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(U) Nonspecific Resistance Induced by an Immunopharmacologic Agent Derived from Bordetella pertussis

12 PERSONAL AUTHOR(S)
Winters, Alvin Leo; LeBlanc, Paul Arthur; Sloan, Gary Lee

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Treatment of mice with a trichloroacetic acid soluble extract of Bordetella pertussis (Boivin antigen) induces resistance to a lethal dose challenge of mouse adenovirus. The resistance develops within 2-3 days after treatment and persists for 10 days and possibly longer. The TCA-soluble extract designated EP-LPS (endotoxin associated proteins and lipopolysaccharide) consists of proteins that may or may not be complexed to the lipopolysaccharide under nondenaturing conditions. Although EP-LPS induced a notable increase of lymphocytes in the blood and peritoneum and a marked decrease of monocytes and macrophages in the peritoneum, investigation of B cell, T cell, NK cell, and macrophage activities failed to demonstrate any striking mechanism that might account for antiviral activity. In a search for an adjuvant to replace alum we discovered that block copolymers L121 and T150R1 in combination exhibited antiviral activity.

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1.0 BACKGROUND. Administration of 250 ug of Bordetella pertussis vaccine (BPV) to mice intraperitoneally (i.p.) renders the animals resistant to a lethal dose challenge of mouse adenovirus (MAdipt4) 7 days after treatment (1). A similar activity had been reported with type 1 herpes simplex was used as the challenging virus (2). These observations provided the background for the objectives of identifying the molecule or complex of molecules in B. pertussis that induced resistance to a MAdipt4 challenge and characterizing their mechanism of action. Investigation of this molecule or complex of molecules will lead to a better understanding of immunomodulation and nonspecific resistance mechanisms.

1.1 FIRST CONTRACT YEAR (3). We established that the antiviral activity of BPV was not associated with a given phase or strain of B. pertussis, but was associated with many strains, including phase I and phase IV strains. Acellular fractions of B. pertussis demonstrated antiviral activity. Acellular fractions included a 1.0 M NaCl extract of whole cells (produced by Dr. R. D. Leamon, Connaught Laboratories, Inc., Swiftwater, PA), a cell surface polysaccharide removed from whole cells by shearing in a Waring blender, and lipopolysaccharide (LPS) extracted by the phenol-water method of Westphal. All of these fractions contained endotoxin as detected by the Limulus amoebocyte assay.

1.2 SECOND CONTRACT YEAR (4). We established that an acellular fraction of B. pertussis (Boivin antigen extracted in 2.5 % trichloroacetic acid from whole cells) induced resistance to mouse adenovirus infection. Doses in the range of 20 ug (approximately 0.9 mg/kg) induced resistance, whereas doses in the range of 2.0 ug (approximately .09 mg/kg) induced resistance when adsorbed to alum. Lipopolysaccharide extracted from boivin antigen by phenol-water precipitation of the proteins induced resistance also. Similar concentrations of gliding bacteria adjuvant (provided by Dr. William R. Usinger) did not induce resistance. Boivin antigen consisted of 5-7 major proteins (detected by SDS-polyacrylamide gel electrophoresis and PAGE blue 83 staining) and lipopolysaccharide (detected by SDS-polyacrylamide gel electrophoresis and silver staining). Boivin antigen will be designated hereafter as EP-LPS, i.e., co-extracted endotoxin-associated proteins and lipopolysaccharide. Two dimensional polyacrylamide gel electrophoresis indicated that several of the proteins of EP-LPS were not closely associated with the LPS. However, Western blot of the two dimensional gels revealed several minor proteins that were immunodominant antigens and that appeared to be associated with at least a portion of the LPS fraction.

2.0 THIRD CONTRACT YEAR.

2.1 CONTINUED VIRUS PROTECTION STUDIES OF EP AND LPS DERIVED FROM EP-LPS BY THE PHENOL-WATER METHOD. Dr. Barnet Sultzer, Downstate Medical Center, SUNY, separated EP-LPS into EP and LPS using phenol-water precipitation of the proteins (4). Over a period of time the LPS preparation appeared to lose potency. Our inital experiments demonstrated activity repeatably at a dosage of 20 ug; however, subsequent experiments demonstrated activity only at higer dosage, i.e., 80 - 160 ug (Table 2.1.1). The LPS was stored at 4°C and the chemical characteristics of the preparation, such as micelle size, may have changed or the molecule may have suffered biochemical degradation. A possible role of EP may be the stabilization of the LPS moiety in an active configuration over a long period of storage. Reconstitution of the separated endotoxin-associated proteins and LPS failed to return activity (Table 2.1.2).

Finally, a protective activity of the Alhydrogel was noted in several experiments. At approximately the same time Dr. P. S. Morahan, Medical College of Pennsylvania, also noted protective activity using the same lot of Alhydrogel in a herpes simplex virus infection model (personal communication). We could not detect bacterial or endotoxin contamination in this lot of Alhyrogel. Due to the intermitent protective activity of Alhydrogel and communication with Dr. R. Bomford, Wellcome Research Laboratories, England, concerning immunomodulation by alum (5), we have discontinued the use of this component in our experimental protocols. In an attempt to find an EP-LPS potentiating agent to replace alum we tested a combination of block copolymers (copolymers of blocks of hydrophobic polyoxypropylene and hydrophilic polyoxyethylene) that has adjuvant and inflammatory activity (6). Block copolymers L121 and T150R1 and EP-LPS in combination demonstrated antiviral activity; however, much to our suprise the combination of block copolymers L121 and T150R1 demonstrated antiviral activity also (Table 2.1.3). Subjectively, the group of mice treated with block polymers alone exhibited more signs of disease, i.e., rough fur, hunched back, and huddling, but health returned. Future studies of EP-LPS immunomodulation will include comparison of block polymer antiviral activity.

Table 2.1.1. Dose-Response of LPS Extracted From EP-LPS.

Treatment[a]	Mortality[b] (deaths/total)
Experiment 1[c]:	
EP-LPS, 40 ug/ml	9/10
EP-LPS, 4.0 ug/ml	11/11
EP-LPS, 0.4 ug/ml	10/10
Alhydrogel	10/10
Experiment 2:	
EP-LPS, 40 ug/ml	9/9
EP-LPS, 4.0 ug/ml	10/10
Alhydrogel	9/10
Experiment 3:	
EP-LPS, 160 ug/ml	4/10
EP-LPS, 80 ug/ml	1/10
EP-LPS, 40 ug/ml	6/10
Alhydrogel	6/10

[a] EP-LPS was suspended in alum at a concentration of 1.0 mg/ml and the designated dose in 0.5 ml of endotoxin-free water was injected i.p. into C3H/HeN mice 7 days prior to challenge with MAdipt4 (3.2 LD_{50}, Exp. 1 and 2; 3.1 LD_{50} Exp. 3)

[b] Deaths were recorded daily. All mice died within 3 to 11 days after infection.

[c] Reported previously (4).

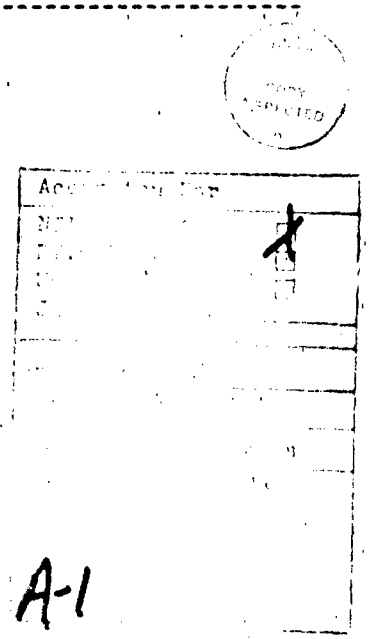


Table 2.1.2 Reconstitution of EP-LPS

Treatment[a]	Mortality[b] (deaths/total)
16 ug EP + 16 ug LPS + Alhydrogel	10/10
8 ug EP + 8 ug LPS + Alhydrogel	9/10
4 ug EP + 4 ug LPS + Alhydrogel	10/10
2 ug EP + 2 ug LPS + Alhydrogel	10/10

[a] EP (endotoxin-associated protein and LPS were each suspended in alum at a concentration of 1.0 mg/ml, incubated at 25°C for 30 min, diluted in endotoxin-free water, and the designated dose in 0.5 ml was injected i.p. into C3H/HeN mice 7 days prior to challenge with MAdipt4 (3.2 LD_{50}, Exp. 1 and 2; 3.1 LD_{50}, Exp. 3)

[b] Deaths were recorded daily. All mice died within 3 to 11 days after infection.

Table 2.1.3. Potentiation of EP-LPS Antiviral Activity by Block Copolymers L121 and T150R1

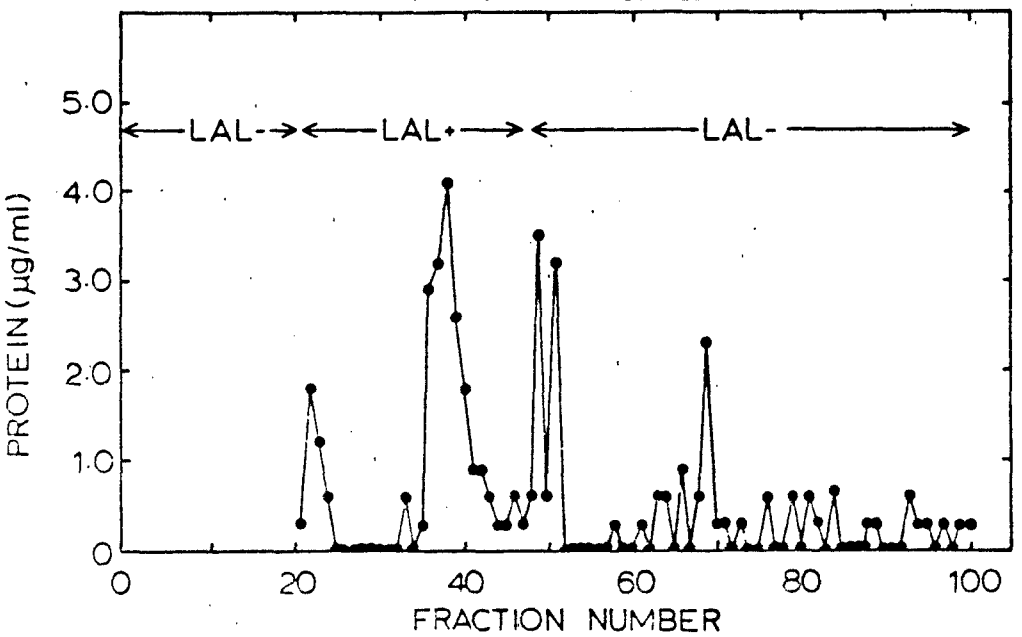
Treatment[a]	Mortality[b] (Deaths/Total)
None	9/10
10 ug EP-LPS	10/10
10 ug EP-LPS + 5 ul Drakeol + 1.25 mg L121 + 1.25 mg T150R1	0/10
5 ul Drakeol + 1.25 mg L121 + 1.25 mg T150R1	0/10

[a] EP-LPS alone was resuspended in endotoxin-free PBS containing 0.2% Tween 80. EP-LPS was combined with drakeol, L121, and T150R1. The materials were mixed thoroughly using a Kontes disposable micropestle and mortar. A uniform emulsion was formed by adding endotoxin-free PBS containing 0.2% Tween 80 and mixing in the micromortar. The emulsion (50 ul) was injected immediately i.p. into C3H/HeN mice 7 days prior to challenge with MAdipt4 (5.7 LD_{50}). An emulsion of the block polymers alone was formed in the same manner.

[b] Deaths were recorded daily. All mice died within 3 to 12 days after infection.

2.2 ADDITIONAL BIOCHEMICAL CHARACTERIZATION OF EP-LPS. An attempt was made to separate EP-LPS into a protein and lipopolysaccharide fractions under nondenaturing conditions using gel filtration. Ultrogel AcA 34 separates proteins in the range of 20,000 - 300,000 molecular weight. Under nondenaturing conditions major peaks of protein were associated with LPS (Figure 1). These data support our previous findings using two dimensional gel electrophoresis and Western blot (4). Pools of fractions 21-34, 35-4, 48-100 were lyophilized. Recovery of material from the column was poor and was insufficient to test in the MAdipt4 infection model. We will proceed to separate all detectable protein from LPS in the EP-LPS complex by isoelectric focusing. Dr. David Morrison, University of Kansas Medical Center, Kansas City, KS has agreed to separate the EP and LPS in preparative quantities on his newly developed isoelectric focusing apparatus. The separated fractions will be stored in a lyophilized state until immediately before testing. Testing of the highly purified fractions will answer definitively whether all of the antiviral activity resides in the LPS moiety of EP-LPS. In addition, Dr. B. Sultzer, Downstate Medical Center SUNY, has developed a technique for separating two species of LPS derived from B. pertussis and has reported differing immunomodulatory activities for each species (personal communication). We have acetone-dried approximately 200 g of B. pertussis cells and Dr. Sultzer is currently extracting the two species of LPS. We will test the antiviral activity of each LPS species when it becomes available.

Figure 1. Protein and Endotoxin Profile of EP-LPS Chromatographed on an Ultrogel AcA 34 Column.



EP-LPS (1.0 mg) was applied to an Ultrogel AcA 34 column (1.0 cm x 50 cm) in endotoxin-free, azide-free PBS, pH 7.2. Fractions (approximately 10 ml) were collected and assayed for the presence of LPS [Limulus amoebocyte assay, (7)] and protein [fluorescamine assay, (8)].

2.3 DEVELOPMENT OF RESISTANCE TO VIRUS INFECTION AFTER EP-LPS TREATMENT. The protective effect of BPV appears five days after treatment and susceptibility returned to a portion of the test population after 35 days (10). The protective effect of EP-LPS appeared two to three days after treatment (Table 2.3.1). EP-LPS may not require the extent of processing that whole cell BPV would require to release the active antiviral substance and consequently the extracted material can immunomodulate the animal in a shorter time period. In addition, the EP-LPS induced antiviral state may decay more rapidly than BPV induced resistance.

Table 2.3.1. Kinetics of Resistance to Virus Challenge After EP-LPS Treatment.

Treatment[a]	Mortality[b] (deaths/total)
Experiment 1:	
Day 0	5/5
Day -3	1/10
Day -5	4/10
Day -7	2/5
Day -10	1/5
Endotoxin-free PBS	5/5
Experiment 2:	
Day 0	5/5
Day -1	8/9
Day -2	4/10

[a] EP-LPS was suspended in endotoxin-free PBS at a concentration of 1.0 mg/ml and 50 ug in 0.5 ml was injected i.p. into C3H/HeN mice on the day indicated prior to challenge with MAdIpt4 (5.7 LD_{50}).

[b] Deaths were recorded daily. All mice died within 3 to 11 days after infection.

2.4 COMPARISON OF THE ANTIVIRAL ACTIVITY OF Bordetella pertussis AND Escherichia coli. Previous studies comparing LPS derived from E. coli and B. pertussis demonstrated that B. pertussis derived LPS was unique in its ability to induce an antiviral state (3,11). Boivin antigen prepared from E. coli (EP-LPS/EC) did not induce resistance to a mouse adenovirus challenge; however, a whole cell vaccine induced resistance (Table 2.4.1). These data suggest that components of the whole bacterial cell other than the LPS moiety can induce resistance to a mouse adenovirus challenge.

2.5 BPV TREATMENT AND PERITONEAL CIRCULATION. One possible mechanism of resistance to virus challenge following BPV treatment is the blockage of peritoneal-blood circulation by an inflammatory response. Our previous studies indicated that MAdIpt4 appeared in the circulating blood 5.0 min after i.p. injection (3). BPV-treatment did not impair the circulation of ^{125}I-labeled proteins from the peritoneum to the circulating blood (Table 2.5.1). The percentage of label appearing in the circulating blood after i.p. inoculation correlated well with the percentage of virus appearing in the circulating blood after i.p. inoculation as observed previously.

Table 2.4.1. Antiviral Activity of *E. coli* and *B. pertussis* Bovin Antigens

Treatment[a]	Mortality[b] (deaths/total)
Experiment 1:	
E. coli 201 boivin antigen, 50 ug	5/5
B. pertussis boivin antigen, 50 ug	3/5
Endotoxin-free water	5/5
Experiment 2:	
E. coli 201 vaccine, 250 ug	0/10
E. coli 410 vaccine, 250 ug	0/10
Saline-merthiolate diluent	7/10

[a] The designated dose in 0.5 ml of endotoxin-free water (Experiment 1) or saline-merthiolate diluent (Experiment 2) was injected i.p. into C3H/HeN mice 7 days prior to challenge with HAdipt4 (Exp. 1, 7.4 LD_{50}; Exp. 2, 4.2 LD_{50})

[b] Deaths were recorded daily. All mice died within 3 to 11 days after infection.

Table 2.5.1. Migration of Mouse Serum Proteins from the Peritoneum to the Circulating Blood After BPV Treatment.

Treatment[a]	Mouse	Radioactivity in the Circulating Blood[b] 30 min	90 min	300 min
None	A	31,665	134,514	123,621
	B	5,470	41,619	74,493
	C	26,535	101,933	97,736
	average	21,223	92,687	98,617
BPV	D	31,035	57,244	28,518
	E	21,699	42,136	62,224
	F	20,915	56,767	81,921
	average	24,550	52,049	57,554
SM	G	12,840	28,518	46,601
	H	27,139	46,875	72,822
	I	9,596	28,965	81,180
	average	16,525	34,786	66,868

[a] Dialyzed normal mouse serum proteins were labeled with ^{125}I using chloramine-T. Unincorporated label was removed by passage of the label proteins over a G-25 Sephadex column. Mice were inoculated i.p. with 1.2×10^7 cpm in 0.5 ml.

[b] Samples (50 ul) were removed by retroorbital bleeding at the times designated and the radioactivity was determined in a gamma counter.

2.6 IMMUNOMODULATION OF CELLULAR ACTIVITIES FOLLOWING TREATMENT WITH EP-LPS. The white blood cells in both the peritoneum and circulating blood were examined after treatment with BPV and EP-LPS to obtain an initial characterization of the cellular changes that occur seven days after immunomodulation, i.e., the state of white blood cells at the time of virus challenge. EP-LPS treatment induced a decreased level of leukocytosis and elicited fewer white blood cells in the peritoneum, as compared to BPV (Table 2.6.1). Differential counts of the the blood indicated that BPV and EP-LPS induced increased numbers of different cell populations. The notable changes in absolute counts induced by BPV in the peripheral blood was an increase in the numbers of neutrophils, transformed lymphocytes, and monocytes, whereas the notable changes induce by EP-LPS was an increase in the number of lymphocytes (Table 2.6.2). A similar picture of cell population changes was obtained in the peritoneum (Table 2.6.3). In addition, EP-LPS treatment appeared to deplete the monocyte/macrophage population in the peritoneum. Thus, the difference between BPV and EP-LPS immunomodulation was both quantitative and qualitative.

Activities of those cells classified as lymphocytes by the Wright-Giemsa stain were examined. The spleen was used as the source of cells. EP-LPS treatment did not induce a notable increase in the number of antibody-forming cells (IgM) in the spleen seven days after treatment (Table 2.6.4). EP-LPS treatment did not induce a notable increase in the number of cytolytic T-cells in the spleen seven days after treatment (Table 2.6.5). EP-LPS treatment induced a slight increase of natural killer cell activity in the spleen seven days after treatment, whereas LPS derived from E. coli decreased natural killer cell activity in the spleen (Table 2.6.6) and does not induce antiviral activity (11). Additional assay of natural killer cell activity in the spleen confirmed only marginal increases following seven days of EP-LPS treatment (Table 2.6.7). Natural killer cell activity appearred to be induced earlier than seven days had decayed to nearly normal levels by this time (Table 2.6.8). Assay of natural killer activity present in the peritoneal cavity four days after treatment with EP-LPS did not reveal increased activity (data not shown). Bukovski and coworkers reported that treatment of mice with anti-asialo GM1 rabbit serum decreased the LD_{50} of mouse cytomegalovirus (12). Using the same conditions, BPV induced resistance was not abrogated by anti-asialo GM1 serum treatment (results not shown).

Activities and regulation of those cells classified as macrophages by the Wright-Giemsa stain were examined. The system chosen for study was the activiation of bone marrow derived macrophages. Bone marrow derived macrophages can be activated by treatment with E. coli derived LPS (LPS/EC) and gamma interferon to become cytolytic. Dr. Paul A. LeBlanc has demonstrated that activated bone marrow derived macrophages can effect cytolysis of virus (vesicular stomatitis virus) infected cells. LPS/EC induces bone marrow derived macrophages to synthesize interferon also. The ability of EP-LPS to induce interferon production by bone-marrow derived macrophages was examined. Bone-marrow derived macrophages responded to EP-LPS treatment in a manner similar to LPS/EC (Table 2.6.9). However, the bone-marrow derived macrophages did not respond to similar concentrations of EP-LPS/EC. As mentioned previously, EP-LPS/EC did not induce resistance to a MAdipt4 challenge (Table 2.3.1); however, the preparation retained full activity in the Limulus amoebocyte assay. EP-LPS was not as effective as LPS/EC in activating the cytolytic activity of macrophages via the gamma interferon pathway (Table 2.6.10) and EP-LPS did not activate the cytolytic activity of macrophages directly. Incubation of EP-LPS with macrophages resulted in culture supernatants that activated the cytolytic activity of macrophages by a pathway different from LPS/EC activation (Table 2.6.11). Subsequent experiments suggest that the EP-LPS is activating the cytolytic activity of macrophages directly, i.e., the pre-incubation with macrophages is not necessary.

Table 2.6.1 Leukocytosis after Treatment with BPV and EP-LPS.

White Blood Cell Count[a]	Treatment[b] Diluent	BPV	EP-LPS
Blood (x 10^4 cells/ml)	9.7 ± 1.3	27.5 ± 3.8	17.2 ± 1.6
Peritoneal Lavage (cells/ml)	2,427 ± 104	2,971 ± 238	2,703 ± 472

[a] The total number of white blood cells were enumerated using a Coulter Counter, Model ZBi.

[b] BPV (250 ug dry weight) was suspended saline-thimerosal diluent. EP-LPS (20 ug) was suspended in endotoxin-free water. Treatment was initiated by i.p. injection of the designated amount of material in 0.5 ml and the appropriate samples were obtained seven days after treatment.

TABLE 2.6.2. White Blood Cells in Peripheral Blood after Treatment with BPV and EP-LPS.

Cell Type[a]	Treatment[b] Diluent (cells/ul)	BPV (cells/ul)	EP-LPS (cells/ul)
Band Neutrophil	214 ± 71	754 ± 289	356 ± 254
Segmented Neutrophil	3,608 ± 972	16,598 ± 2,494	4,482 ± 1,588
Eosinophil	290 ± 25	443 ± 248	466 ± 506
Lymphocyte	4,846 ± 129	6,502 ± 581	10,494 ± 1,668
Transformed Lymphocyte	159 ± 64	1,133 ± 595	436 ± 104
Monocyte	660 ± 210	2,827 ± 336	1,244 ± 173

[a] Cells were stained with Wright-Giemsa stain and differential counts were made using the criteria of cell identification as described by Diggs, Wintrobe, and coworkers (13, 14).

[b] BPV (250 ug dry weight) was suspended saline-thimerosal diluent. EP-LPS (20 ug) was suspended in endotoxin-free water. Treatment was initiated by i.p. injection of the designated amount of material in 0.5 ml and the appropriate samples were obtained seven days after treatment.

Table 2.6.3. White Blood Cells in Peritoneal Lavage after Treatment with BPV and EP-LPS.

Cell Type[a]	Treatment[b] Diluent (cells/ul)	BPV (cells/ul)	EP-LPS (cells/ul)
Band Neutrophil	53 ± 41	26 ± 26	13
Segmented Neutrophil	55 ± 56	822 ± 130	104 ± 36
Eosinophil	23(± 108	0	213 ± 298
Lymphocyte	685 ± 151	707 ± 66	1,837 ± 178
Transformed Lymphocyte	49 ± 22	66 ± 15	33 ± 6
Monocyte	1296 ± 231	1,283 ± 209	494 ± 69
Macrophage	53 ± 2	65 ± 6	20 ± 8

[a] Cells were stained with Wright-Giemsa stain and differential counts were made using the criteria of cell identification as described by Diggs, Wintrobe, and coworkers (13, 14).

[b] BPV (250 ug dry weight) was suspended saline-thimerosal diluent. EP-LPS (20 ug) was suspended in endotoxin-free water. Treatment was initiated by i.p. injection of the designated amount of material in 0.5 ml and the appropriate samples were obtained seven days after treatment.

Table 2.6.4. Immunomodulation of B-cell Activity by EP-LPS

Treatment[a]	Plaques/10^6 cells[b] Day 3 Mean	Day 3 Spleens	Day 4 Mean	Day 4 Spleens	Plaques/spleen[c] Day 3 Mean	Day 4 Mean
Experiment 1:						
PBS	133	2	714	3	10,700	88,000
EP-LPS	48	2	350	3	7,600	109,600
Experiment 2:						
PBS			317	4		62,300
EP-LPS			53	4		16,000

[a] EP-LPS (100 ug) was suspended in endotoxin-free PBS. Treatment was initiated by i.p. injection of the designated amount of material in 0.5 ml. After seven days the mice were injected i.p. with 0.2 ml of a 10% (v/v) sheep red blood cell suspension.

[b] Mice were sacrificed at the designated day after sheep red blood cell injection, spleens were removed, and a direct Jerne plaque assay was performed with cell suspensions derived from individual spleens using the slide modification of Cunningham. The number of plaques were determined as the mean number of plaques per 10^6 cells from four slides; two slides plated at 10^6 cells per slide and two slides plated at 5×10^5 cells per slide.

[c] Total number of nucleated cells per spleen was determined by hemacytometer counts of the spleen cell suspension.

Table 2.6.5 Immunomodulation of T-Cell Cytolytic Activity by EP-LPS

Treatment[a]	Percent Specific Release[b] Effector:Target (5:1)	(80:1)	Cytotoxic Units[c]/Spleen (x 10^6)
PBS	29.8 ± 6.9	86.0 ± 14.6	6.48 ± 4.5
EP-LPS	26.6 ± 12.5	81.8 ± 18.9	6.66 ± 2.3

[a] EP-LPS (100 ug) was suspended in endotoxin-free PBS. Treatment was initiated in groups of three mice by i.p. injection of the designated amount of material in 0.5 ml. After seven days all groups of mice were injected i.p. with 10^7 P815 mastocytoma cells.

[b] Mice were sacrificed 12 days after immunization with P815 cells, spleens were removed, and the cells were recovered from individual spleens. Cell number was determined by hemacytometer counts. ^{51}Cr-labelled P815 target cells were incubated for 16 h with varying concentrations of spleen cells to yield the designated effector to target ratios. Specific release was calculated with the following formula using the mean number of counts in the supernatants of triplicate coculture:

$$\% \text{ specific release} = \frac{\text{experimental - spontaneous release}}{\text{freeze/thaw - spontaneous release}} \times 100$$

[c] One cytotoxic unit represents the number of effector cells per target to yield 50% specific release.

Table 2.6.6. Comparison of the Modulation of NK Activity by EP-LPS and phenol-extracted E. coli LPS.

TreatmentA	Percent Specific Release[b] (Effector:Target) (12.5:1)	(100:1)	Cytotoxic Units[c]/Spleen (x 10^6)
Medium	2.1	10.5	8.9
Medium	3.5	13.5	10.7
EP-LPS, 50 ug	4.6	15.3	18.1
EP-LPS, 50 ug	10.6	17.4	40.5
LPS (E. coli), 50 ug	1.5	8.3	7.3
LPS (E. coli), 50 ug	1.6	7.3	7.7

[a] EP-LPS and LPS (water-phenol extracted from E. coli O111:B4) was suspended in endotoxin-free medium. Treatment was initiated in groups of two mice by i.p. injection of the designated amount of material in 0.5 ml.

[b] Mice were sacrificed seven days after treatment, spleens were removed, and the cells were recovered from individual spleens. Cell number was determined by hemacytometer counts. ^{51}Cr-labelled YAC-1 target cells were incubated for 16 h with varying concentrations of spleen cells to yield the designated effector to target ratios. Specific release was calculated as described in Table 2.6.5.

[c] One cytotoxic unit represents the number of effector cells per target to yield 50% specific release.

Table 2.6.7. Modulation of Natural Killer Cell Activity by EP-LPS

Treatment[A] Treatment[a]	Percent Specific Release[b] (Effector:Target) (50:1)	(200:1)	Cytotoxic Units[c]/Spleen (x 10^6)
Medium	31.7 ± 8.6	49.1 ± 9.0	7.9 ± 4.4
EP-LPS, 100 ug	38.1 ± 6.9	61.3 ± 5.5	10.5 ± 3.6

[a] EP-LPS was suspended in endotoxin-free medium. Treatment was initiated in groups of four mice by i.p. injection of the designated amount of material in 0.5 ml.

[b] Mice were sacrificed seven days after treatment, spleens were removed, and the cells were recovered from individual spleens. Cell number was determined by hemacytometer counts. ^{51}Cr-labelled YAC-1 target cells were incubated for 16 h with varying concentrations of spleen cells to yield the designated effector to target ratios. Specific release was calculated as described in Table 2.6.5.

[c] One cytotoxic unit represents the number of effector cells per target to yield 50% specific release.

Table 2.6.8. Development of NK Activity after Treatment with EP-LPS

Treatment[a]	Percent Specific Release[b] (Effector:Target) (12.5:1)	(100:1)
0 (no treatment)	0.6	14.0
2 days	16.0	42.0
3 days	11.0	37.5
6 days	6.0	14.0

[a] Two mice per group were inoculated intraperitoneally with 0.5 ml of endotoxin-free PBS containing 50 ug of EP-LPS and sacrificed at the days indicated. Spleen cells from each pair of mice were pooled for the assay. All mice were assayed at the same time.

[b] ^{51}Cr-labeled YAC-1 target cells were incubated 16 h with varying concentrations of spleen cells to yield different effector to target cell ratios. E:T ratios of 12.5:1 and 100:1 are shown here. Specific release was calculated as described in Table 2.6.5.

Taken together the experiments suggest that the immunomodulation relevant to the antiviral resistance observed following B. pertussis induced resistance may be difficult to identify. One has to consider that the immunomodulated activities measured in vitro may be marginal as assessed, but the same immunomodulation of activity expressed in the microenvironment of the immune system may reflect a substantial increase in activity. In addition, the antiviral state might result from marginal immunomodulaton of several activities that act in concert.

2.7. BPV INDUCED ANTIVIRAL ACTIVITY AGAINST VIRUSES OTHER THAN MOUSE ADENOVIRUS. BPV was tested for antiviral activity in virus infection models other than mouse adenovirus. BPV treatment did not prevent death after Venezuelan encephalitis virus of mice (Table 2.7.1). BPV treatment did not prevent death after Pichinde virus challenge of quinea pigs, but the treatment may have extended life (Table 2.7.2).

2.8. MICROBIOLOGICAL STATUS OF THE C3H/HeN MOUSE COLONY. Our C3H/HeN mouse colony was derived from two pairs of breeders obtained from the National Cancer Institute, January 24, 1981. The mouse colony was tested serologically for the presence of mycoplasma and viruses, January-June, 1986 . Dr. Gail Cassel, University of Alabama in Birmingham, testd the individual antisera of 53 retired breeders from our C3H/HeN colony by an ELISA test. All of the mice were negative for antibodies in the IgG class. Seven of the mice were positive, 41 were weakly reactive, and 5 were negative for antibodies in the IgM class. These data indicate the colony is infected with mycoplasma; however, the ELISA test devised by Dr. Cassell does not distinguish between M. pulmonis or M. arthritidis. The role of this "hidden" infection of the colony in immunomodulation studies is not clear at this time.

Microbiological Associates, Inc., Bethesda, MD, tested pools of sera obtained from retired breeders (five animals per pool). The colony was negative for Reovirus 3, K virus, Polyoma virus, murine cytomegalovirus, lymphocytic choriomeningitis virus, Sendai virus, ectromelia virus, mouse encephalomyelitis virus, and most significantly, mouse adenovirus. The absence of mouse adenovirus antibodies in the colony coupled with the presence of high titer MAdipt4 in the virology laboratory indicates our containment procedures are effective. However, all of the serum pools tested positive for mouse hepatitis virus. The mice did not have evidence of mouse hepatitis virus infection at the time of shipment from the National Cancer Institue Contractor. Mouse hepatitis virus infection in a colony is known to be associated with abnormal immune reactions. This infection of the colony may be associated with the development of erratic results obtained with the Alhydrogel protocol. However, Dr. P. Morahan has noted the partial protective activity of Alhydrogel in mice that were raised in isolators. We immediately initiated a new breeding colony of C3H/HeN mice and are reviewing possible sources of infection from within the animal facility.

2.9 MOUSE ADENOVIRUS CONCENTRATION. A constant problem in our antiviral studies has been the growth of high titer stocks of MAdipt4 for the challenging infection. These stocks should be 4.0×10^7 PFU/ml or higher to obtain death in 100% of the test population. We attempted to concentrate low titer stocks of MAdipt4 with tangential flow ultrafiltration. Our initial experiment demonstrated that tangential flow ultrafiltration does not inactivate the virus and can be used to concentrate the virus for lethal dose virus stocks with excellent levels of recovery (Table 2.9.1). The 0.1 µM pore size was too large to retain all of the virus, but filters of smaller pore size are available.

Table 2.6.9. Interferon Production by Macrophages Treated with EP-LPS

Concentration[a] (ng/ml)	Interferon Titer[b] of Supernatants from Macrophages Treated with: EP-LPS	LPS/EC	EP-LPS/EC
Experiment 1:			
100	>8	4	
10	4	4	
1.0	4	<2	
0.1	<2	<2	
Experiment 2:			
100	>8	>8	<2
10	>8	>8	<2
1.0	<2	<2	<2
0.1	<2	<2	<2

[a] Macrophages were derived from 10 day cultures of murine bone marrow cells grown in the presence of colony stimulating factor-1 (L929 cell-conditioned medium). The cultures were incubated in the designated concentrations of immunomodulators for 2.0 h, washed, and incubated an additional 24 h. Culture supernatants were removed and assayed for interferon activity.

[b] The titer is endpoint is defined as that amount of interferon sufficient to inhibit plaque production by a standard dose of vesicular stomatitis virus by 50 %.

Table 2.6.10. Activation of Cytolytic Activity in Macrophages by EP-LPS

Treatment[a] (ng/ml)	Percent Specific Release[b] [-IFN]	[+IFN][c]
EP-LPS		
2.0	-4.4	13.7
1.0	0.9	-5.7
0.5	3.1	-8.7
0.25	5.2	-6.8
0.125	2.0	-4.4
0.06	-1.0	-3.9
LPS/EC		
2.0	5.5	67.7
1.0	1.6	64.3
0.5	2.5	32.6
0.25	2.2	-0.3
0.125	5.0	-4.1
0.06	8.1	-4.0

[a] Macrophages were derived from 10 day cultures of murine bone marrow cells were grown in the presence of colony stimulating factor-1 (L929 cell-conditioned medium). The cultures were incubated for 4.0 h with the designated concentration of immunomodulators to obtain partial activation and then incubated in the presence or absence of gamma interferon to obtain full activation as effector cells.

[b] ^{51}Cr-labeled P815, mastocytoma cells were incubated 16 h with macrophages at a single effector to target cell ratio of 2.5:1. Specific release was calculated as described in Table 2.6.5.

[c] Gamma interferon at a final concentration of 0.4 IRU/ml was supplied by a supernatant from a 72 h culture of Con A stimulated spleen cells.

Table 2.6.11. Activation of Cytolytic Activity in Macrophages by Supernatants from EP-LPS Treated Macrophage Cultures.

Treatment of Macrophage Culture[a]	Dilution of Conditioned Supernatant[b]	Percent Specific Release[c] Experiment 1	Experiment 2
EP-LPS, 100 ng/ml	1:2	70 ± 6	24 ± 4
	1:4	63 ± 7	18 ± 1
	1:8	61 ± 4	11 ± 3
	1:16	49 ± 1	6 ± 2
EP-LPS, 10 ng/ml	1:2	49 ± 1	
	1:4	31 ± 6	
	1:8	31 ± 4	
	1:16	25 ± 6	
LPS/EC, 100 ng/ml	1:2	19 ± 2	4 ± 2
	1:4	5 ± 2	3 ± 1
	1:8	4 ± 3	4 ± 1
	1:16	3 ± 2	1 ± 1
LPS/EC, 10 ng/ml	1:2	8 ± 1	
	1:4	2 ± 1	
	1:8	5 ± 2	
	1:16	0 ± 1	
EP-LPS/EC, 100 ng/ml	1:2	15 ± 12	3 ± 2
	1:4	8 ± 6	2 ± 1
	1:8	8 ± 7	3 ± 2
	1:16	8 ± 1	0 ± 1
EP-LPS/EC, 10 ng/ml	1:2	16 ± 5	
	1:4	7 ± 1	
	1:8	11 ± 2	
	1:16	3 ± 2	
None	1:2	14 ± 18	2 ± 1
	1:4	7 ± 12	2 ± 1
	1:8	11 ± 15	2 ± 0
	1:16	9 ± 15	1 ± 1

[a] Macrophages were derived from 10 day cultures of murine bone marrow cells were grown in the presence of colony stimulating factor-1 (L929 cell-conditioned medium). The cultures were incubated for 2.0 h with medium containing the designated concentration of immunomodulators.

[b] The supernatants from each culture were harvested separately and designated dilutions tested for their ability to activate cytolytic activity in new macrophage cultures. The medium in the new macrophage cultures contained 10^{-6} M indomethacin to prevent down regulation of the macrophages by endogenous production of prostaglandins and LPS/EC (100 ng/ml) to partially activate the macrophages. Cultures were then incubated for 24 h and washed.

[c] ^{51}Cr-labelled P815 mastocytoma cells were added at a single effector to target cell ratio of 2.5:1. After an additional 16 h of incubation the release of ^{51}Cr was assayed and specific release was calculated as described in Table 2.6.5.

Table 2.7.1 Effect of BPV Treatment on Resistance to Venezuelan Virus Infection in Mice.

Treatment[a]	Deaths (day after infection) 5	6	7	8	9	10	11	12	Deaths/Total
Experiment 1[a]:									
None		1	2	2	5				10/10
BPV, 250 ug		2	2	3	2	1			10/10
Experiment 2[b]									
None		1		2	1	5	1.		10/10
EP-LPS, 50 ug		1		1		3	4	1	10/10

[a] BPV was suspended in saline-merthiolate at a concentration of 500 ug/ml and the designated dose in 0.5 ml was injected i.p. into C3H/HeN mice seven days prior to challenge with Venezuelan equine encephalitis virus, strain 68U201 (400 PFU in 0.2 ml), by subcutaneous injection.

[b] EP-LPS was suspended in endotoxin-free water at a concentration of 1.0 mg/ml and the designated dose in 0.5 ml was injected i.p. into C3H/HeN mice seven days prior to challenge with Venezuelan equine encephalitis virus, strain 68U201 (400 PFU in 0.2 ml), by subcutaneous injection.

Table 2.7.2. Effects of BPV Treatment on Resistance to Pichinde Virus Infection in Guinea Pigs

Treatment [a]	Deaths (day after infection) 13	14	15	16	17	18	19	Deaths/Total
Diluent	2	3	1					6/6
BPV, 4.0 mg (10 mg/kg)			2	3			1	6/6

[a] BPV suspended in saline-merthiolate was used at a concentration of 2.0 mg/ml. The designated dose in 2.0 ml was injected i.p. into guinea pigs (400 g) seven days prior to challenge with Pichinde virus (40 PFU in 0.2 ml) by subcutaneous injection.

Table 2.9.1 Concentration of HAdipt4 by Tangential Flow Ultrafiltration

Sample	Titer (PFU/ml)	% Recover	Fold Concentration	Deaths/Total
Stock	1.9×10^4	--	---	5/5
Retentate	6.0×10^4	65	3.15	5/5
Filtrate	4.4×10^3	35	0.18	0/5

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[1] Department of Microbiology, University of Alabama, University, Alabama 35486, U.S.A.

[2] Analytical Biochemistry, Connaught Laboratories, Inc., Swiftwater, Pennsylvania 18370, U.S.A.

IMMUNOMODULATION BY *BORDETELLA PERTUSSIS*: ANTIVIRAL EFFECTS

A.L. Winters[1], *D.W. Baggett*[1], *J.D. Lee*[1], *G.L. Sloan*[1], *R.D. Lemmon*[2] *and R.S. Stinson*[1]

ABSTRACT

Treatment of mice by intraperitoneal inoculation of pertussis vaccine or lipopolysaccharide extracted from *B. pertussis* will effect resistance to rabies virus, encephalomyocarditis virus, Semliki Forest virus, and Herpes simplex virus. Our previous observations indicated that treatment of C3H/HeN (+/*nu*) and BDF_1 mice with pertussis vaccine injected i.p. five days prior to a mouse adenovirus lethal dose i.p. challenge elicited resistance to clinical disease and death. Susceptibility returned to a portion of the test population 35 days after pertussis vaccine treatment. The pertussis vaccine induced resistance developed in athymic (nude) mice also; however, the population succumbed to infection 35 days later. Titration of pertussis vaccine with respect to induction of resistance indicated the median effective dose (ED_{50}) was approximately 25 µg dry weight.

This report describes the antiviral activity of acellular components extracted from pertussis vaccine. Extraction of *B. pertussis* cells with 1.0M NaCl and ammonium sulfate fractionation (20-40% saturation) of the extract resulted in an acellular preparation that induced resistance to lethal dose mouse adenovirus infection. The resistance inducing activity was retained after treatment of the extract with detergent (GAF Emulphogene BC 720) to remove lipopolysaccharide and adsorption to alum gel. Comparison of endotoxin content of pertussis vaccine acellular fractions, polysaccharide fraction and purified lipopolysaccharide suggested that endotoxin probably plays a role in the induction of resistance. The endotoxin content of a Emulphogene-treated preparation that protected 80% of a test population was 39 ng. The lipopolysaccharide extracted from *Escherichia coli, Vibrio cholerae, Salmonella typhimurium,* and *Salmonella minnesota* did not induce a resistant state seven days after administration; however, lipopolysaccharide extracted from *B. pertussis* induced a resistant state. Treatment of pertussis vaccine with periodate (0.1 M $NaIO_4$ at 25°C for 48 h) destroyed the resistance inducing activity. Our data suggest that either *B. pertussis* endotoxin is unique from other bacterial endotoxins and is an extremely long-acting immunomodulator or the *B. pertussis* lipopolysaccharide preparation (modified Westphal phenol-water extraction from T.W. Klein, University of South Florida, Tampa, FL) was contaminated with an unidentified periodate-sensitive molecule with immunomodulating activity.

INTRODUCTION

Numerous immunomodulatory activities have been observed following the administration of pertussis vaccine (PV) (1-4). Several of these immunomodulatory

activities have been associated with specific components of the microorganism (5-11).

Immunomodulation by PV can modify the pathogenesis of viral infections. Treatment of mice by intraperitoneal (i.p.) injection of PV increased susceptibility to intranasal influenza virus challenge five to seven days later (12). Administration of PV by the intracranial, intravenous, or i.p. route increased resistance to an intracranial challenge of rabies virus when the vaccine was administered simultaneously with the virus (3). Increased resistance to Herpes simplex virus inoculated by the i.p. route developed seven days after PV treatment by the i.p. route, but resistance was not observed when the mice were treated with PV for only three days (13). Increased resistance to mouse adenovirus inoculated by the i.p. route developed seven days after PV treatment by the i.p. route (1), but not before five days. The resistance developed in nude mice and was retained approximately 35 days.

Acellular fractions of *B. pertussis*, namely lipopolysaccharide (LPS), glycolipid, lipid A, or lipid X, induce resistance to an i.p. challenge of encephalomyocarditis virus or a subcutaneous challenge to Semliki Forest virus when the acellular fraction of *B. pertussis* was administered 24 h before the virus challenge (5, 14). An acellular fraction developed from a high salt wash of *B. pertussis* cells induced resistance to mouse adenovirus challenge seven days later. A preliminary characterization of the factor in PV that induces resistance to mouse adenovirus infection is described in this report.

MATERIALS AND METHODS

Animals

A colony of C3H/HeN (mammary tumor virus negative) mice was maintained at the University of Alabama. C3H/HeJ mice were obtained from Jackson Laboratory, Bar Harbor, Me.. Male and female mice were used in this study. All animals were treated by i.p. injection of the test material and after seven days challenged with a lethal dose of virus inoculated i.p..

Vaccines

PV was provided by Connaught Laboratories, Inc., Swiftwater, Pa.. Vaccines were made also from Strain 18323 and Tohama III (Dr. James L. Cowell, Center for Drugs and Biologics, Bethesda, Md.), Tohama I, Tohama I-derived BP347, and Tohama I-derived BP359 (Dr. Stanley Falkow, Stanford University, Stanford, Ca.), and Strain 11615 (American Type Culture Collection, Rockville, Md.). The organisms were maintained on BG agar base (Difco Laboratories, Detroit, Mich.) supplemented with 17% defibrinated sheep blood. Vaccines were made by harvesting four-day growth from Cohen-Wheeler agar (15) in phosphate buffered saline (pH 7.2) and inactivating the cells by heating (56°C for 30 min) in the presence of 0.02% thimerosal. The vaccines were adjusted to approximately 4.0 mg dry weight per ml in saline-thimerosal diluent (0.15M NaCl in 0.02% thimerosal) and stored at 4°C.

Acellular fractions

Fraction 15A-1B. Four day growth of phase I *B. pertussis* (Connaught Laboratories, Inc. vaccine strain) was harvested from Cohen-Wheeler agar medium and the cells were extracted with 1.0 M sodium chloride containing 0.05 M sodium phosphate buffer (pH 7.2) and 0.02% thimerosal for four days at 4°C. The cells were sedimented by centrifugation and the supernatant was decanted. The fractional precipitate (20-40% ammonium sulfate saturation) was collected by centrifugation, resuspended in buffered saline, dialyzed and designated 15A-1B. Protein content was determined by the method of Lowry and coworkers (16).

Fraction 15A-108A. Emulphogene BC 720 (General Aniline & Film Corp., New York, N.Y.) was added to a portion of fraction 15A-1B to give a final concentration of 1.0% (v/v). The mixture was incubated for 60 min at 4°C and the precipitate was sedimented at 100,000 × g for 60 min. The pellet was resuspended in phosphate buffered saline and the suspension was adsorbed to a 1.0% aluminum hydroxide gel. This alum-stabilized preparation was designated as fraction 15A-108A.

LPS. Purified *B. pertussis* LPS was provided by Dr. Thomas W. Klein, University of South Florida, Tampa, Fl. The LPS was extracted from *B. pertussis*, 3779 BL_2S_1 by a modified Westphal phenol-water procedure (17, 18). Lipopolysaccharides extracted by the Westphal procedure from microorganisms other than *B. pertussis* and the Limulus amoebocyte lysate test for endotoxin were obtained from Sigma Chemical Company, Inc., St. Louis, Mo.. The efficacy of endotoxin detection was assumed to be similar for free- and bound-LPS (19).

Polysaccharide. Cell surface polysaccharide was harvested and partially purified as described by another worker (20). Four day growth of phase I *B. pertussis* 18323 was harvested from Cohen-Wheeler agar medium using 0.01 M potassium phosphate buffer (pH 7.0) and the cells sedimented by centrifugation. The cells were resuspended in buffer and the capsule (slime) polysaccharide was removed by brief shearing treatment in a Waring blender set at top speed for 45 sec. The cells were removed by centrifugation and the supernatant was combined with the previous wash buffer. After precipitation with acetone at pH 2.0, and resuspension in aqueous solution at pH 9.0, protein was removed by a second precipitation at pH 3.0. The supernatant was dialyzed and concentrated in a flash evaporator at 30°C. Hexose content of the polysaccharide was estimated by the anthrone reaction (21, 22).

Virus

A plaque-type variant of mouse adenovirus, designated as mouse adenovirus plaque type 4 (Mad1*pt*4), was used in this study. The median lethal dose of the virus is 5.0×10^6 PFU in C3H/HeN mice. Lethal infections are accompanied by hemorrhagic pneumonia (23). The virus was propagated in L cells (NCTC Clone 929, American Type Culture Collection, Rockville, Md.). Virus titers were determined by a plaque assay (24).

RESULTS

Acellular fractions 15A-1B and 15A-108A were developed in an attempt to eliminate the deleterious side effects of immunization with PV. These fractions were formed by extraction of whole *B. pertussis* with a high salt solution. Polyacrylamide gel electrophoresis of 15A-1B revealed at least 13 proteins ranging in size from 16,500 to 139,000 and the gel patterns were similar to outer membrane proteins extracted by other researchers (25, 26). The acellular fractions retained significant protective activity in the mouse protection test (27) and induced resistance to mouse adenovirus infection also (Table I).

Table I. Activities of *B. pertussis* vaccine and acellular extracts

Treatment	Dry Weight (ug)	Protein (ug)	Hexose (ug)	LPS (ug)	Challenging Dose* (PFU)	Mortality (deaths/total)
PV, Connaught	250	51	---	2.34	1.0×10^8	0/21
PV, Connaught	25	5.1	---	0.23	1.0×10^8	19/47
15A-1B	---	46	---	147	2.8×10^7	3/16
15A-108A	---	12	---	0.039	2.8×10^7	4/20
LPS	20	< 0.2	1.1	> 40	2.8×10^7	0/5
Polysaccharide	415	11.5	480	100	2.1×10^7	0/5

* All virus stocks were lethal to 100% of diluent-treated animals, except in the polysaccharide test, where the virus stock initiated lethal infection in three of five mice.

Examination of the protein content of PV and the acellular fractions indicated that a proteinaceous factor might be involved in the induction of resistance. Several of the virulence factors of *B. pertussis* are associated with the cell surface and are known to have immunomodulatory activity. Mutants of *B. pertussis* and strains that are lacking or greatly deficient in virulence factors were examined for their ability to induce resistance to mouse adenovirus infection. Strains that do not exhibit virulence factors, Tohama III (28) and a prototype phase IV ATCC 11615 (29) induced resistance to adenovirus infection. Mutants of *B. pertussis*, which were induced by a single transposon insertion and are deficient in at least five of the virulence-associated factors (30), induced resistance also.

Examination of the LPS content of PV and the acellular fractions indicated LPS was present in the vaccine and its fractions. However, no quantitative relationships between the amount of LPS present and the degree of resistance inducing activity was recognized. Although fractions 15A-1B and 15A-108A varied several thousandfold in LPS content, the fractions induced similar degrees of resistance (Table I). The role of LPS in the induction or resistance was examined further by assaying the immunomodulatory activity of PV in the endotoxin unresponsive C3H/HeJ mouse strain. Resistance did not develop in PV treated C3H/HeJ mice (Table II). However, LPS extracted from *B. pertussis* induced a virus resistant state in the endotoxin responsive C3H/HeN mice and the resistance inducing activity of *B. pertussis* LPS appeared to be unique among several gram-negative bacteria tested (Table III).

Table II. Activity of Connaught PV in C3H/HeN and C3H/HeJ mice.

Treatment	Mouse Strain	Challenging Dose (PFU)	Mortality (deaths/total)
Diluent	C3H/HeN	2.1×10^7	3/5
PV, 250 ug	C3H/HeN	2.1×10^7	0/5
Diluent	C3H/HeJ	2.1×10^7	9/10
PV, 250 ug	C3H/HeJ	2.1×10^7	10/10

Table III. Activity of LPS extracted from several bacterial species.

Treatment	Mortality* (deaths/total)
Diluent	5/5
Escherichia coli, serotype 055:B5, 20 ug	5/5
Vibrio cholerae, serotype Inaba 569B, 20 ug	5/5
Salmonella typhimurium, 20 ug	5/5
Salmonella minnesota, 20 ug	5/5
Bordetella pertussis, 20 ug	0/5

* Challenged with 2.8×10^7 PFU seven days after treatment.

A carbohydrate moeity in PV was involved in the induction of resistance. The protective activity of PV was abrogated when modified by treatment with 100 mM periodate (Table IV). However, milder treatment with periodate did not affect PV activity. The purification of LPS by the phenol-water procedure does not specifically separate polysaccharide from LPS. Therefore, capsule (slime) polysaccharide was extracted from *B. pertussis* cells and tested for resistance inducing activity. The polysaccharide fraction induced resistance to mouse adenovirus infection (Table I). The fraction contained a greater amount of anthrone reactive sugar residues on a basis of dry weight as compared to the purified LPS fraction, however the polysaccharide fraction contained substantial amounts of LPS and protein also.

Table IV: Periodate treatment of PV.

Treatment*	Challenging Dose (PFU)	Mortality (deaths/total)
Exp. 1		
Diluent	2.8×10^7	5/5
PV + 100 mM $NaIO_4$ for 72 h at 25°C	2.8×10^7	5/5
PV + H_2O for 72 h at 25°C	2.8×10^7	0/5
100 mM $NaIO_4$ for 72 h at 25°C	2.8×10^7	5/5
Exp. 2		
Diluent	2.8×10^7	5/5
PV + 0.5 mM $NaIO_4$ for 30 min at 37°C	2.8×10^7	0/5
Exp. 3		
None	2.1×10^7	5/5
PV + 10 mM $NaIO_4$ for 30 min at 37°C	2.1×10^7	0/5
PV + 2.0 mM $NaIO_4$ for 30 min at 37°C	2.1×10^7	0/5

* After the indicated reaction of periodate with PV mice were inoculated i.p. with the mixtures and challenged with virus seven days later.

DISCUSSION

The observation that acellular fractions of PV could induce resistance to mouse adenovirus infection demonstrated definitively that a subcellular component(s) of the *B. pertussis* cell was involved in the triggering of an acquired resistance to adenovirus infection. The role of protein in induction of resistance to mouse adenovirus infection is probably minor since the purified LPS fraction containing relatively little protein (< 0.2 μg) induced resistance. This conclusion was supported also by the fact that *B. pertussis* strains, which lack several virulence factors associated with the cell surface, induced resistance. However, small amounts of protein might play a synergistic role in the induction of resistance (51).

The Limulus amoebocyte lysate assay for endotoxin detected approximately one third of the endotoxin estimated to be in unfractionated PV (32, 33). A lack of correlation between LPS concentration and resistance inducing activity of the vaccine and its acellular fractions may have been due to substances in each fraction that interfered with the amoebocyte lysate assay to varying degrees. The observation that C3H/HeJ mice did not respond to PV with induction of resistance indicates that LPS plays an important role in the triggering of the antiviral mechanism.

The role of polysaccharide in the resistance inducing mechanism remains to be questioned. Mild conditions of periodate treatment (0.5 mM $NaIO_4$ for 30 min at 37°C), which abrogates the adherence of *B. pertussis* glycocalyx to bronchial ciliated epithelial cells (34), did not decrease the resistance inducing activity. However, treatment of PV with an increased concentration of periodate and increased reaction time destroyed the resistance inducing activity. The terminal octulosonate residue of polysaccharide I derived from *B. pertussis* LPS has been reported to be sensitive to the more stringent periodate treatment (35). Comparison of the LPS and polysaccharide content in both the LPS and polysaccharide fractions in conjunction with the titration of the resistance inducing factor in both fractions should illuminate an association between a given component and the resistance inducing activity.

The LPS of *B. pertussis* appears to be unique, since LPS extracted from other gram negative miroorganisms did not induce resistance to mouse adenovirus. These observations would imply also that the mechanism of resistance induction probably does not involve the numerous immunomodulatory mechanisms activated by the LPS of other gram negative microorganisms (36). We may be examining, using a different functional assay, the same compensatory resistance mechanism to *Listeria monocytogenes* described by others (33). It is interesting that both this bacterium and virus are cleared by a cell-mediated immune response (37, 38). Although many of the immunomodulating reactions of LPS are observed within 72 h after treatment, LPS is retained in a biologically active form in lymphoid tissues for much longer periods of time (39) and therefore could serve possibly to stimulate the antiviral resistance mechanisms for several weeks. The PV induced altered immune state (revealed as resistance to virus infection that persists several weeks) might play a role in the continued pathogenesis of *B. pertussis* infection after the detectable organism is cleared.

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ANTIVIRAL ACTIVITY OF BORDETELLA PERTUSSIS VACCINE-ELICITED PERITONEAL EXUDATE CELLS

D. W. Baggett, P. A. LeBlanc, F. S. Allison,
M. J. Thomley and A. L. Winters*
Department of Microbiology, University of Alabama
University, Alabama 35486

ABSTRACT

Peritoneal exudate cells collected from mice 7 days after treatment with Bordetella pertussis vaccine exhibited significant in vitro antiviral activity against vesicular stomatitis virus (VSV). Vaccine-induced peritoneal exudate cells exhibited both intrinsic and extrinsic antiviral activity in culture with target VSV-infected L cells. Virus replication was poor in the vaccine-induced exudate cells. Coculture of vaccine-induced exudate cells and VSV-infected L cell targets decreased virus yield. The activity appeared specific for infected cells and at least a portion of the antiviral activity was directed against the initial infection cycle. Nonadherent vaccine-induced exudate cells showed an increase in antiviral activity over total vaccine-induced exudate cells.

INTRODUCTION

A number of immunomodulatory activities has been associated with Bordetella pertussis vaccine (BPV) (1,2,3). Several of these activities have been associated with specific components of B. pertussis cells (4-11). Treatment of mice with either BPV or extracts of the organism have been shown to alter the response to virus infections. An increase in the susceptibility to intranasal influenza virus challenge resulted 5 to 7 days following treatment with BPV (12). An increase in resistance to intracranial rabies virus challenge was observed when an extract of B. pertussis was

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administered by the subcutaneous, intraperitoneal, or intravenous route at the same time as the virus (2). Kirchner and coworkers demonstrated that pretreatment of mice with BPV 7 days prior to intraperitoneal challenge with herpes simplex type 1 virus conferred protection to the mice; however, administration of BPV 3.0 days prior to challenge did not result in protection (13). Similar protection has been observed with foot and mouth disease virus (14) and mouse adenovirus (1,15). Resistance to challenge with Semliki Forest virus and encephalomyocarditis virus has been reported when acellular fractions of B. pertussis were administered 24 hours earlier (4,16). While several reports on the ability of BPV or components of B. pertussis to alter the murine response to a variety of virus infections exist, few have attempted to characterize or define the mechanism of antiviral activity. This report initiates characterization of peritoneal exudate cell (PEC) antiviral activity elicited by BPV treatment.

MATERIALS AND METHODS

Vaccine Treatment. BPV was provided by Connaught Laboratories, Swiftwater, Pa., and stored at 4°C. BPV was adjusted to 500 ug (dry weight) per ml in a thimerosal-saline diluent (0.02% thimerosal in 0.15M NaCl) and 0.5 ml was injected intraperitoneally immediately after dilution to initiate treatment.

Mice. A colony of mice (C3H/HeN, MTV⁻) was maintained at the University of Alabama using a modification of the aseptic techniques of Giovanella and Stehlin (11). The initial breeding stock for the colony was obtained from Dr. Carl Hansen (National Cancer Institute, Bethesda, Md.).

Cells and Culture. L cells (NCTC clone 929; American Type Culture Collection, Rockville, Md.) were maintained in Dulbecco modified Eagle medium (DME) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 ug/ml streptomycin (K.C. Biological Inc., Lenexa, Ks.). All incubations were carried out at 37°C under 5% CO_2-95% humidified air mixture.

Virus. Vesicular stomatitis virus (VSV), Indiana serotype, was obtained from Dr. Jack W. Frankel (Dept. of Health and Rehabilitative Services, Tampa, Fl.) and propagated in L cells. Virus infectivity was determined using a plaque assay on L cell monolayer cultures under DME containing 0.5% agar. Thirty hours after virus adsorption, plaque forming units (PFU) were enumerated after monolayers cultures were fixed with buffered formalin and stained with crystal violet solution (1 mg/ml).

Effector Cells. Peritoneal exudate cells (PEC) were collected 7.0 days after treatment with either BPV (BPV-PEC) or vaccine diluent (DIL-PEC). The elicited cells were removed by peritoneal lavage using iced unsupplemented medium. PEC were centrifuged (175 x g, 10 minutes, 4°C) and washed twice with phosphate buffered saline (4°C). The PEC were adjusted to 4.0×10^6 cells/ml in supplemented DME for subsequent culture.

Nucleated Cell Enumeration and Differentiation. Mice were treated 7 days prior to use with BPV or diluent. Animals were sacrificed by decapitation and the peritoneum was lavaged with 5.0 ml Hank's balanced salt solution (25°C). To provide consistency only 3.0 ml of the 5.0 ml lavage was recovered and placed in 12 x 75 mm glass

tubes for centrifugation, 100 x g for 3.0 min at 25°C. The supernatant was discarded and the cell pellet resuspended in three drops of 25% albumin. Wedge-shaped smears for Wright-Giemsa staining and nucleated cell differentiation were made from this suspension and evaluated using the criteria for cell identification as described by Diggs et al. and Wintrobe et al. (18,19). In addition 20 ul of the original lavage was diluted in particle-free dilution vials containing 10 ml of azide-free Isoton II (Coulter Diagnostics, Hialeah, FL) for electronic counting. Three drops of azide-free Zapoglobulin II (Coulter Diagnostics, Hialeah, FL) were added to lyse erythrocytes, the vials were mixed by inversion, and the total number of white blood cells in the peritoneal lavage enumerated using a Coulter Counter, Model ZB1 (Coulter Electronics, Hialeah, FL).

Growth Curve of VSV in Target Cells or Effector Cells. One tenth milliliter of L cell suspension (4.0×10^4 cells/ml) was placed in each well of a flat bottom microtiter plate and incubated overnight. The cultures were infected with VSV by addition of 0.025 milliliters of virus stock to obtain a calculated multiplicity of infection (MOI) of 5×10^{-3} PFU/cell. One hour after virus adsorption, 0.05 ml of supplemented DME was added to each well. The plates were incubated and samples taken at appropriate time intervals. Each data point represents the virus concentration from 12 pooled microtiter well cultures.

The permissiveness of DIL-PEC and BPV-PEC for VSV was examined in a similar manner. Each well of separate sets of microtiter

plates received 1.0×10^5 PEC (either BPV- or DIL-elicited). The PEC were infected with the number of PFU of VSV as used with target L cells. This reduced the calculated MOI to 0.0002 PFU/cell yet maintained the same PFU to PEC ratio as used in coculture experiments (see below).

Coculture of Effector and Target Cells. L cells (target cells) were added to microtiter plates as described earlier. After 24 h, the cells were infected by adsorption of VSV at a calculated MOI of 5×10^{-3} PFU/cell. One hour after virus adsorption, 0.05 ml DME was added to each well. Two hours after virus adsorption, either BPV-PEC, DIL-PEC, nonadherent BPV-PEC, or nonadherent DIL-PEC (effector cells) were added to the cultures at 25:1 effector:target cell ratio. Nonadherent PEC were obtained by planting PEC suspension in plastic tissue culture dishes and incubating 2.0 h to allow attachment of the adherent cell population (20). After 2.0 h the medium was removed and the plates washed twice to resuspend nonadherent cells. The pooled washings were centrifuged and the cell concentration adjusted to 4.0×10^6 cells/ml for subsequent culture. The plates were incubated and samples taken at appropriate intervals.

Infectious Center Development. Thirty-five mm dishes were seeded with 1.2×10^5 target cells. After 24 h incubation, the medium was aspirated and VSV added at a calculated MOI of 5×10^{-3} PFU/cell. Two hours after virus adsorption, the medium was removed and the infected cells washed twice with medium. Either BPV-PEC or DIL-PEC were added at an effector to target cell ratio of 25:1. Four hours

after virus adsorption, the medium was aspirated to remove non-adherent PEC, and a suspension of L cells sufficient to form a monolayer within 24 h was added to each dish. Five hours after virus adsorption, the medium was aspirated and agar overlay was added as previously described. Thirty hours after virus adsorption, PFUs were enumerated after monolayer cultures were fixed and stained.

L Cell Colony Formation. L cells were added to 35 mm dishes at a density of 40 cells per dish. After 24 h, the medium was removed and either BPV-PEC or DIL-PEC added at the previously described planting density. Due to the lower number of L cells, an effector to target cell ratio of 75,000:1 was obtained. Two hours after addition of the effector cells, the medium was aspirated and agar overlay medium added to each dish. The plates were incubated for 6.0 days, the monolayer cultures were fixed and stained, and colonies enumerated.

Cytolytic (Chromium Release) Activity Assay. Direct cellular toxicity was determined as described by Russell (21) with the following modifications. Effector cells in the cytolysis assays were 5×10^5 BPV-PEC or DIL-PEC. Target cells were ^{51}Cr-labelled VSV-infected L cells and uninfected L cells. Effector cells were treated for 4 h with either medium or medium containing activators. Activators used were lipopolysaccharide (E. coli 0111:B4, 3.0 ng/ml courtesy of Dr. D. C. Morrison, Kansas Medical Center, Kansas City, KS), lymphokine-rich supernatant from Con A-stimulated spleen cell culture (1 IRU gamma interferon/ml), or a mixture of lipopoly-

saccharide and lymphokine. Specific chromium release was calculated using the formula:

$$\%\text{Specific } {}^{51}\text{Cr Release} = \frac{\text{Experimental CPM} - \text{Spontaneous Release CPM}}{\text{Total Release CPM} - \text{Spontaneous Release CPM}} \times 100$$

Statistical Analysis: Results from the experiments were expressed as mean values and analyzed using the Student's t test.

RESULTS

Differences in the amount of virus produced in cocultures of L cells and DIL-PEC as compared to L cells alone, were not apparent (Figure 1). However, cocultures of L cells with BPV-PEC decreased the ability of L cells to synthesize VSV approximately 90%.

To establish the contribution of PEC to viral replication in the cocultures, separate cultures were infected with VSV with the usual virus concentrations and the usual cell concentrations. These conditions resulted in a calculated MOI of 5×10^{-3} PFU per L cell, and a calculated MOI of 2×10^{-4} PFU per PEC. Although there were more cells in the PEC populations than in the L cell cultures, infection of BPV-PEC or DIL-PEC resulted in the production of 2-3.5 $\log_{10}$ less virus. Thus, both BPV-PEC and DIL-PEC were less permissive than L cells (Figure 2). Furthermore, BPV-PEC were less permissive for VSV replication than were DIL-PEC (4.0 versus 2.5 $\log_{10}$ PFU/ml at 72 h). These results, considered with those in Figure 1, demonstrated that in combination cultures of PEC and infected L cells, the PEC were responsible for less than

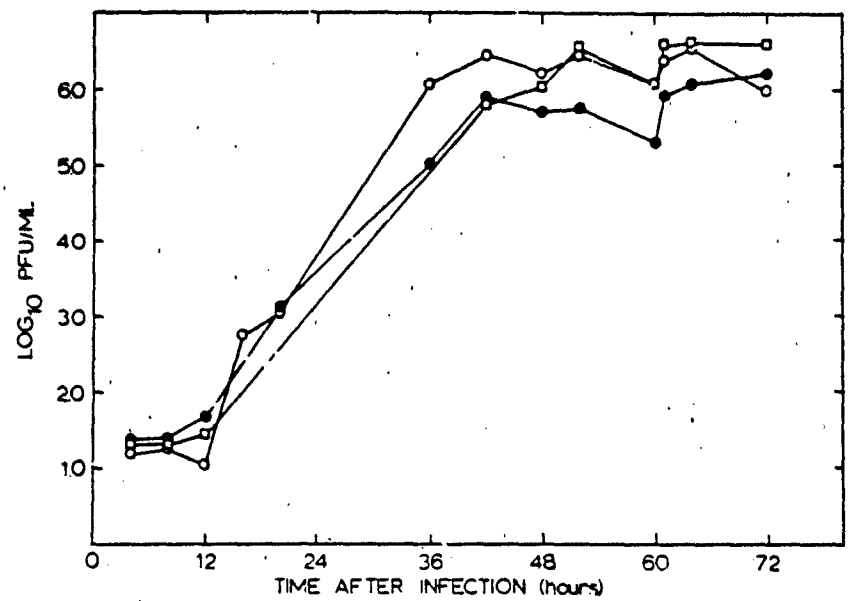

FIG. 1. Growth Curve of VSV in L Cells and Peritoneal Exudate Cell Cocultures. VSV was adsorbed to L cell cultures. After 2 h, either medium (□), Diluent-elicited PEC (O), or BPV-elicited PEC (●) were added and VSV infectivity in the cocultures was assayed at the times indicated.

1 percent of the virus synthesized and that BPV-PEC were inhibiting virus replication in the L cells.

To establish the significance of the antiviral effect, ten separate pools of supernatants of VSV-infected L cell cultures, alone and in coculture with BPV-PEC and DIL-PEC, were collected. Samples taken 60 h after infection confirmed the antiviral activity of BPV-PEC and showed that cocultures of BPV-PEC and infected L cells produced significantly less virus (approximately 80%) than cultures of DIL-PEC and infected L cells (Table 1).

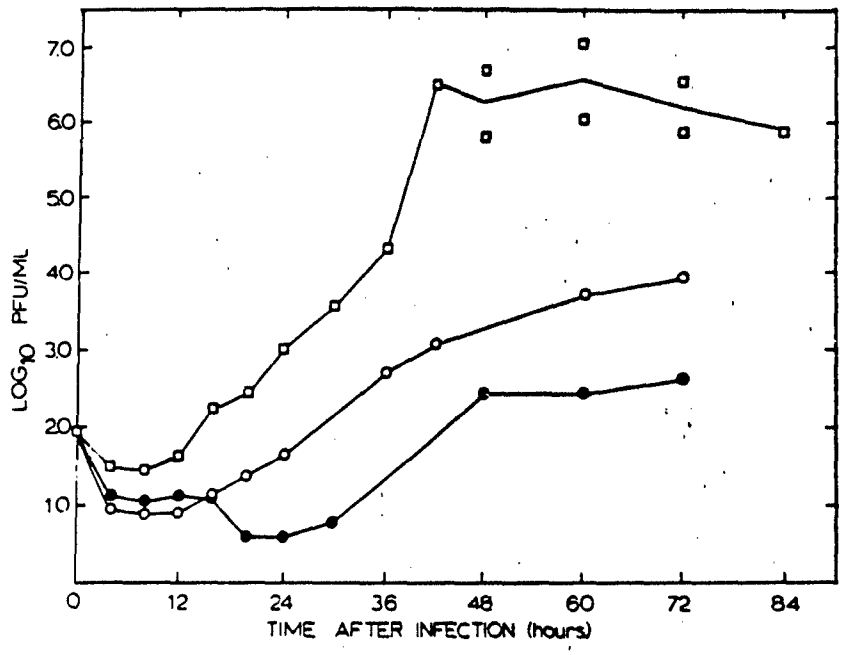

FIG. 2. Growth Curve of VSV in L Cells and Peritoneal Exudate Cells. VSV was adsorbed to cultures of L cells (□), Diluent-elicited PEC (○), or BPV-elicited PEC (●), and virus infectivity assayed.

TABLE 1

Activity of BPV-induced Peritoneal Exudate Cells on VSV Synthesis in L-929 Cells

Infected Culture Treatment	Virus titer after 60 h (PFU x 10^{-6}) Mean	SD
Control (no PEC addition)	18.4[a,c]	7.8
DIL-PEC Coculture	37.1[a,b]	28.8
BPV-PEC Coculture	2.6[b,c]	0.9

a. Student's *t* test, d.f. = 18, p = .059.

b. Student's *t* test, d.f. = 18, p = .001.

c. Student's *t* test, d.f. = 18, p < .001.

The effect of BPV-PEC coculture on infectious center development was examined to see if inhibition of VSV synthesis occurred in the initial cycle of infection. BPV-PEC resulted in a 15% decrease in infectious centers as compared to control cultures and a 32% decrease in infections centers as compared to DIL-PEC (Table 2). At least a portion of the antiviral activity resulted from the interaction of BPV-PEC and the initially infected L cells.

Colony formation of L cells in the presence of PEC was examined to determine whether BPV-induced PEC were toxic toward L cells independent of virus infection. While BPV-PEC exhibited some toxicity toward uninfected L cells (Table 3), BPV-PEC reduced the number of colonies only 22% while DIL-PEC caused a 69% decrease in L cell colonies. The notable decrease in colony formation resulting from coculture with DIL-PEC suggested a nonspecific toxicity toward uninfected L cells. However, microscopic examination of the cultures suggested that the decrease was due to enhanced dispersion of L cells, thereby reducing the number of discrete colonies observed.

The cytotoxic activities of BPV-PEC and DIL-PEC were examined to establish whether other qualitative differences between the two PEC populations could be observed at the cellular level. The cytotoxic activity of both cell populations was assayed using untreated cells and cells exposed to two known biological response modifiers. In medium without immunomodulators, neither BPV-PEC nor DIL-PEC exhibited cytolytic activity against uninfected L cells, while both showed moderate activity against VSV-infected L cells.

TABLE 2

Activity of BPV-induced Peritoneal Exudate Cells on VSV infectious Centers

Infected Culture Treatment	Infectious Centers in L Cell Culture	
	Mean	SD
Control (no PEC addition)	62.8[a,c]	8.6
DIL-PEC Coculture	77.4[a,b]	11.0
BPV-PEC Coculture	53.0[b,c]	6.4

a. Student's t test, d.f. = 18, p = .004.
b. Student's t test, d.f. = 18, p = .001.
c. Student's t test, d.f. = 18, p = .009.

TABLE 3

Activity of BPV-Elicited Peritoneal Exudate Cells on L Cell Colony Formation

Sample Treatment	Colonies After 6 days	
	Mean	SD
Control (no PEC addition)	30.3[a,c]	4.9
DIL-PEC Coculture	9.3[a,b]	2.8
BPV-PEC Coculture	23.6[b,c]	4.1

a. Student's t test, d.f. = 18, p < .001.
b. Student's t test, d.f. = 18, p < .001.
c. Student's t test, d.f. = 18, p = .003.

Pretreatment of PEC with lipopolysaccharide potentiated the cytotoxic activity against virus-infected cells of BPV-PEC to 100% and DIL-PEC to only 63% (Table 4). Pretreatment of PEC with gamma interferon did not increase the cytotoxic activity of BPV-PEC, but increased the activity of DIL-PEC two-fold.

Seven days after treatment of mice with BPV there was a 20% increase in leucocytes in the peritoneum. Differential analysis of BPV-PEC further indicated not only a quantitative difference, but also a qualitative difference in the PEC populations obtained (Table 5). BPV-PEC demonstrated a significant increase in the proportion of segmented neutrophils over DIL-PEC. Also, the levels of eosinophils observed in DIL-PEC were not detected in BPV-PEC. There were not significant differences between the proportion of macrophages and monocytes in BPV-PEC and DIL-PEC.

An initial characterization of the PEC population which displayed antiviral activity was performed by examining the activity of PEC and a nonadherent population in PEC. Adherence to plastic was used to deplete the PEC population of adherent cells. Nonadherent cells were collected and adjusted to the same concentration as total PEC. Removal of the adherent cell population appeared to increase the antiviral activity in a BPV-PEC coculture (Table 6). Statistical analysis confirmed that removal of the adherent population from BPV-PEC increased the antiviral activity, while removal of the adherent cells from DIL-PEC did not result in a significant virus of antiviral activity.

TABLE 4

Cytotoxicity of BPV Elicited Peritoneal Exudate Cells for VSV-Infected and Uninfected L Cells

	Specific ^{51}Cr Release [% release (MEAN ± SD)]			
	ACTIVATORS			
Effector/Target Cell	Medium	[a] Gamma Interferon	[b] E. coli LPS	[c] Interferon LPS
BPV-PEC/L cell	0 (1328 ± 16)	2 (1437 ± 56)	28 (2621 ± 39)	7 (1661 ± 42)
BPV-PEC/VSV-Infected L cell	44 (3480 ± 123)	43 (3467 ± 122)	100 (5773 ± 285)	95 (5348 ± 260)
DIL-PEC/L cell	0 (1151 ± 35)	0 (1158 ± 20)	0 (1101 ± 46)	0 (1054 ± 77)
DIL-PEC/VSV-Infected L cell	31 (3014 ± 283)	62 (4167 ± 419)	63 (4199 ± 112)	65 (4269 ± 215)

a. 1.0 IRU/ml.

b. 3.0 ng/ml preparation of E. coli 0111:B4.

c. 3.0 ng/ml lps + 1.0 IRU/ml gamma interferon preparation.

TABLE 5

Differential Analysis of Peritoneal Lavage Cells

Cell Type	Cell count per microliter (mean ± SD) DIL	BPV
Band Neutrophil	52.6 ± 40.5	26.1 ± 25.6
Segmented Neutrophil	55.1 ± 55.8	821.6 ± 129.6
Eosinophil	237.7 ± 108.2	0
Lymphocyte	684.7 ± 151.4	706.9 ± 65.5
Transformed Lymphocyte	48.6 ± 22.0	65.6 ± 14.7
Monocyte	1295.8 ± 231.2	1282.9 ± 209.2
Macrophage	52.7 ± 1.7	65.1 ± 6.4

TABLE 6

Activity of Nonadherent BPV-induced Peritoneal Exudate Cells on VSV Synthesis in L-929 Cells

Infected Culture Treatment	Virus Titer After 60 h (PFU x 10^{-6}) Mean	SD
Control (no PEC addition)	10.6	3.8
DIL-PEC Coculture	3.5[a]	1.5
Nonadherent DIL-PEC Coculture	1.8[a]	1.3
BPV-PEC Coculture	2.5[b]	0.6
Nonadherent BPV-PEC Coculture	1.3[b]	0.4

a. Student's t test, d.f. = 8, p = .083.

b. Student's t test, d.f. = 8, p = .004.

DISCUSSION

Our data indicated that BPV treatment resulted in both quantitative and qualitative changes in the peritoneum. We obtained a 20% increase in PEC following treatment with BPV. BPV also induced qualitative differences in the cell populations of the peritoneum. BPV-elicited PEC demonstrated an increase in segmented neutrophils, and dramatic decrease in eosinophils. In contrast Fishel and coworkers have reported that intraperitoneal injection of mice with BPV results in a significant increase in leucocytes in the peritoneal cavity, due primarily to an influx of macrophages and their precursors, along with neutrophils (22). They observed that seven days after treatment with BPV, leucocyte counts and protein concentrations increased approximately sixfold over those of either uninjected controls or vaccine-diluent treated animals.

PEC from mice pretreated with either vaccine diluent or BPV supported VSV replication poorly when compared to L cells. Furthermore, BPV-PEC seemed to be less permissive for virus replication than DIL-PEC. Monocytes and macrophages that make up nearly 50% of the cells in PEC are intrinsically resistant to the replication of many viruses. Generally they adsorb and phagocytize viruses and render the internalized virus incapable of infecting other host cells (23).

BPV elicited PEC also demonstrated extrinsic antiviral activity. At least a portion of the antiviral activity of BPV-PEC was directed against the initial infection cycle of L cells, as shown by a reduction in infectious center development. Since the

PEC were added after virus adsorption to the cells, it is unlikely that inhibition occurred through direct phagocytosis and destruction of the viral inoculum by cells in the PEC population. Therefore, a portion of the antiviral activity associated with BPV-PEC appeared to be of an extrinsic nature. One possible mechanism was described by Kirchner and coworkers, who demonstrated enhanced induction of interferon in mice after BPV treatment (24,25).

Our observations were similar to reports on the kinetics of the antiviral activity of Corynebacterium parvum elicited PEC. Morahan and coworkers reported that the protective effect of C. parvum activated PEC against vaccinia virus and herpes simplex type 2 virus was greatest in experiments using a low MOI and allowing multiple cycles of virus replication (26). On the other hand, Morahan and coworkers reported a limited effect against single cycle virus yields following a high multiplicity of infection (40 PFU/cell), whereas our results obtained with a low multiplicity of infection (5×10^{-3} PFU/cell) demonstrated significant activity against single cycle virus yields.

Although examination of L cell colony formation suggested some toxicity of BPV-PEC toward uninfected L cells, chromium release studies revealed that while neither BPV-PEC nor DIL-PEC were cytolytic against uninfected L cells, both exhibited activity toward VSV-infected L cells. The extrinsic antiviral mechanism could occur either through a change in the target cell metabolism so that virus yield per cell was reduced, or by the reduction of

virus synthesizing cells due to cytolysis. Increased cytotoxic activity of a cell population in BPV-PEC was demonstrated. In addition, the undetermined cytotoxic cells could be stimulated to greater activity by the addition of lipolysaccharide. Furthermore, BPV-PEC can be potentiated to higher levels of activity than DIL-PEC. It was not possible to determine whether the pertinent cells in BPV-PEC may be more efficient at cytolysis of virus infected cells, or whether there may have been an increased number of a particular effector population resulting from treatment with BPV.

Several investigators have shown that depletion or removal of the adherent cell population from C. parvum treated mice by adherence to glass or plastic (27,28) or inactivation with anti-macrophage sera (23) decreased or abolished resistance to a variety of infectious agents, suggesting that macrophages are key effector cells. In contrast to these studies, our results indicated that removal of adherent cells increased the antiviral activity of BPV-PEC. Nonadherent BPV-PEC showed an approximate 48% increase in antiviral activity over total BPV-PEC. Assuming the peritoneal exudate population is 50% macrophages, this experiment enriched the nonadherent population approximately twofold. Whether the increase in activity was due to this enrichment or the removal of an inhibitor normally in the peritoneal exudate population was not determined. Similar results were obtained in a study by Tracey using Bacille Calmette-Guerin (BCG) elicited-PEC (29). Mice were injected intraperitoneally with BCG and PEC collected 3 days later

and injected into syngeneic untreated mice. After 4 days, the PEC from recipient mice were collected and depleted of adherent cells. Approximately 50% of the cells were nonadherent and were twice as cytotoxic as total PEC. The active cells were identified as natural killer cells; however, macrophages appeared to be involved in the regulation of natural killer cell activity in BCG treated mice by secreting both NK-enhancing factors and NK-inhibiting factors, including Prostaglandin E_2 (30). We are currently investigating the role of natural killer cells and prostaglandins in BPV induced antiviral activity.

While the exact mechanism(s) of the antiviral activity associated with BPV-PEC remains unclear, it is evident that the vaccine is capable of significantly altering the antiviral activity of peritoneal cells. Determination of the mechanism by which BPV confers resistance to infection will not only allow us to manipulate the immune response, but will aid in our basic understanding of the pathogenesis of virus infections.

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Removal of lipopolysaccharide from acellular *Bordetella pertussis* vaccine by detergent treatment*

Robert S. Stinson,†‡ *Robert D. Lemmon*,§ *David W. Baggett*,‡ *Timothy L. Huff*,‡ *Jeffery A. Malone*,‡ *Gary L. Sloan*,‡ *Alvin L. Winters*†‡‖

Protective antigen was extracted from *Bordetella pertussis* cells with 1·0 M NaCl and precipitated with ammonium sulfate, 20–40% saturation (designated fraction 15A-1B). The protective antigen was purified further by detergent (Emulphogene BC720) treatment and adsorption to aluminum hydroxide gel (designated fraction 15A-108A). Compared with *B. pertussis* vaccine and fraction 15A-1B, fraction 15A-108A retained protective activity as assessed by the mouse protection test, but had reduced protein and markedly reduced endotoxin content. Fraction 15A-108A also had reduced leukocytosis-promoting, histamine sensitizing splenomegaly-inducing, and adjuvant activities. Emulphogene treatment provided a relatively simple method for removing endotoxin from a potential acellular *B. pertussis* vaccine.

INTRODUCTION

Immunization with *Bordetella pertussis* vaccine (BPV) has been accepted as the single most effective method in preventing *B pertussis* infections; however, BPV continues to

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† Present address: SERVAS, Inc., Storrs, CT 06268, U.S.A.

‡ Department of Microbiology, The University of Alabama, P.O. Box BM, University, AL 35486, U.S.A.

§ Analytical Biochemistry, Connaught Laboratories, Inc., Swiftwater, Pennsylvania 18370, U.S.A.

‖ To whom correspondence should be addressed.

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be one of the more troublesome vaccines to produce and standardize.[1-6] A central problem with BPV production is standardization. One has to contend with genetic shifts from virulent Phase I to avirulent Phase IV organisms[7] and variable viability of cultures.[8,9] For these reasons the vaccine has one of the highest failure rates of any vaccine currently produced.[10]

The public perception that immunization with the vaccine may be of greater risk than the disease[11] has provided an impetus to develop a nontoxic acellular vaccine with a standardized potency. An effort has been made to separate the component(s) of BPV responsible for immunization from the components that cause serious side reactions.[4,6,12] Investigators have isolated several factors, but no consensus exists as to the role of each factor in infection or immunization. A potentially toxic fraction that has been isolated is lipopolysaccharide (LPS) with its associated endotoxin activity.[13,14] All vaccines used presently for immunization contain LPS, and a proportion of the adverse reactions following administration of BPV are attributable to this component.

Antigens that appear to be responsible for induction of resistance to *B. pertussis* infection are found on or near the surface of the cell envelope[12] and cell-free protective antigens have been extracted by a variety of methods.[15-24] This report describes a method for removing lipopolysaccharide from an acellular BPV.

MATERIALS AND METHODS

Reagents were obtained from the following sources: *Bordetella pertussis* vaccine (Lot 32340), Connaught Laboratories, Inc., Swiftwater, Pennsylvania; Emulphogene BC 720, GAF Corporation, Chemical Group, New York, New York; Alhydrogel, E. M. Sergeant Pulp and Chemical Co., Inc., Hoboken, New Jersey; histamine diphosphate and E-Toxate (*Limulus* amoebocyte lysate assay), Sigma Chemical Co., St. Louis, Missouri; *Brucella abortus* antigen, Difco Laboratories, Detroit, Michigan.

Vaccine and acellular extractions

BPV consisted of heat-inactivated Phase I cells (Connaught Laboratories vaccine strain derived from ATCC 9797) suspended in diluent (0·02% thimerosal in 0·15 M NaCl) to a concentration of 4·0 mg/ml (dry weight).

Fractions derived from *B. pertussis* cells (Connaught Laboratories vaccine strain) were prepared as follows. Cells were grown on Cohen–Wheeler agar medium for 96 h at 36°C, harvested, and washed in phosphate buffered saline (PBS; 0·15 M sodium chloride and 0·05 M sodium phosphate buffer, pH 7·0). The culture was inactivated and extracted by overnight incubation in 0·75 M sodium chloride, 0·05 M sodium phosphate buffer (pH 7·0), and 0·02% thimerosal at 4°C. The cells were removed by centrifugation and 0·106 g ammonium sulfate per milliliter of supernatant was added (20% saturation). After incubation at 4°C for 30 min, the precipitate was sedimented (10 000 *g*, 4°C, 10 min). The supernatant was decanted and 0·113 g ammonium sulfate per milliliter of supernatant was added (40% saturation). The mixture was incubated and centrifuged as before. The precipitate was resuspended in PBS and dialysed against PBS. This acellular fraction was designated 15A-1B. Lipopolysaccharide was removed from 15A-1B by treatment with 1% (v/v) Emulphogene BC 720 at 4°C for 60 min. A precipitate formed and this mixture was then centrifuged at 100 000 *g* for 1·0 h at 4°C. The sediment was resuspended in PBS, centrifuged as before, resuspended, and absorbed to Alhydrogel (1·0%, w/v, aluminum hydroxide gel) to prevent precipi-

tation. The alum-absorbed preparation was designated 15A-108A. Potency of BPV, 15A-1B, and 15A-108A was assessed using the mouse protection test.[25] Protein content was determined by the method of Lowry and coworkers.[26] LPS (endotoxin) was assayed by the *Limulus* amoebocyte lysate assay[27] using *Shigella flexneri* LPS standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The preparations were denatured by boiling in 1·0% SDS and 1·0% beta-mercaptoethanol. The denatured proteins (100 μg) were separated by electrophoresis iun 6% polyacrylamide gels containing 1·0% SDS.[28]. Proteins were fixed and stained with 0·25% Comassie blue R250 in 9·6% acetic acid. Gels were scanned in a Varian Cary 210. Molecular weights were calculated using crosslinked hemocyanin, hemocyanin, bovine serum albumin, ovalbumin, pepsin, trypsinogen and β-lactoglobin (140, 70, 66, 45, 33, 24 and 18 kDa, respectively) as standards.

Animals

All studies, except histamine sensitization and pertussis vaccine potency tests, were carried out with C3H/HeN mice (4–5 weeks of age) derived from a breeding colony maintained in the Animal Care Facility, University of Alabama, as described previously.[29] All mice were supplied with autoclaved mouse chow and acidified water *ad libitum*. CFW mice used for histamine sensitization tests were obtained from Charles River Laboratories, Inc., Wilmington, Massachusetts. Female ICR mice used for pertussis vaccine potency were obtained from Buckberg Lab Animals, Inc., Tomkins Cove, New York, and were maintained at Connaught Laboratories, Inc., Swiftwater, Pennsylvania, in accordance with the N.I.H. Guide for the Care and Use of Laboratory Animals (DHEW Pub. No. 78-23).

Treatment with BPV, 15A-1B, or 15A-108A was initiated by intraperitoneal inoculation of 0·5 of each preparation at the concentration indicated in Table 1. Mice were inoculated with the vaccine diluent also to serve as controls.

Mouse protection test

The potency of BPV and the fractions was assessed using the standard mouse protective test.[25] Simultaneous intracerebral mouse protection tests of the test preparations and the US Standard pertussis Vaccine were performed according to established procedures (Code of Federal Regulations, Title 21, Part 620.4). Briefly, three fivefold dilutions were prepared of each test preparation and the standard. Three groups of mice, consisting of 16 animals each, were injected intraperitoneally with 0·5 ml of diluted material. Fourteen days after vaccination the challenge inoculum was prepared by harvesting *B. pertussis* strain 18323 from a 24–h culture grown on Bordet–Gengou medium and uniformly suspending the bacteria in a solution containing 1·0% casein peptone and 0·6% sodium chloride at pH 7·1. The suspension was adjusted to 160 units on a Klett–Summerson Colorimeter (number 54 green filter) and further diluted 1:3000 (v/v). The mice were lightly anesthetized with ether and injected intracerebrally with 0·03 ml of this challenge suspension. The mice were observed daily for 14 days. The total number of mice in each group, as well as the number active, paralysed, and dead were recorded for purposes of determining the relative potency of the test preparations relative to the standard vaccine. The standard vaccine contained 8 potency units per milliliter.

Leukocytosis

Blood samples were obtained by tail bleed at designated times after treatment. Counts were obtained by mixing the blood with Turk's solution and counting the nucleated cells in a hemocytometer.[30] Differential blood cell counts were obtained from duplicate smears made and air-dried at each bleeding. Smears were stained with Wright–Giemsa stain and at least 100 cells were counted per slide and scored as mononuclear (monocytes and lymphocytes) or polymorphonuclear leukocytes (heterophils, eosinophils and basophils).

Histamine sensitization

Four days after treatment CFW mice were challenged with 10 mg of histamine diphosphate by intraperitoneal inoculation.[4] Deaths were recorded 30 min after histamine challenge.

Splenomegaly

Seven days after treatment mice were weighted and killed by cervical dislocation. The spleen was removed, excised from excess fatty tissue, washed in saline, blotted to remove excess saline and weighed. A splenic index was calculated by dividing spleen weight by body weight.

Adjuvancy

Mice were injected intraperitoneally with a mixture consisting of 0·1 ml of heat-killed *Brucella abortus* (BA) antigen and 0·5 ml of BPV or the appropriate *B. pertussis* fraction. A control group was given *B. abortus* antigen only. Serum was obtained seven days after combined treatment and immunization and the anti-BA titer determined. Test serum was diluted in microtiter plates with round bottom wells using twofold serial dilutions. *Brucella abortus* antigen (0·1 ml, 1:10, v/v) was then added to each well. Control wells contained *B. abortus* antigen and saline diluent. Plates were incubated at room temperature overnight and the endpoint recorded at the highest dilution with visible agglutination.

Statistical analysis

Differences in the mean values of the appropriate experimental systems were assessed by Student's *t*-test.

RESULTS

Characterization of cell extract and fractions

Ammonium sulfate fractionation of the salt extract from *B. pertussis* cells selectively decreased the content of several proteins. SDS-PAGE of the salt extract revealed at least 13 proteins that ranged in size from 139 kDa (protein 1) to 16·5 kDa (protein 13) [Fig. 1(a)]. Fractionation of the salt extract by ammonium sulfate precipitation to form the preparation designated 15A-1B removed most of the polypeptides with molecular weights of 80 kDa or greater [Fig. 1(b)]. Solubilization of LPS by detergent treatment of 15A-1B to form the preparation designated 15A-108A resulted in precipitation of protein. The precipitation either concentrated two high molecular weight proteins that were not detected previously, or caused an aggregation of lower molecular weight

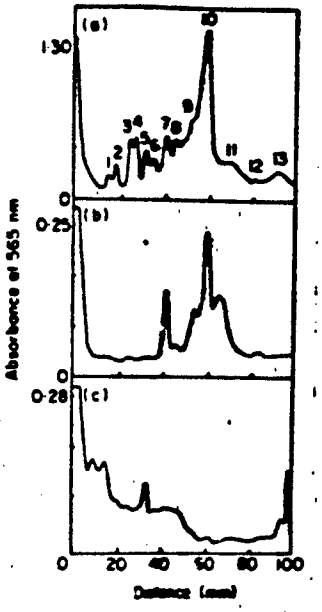

Fig. 1. Tracings of scans of coomassie blue stained SDS-PAGE of *Bordetella pertussis* salt extract and fractions. (a) Salt extract. (b) Fraction 15A-1B. (c) Fraction 15A-108A.

proteins that was not reversible in the conditions of SDS denaturation. Decreased amounts of proteins with molecular weights less than 58 000 kDa were observed also (Fig. 1(c)).

Ammonium sulfate precipitation of the salt extract enriched the relative concentration of lipopolysaccharide in 15A-1B (Table 1). Precipitation of material by Emulphogene treatment of fraction 15A-1B and adsorption of the material to alum decreased the relative lipopolysaccharide content with respect to protein approximately 4000-fold, while reducing the mouse protection potency eightfold.

Comparison of the biological effects of BPV and extracted fractions

Although fractions 15A-1B and 15A-108A retained activity in the mouse protection test the fractions differed in biological activities.

TABLE 1. The characteristics of *Bordetella pertussis* vaccine (BPV) and extracted fractions

Preparation	Protein (μg)	Lipopoly-saccharide (μg)	Mouse protection (units)
BPV*	51	2·34	1·0
15A-1B	46	74·0	4·1
15A-108A	12	0·039	0·5

* 250 μg dry weight.

Leukocytosis

White blood cell counts averaged 8700 mm^3 after treatment with vaccine diluent. Leukocyte counts in BPV-treated mice increased to a maximum values three to six days after treatment. The means of the elevated leukocyte counts obtained four days after treatment with BPV and 15A-1B were not significantly different [Fig. 2(a)]. However, the means of the leukocyte counts obtained four days after treatment with BPV and 15A-108A were significantly different. Differential counts of blood smears indicated that the leukocytosis consisted of approximately equal numbers of mononuclear and polymorphonuclear cells. A slight, transient increase in leukocytosis was observed 12 h after treatment with BPV and fraction 15A-108A (Fig. 2(b)]. The response was short-lived and leukocyte counts decreased before the major leukocytosis response initiated 48 h after treatment. The majority of cells during the period of transient leukocytosis were polymorphonuclear.

Sensitization to histamine

The lethal dose of histamine diphosphate for normal CFW mice was approximately 200 mg per 20 g body weight (data not shown). To determine whether *B. pertussis*-derived fractions could induce sensitization to histamine, groups of mice were treated with BPV, 15A-1B, 15A-108A or vaccine diluent. All mice treated previously with vaccine diluent survived (Table 2). All mice treated previously with BPV or 15A-1B died within 10–30 min after histamine challenge. In contrast, acellular fraction 15A-108A induced histamine sensitivity in only a portion of the treated mice (55%).

Splenomegaly

Mice treated with BPV showed a significant increase in spleen weight over controls (Table 3). Mice treated with 15A-1B developed significant levels of splenomegaly also. In contrast, mice treated with 15A-108A did not develop a significant increase in spleen weight as compared to animals treated with vaccine diluent.

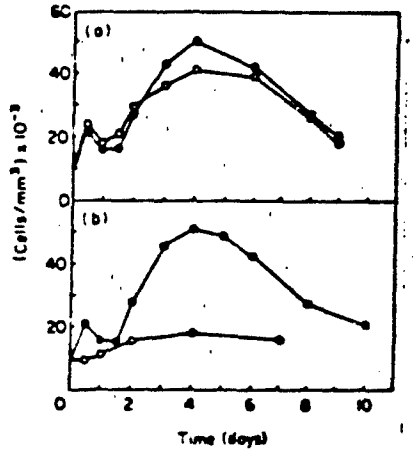

Fig. 2. Leukocytosis after treatment with *Bordetella pertussis* vaccine (BPV), fraction 15A-1B, or fraction 15A-108A. (a) Treatment with BPV (●) and fraction 15A-1B (○). (b) Treatment with BPV (●) and fraction 15A-108A (○).

TABLE 2. The effects of treatment with *Bordetella pertussis* vaccine (BPV), vaccine diluent, fraction 15A-1B, or fraction 15A-108A on histamine sensitization in CFW mice

Treatment	Mortality after histamine challenge (deaths/total)
Experiment 1	
Diluent	0/8
BPV	5/5
Fraction 15A-1B	10/10
Experiment 2	
Diluent	0/16
BPV	18/18
Fraction 15A-108A	6/11

Adjuvancy

Adjuvancy effects of BPV and *B. pertussis*-derived fractions on a T-cell independent antigen were assessed using heat-killed *B. abortus* as antigen. Mice injected with BPV and *B. abortus* produced significantly higher titers of antibody against *B. abortus* than mice treated with either a combination of *B. abortus* and 15A-1B or *B. abortus* and 15A-108A (Table 4).

DISCUSSION

Morse and coworkers observed a transient leukocytosis 12 h after treatment of mice with BPV and they attributed the leukocytosis to the activity of endotoxin in their BPV preparation.[32,33] In addition, splenomegaly has been associated with the presence of

TABLE 3. Splenomegaly after treatment with *Bordetella pertussis* vaccine (BPV), vaccine diluent, fraction 15A-1B, or fraction 15A-108A on histamine sensitization in CFW mice

Treatment	No. of mice	Spleen weight/body weight	
		Mean	SD
Experiment 1			
Diluent	6	4·4	1·1
BPV	10	12·6	3·8*
Fraction 15A-1B	10	6·9	1·7*
Experiment 2			
Diluent	21	4·5	0·9
BPV	15	12·6	2·1†
Fraction 15A-108A	28	5·1	1·1†

* Means were significantly different from diluent control by Student's *t*-test, $P < 0.05$.

† Means were significantly different from diluent control by Student's *t*-test, $P < 0.05$.

TABLE 4. Adjuvant activity of *Bordetella pertussis* vaccine (BPV), vaccine diluent, fraction 15A-1B or fraction 15A-108A

Treatment	Anti-*B. abortus* titer	
	Mean	SD
Experiment 1		
Diluent	3·9	1·1
BPV	6·6	2·5*
Fraction 15A-1B	5·5	0·5*
Experiment 2		
Diluent	4·7	1·9
BPV	6·9	2·9†
Fraction 15A-108A	5·0	2·0

* Means were significantly different from diluent control by Student's *t*-test, $P < 0{\cdot}05$

† Means was significantly different from diluent control by Student's *t*-test, $P < 0{\cdot}05$

endotoxin.[4] Our data support their hypothesis, since the endotoxin-deficient 15A-108A did not elicit these responses.

BPV acts as an adjuvant to many antigens in various animal species.[34] It is usually most effective when given simultaneously with the antigen,[35,36] although there are exceptions.[37] The mechanism responsible for the enhancing effect of BPV on antibody production is unclear. Reed and coworkers[38] suggested BPV exerts its effects directly on the precursor of antibody-forming cells by increasing their rate of division. Finger and coworkers[39] concluded that the adjuvancy response was due to an increased recruitment of antigen responding cells and proliferation of memory cells. The adjuvancy activity of *B. pertussis* endotoxin may be dependent on the type of antigen. Vogel & Klein reported that endotoxin derived from *B. pertussis* has adjuvant activity in an *in vitro* plaque assay with sheep red blood cells.[40] Sultzer and coworkers have shown recently that the endotoxin-associated proteins of *B. pertussis* have marked adjuvant activity with cholera enterotoxin.[41] In contrast, Monji and coworkers did not observe adjuvant activity of *B. pertussis* endotoxin with *Haemophilus influenzae* type b capsular polysaccharide.[42] One has to consider that removal of the oligomeric protein called pertussigen (lymphocytosis promoting factor)[43,44] from 15A-1B might decrease the adjuvant activity. However, the adjuvant activity of pertussigen remains unclear also. Both enhancement and suppression of an antibody response by pertussigen has been reported.[45] Our findings that the detergent-treated fraction 15A-108A had decreased adjuvancy activity supports a role for endotoxin and possibly endotoxin-associated proteins in the adjuvancy phenomenon.

The development of a relatively inexpensive acellular *B. pertussis* vaccine with decreased side reactions represents a solution to many of the current problems encountered with the clinical usage of BPV. One step toward this goal is the preparation of an acellular vaccine without the deleterious immunomodulatory activities of whole cell BPV. Mechanisms involved in immunomodulation by BPV have been defined, but the overall activities of these mechanisms in the host-parasite relationship remains unclear.

We have demonstrated that an acellular fraction of BPV designated 15A-1B elicited several of the immunomodulatory reactions associated with BPV. Detergent treatment of this fraction resulted in a preparation with reduced leukocytosis and histamine sensitization associated with pertussis toxin and reduced splenomegaly and adjuvant activity associated with lipoplysaccharide, yet the preparation retained protective activity against *B. pertussis* infection of the mouse. A portion of the decreased pertussis toxin activity and the mouse protective activity of 15A-108 may be due to adsorption of 15A-108A to aluminum hydroxide gel. Fraction 15A-108A retained the antiviral activity associated with *B. pertussis* also.[46,47] Emulphogene treatment provides a relatively simple method for removing endotoxin from a potential acellular *B. pertussis* vaccine.

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Investigators:

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Dr. John D. Clements
Department of Microbiology
and Immunology
Tulane University Medical Center
1430 Tulane Avenue
New Orleans, LA 70112

Francis A. Ennis, M.D.
Department of Medicine
University of Massachusetts
Medical School
55 Lake Avenue
Worcester, MA 01605

Dr. Matthew J. Kluger
Department of Physiology
University of Michigan Med. School
7620 Medical Science II Bldg.
Ann Arbor, MI 48109

Dr. James M. Krueger
Department of Physiology
University of Tennessee
894 Union Street
Memphis, TN 38163

Dr. Roger M. Loria
Dept. of Microbiology and Immunology
Virginia Commonwealth University
Box 678, MCV Station
Richmond, VA 23298-0001

Dr. Robert I. Mishell
Dept. of Microbiology
and Immunology
Univ. of California, Berkeley
Berkeley, CA 94720

James J. Mond, M.D.
Department of Medicine
Uniformed Services University
of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814

Dr. Page S. Morahan
Department of Microbiology
Medical College of Pennsylvania
3300 Henry Avenue
Philadelphia, PA 19129

David A. Stevens, M.D.
Department of Medicine
Santa Clara Valley Medical Center
Stanford University
751 S. Bascom Avenue
San Jose, CA 95128

Dr. Barnet M. Sultzer
Department of Microbiology
and Immunology
Downstate Medical Center
450 Clarkson Avenue
Brooklyn, NY 11203

G. Jeanette Thorbecke, M.D.
Department of Pathology
New York University School of Medicine
550 First Avenue
New York, NY 10016

Dr. Alvin L. Winters
Department of Microbiology
University of Alabama
University, AL 35486

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Administrators:

Dr. Jeannine A. Majde, Code 1141SB
Scientific Officer, Immunology Program
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

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Defense Technical Information Center
Building 5, Cameron Station
Alexandria, VA 22314

Thomas A. Bryant
Administrative Contracting Officer
ONR Resident Representative
Georgia Institute of Technology
206 O'Keefe Building
Atlanta, GA 30332-0490

Project Manager, Life Science Technology
Office of Naval Research, Code 125
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