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Inhibition of Mononuclear Phagocyte Elongation, Migration, and Cellular Exudate Formation following *Bordetella pertussis* Vaccine Administration (41054)[1]

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Abstract. The effects of the iv administration of phase I *Bordetella pertussis* vaccine (BPV) on several functions of murine peritoneal macrophages were examined. Macrophages obtained from mice on Days 2 and 6 after BPV administration demonstrated a significant reduction in elongation and random migration activities *in vitro*. Mice injected iv with BPV 2 or 6 days prior to an ip challenge with proteose peptone broth exhibited a reduced peritoneal cellular exudate response. The component of BPV responsible for all three activities was labile to heating at 80° for 30 min. These results demonstrate that the systemic administration of BPV alters peritoneal macrophage function and suggest that the same component of BPV is responsible for these alterations.

Bordetella pertussis vaccine (BPV) possesses a number of immunomodulatory activities. Administration of BPV to experimental animals enhances antibody responses to foreign antigens (1, 2), increases resistance to bacterial infections (3, 4), and enhances or reduces resistance to tumors (5, 6). Since macrophages are involved in both afferent and efferent phases of immune responses, several investigators have suggested that alterations of macrophage function by BPV may account for several of its immunomodulatory activities (4, 7). However, very little information is available concerning the alteration of specific macrophage functions after mice are injected with BPV. Evidence is accumulating in our laboratory that murine peritoneal macrophage phagocytic activity and lysosomal enzyme levels are increased following iv BPV administration (submitted for publication). However, we have preliminary evidence that macrophages from BPV treated mice do not demonstrate increased spreading following attachment to glass surfaces. Hence, the purpose of the present study was to characterize the spreading capabilities and associated activities of peritoneal macrophages following the systemic administration of BPV. In addition, since heating pertussis vaccine at 80° for 30 min inactivates several of its biological activities (8), we examined the heat lability of the factor responsible for the effects observed.

Materials and Methods. Female mice, strain BDF_1/Cox (C57B1/6 Cox × DBA/2 Cox), weighing 17–20 g were obtained from Laboratory Supply Company, Indianapolis, Indiana. *Bordetella pertussis* vaccine (Lot 26663) containing 4 mg dry weight of inactivated pertussis organisms per milliliter of saline–merthiolate (SM) diluent was provided by Connaught Laboratories, Swiftwater, Pennsylvania. Vaccine diluent consisted of 0.9% NaCl containing 0.02% merthiolate. The tissue culture media used in these studies were Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640. Both media were supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% heat-inactivated (56°, 30 min) fetal bovine serum (Kansas City Biological, Inc., Lenexa, Kan.; Lot 309459). All cultures were incubated at 37° in a humidified atmosphere of 95% air and 5% CO_2.

Experimental mice were injected iv via a lateral tail vein with 0.2 ml BPV or vaccine heated at 80° for 30 min (HPV). Control mice were injected with 0.2 ml SM. In some

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experiments uninjected mice also served as controls. The BPV and HPV were adjusted with SM so that each mouse received 40 μg vaccine/g body wt. On Days 2, 6, or 14 after injection, the mice from control and experimental groups were sacrificed by cervical dislocation and the peritoneal leukocytes were removed by lavage. Peritoneal lavage consisted of injecting 4 ml DMEM into the peritoneal cavity, uniformly agitating the cavity, and withdrawing the medium containing the leukocytes. The cells were washed and were resuspended to 1.5×10^6 viable leukocytes/ml in DMEM. Viable leukocyte counts were determined by trypan blue dye exclusion.

Adherent peritoneal leukocyte elongation assays were performed as follows. Adherent cell monolayers were established by placing 100-μl aliquots of cell suspensions onto 12-mm glass coverslips and incubating these preparations for 30 min at 37°. The coverslips were then rinsed with culture medium to eliminate nonadherent leukocytes and were incubated for an additional 1.5 or 23.5 hr in 35 × 10-mm tissue culture dishes containing 2 ml DMEM. The coverslip preparations were removed, fixed, and stained with buffered differential Wright's stain (Cambridge Chemical Products, Inc., Ft. Lauderdale, Fla.), and were examined with a light microscope equipped with an ocular micrometer. Two hundred adherent leukocytes were examined from each preparation. Leukocytes possessing diameters of $\geq$20 μm were scored as elongated. In some experiments the ability of these adherent cells to phagocytize carbon particles (India ink) was examined. For these studies, coverslip preparations were incubated with 0.1% India ink in DMEM for 30 min at 37°. The coverslips were then rinsed and stained as described.

Peritoneal leukocyte random migration was assayed by a modification of the agarose droplet technique of Harrington (9). The agarose was prepared by mixing equal volumes of a melted 0.4% agarose solution with 2× DMEM. Peritoneal leukocytes from control and experimental mice were suspended to a final concentration of 2×10^7 viable leukocytes/100 μl agarose–DMEM solution. One-microliter drops of agarose, containing peritoneal leukocytes, were then placed into individual wells of a 96-well microtiter plate, allowed to solidify for 5 min at 4°, and overlaid with 100 μl RPMI 1640. The distance of macrophage migration from each agarose droplet was determined after 24 and 48 hr of incubation at 37°. The migration index was determined by the formula

$$\text{migration index} = \frac{\text{distance of migration of experimental leukocytes}}{\text{distance of migration of control leukocytes}}.$$

A migration index of $\leq$0.80 was taken as inhibition of migration (9).

Several experiments were performed to determine the effect of BPV administration on peritoneal cellular exudate formation. Peritoneal exudates were elicited by the ip administration of either 1 ml sterile 10% proteose peptone broth or 2.98% thioglycollate broth. On various days after proteose peptone or thioglycollate administration, the mice were sacrificed by cervical dislocation and the peritioneal leukocytes were removed by lavage and counted as described.

Results. The effect of iv administration of BPV on adherent peritoneal macrophage elongation is shown in Fig. 1 and Table I. Following 2 hr of *in vitro* cultivation, there was no significant difference between control and experimental animals in the ability of their macrophages to elongate. However, after 24 hr *in vitro,* the macrophages obtained from BPV-treated mice exhibited significantly fewer elongated forms than those from SM-treated animals. Examination of cell preparations from both groups at time points between 2 and 24 hr (data not shown) revealed that cells from SM-treated animals gradually spread over the 24-hr period in culture while those from the BPV-treated group failed to spread during the course of incubation. The greatest reduction in elongation was observed on Days 2 and 6 after BPV administration. Heating the vaccine at 80° for 30 min prior to its admini-

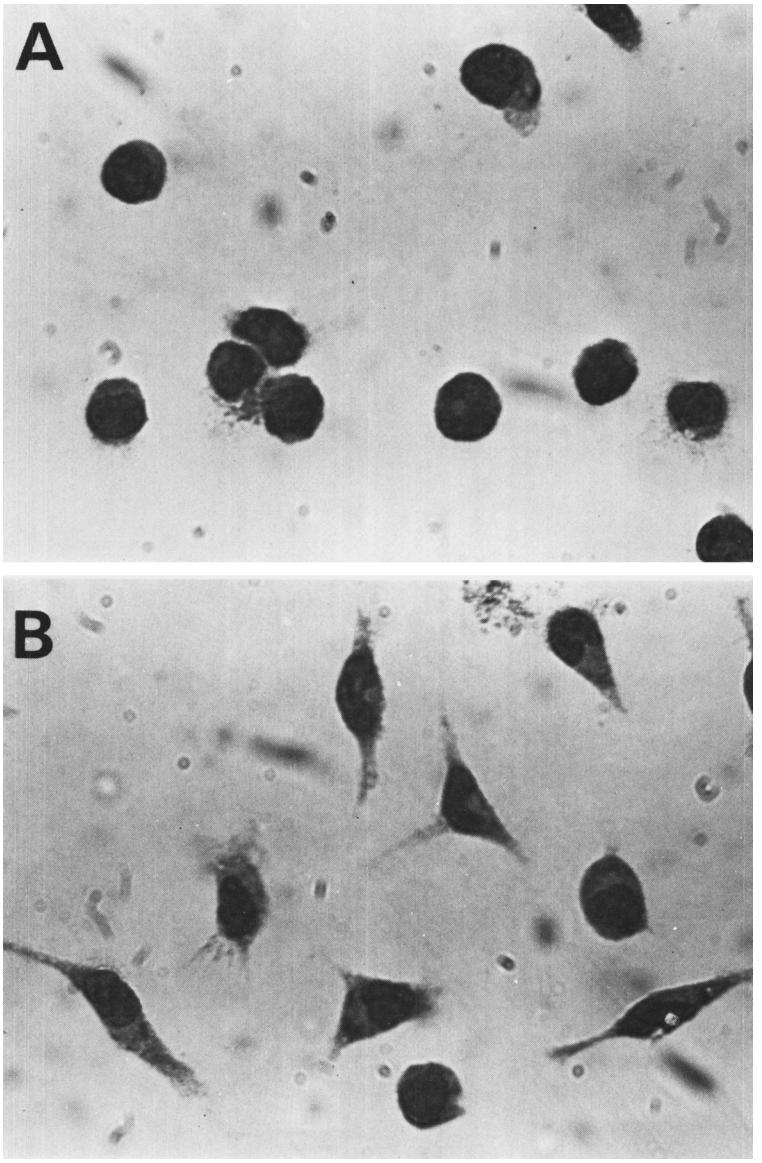

FIG. 1. The effect of *Bordetella pertussis* vaccine (BPV) on elongation of murine peritoneal macrophages. Adherent peritoneal leukocytes obtained from mice on Day 6 after BPV or saline–merthiolate (SM) diluent administration. Leukocytes from (A) SM-treated mice after 2 hr, (B) 24 hr of *in vitro* culture, (C) BPV-treated mice after 2 hr, and (D) 24 hr of culture. (×590).

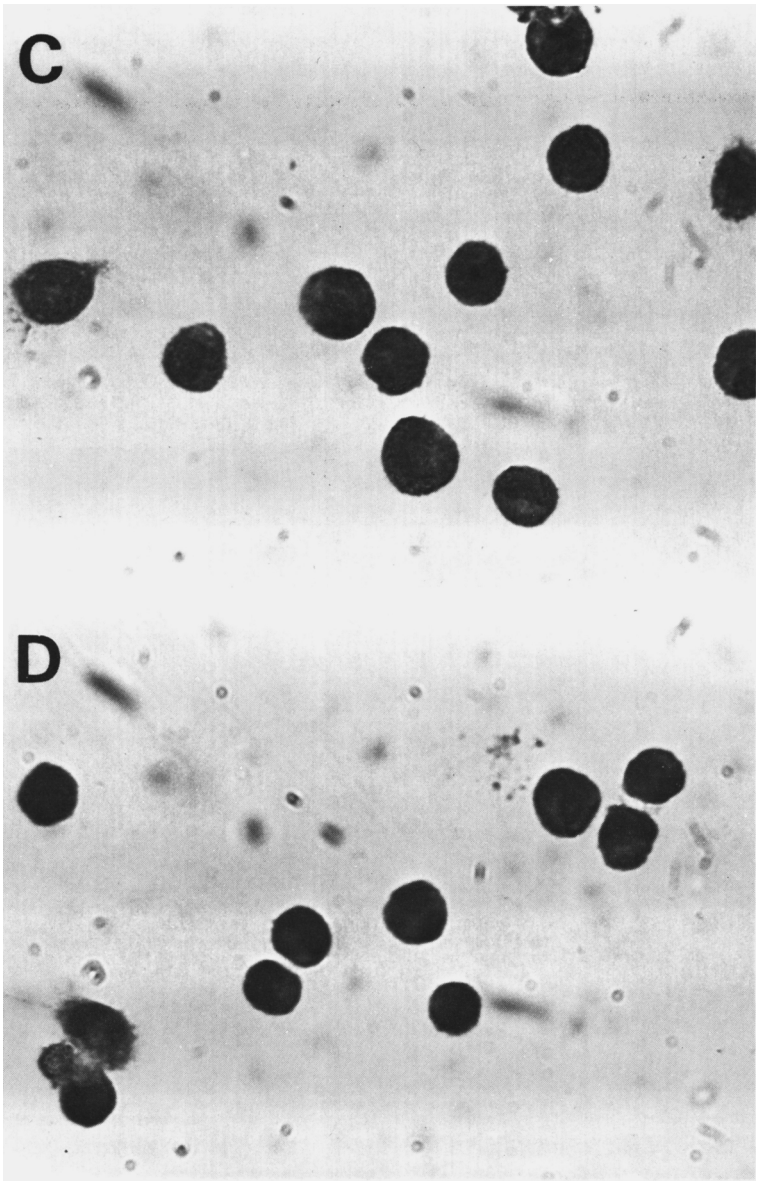

FIG. 1—*Continued*

stration reduced the vaccine's inhibitory activity. There were no detectable differences in the percentage of control and experimental adherent cells capable of phagocytizing carbon particles, indicating that the adherent macrophages from all groups were viable and still functional in this regard (data not shown). In addition, there were no differences in the elongation properties of macrophages obtained from uninjected mice and those injected with SM.

In order to determine if the inhibition of macrophage elongation represented an alteration of random migration activity, the migration of peritoneal leukocytes obtained from BPV-, HPV-, and SM-treated mice was examined *in vitro*. The results in Table II show that peritoneal leukocytes obtained

TABLE I. ELONGATION OF ADHERENT PERITONEAL LEUKOCYTES FOLLOWING THE iv ADMINISTRATION OF *Bordetella pertussis* VACCINE (BPV) OR SALINE–MERTHIOLATE DILUENT (SM)

Days after injection	Percentage elongated adherent cells					
	SM (2 hr)[a]	BPV (2 hr)	HPV[b] (2 hr)	SM (24 hr)	BPV (24 hr)	HPV (24 hr)
2	6 ± 1[c]	2 ± 1	—	59 ± 8	8 ± 1[d]	—
6	5 ± 1	4 ± 1	6 ± 2	49 ± 5	6 ± 1[d]	35 ± 6
14	7 ± 2	6 ± 1	—	56 ± 7	20 ± 2[d]	—

[a] Incubation time of adherent cell cultures *in vitro*.
[b] Pertussis vaccine heated at 80°, 30 min.
[c] Mean ± SEM, $N = 3 - 8$; Uninjected values: 2 hr = 5 ± 1, 24 hr = 56 ± 8.
[d] $P < 0.005$ when compared to SM by Student's t test.

from mice on Days 2 and 6 after BPV administration demonstrated a significant reduction of random migration when compared to leukocytes obtained from SM-treated control mice. By Day 14 after injection, leukocytes from BPV-treated mice exhibited random migration activity similar to that of cells from SM control mice. This inhibition of random migration was not the result of an increased number of polymorphonuclear leukocytes since >95% of the peritoneal leukocytes were mononuclear. Administration of HPV did not alter significantly the random migration characteristics of the peritoneal leukocytes.

We wanted to determine whether the inhibition of migration observed might be an indication of altered *in vivo* emigration properties of macrophages. The ip injection of proteose peptone into mice results in a marked exudation of mononuclear phagocytes by the third day following injection. We reasoned that if BPV treatment altered the migration pattern of macrophages then the prior injection of BPV might suppress the influx of these cells in response to proteose peptone. Therefore, the effect of prior iv administration of BPV on the peritoneal exudate response 3 days following the ip injection of proteose peptone broth was examined. The Day 3 exudate response was chosen because, in our hands, the exudate leukocyte population at this time had approximately doubled in size and consisted of greater than 95% mononuclear cells. The results of these experiments are shown in Table III. There was a significant inhibition ($P < 0.05$) of peritoneal cellular exudate formation in BPV-treated mice when proteose peptone broth was injected on Day 2 after BPV administration. By Day 14 after BPV, the cellular exudate response to proteose peptone broth returned to SM control levels. Administration of HPV was not as effective as pertussis vaccine in inhibiting exudate formation in response to proteose peptone challenge. Similar results were observed when thioglycollate broth was used as the eliciting agent (data not shown).

In order to examine if the inhibition of cellular exudate formation was the result of an altered kinetics of influx, mice were injected with BPV and on Day 2 challenged with proteose peptone. The cellular exudate was examined over the following 4 days. The results of this experiment are

TABLE II. RANDOM MIGRATION OF PERITONEAL LEUKOCYTES FOLLOWING THE iv ADMINISTRATION OF *Bordetella Pertussis* VACCINE (BPV), HEATED PERTUSSIS VACCINE (HPV) OR SALINE–MERTHIOLATE DILUENT (SM)

Treatment	Days after injection	Migration index[a]	
		24 hr	48 hr
BPV	2	0.58 ± 0.14[b]	0.66 ± 0.02
	6	0.47 ± 0.04	0.35 ± 0.08
	14	1.02 ± 0.17	0.94 ± 0.15
HPV[c]	6	0.88 ± 0.06	0.83 ± 0.08

[a] Migration index = distance migrated by BPV or HPV leukocytes/distance migrated by SM leukocytes.
[b] Mean ± SEM, $n = 3$.
[c] Pertussis vaccine heated at 80°, 30 min.

shown in Fig. 2. The inhibition of peritoneal exudate formation in BPV-treated mice was not due to an alteration in the kinetics of the exudate response, since leukocyte influx was inhibited by prior injection of BPV on Days 1 to 4 following proteose peptone administration.

Discussion. The present study indicates that the systemic administration of BPV depresses several functions of peritoneal macrophages. The iv administration of BPV results in a decreased ability of adherent peritoneal mononuclear phagocytes to elongate after attaching to a glass substratum (Fig. 1, Table I). These leukocytes also exhibit reduced *in vitro* random migration (Table II). The leukocyte population which is inhibited from migrating following BPV treatment appears to be the mononuclear phagocyte population. This is suggested by the fact that iv injection of BPV does not appreciably alter the adherent (60–70%), mononuclear (>90%), nonspecific esterase positive (40–50%) cell content of the peritoneal cavity. Therefore, a significant contribution of polymorphonuclear leukocytes to the migrating pool can be excluded. Furthermore, in the migration assay employed, the random migration of macrophages is thought to be more extensive than the migration of lymphocytes, and therefore an involvement of the latter cell type would be minimized. The component of BPV responsible for inducing these alterations is partially labile to heating at 80° for 30 min. In contrast to the activities of BPV, the iv administration of *Corynebacterium parvum* vaccine or viable bacillus Calmette–Guerin (BCG) increases peritoneal macrophage spreading (10, 11). Furthermore, peritoneal macrophages obtained from experimental animals injected with BCG exhibit increased random migration (12). Thus, in comparison to these bacterial agents, BPV appears to be unique in its ability to produce depressed macrophage elongation and random migration following its *in vivo* administration.

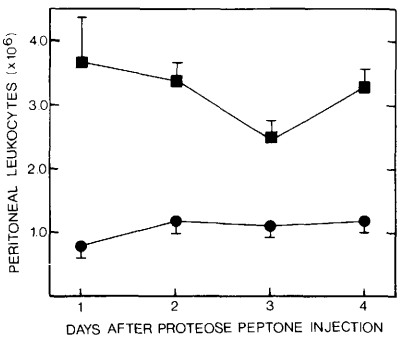

FIG. 2. Kinetics of peritoneal leukocyte influx induced by proteose peptone. Mice were injected iv with *Bordetella pertussis* vaccine (●) or vaccine diluent (■) 2 days prior to ip administration of proteose peptone broth. The number of peritoneal exudate leukocytes per milliliter of lavage fluid was determined on Days 1, 2, 3, and 4 after proteose peptone inoculation. Peritoneal leukocyte counts in vaccine-treated and diluent-treated groups prior to proteose peptone treatment were $1.2 \times 10^6 \pm 0.1$ and $1.5 \times 10^6 \pm 0.1$, respectively. Each point represents mean ± SEM of three determinations.

The possibility that alterations of macrophage migration similar to those observed *in vitro* might also be demonstrated *in vivo* was examined by determining the ability of the monocyte/macrophage system to respond to the ip administration of a sterile inflammatory agent. In our hands, the Day 3 proteose peptone induced exudate response consists of greater than 95% mononuclear cells. Administration of BPV iv depresses the ability of these cells to form a peritoneal cellular exudate in response to the ip injection of proteose peptone broth (Table III). In addition, this activity of BPV was partially labile to heat inactivation. The inhibition of cellular exudate formation was not due to an altered kinetics of influx to proteose peptone broth challenge (Fig. 2) or to an increased adhesion of macrophages to the peritoneal cavity (unpublished observations). Moreover, the temporal development of exudate inhibition was similar to the development of the inhibition of elongation and random migration and all three activities of BPV were partially inactivated by heating the vaccine at 80° for 30 min. Thus, the alteration of these three activities may be the effect of the same vaccine component.

One of the heat labile components of BPV is leukocytosis-promoting factor

TABLE III. DAY 3 PROTEOSE PEPTONE PERITONEAL EXUDATE RESPONSE OF MICE PREVIOUSLY INJECTED INTRAVENOUSLY WITH EITHER *Bordetella pertussis* VACCINE (BPV), SALINE–MERTHIOLATE DILUENT (SM), OR HEATED PERTUSSIS VACCINE (HPV)

Prior treatment	Day of proteose peptone injection after BPV, SM, or HPV	Peritoneal leukocytes ($\times 10^{-6}$)[a]	Percentage of SM control	P[c]
BPV	2	1.1 ± 0.1[b]	33	≤0.001
SM		3.3 ± 0.3		
BPV	6	1.7 ± 0.1	66	NS[d]
SM		2.6 ± 0.4		
BPV	14	3.4 ± 0.1	113	NS
SM		3.0 ± 0.4		
HPV	2	2.6 ± 0.3	63	NS
SM		4.1 ± 0.5		

[a] Number of leukocytes/ml of lavage fluid three days after proteose peptone injection.
[b] Mean ± SEM, four determinations per point.
[c] P value comparing BPV to SM by Student's t test.
[d] NS, not significant; $P > 0.05$.

(LPF). This factor possesses the activity of inducing a leukocytosis consisting predominantly of a lymphocytosis (13, 14). We have fractionated *Bordetella pertussis* culture supernatants by means of hydroxyapatite column chromatography according to previously described methods (15). One of the resulting fractions possessed potent LPF activity in that injection of as little as 10 μg protein into mice increased the blood leukocyte count from approximately 10,000/mm^3 to 80,000/mm^3. This LPF activity was suppressed >95% by heating the fraction. Of interest is our preliminary finding that prior injection of this fraction (5 μg protein) into mice followed by proteose peptone administration reduces the peritoneal exudate response to 30% of control. Furthermore, heating the fraction prior to injection removes the suppressive effect. Morse suggested that one mechanism by which BPV and LPF induce leukocytosis is by decreasing the ability of lymphocytes to emigrate from the blood to peripheral organs (14). The present results suggest that BPV (and possibly LPF) may have a similar effect on cells of the monocyte/macrophage series. Furthermore, our observations concerning the *in vitro* inhibition of macrophage migration and elongation suggest that the failure of mononuclear phagocytes to emigrate *in vivo* may be due in part to a suppression of their migration ability. In addition, our results may also explain the observation of Levine (16) that a heat labile component of BPV inhibits the macrophage response to a zone of thermal coagulation necrosis in the brain.

We have observed that the phagocytic activity (E–IgM–C) and lysosomal enzyme activity (acid phosphatase) of peritoneal macrophages are increased 6 days following iv injection of BPV (submitted for publication). In the present report, we have demonstrated a decrease in macrophage function at this time following BPV injection. These results appear conflicting if it is assumed that the functional repertoire of the stimulated cell at a specific point in time is either 100% enhanced or 100% suppressed. Recent evidence, however, has indicated that this is not the case. It has been demonstrated that a given point in time following administration of a stimulus the mouse peritoneal macrophage population displays simultaneous enhancement of certain functions and suppression of others (17, 18). The mechanisms of this functional limitation are unknown but could result from cell surface regulatory events or the inherent limitations of certain cellular capacities such as the microtubule–microfilament system. The limited capacity of this system might account for the simulta-

neous enhancement of immune phagocytosis coupled with the depressed ability to spread or migrate. Further studies are needed which are designed to examine a variety of macrophage functions at specific times following stimulation for a better understanding of these mechanisms.

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