* bacteria, or thioglycolate medium influenced not only the number but also adherence properties of peritoneal cells.

**MATERIALS AND METHODS**

**Mice.** Female mice of the Dub/ICR strain weighing 16 to 18 g when received were obtained from Flow Research Animals, Inc. (Dublin, Va.) and maintained in the laboratory for 1 week before use. These animals would develop hypersensitivity to histamine (1.0-mg histamine base) 3 to 4 days after the injection of 125 μg of PE or 5 days after PV.

**Bacterial preparations.** Pertussis vaccine was kindly supplied by Robert J. Hosley, Eli Lilly and Co. (Indianapolis, Ind.). This material (lot no. 7BB9), containing 40 billion organisms per ml, was dialyzed 24 h with several changes of physiological saline to remove merthiolate used as preservative. A portion of the vaccine was heated at 80 C for 30 min to inactivate the histamine-sensitizing factor. Strain O111-B4 of *E. coli* was grown over-night in Trypticase soy broth. After dilution plating to determine number of viable cells, the suspension was centrifuged and the sedimented cells were washed three times and suspended in saline. The latter was placed in a boiling-water bath for 3 min, cooled, and adjusted to contain 40 billion organisms per ml with saline. Both pertussis vaccine and *E. coli* preparations were diluted 1:2 in saline just before intraperitoneal injection (0.5 ml) into mice.

A saline extract of *B. pertussis* was prepared and generously supplied by John J. Munoz, U.S. Public Health Service (Hamilton, Mo.). This lyophilized material was dissolved in saline immediately before use.

**Harvest of cells.** Mice were killed by cervical dislocation and a slit was made into the peritoneal cavity. Three milliliters of Hanks balanced salt solution (HBSS) (Flow Laboratories, Rockville, Md.) was introduced into the peritoneum and, after a thorough flushing by aspiration, 2 ml was withdrawn. The peritoneal fluid was centrifuged, and the sedimented cells were washed two times with HBSS and suspended in 2 ml of incubation medium. The latter consisted of Eagle basal medium with HBSS plus 5% heat-inactivated newborn calf serum and streptomycin (50 μg/ml), penicillin (50 Us/mI), neomycin (50 μg/ml), and 0.05% sodium bicarbonate. Cell counts were made using an electronic counter (Bio/Physics Systems, Inc., Mahopac, N.Y.).

**Culture conditions.** After the appropriate numbers of peritoneal cells (1 × 10⁶ to 2 × 10⁶) were seeded over glass cover slips in plastic tissue culture dishes (35 by 10 mm) (Falcon, Oxnard, Calif.), these chambers were incubated at 37 C in a humidified atmosphere containing 5% CO₂ and 95% air. At the end of incubation time, usually 2 h, the cover slips were washed three times with 2 ml of HBSS, fixed in 2.5% glutaraldehyde in phosphate buffer at pH 7.4, air dried, and stained with Wright stain. The preparations were examined routinely by phase-contrast and, in certain instances, by electron microscopy.

**Test for phagocytic activity.** Phagocytosis of yeast cells was employed in testing the activity of adherent cells. After a 2-h incubation and removal of nonadherent cells, 0.25 ml of a 1:10 dilution of 5% yeast cell suspension was added to 2-ml incubation medium and placed over the cover slips. After 30 min at 37 C, the preparations were washed with HBSS, fixed in glutaraldehyde, and stained as mentioned in preceding section.

**Electron microscopy.** Cover slip preparations were fixed with 2.5% glutaraldehyde in phosphate buffer, pH 7.4. After a wash with the buffer, they were placed in 2% osmium tetroxide in veronal acetate buffer, pH 7.4, and, after 1 h, were rinsed in 10% aceton. The cover slips were then placed in 2% uranyl acetate in 10% acetone and dehydrated with increasing concentrations of acetone followed by a 1:1 mixture of 100% acetone and Epon 812. The cover glasses were inverted over small petri dishes containing Epon 812 and placed at 60 C overnight to polymerize the resin. The cover glass was removed by dipping into liquid nitrogen, and the appropriate areas were cut out and sectioned at approximately 50 to 60 nm. The sections were placed on 200-mesh copper grid and stained with Venable lead acetate. These were examined with a Philips 301 electron microscope.

**RESULTS**

The intraperitoneal injection of mice with any of the agents used in these studies was followed within 24 h by a marked increase in the number of cells in the peritoneal cavity as shown in Table 1. This increase was maintained for 5 days in those animals which had received PV or heated PV. However, the numbers were approaching control levels by day 5 in preparations obtained from PE- and *E. coli*-injected mice. The administration of thioglycolate medium also induced an increase in the number of cells in peritoneal fluid.

It can also be seen from the data presented in Table 1 that glass-attachment properties of at least certain populations of cells were affected by prior administration of pertussis preparations or *E. coli* but not by thioglycolate medium. After a 2-h incubation period, the increase in number of nonadherent cells was particularly noticeable in those preparations obtained from PV- and PE-injected animals.
The increase was significant over the 5-day period with PV as the inducing agent and for 3 days after PE. Heating the vaccine appeared to reduce the effect on adherence and the pattern resembled that obtained with *E. coli* organisms.

The percentage of cells that detached from glass between 2 and 24 h was also of interest to us. It seemed that approximately 2% of the cells of normal animals detached during this time, whereas 9 to 11% of those obtained from PV or *E. coli* and 3 to 7% from PE-treated mice were affected. This suggests that these materials not only influence initial attachment processes but also have an effect on the ability of certain cells to remain attached during the 24-h period.

**Characteristics of adherent cells.** After a 2-h incubation in vitro, the adherent cells obtained from the peritoneal fluid of nonstimulated mice appeared by phase-contrast microscopy to consist mainly of mononuclear elements, with polymorphonuclear leukocytes and other granulocytes contributing less than 5% to the total population (Fig. 1A, 2A). By electron microscopy examination, it was found that the mononuclear forms were macrophages (95%) and lymphocytes (5%) with identification as shown in Fig. 3. Lymphocytes were not observed in the adherent population after a 24-h incubation period. There were very few macrophages in the "activated" form as defined by Mackaness (16) in the peritoneal fluid of nonstimulated mice.

Within 24 h after the administration of PE or *E. coli*, there was a striking influx of polymorphonuclear leukocytes (neutrophils) into the peritoneal cavity and an increase in the number of mononuclear cells in the activated form (Fig. 2B, C). Injected PV also induced a rapid response which was characterized mainly by an increase in mononuclear forms although more neutrophils were present in the 24 h preparation than noted in control counterparts. Two days after the injection of thioglycolate, the glass-adherent cells were almost entirely mononuclear and 85% of these appeared to be macrophages in the activated state; only a few neutrophils were observed at this time. Marked influx of neutrophils and activation of macrophages were noted in preparations obtained from mice 3 days after the injection of PV (Fig. 1B). By day 5, the characteristics of the adherent cells obtained from PV- and *E. coli*-injected mice were quite different. With *E. coli*, the number of neutrophils appeared to be reduced and the pattern was one of activated and amoeboid forms in macrophage elements as shown in Fig. 2D. By contrast, cells in the peritoneal fluid of PV-injected mice appeared to consist primarily of neutrophils with lesser numbers of macrophages (Fig. 1C), and it was
not until 10 days after the injection that the cell types resembled those of the 5-day *E. coli* (Fig. 1D).

To obtain further information on the type(s) of cells in the peritoneal cavity of PV-injected mice 3 days after vaccination, components were recovered from washings (after 2 h of incubation) and examined by the electron microscope (Fig. 4). The nonadherent cells seemed to be macrophages and neutrophils with very few of other types present.

**Phagocytosis of yeast.** With the indications that the adherence properties of macrophages and/or neutrophils were altered after PV ad-
ministration, it was our interest to determine whether phagocytic activity might also be influenced by *B. pertussis*. As shown in Fig. 5A, glass-adherent cells from the peritoneal cavity of nonstimulated mice appeared to be, as expected, mainly mononuclear forms with 20 to 30% of these inactive in terms of ability to phagocytize yeast. Preparations derived from PV-injected mice on day 3 after vaccination seemed to contain only the active form of the mononuclear cells (Fig. 5B).

**DISCUSSION**

As mentioned in the first section of this report, extensive studies by other investigators have established the pattern of response in

![Fig. 2. Adherent cells obtained from nonstimulated mice (A), and from peritoneal fluids 5 days after the injection of 125 μg of pertussis extract (B), and 1 (C) and 5 days (D) after treatment with E. coli organisms. Phase contrast. ×400.](http://iai.asm.org/)

Downloaded from http://iai.asm.org/ on December 21, 2017 by guest
mice to *B. pertussis* in terms of increased numbers and types of cells present in the circulation. Results obtained in the present studies pointed to increased numbers of leukocytes in the peritoneal cavity of *B. pertussis*-injected mice (Table 1). However, by contrast to lymphocytosis, this increase appeared to be the result of influx of macrophages (or precursors) and neutrophils. It might be argued that present evidence was based upon glass-adherence
characteristics and that, with a few exceptions (28), lymphocytes do not have this property. Potential increases in this type of cell would therefore not be detected under present experimental conditions. That such was probably not the case was shown in experiments not included in this report. Examination by electron microscopy of the peritoneal fluid of normal and

Fig. 4. Electron micrographs of cells present in the washings (nonadherent) of cultures prepared from peritoneal fluid of pertussis-vaccinated (day 3) mice and incubated for 2 h before the wash. M, Macrophage; P, neutrophil. ×5510.
B. pertussis-injected animals did not reveal significant differences in lymphocyte populations. Furthermore, cells present in the washings of 2-h cultures of pertussis preparations were predominately macrophages with no evidence of increased numbers of lymphocytes (Fig. 4). Thus it is believed that the leukocytosis, where observed, was due to influx of macrophages and neutrophils. Actually, the failure to detect increased numbers of lymphocytes in the peritoneal fluid adds support to the concept of pertussis influence on emigration of lymphocytes from the circulation.

In terms of types of cells in the peritoneal cavity that are affected by induced acute inflammation, the general pattern of response appears to be rather constant and independent of the nature of the inciting agent. For example, an early increase followed by a rapid decrease in numbers of polymorphonuclear leuco-
cytes has been observed subsequent to the intraperitoneal administration of latex particles (29). Data presented in the same report showed that macrophages followed the same, although delayed, sequence and those cells late in the response were quite heterogenous in morphology. It has also been reported that, within 24 h after the intraperitoneal injection of mice with endotoxin, circulating polymorphonuclear leukocytes and possibly other blood cells emigrate into the peritoneal cavity. For 72 h, the peritoneal cell population consisted of a mixture of polymorphonuclear leukocytes and mononuclear elements while thereafter changes occurred in the morphology of the mononuclear phagocytes. At 48 h, large macrophages appeared which, in settle preparations, flattened on glass surfaces and demonstrated amoeboid motion (4). Others have found the response to thioglycolate consisted of an extensive infiltration of polymorphonuclear leukocytes into the peritoneal exudate within 1 day after injection with disappearance of this type of cell by day 3 (30). Replacement elements resembled macrophages. With this background, the types of cells influenced by the materials used in the present studies are consistent with the observations of others, with variation seemingly associated with time intervals. A point of interest to us was the delay in influx of neutrophils observed with PV and the prolonged phase of macrophage activation (Fig. 1B, C, D) with an indication of a similar pattern with PE. The presence of histamine-sensitizing factor in these preparations may play a role in this process.

It perhaps should also be mentioned that macrophage activation by the materials employed in the present investigation is not a unique property of these substances. Activated macrophages have been obtained from peritoneal fluids of mice infected with certain protozoan parasites (13, 15), BCG (1, 12), Listeria monocytogenes (26, 31), and from mice stimulated with killed Corynebacterium parvum (3, 10, 22). Thus any single property possessed by B. pertussis cannot account for the activation process alone.

In terms of glass-adherence properties of peritoneal cells, present data are in agreement with those reported by Krahnebuilh and Lambert (14), who found that 60 to 70% of cells obtained from normal mice adhered to culture surfaces. On the other hand, Stewart et al. (30) observed that only 15% of the peritoneal cells appeared to have the attachment property. This variation in results may be related to differences in types of culture vessels employed in the various studies.

The present observations (Table 1) concerning adherence characteristics of peritoneal cells also represent to our knowledge the first description of suppression of this activity by B. pertussis. Mechanistically, the increase in number of nonadherent cells might revolve around the same process as proposed to define pertussis-induced leukocytosis in the circulation referred to in a previous section. However, demonstration of the presence of a bacterial component(s) on the cells of peritoneal fluid has not, as yet, been accomplished.

Within experimental limitations, the type(s) of cells that would appear to be potential subjects for pertussis influence are macrophages and neutrophils. There is some evidence that favors the participation of macrophages rather than neutrophils. By electron microscopy examination, the adherent population of peritoneal cells obtained from normal mice consisted of 90 to 95% macrophages, 5 to 8% lymphocytes, and a few granulocytes (Fig. 3). However, in testing phagocytosis with yeast particles, 20 to 30% of the mononuclear cells present were inactive (Fig. 5A), suggesting that some of the nonphagocytic cells were macrophages. The absence of nonphagocytizing adherent cells in the peritoneal fluid of B. pertussis-injected mice (Fig. 5B) would suggest these as potential targets of pertussis influence. The 3-day period for examination was selected because it preceded the time of initiation of activation that would also influence the phagocytic activity of macrophage units.

With the above considerations in mind, present results serve to emphasize a potential influence of B. pertussis on cells capable of participating in the immune response yet differing from those generally recognized to be subject to pertussis action, namely, lymphocytes. For example, pertussis has been shown to potentiate the immune response to sheep erythrocytes by a stimulation of helper T lymphocytes at low antigen doses (5). It has also been suggested that the effect of pertussis on the response to PhiX is by potentiation of B cells and a suppression of helper T cells (6). Pertussis vaccine seems to exert a differential effect on subpopulations of thymus-derived lymphocytes (24), possibly those which participate in the development of cellular hypersensitivity (21) and those involved in the regulation of immunoglobulin E antibody response (32). The participation of macrophages in immune induction is well recognized, but there remain several facets of their activity that need clarification (34). The present demonstration of pertussis action on these cells or types thereof may provide a new method of approach
in further defining the role of macrophages in immune responses.

ACKNOWLEDGMENTS

These studies were supported in part by National Science Foundation grant no. 27314.

Appreciation is expressed to David W. Talmage for the many valuable suggestions in planning and conducting these studies. Farhad Mostamed for the electron microscopy information, and to Betty Nager for the excellent technical assistance.

LITERATURE CITED


