(U)
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

REPORT SECURITY CLASSIFICATION (U)	1b RESTRICTIVE MARKINGS (U)
SECURITY CLASSIFICATION AUTHORITY NA	3 DISTRIBUTION / AVAILABILITY OF REPORT Distribution Unlimited
DECLASSIFICATION / DOWNGRADING SCHEDULE NA	
PERFORMING ORGANIZATION REPORT NUMBER(S) University of Alabama #2	5 MONITORING ORGANIZATION REPORT NUMBER(S) NA
NAME OF PERFORMING ORGANIZATION University of Alabama 6b OFFICE SYMBOL (If applicable) NA	7a NAME OF MONITORING ORGANIZATION Office of Naval Research
ADDRESS (City, State, and ZIP Code) Department of Microbiology Box BM University, AL 35486	7b ADDRESS (City, State, and ZIP Code) 800 N. Quincy St. Arlington, VA 22217-5000
NAME OF FUNDING SPONSORING ORGANIZATION Office of Naval Research 8b OFFICE SYMBOL (If applicable) ONR	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-83-K-0597
8c ADDRESS (City, State, and ZIP Code) 800 N. Quincy St. Arlington, VA 22217-5000	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 61153N PROJECT NO: RR04108 TASK NO: NR 666-028 WORK UNIT ACCESSION NO:

11 TITLE (Include Security Classification)
(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

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM 8/84 TO 8/85	12/17/85	13 plus reprint

16 SUPPLEMENTARY NOTATION

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Key Words: antiviral agent, Bordetella pertussis, lipopoly-saccharide, endotoxin, endotoxin-associated proteins, Boivin antigen, immunomodulation, two dimensional polyacrylamide
08			

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Administration of Bordetella pertussis vaccine intraperitoneally induces resistance to mouse adenovirus infection. Subcellular fractions of B. pertussis are capable of inducing resistance also. Boivin antigen, a trichloroacetic acid-soluble extract of whole cells, is capable of inducing resistance in the dose range of 0.2-2.0 ug when adsorbed to alum. Biochemical analysis of Boivin antigen indicated that the lipopolysaccharide and protein components were not complexed, but were co-extracted. The lipopolysaccharide (endotoxin) moiety was responsible for the antiviral activity and the endotoxin-associated proteins potentiated the antiviral effect.

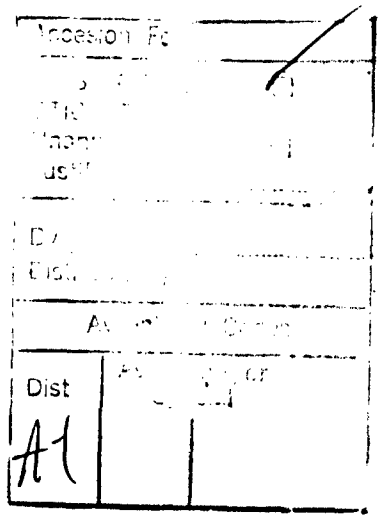
20 DISTRIBUTION / AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	(U) A

22a NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Dr. J. A. Majde	202-696-4055	ONR

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted
All other editions are obsolete

SECURITY CLASSIFICATION OF THIS PAGE

18. gel electrophoresis, Western Blot



1.0 BACKGROUND. Administration of Bordetella pertussis vaccine (BPV) intraperitoneally renders a mouse resistant to a subsequent lethal dose challenge of mouse adenovirus. A similar activity has been reported when type 1 herpes simplex virus was used as the challenging virus. During the first contract year we established that the antiviral activity of BPV was not associated with a given phase of B. pertussis, but was associated with a variety of phase I and phase IV B. pertussis strains (1, see attached reprint). Several subcellular fractions of B. pertussis were shown to have antiviral activity, i.e., a 1.0 M NaCl extract of whole cells (produced by Dr. R. D. Lennon, 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 (2).

2.0 IDENTIFICATION OF A BPV COMPONENT WITH ANTIVIRAL ACTIVITY. Drs. Barnet Sultzer, John Craig, and R. Castagna, Downstate Medical Center, SUNY, Brooklyn, NY, reported a marked adjuvancy activity following treatment of mice with endotoxin-associated proteins (EP) derived from B. pertussis (3). EP is derived from Boivin antigen, a trichloroacetic acid soluble LPS-rich extract of whole cells. We extracted Boivin antigen from B. pertussis cells and observed antiviral activity. The EP were co-extracted with LPS, but not complexed with LPS. LPS alone induced antiviral activity, but the presence of EP potentiated the activity tenfold or more. Hereafter, Boivin antigen will be designated as EP-LPS to indicate that the antigen contains both lipopolypolysaccharide and endotoxin-associated proteins.

EP-LPS was extracted in the following manner. B. pertussis cells, strain 10536, were obtained from Dr. Lance Gordon, Connaught Research Institute, Ontario, Canada. Trichloroacetic acid was added to obtain a final concentration of 2.5 % and incubated at 4°C for 3 h. Cell debris was removed by centrifugation at 4,000 x g for 30 min. The supernatant was decanted and the cells were extracted twice with 2.5 % trichloroacetic acid. The supernatants, bright yellow in color, were pooled and filtered through Whatman No. 40 filter paper to remove floating debris, and dialyzed (molecular weight cutoff 12,000-14,000) against chilled tap water overnight. The dialyzed supernatants were concentrated approximately tenfold by flash evaporation. A slight precipitate developed and was removed by centrifugation at 4,000 x g. EP-LPS was precipitated by addition of ethanol (68% final concentration) and incubation overnight at -18°C. The precipitate was sedimented by centrifugation at 4,000 x g for 30 min and washed with 95% ethanol. The sediment was resuspended in endotoxin-free water and dialyzed against distilled water overnight. The solution was frozen and lyophilized. The resulting light brown crystalline powder (EP-LPS) was stored at -18°C.

The EP-LPS antigen contained 1-10 mg endotoxin per mg (dry weight) as measured by the Limulus amoebocyte assay and 680 ug of protein per mg (dry weight) as measured by the Lowry method. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) followed by fixation and staining with PAGE blue 83 revealed seven major polypeptides (62K, 57K, 44K, 39K, 34K, 23.5K, and 18K) and 2 minor polypeptides (90K and 32K). Silver staining of gels by the method of Hitchcock and Brown (4) revealed two species of LPS similar to other B. pertussis strains (5).

Treatment of C3H/HeN mice with B. pertussis-derived EP-LPS antigen rendered the mice resistant to mouse adenovirus infection (Table 1). A dose of 20 ug induced resistance, whereas, a dose of 2.0 ug was not effective (Table 1).

Table 1. Antiviral Activity Associated with EP-LPS Extracted from Bordetella pertussis

Treatment*	Mortality (Deaths/Total)
EP-LPS, 20 ug	3/10
EP-LPS, 2.0 ug	10/10
Water (endotoxin-free)	9/10

* EP-LPS was suspended (1.0 mg/ml) in endotoxin-free water and diluted tenfold in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally seven days prior to intraperitoneal inoculation of mouse adenovirus. Experiments were terminated 21 days after infection.

Previous observations obtained with a subcellular fraction of B. pertussis prepared by Dr. Robert Lennon, Connaught Laboratories, (1.0 M NaCl extraction, ammonium sulfate precipitation, and Emulphogene treatment) indicated that lower doses of a subcellular fraction (12 ug) could effect resistance against a virus challenge (2). Emulphogene treatment to reduce the LPS content resulted in a hydrophobic, insoluble precipitate; therefore, Dr. Lennon's subcellular fraction was adsorbed to alum for administration to test animals. The slow release of Dr. Lennon's fraction from the alum might have increased the potency of the fraction. We adsorbed EP-LPS antigen to the same alum preparation (Alhydrogel) and administered the complex i.p. seven days before challenge with a lethal dose of virus. An increase in potency of EP-LPS antigen was observed (Table 2). As little as 0.02 ug of the alum-adsorbed preparation induced resistance in a portion of the test population.

Table 2. Antiviral Activity Associated with EP-LPS Extracted from *Bordetella pertussis* and Adsorbed to Alum

Treatment*	Mortality (Deaths/Total)
Experiment 1:	
EP-LPS (20 ug) + Alum	0/10
EP-LPS (2.0 ug) + Alum	1/10
Alum (control)	9/9
Experiment 2:	
EP-LPS (2.0 ug) + Alum	1/10
EP-LPS (0.2 ug) + Alum	4/10
EP-LPS (0.02 ug) + Alum	6/10
Alum (control)	8/10

*EP-LPS was suspended (1.0 mg/ml) in Alhydrogel, E.M. Sergeant Pulp and Chemical Co., Inc., Hoboken, N.J., and diluted to the appropriate concentration in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally 7 days prior to intraperitoneal inoculation of the mouse adenovirus. Experiments were terminated 21 days after infection.

Dr. Bernet Sultzer separated EP-LPS into EP and LPS using phenol-water precipitation of the proteins. Antiviral activity was retained in the LPS fraction, but not in the EP fraction (Table 3). Twenty micrograms of EP adsorbed to alum induced resistance in only a small portion of the test population; whereas 20 ug of LPS induced resistance in most of the test population. A second experiment with 20 ug of LPS did not demonstrate a great degree of protection; thus a 20 ug dose of LPS may be near the dilution endpoint. Additional testing near the 20 ug dose of LPS is planned to confirm the dilution endpoint of LPS.

Table 3. Antiviral Activity Associated with Endotoxin-associated Proteins (EP) or Lipopolysaccharide (LPS) Derived from Bordetella pertussis EP-LPS and Adsorbed to Alum

Treatment*	Mortality (Deaths/Total)
Experiment 1:	
EP (20 ug) + Alum	9/10
LPS (20 ug) + Alum	1/10
Alum	8/10
Experiment 2:	
LPS (20 ug) + Alum	9/10
LPS (2.0 ug) + Alum	11/11
LPS (0.2 ug) + Alum	10/10
Alum	10/10

*EP or LPS was suspended (1.0 mg/ml) in Alhydrogel, E.M. Sergeant Pulp and Chemical Co., Inc., Hoboken, N.J., and diluted to the appropriate concentration in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally 7 days prior to intraperitoneal inoculation of the mouse adenovirus. Experiments were terminated 21 days after infection.

Adjuvancy activity alone did not account for the antiviral activity. Dr. Barnet Sultzer observed that the EP extracted from EP-LPS possessed immunomodulatory activities associated with adjuvancy, i.e., both polyclonal activation and mitogenicity. In addition, we have examined a gliding bacteria adjuvant (GBA) for antiviral activity. GBA, provided by Dr. William R. Wainger, University of California, Berkley, CA, has notable adjuvancy activity both *in vitro* and *in vivo* (6). The complex polysaccharide is active in picomolar concentrations, stimulates the production of macrophage-derived hormones, IL-2 colony stimulating factor, and tumor necrotizing factor, and stimulates B cell proliferation and immunoglobulin secretion; however, the polysaccharide failed to induce resistance to mouse adenovirus under our test conditions (Table 4). We plan to test higher concentrations of GBA (20-200 ug) for antiviral activity.

Table 4. Lack of Antiviral Activity Associated with Gliding Bacteria Adjuvant Derived from Cytophaga

Treatment*	Mortality (Deaths/Total)
GBA (20 ug) + Alum	10/10
GBA (2.0 ug) + Alum	9/9
GBA (0.2 ug) + Alum	10/10
Alum	9/10

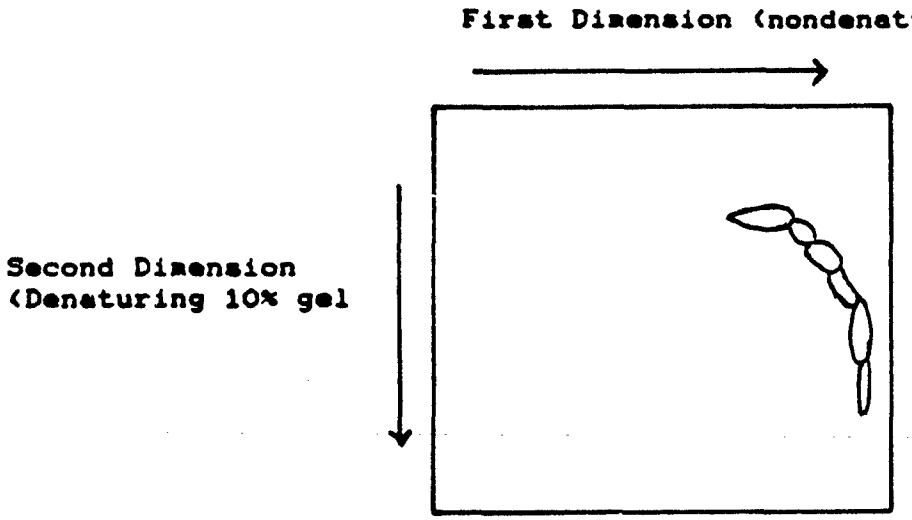
* GBA was suspended (1.0 mg/ml) in Alhydrogel, E.M. Sergeant Pulp and Chemical Co., Inc., Hoboken, N.J., and diluted to the appropriate concentration in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally 7 days prior to intraperitoneal inoculation of the mouse adenovirus. Experiments were terminated 21 days after infection.

It appears that the EP in EP-LPS potentiates the antiviral activity of LPS approximately 10- to 100-fold. We are currently examining the activity of both EP and GBA in reconstruction experiments to determine whether the interaction of these adjuvants with B pertussis-derived LPS will potentiate the antiviral activity.

We have examined EP-LPS to determine whether a unique complex of LPS and protein existed. EP-LPS was electrophoresed in a two-dimensional gel. The first dimension consisted of nondenaturing conditions in a cylindrical 5% acrylamide gel and discontiuous buffers (stacking gel, pH 6.8; resolving gel pH 8.8) to separate the proteins of EP-LPS by charge and not by size (7). After a short equilibration of the first dimension cylindrical gel in SDS-buffer (pH 6.8) the proteins were then electrophoresed in a second dimension consisting of denaturing conditions in a slab 10% acrylamide gel and SDS-buffer (pH 8.8) to separate the proteins by molecular size (8). If EP-LPS existed as a complex then the proteins would be detected in a vertical line parallel with the direction of electrophoresis in the second dimension. PAGE Blue 83 staining of the two dimensional gel revealed the proteins of EP-LPS in the righthand side of the gel and most of the proteins were not oriented in a straight line parallel to the direction of electrophoresis in a denaturing SDS gel (Figure 1). Under the conditions use for electrophoresis the proteins of EP-LPS do not appear to be extracted as a complex.

Figure 1. Two Dimensional Gel Electrophoresis of EP-LPS followed by Protein Stain

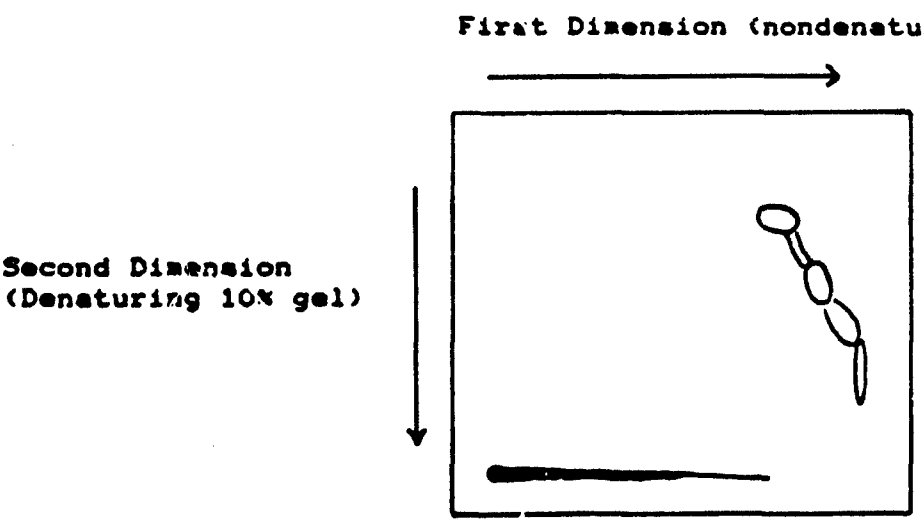
---



Silver staining of LPS followed by PAGE Blue 83 staining revealed the LPS of EP-LPS in the lower lefthand corner of a two dimensional gel and the LPS was well-separated from the proteins (Figure 2).

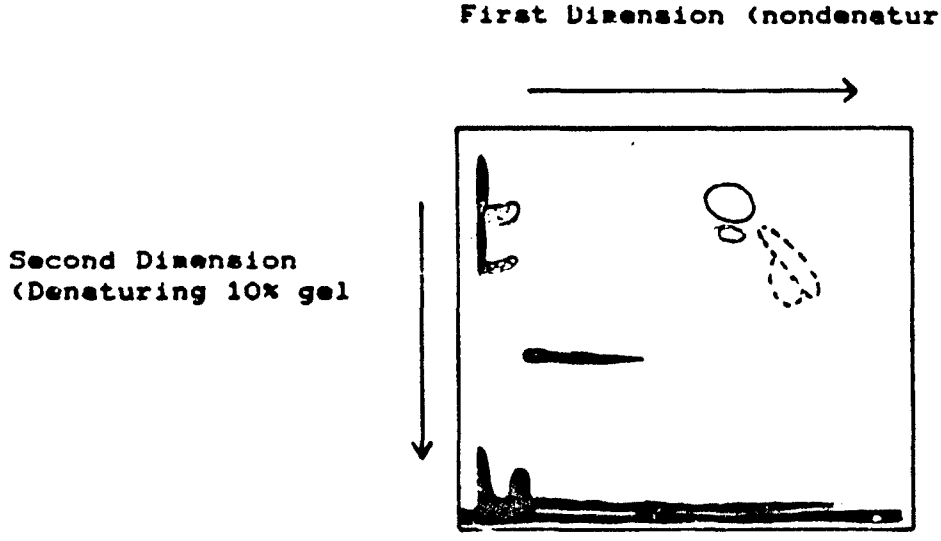
Two Dimensional Gel Electrophoresis of EP-LPS followed by Silver Staining and Protein Staining

---



EP-LPS was separated by two dimensional electrophoresis and transferred to nitrocellulose paper by electrophoretic transfer at a pH of 8.3 (9). The nitrocellulose paper was blocked with 10% nonfat dry milk. The blocked paper was then incubated with rabbit antiserum raised against BPV (Connaught Laboratories, Inc.), washed, and incubated with goat anti-rabbit IgG conjugated with horse radish peroxidase. The immunoblot was developed with 4-chloro-1-naphthol and hydrogen peroxide (10). LPS was the immunodominant antigen in EP-LPS. As observed with the silver stain, the two species of B. pertussis LPS was observed in the lower lefthand corner of the two dimensional gel and well separated from the proteins (Figure 3). A single immunodominant protein may have been complexed with LPS during the extraction. This protein migrate poorly in the first dimension and was marginally visible on the PAGE Blue 83 stained two dimensional gels. Taken together, these data indicate that EP-LPS is probably not extracted as a complex, but the components are co-extracted in the 2.5% trichloroacetic acid. Additional two dimensional gels will be silver stained for protein to ascertain the number and characteristics of minor proteins in the EP-LPS extract. We also will examine the purified LPS by Western blot analysis to determine whether the single immunodominant protein observed in EP-LPS was removed by phenol-water precipitation.

Figure 3. Two Dimensional Gel Electrophoresis of EP-LPS followed by Electrophoretic Transfer To Nitrocellulose Paper and Staining with Enzyme-linked Antibody to BPV (Western Blot).



LPS, derived from strains 3779 BL_2S_4 (2) and 10536 and purified by two different methods, elicits antiviral activity in C3H/HeN mice. These observations support a hypothesis that LPS plays an important, if not a singular role, in the antiviral activity associated with B. pertussis. Using purified LPS we can now begin to examine in detail the immunomodulatory mechanisms that develop following administration of LPS and hopefully associate given mechanisms with the virus resistant state. In addition we can begin to assess the role of adjuvants in this phenomenon.

4.0 REFERENCES

1. Winters AL, Baggett DW, Benjamin WR, Brown HK, Klein TW: Resistance to adenovirus infection after administration of Bordetella pertussis vaccine in mice. Infect Immun 47:587-591, 1985.

2. Winters AL, Baggett DW, Lee JD, Sloan GL, Lemmon RD, Stinson RS: Immunomodulation by Bordetella pertussis: Antiviral effects. (in press). In CR Manclark and W Hennessen (eds). Proceedings of the Fourth International Symposium on Pertussis. S. Karger, NY, 1985.

3. Sultzer BM, Craig JP, Castagna R: The adjuvant effect of pertussis endotoxin protein in modulating the immune response to cholera toxoid in mice. (in press). In CR Manclark and W Hennessen (eds). Proceedings of the Fourth International Symposium on Pertussis. S. Karger, NY, 1985.

4. Hitchcock PJ, Brown TM: Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J Bacteriol 154:269-277, 1983.

5. Peppler MS: Two physically and serologically distinct lipopolysaccharide profiles in strains of Bordetella pertussis and their phenotype variants. Inf Immun 43:224-232, 1984.

6. Usinger WR, Mishell RI: Murine macrophages specifically recognize a polysaccharide polymer from Cytophaga that is an immunological adjuvant. Fed Proc 44: 598 (Abst 1097), 1985.

7. Reisfeld RA, Lewis UJ, Williams DE: Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195:281-283, 1962.

8. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

9. Burnette WN: "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195-203, 1981.

10. Hawkes R, Niday E, Gordon J: A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 119: 142-147, 1982.

5.0 PUBLICATIONS/PRESENTATIONS RELATED TO THIS PROJECT.

Winters AL, Baggett DW, Benjamin WR, Brown HK, Klein TW: Resistance to adenovirus infection after administration of Bordetella pertussis vaccine in mice. Infect Immun 47:587-591, 1985.

Winters AL, Baggett DW, Lee JD, Sloan GL, Lemmon RD, Stinson RS: Immunomodulation by Bordetella pertussis: Antiviral effects. (in press). In CR Manclark and W Hennessen (eds). Proceedings of the Fourth International Symposium on Pertussis. S. Karger, NY, 1985.

Stinson RS, Lemmon RD, Baggett DW, Huff TL, Malone JA, Sloan GL, Winters AL: Removal of lipopolysaccharide from acellular Bordetella pertussis vaccine by detergent treatment. J Biol Standard (submitted and returned for revision).

Winter AL, Baggett DW, Lee JD, Sloan GL, Lemmon RD, Stinson RS: Immunomodulation by Bordetella pertussis: Antiviral effects. Fourth International Symposium on Pertussis, Geneva, Switzerland, September 25-27, 1984.

Baggett DW, LeBlanc PA, Winters AL: Antiviral activity of Bordetella pertussis: Vesicular stomatitis virus. Charter Conference of the Inter-American Society for Chemotherapy, Inc., St. Petersburg, FL, December 9-12, 1984.

INFECTION AND IMMUNITY, Mar. 1985, p. 587-591
0019-9567/85/030587-05$02.00/0

Vol. 47, No. 3

Resistance to Adenovirus Infection After Administration of *Bordetella pertussis* Vaccine in Mice

ALVIN L. WINTERS,[1]* DAVID W. BAGGETT,[1] WILLIAM R. BENJAMIN,[2]† H. KEITH BROWN,[3] AND THOMAS W. KLEIN[2]

Department of Microbiology, University of Alabama, University, Alabama 35486,[1] and Departments of Medical Microbiology and Immunology[2] and Anatomy,[3] University of South Florida, Tampa, Florida 33612

Received 27 August 1984/Accepted 13 November 1984

Treatment of mice with *Bordetella pertussis* vaccine rendered mice resistant to mouse adenovirus infection. The resistant state took at least 5 days to develop, and susceptibility returned to a portion of the test population 35 days after treatment. Transient resistance developed in congenitally athymic mice also. Treatment with a dose of 25 μg (dry weight) of *B. pertussis* vaccine protected approximately 50% of the test population. Vaccines prepared from several different strains of *B. pertussis* were capable of inducing resistance, and the induction of resistance was not dependent on the mouse strain used for testing. Cross-reacting antibodies capable of neutralizing the virus or protecting against a challenging infection were not induced by treatment with *B. pertussis* vaccine.

A variety of immunomodulatory activities have been associated with *Bordetella pertussis* vaccine (BPV) (14, 17, 19, 20). Acellular components extracted from the *B. pertussis* cell can mimic some of these immunomodulatory activities (3, 4, 7, 8, 18, 22, 23). Treatment of mice with BPV or components extracted from *B. pertussis* can modify the pathogenesis of virus infections. Increased susceptibility to intranasal influenza virus challenge was observed 5 to 7 days after intraperitoneal (i.p.) injection of BPV (21). Increased resistance to intracranial rabies virus challenge was observed when an extract of *B. pertussis* was administered by the subcutaneous, intravenous, or i.p. route at the same time as the virus (19). Increased resistance to i.p. herpes simplex virus challenge was observed 7 days after i.p. injection of BPV; however, resistance was not observed when BPV was administered 3 days before virus challenge (13). Several acellular fractions of *B. pertussis*, namely, lipopolysaccharide, glycolipid, lipid A, or lipid X, induce a state of resistance to an i.p. challenge of encephalomyocarditis virus or a subcutaneous challenge of Semliki Forest virus when the *B. pertussis*-derived fractions were administered i.p. 24 h previously (1, 3). Discussions with the late Charles W. Fishel concerning the immunomodulatory activities of *B. pertussis* and the reports of antiviral activity of BPV suggested that BPV might modulate mouse adenovirus infection by a plaque-type variant of mouse adenovirus strain FL. The plaque-type variant strain, designated MAd1*pt*4, induces an interstitial pneumonia and death (25). Increased resistance to an i.p. challenge of mouse adenovirus was observed when BPV was injected i.p. 7 days before virus challenge (14). This report extends the characterization of BPV-induced resistance to mouse adenovirus infection.

MATERIALS AND METHODS

Animals. Female mice, strain BDF_1/Cox (C57Bl/6 Cox × DBA/2 Cox) were obtained from Laboratory Supply Co., Indianapolis, Ind. A colony of C3H/HeN (mammary tumor virus positive [MTV^+]) mice with the nude gene mutation was obtained from Carl Hansen, National Institutes of Health, Bethesda, Md., and bred and maintained at the University of South Florida, Tampa, Fla., as described previously (24). Male mice heterozygous for the nude gene and female mice homozygous for the nude gene were used in this study. A colony of C3H/HeN (MTV^-) mice was obtained from Carl Hansen and bred and maintained at the University of Alabama, University. Both male and female mice were used in this study.

Vaccines. BPV was provided by Connaught Laboratories, Swiftwater, Pa., and was adjusted to approximately 4.0 mg (dry weight) per ml in saline-thimerosal diluent (0.15 M NaCl in 0.02% thimerosal). Vaccines were made also from *B. pertussis* strains 18323 (James L. Cowell, Food and Drug Administration, Bethesda, Md.) and Tohama I (Stanley Falkow, Stanford University, Stanford, Calif.). The organisms were maintained on BG agar base (Difco Laboratories, Detroit, Mich.) supplemented with 17% defibrinated sheep blood. Vaccines were made by harvesting 4-day growth from Cohen-Wheeler agar 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 4.0 mg (dry weight) in saline-thimerosal diluent and stored at 4°C.

Virus. MAd1*pt*4 was propagated in L cells (NCTC clone 929; American Type Culture Collection, Rockville, Md.) The virus titer of infecting stocks was determined by a plaque assay (24).

Leukocytosis. Mice were bled from the retroorbital plexus with heparinized capillary tubes. Samples (10 μl) of peripheral blood from each mouse were placed into tubes containing 490 μl of Turk solution (9), mixed, and counted with a hemocytometer.

Electron microscopy. Cells obtained by peritoneal lavage were sedimented by centrifugation at 450 × *g* for 10 min. The cell pellet was covered with fixative (2% paraformaldehyde–3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3) and postfixed with osmium tetroxide. After the cell pellet was embedded in epon, ultrathin sections (60 to 70 nm) were stained as previously described (24).

* Corresponding author.
† Present address: Hoffmann-LaRoche, Inc., Nutley, N.J. 07110.

TABLE 1. Induction of resistance to MAd1*pt*4 challenge by BPV administration

Mouse strain	Treatment[a]	Virus dose (PFU)	Mortality (deaths/ total)
C3H/HeN (+/*nu*) (MTV$^+$)	Diluent	1.0×10^8	25/25
C3H/HeN (+/*nu*) (MTV$^-$)	BPV (Connaught)	1.0×10^8	0/20
BDF_1	Diluent	1.0×10^8	20/20
BDF_1	BPV (Connaught)	1.0×10^8	0/20
C3H/HeN (MTV$^-$)	Diluent	2.2×10^7	21/21
C3H/HeN (MTV$^-$)	BPV (Connaught)	2.2×10^7	0/21
C3H/HeN (MTV$^-$)	Diluent	2.8×10^7	10/10
C3H/HeN (MTV$^-$)	BPV (13323)	2.8×10^7	0/10
C3H/HeN (MTV$^-$)	Diluent	2.8×10^7	5/5
C3H/HeN (MTV$^-$)	BPV (Tohama I)	2.8×10^7	0/5

[a] Animals were injected i.p. with either saline-thimerosal diluent or 250 μg (dry weight) of BPV 7 days before i.p. virus challenge.

TABLE 3. Kinetics of BPV-induced resistance to MAd1*pt*4

Expt	Mouse strain	Time of treatment[a] (days)	Virus dose (PFU)	Mortality (deaths/ total)
1	BDF_1	0.02	1.0×10^8	5/5
	BDF_1	7	1.0×10^8	0/10
	BDF_1	14	1.0×10^8	0/10
	BDF_1	21	1.0×10^8	0/10
	BDF_1	35	1.0×10^8	4/10
2	C3H/HeN (MTV$^-$)	1	2.1×10^7	5/5
	C3H/HeN (MTV$^-$)	2	2.1×10^7	5/5
	C3H/HeN (MTV$^-$)	3	2.1×10^7	5/5
	C3H/HeN (MTV$^-$)	4	2.1×10^7	5/5
	C3H/HeN (MTV$^-$)	5	2.1×10^7	0/5
	C3H/HeN (MTV$^-$)	6	2.1×10^7	0/5
	C3H/HeN (MTV$^-$)	7	2.1×10^7	0/5

[a] Mice were injected i.p. with 250 μg (dry weight) of BPV (Connaught Laboratories) and challenged with virus at the designated times of treatment. Both experiments included diluent injected groups of mice as controls; the control groups exhibited 100% mortality.

RESULTS

Induction of resistance. Treatment of mice with BPV induced resistance to an MAd1*pt*4 challenge (Table 1). Animals were injected i.p. with BPV; 7 days later, lethal doses of MAd1*pt*4 were administered. The animals were observed thereafter for clinical disease and death accompanied by hemorrhagic lungs at necropsy. Treatment of mice with BPV induced a resistant state to as much as 20 50% lethal doses of the virus. The treatment protected the mice also from overt clinical signs of disease, such as hunched back, ruffled fur, marked inactivity, and huddling. The long-term survival of mice after BPV treatment and MAd1*pt*4 infection was at least 65 days. The longest duration of disease and death observed after MAd1*pt*4 infection was 11 days; therefore, experiments were scored 21 days after infection. The 50% effective dose of BPV was approximately 25 μg (dry weight) or approximately 1.2 mg/kg of body weight (Table 2).

The kinetics of the induction of the resistant state suggest that BPV was not interacting directly with the virus. The resistant state did not develop immediately after administration of the vaccine, but was observed initially 5 days after BPV treatment (Table 3). The resistant state was transient, and susceptibility returned to a portion of the test population 35 days after BPV treatment.

A functioning thymus was not necessary for induction of the resistant state. Congenitally athymic mice were very susceptible to MAd1*pt*4 infection (Table 4). The kinetics of disease and death after high-dose virus infection was similar in both thymus-bearing and athymic animals. Infection with low doses of the virus produced no overt disease or death in thymus-bearing animals, whereas low-dose infection of athymic animals produced a chronic wasting disease and death with hemorrhagic lungs after an extended period of time. A transient BPV-induced resistant state was observed also in congenitally athymic mice. In contrast to the thymus-bearing animals, the athymic animals succumbed to high-dose infection and disease after the resistant state decayed (Table 4).

TABLE 2. Dose response of BPV-induced resistance to MAd1*pt*4 in BDF_1 mice

Treatment[a]	Mortality (deaths/ total)
Diluent	10/10
2.5 μg of BPV	10/10
12.5 μg of BPV	10/10
25 μg of BPV	19/47
125 μg of BPV	1/10
250 μg of BPV	0/10

[a] Mice were injected i.p. with the designated amounts (dry weight) of BPV (Connaught Laboratories) 7 days before i.p. virus challenge with 1.0×10^8 PFU.

TABLE 4. Dose response of MAd1*pt*4 in normal, athymic, and BPV-treated athymic mice

Mouse strain	Virus challenge (PFU)	Mortality (deaths/ total)	Time of death (days)
BDF_1	1.0×10^8	10/10	3, 3, 3, 3, 3, 3, 3, 3, 3, 3
BDF_1	1.0×10^7	10/10	3, 3, 3, 3, 3, 3, 3, 3, 4, 4
BDF_1	1.0×10^6	2/10	3, 3
C3H/HeN (+/*nu*)	1.0×10^8	15/15	3, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 5, 5, 5
C3H/HeN (+/*nu*)	1.0×10^7	8/10	6, 8, 8, 8, 8, 10, 10, 10
C3H/HeN (+/*nu*)	1.0×10^6	1/10	9
C3H/HeN (*nu*/*nu*)	1.0×10^8	5/5	5, 5, 5, 5, 6
C3H/HeN (*nu*/*nu*)	1.0×10^7	5/5	8, 10, 16, 21, 33
C3H/HeN (*nu*/*nu*)	1.0×10^6	5/5	18, 18, 22, 26, 31
C3H/HeN (*nu*/*nu*)	1.0×10^5	4/5[a]	35, 38, 38, 38
C3H/HeN (*nu*/*nu*)	1.0×10^4	5/5	26, 30, 33, 35, 39
C3H/HeN (*nu*/*nu*)	1.0×10^3	5/5	25, 29, 33, 35, 36
C3H/HeN (*nu*/*nu*)	1.0×10^2	5/5	33, 33, 36, 36, 45
BPV-treated C3H/HeN (*nu*/*nu*)[b]	1.0×10^8	15/15	7, 11, 37, 38, 39, 40, 43, 43, 46, 46, 48, 51, 54, 54, 63
Diluent-treated C3H/HeN (*nu*/*nu*)[b]	1.0×10^8	15/15	4, 4, 4, 4, 5, 5, 5, 5, 5, 5, 5, 5, 6, 9

[a] The survivor was sacrificed by cervical dislocation 149 days after infection.

[b] Mice were injected with vaccine diluent or 250 μg (dry weight) of BPV (Connaught Laboratories) 7 days before virus challenge.

TABLE 5. Effect of heat treatment on the antiviral activity of BPV

Mouse strain	Treatment[a]	Mortality (deaths/total)
C3H/HeN (+/*nu*)	Diluent	15/15
C3H/HeN (+/*nu*)	BPV	0/10
C3H/HeN (+/*nu*)	Heated BPV[b]	0/15
BDF_1	Diluent	5/5
BDF_1	BPV	0/10
BDF_1	Heated BPV[b]	5/10

[a] Mice were injected i.p. with vaccine diluent or 250 μg (dry weight) of BPV (Connaught Laboratories) 7 days before virus challenge.
[b] BPV (Connaught Laboratories) was incubated at 80°C for 30 min before i.p. injection.

Treatment of BPV to inactivate pertussigen (80°C for 30 min) (19) did not affect resistance-inducing activity when assayed in C3H/HeN (+/*nu*) mice, but decreased activity when assayed in BDF_1 mice (Table 5). The heated vaccine failed to induce lymphocytosis in the peripheral blood of mice (Table 6).

Effects of treatment with BPV. High-dose MAd1*pt*4 infection did not effect a notable generalized immune response (splenomegaly) within 3 days of virus inoculation (Table 7). Treatment of mice with BPV induced a threefold increase in spleen size, and the splenomegaly was retained during 3 days of high-dose virus infection.

High-dose MAd1*pt*4 infection did not induce an observable increase in the number of peritoneal leukocytes 3 days after infection (Table 8). Similar to a previous study of Fishel and coworkers (5), BPV treatment effected a fivefold increase in peritoneal leukocyte number. The combination of BPV treatment and high-dose virus infection had a synergistic effect, and the peritoneal leukocyte number was increased approximately 10-fold. The activity of the BPV-induced peritoneal lavage cells increased markedly. Electron microscopy of diluent-treated peritoneal exudate cells 3 days after high-dose virus infection revealed numerous virus particles in the extracellular medium and in phagolysosomes (Fig. 1A). In contrast, BPV-treated peritoneal exudate 3 days after high-dose virus infection exhibited virtually no cells with virus particles (Fig. 1B).

Antibodies did not play a role in the development of the BPV-induced resistant state. Treatment of mice with BPV for 10 days did not induce MAd1*pt*4 neutralizing antibodies. Studies with monoclonal antibodies indicate that certain classes of antibodies do not neutralize, but will protect the test animal against a challenging virus infection (2, 15, 16). Treatment of mice with BPV for 21 days did not induce protective antibodies. Serum (0.5 ml) from the BPV-treated C3H/HeN (MTV^-) mice was injected i.p. into mice of the same strain and challenged with a high-dose virus inoculum 24 h later. Assuming a plasma volume of 1.1 ml (6), then potentially protective antibodies would have been diluted approximately threefold. A group of 10 mice treated in this manner succumbed to high-dose infection within 7 days.

TABLE 6. Effect of heat on lymphocytosis after BPV treatment of C3H/HeN (+/*nu*) mice

Treatment[a]	Peripheral blood leukocytes (cells/mm^3)	
	Mean	SD
Diluent	6,380	260
BPV	17,600	3,800
Heated BPV[b]	7,600	820

[a] Mice were injected i.p. with vaccine diluent or 250 μg (dry weight) of BPV (Connaught Laboratories) 4 days before bleeding.
[b] BPV (Connaught Laboratories) was incubated at 80°C for 30 min before i.p. injection.

TABLE 7. Effect of BPV treatment and MAd1*pt*4 infection on spleen weight[a]

Treatment		Avg spleen wt on day 10 (g)
Day 0	Day 7	
Diluent	Medium	0.090
Diluent	1.0×10^8 PFU	0.130
BPV	Medium	0.352
BPV	1.0×10^8 PFU	0.338

[a] Groups of three C3H/HeN (+/*nu*) mice were injected i.p. with vaccine diluent or 250 μg (dry weight) of BPV. Seven days later, the mice were injected i.p. with cell culture medium or virus stock. After 3 days the mice were sacrificed by cervical dislocation, and the spleens were removed, blotted to remove excess fluid, and weighed.

DISCUSSION

Mouse adenovirus can be added to the list of virus infections that are modulated by treatment of the test animal with *B. pertussis* or materials derived from the microorganism. Development of the resistant state was not dependent on the strain of the microorganism. Although the *B. pertussis* strain from Connaught Laboratories, Inc., and strain 18323 (12) have a common lineage, strain Tohama I was derived from a completely different source (10). Development of the resistant state also was independent of the strain of mouse used.

The kinetics of development and decay of the resistant state indicated that a relatively long-term modification of the immunological mechanisms occurred. The modification probably did not involve thymus-derived lymphocytes in its mechanism of development, since congenitally athymic mice responded to BPV treatment with resistance. However, thymus involvement in the BPV-induced resistance in the thymus-bearing animal cannot be ruled out due to possible compensatory immune functions operating in congenitally athymic mice, such as increased numbers of natural killer cells. Thymus-derived cytolytic lymphocytes are necessary for final clearance of certain virus infections (11); consequently, the mechanism of BPV-induced resistance in thy-

TABLE 8. Effect of BPV treatment and MAd1*pt*4 infection on peritoneal leukocytes[a]

Treatment		Leukocytes in Peritoneal Lavage (cells × 10^{-6} per ml)			
		Day 7		Day 10	
Day 0	Day 7	Mean	SD	Mean	SD
Diluent		0.9	0.1	1.0	0.2
BPV		5.2	0.8	5.5	0.5
Diluent	1.0×10^8 PFU			1.1	0.2
BPV	1.0×10^8 PFU			10.0	1.0

[a] Groups of six C3H/HeN (+/*nu*) mice were treated by i.p. inoculation of vaccine diluent or 250 μg (dry weight) of BPV. Seven days later three mice from each group were sacrificed by cervical dislocation, and 4.0 ml of Dulbecco modified minimal Eagle medium was injected into the peritoneum. The peritoneal wall was massaged, 3.5 ml of lavage was withdrawn, and the cells were counted. The remaining three mice in each group were inoculated with virus and sacrificed for lavage 3 days later.

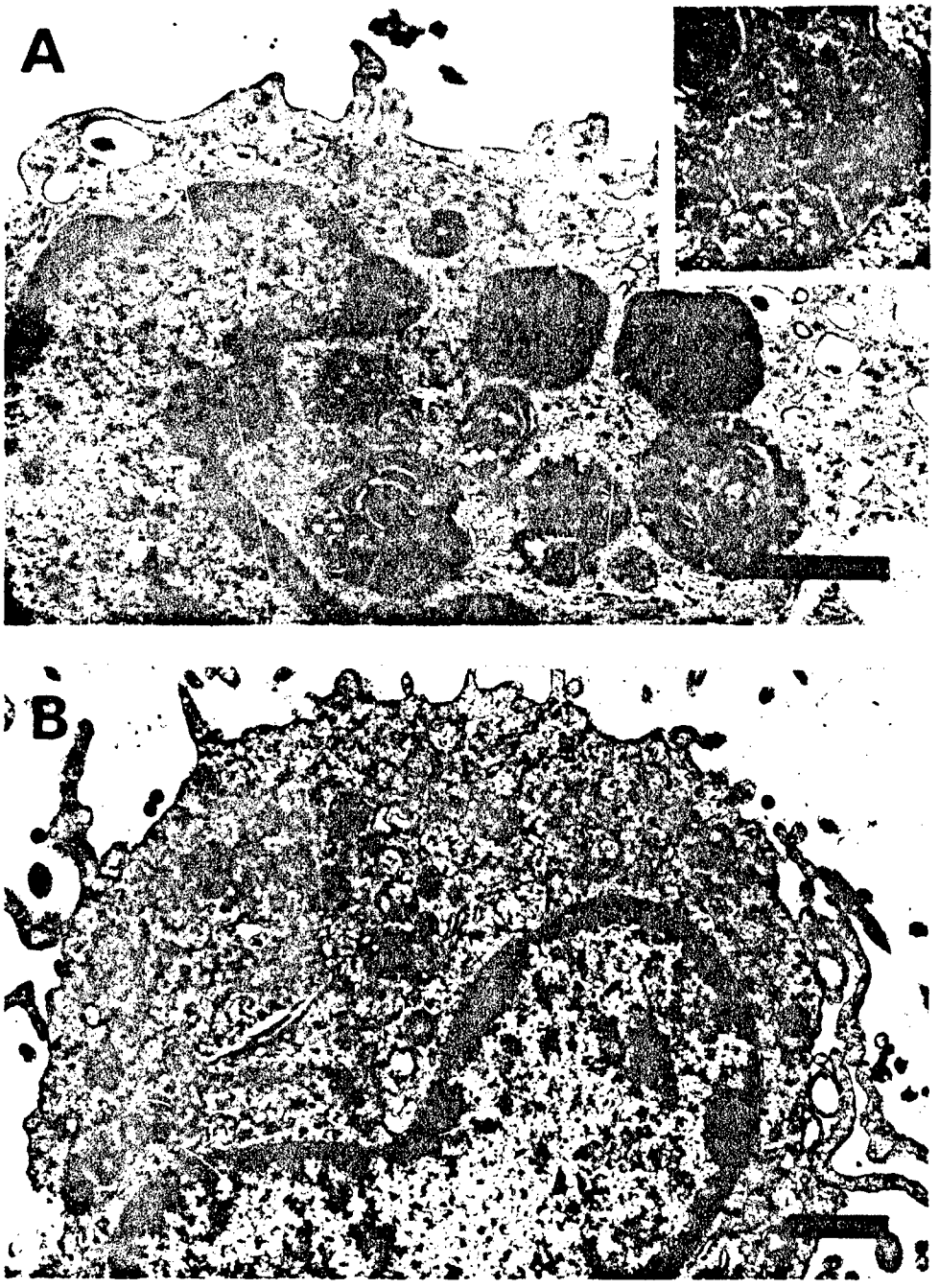


FIG. 1. Electron micrograph of ultrathin sections of peritoneal lavage cells from C3H/HeN (+/*nu*) mice. Bar, 1.0 μm. (A) Representative cell obtained after treatment with vaccine diluent (7 days) followed by MAd1*pt*4 infection (3 days). The inset shows an increased magnification of a phagolysosome containing virus particles. (B) Representative cell obtained after BPV treatment (7 days) followed by MAd1*pt*4 infection (3 days).

mus-bearing mice is probably an expression of retarded virus growth until the proper T-lymphocyte clones can develop. This conclusion is supported by the similarity of disease patterns in BPV-treated and low dose-infected athymic mice.

The immune response of mice appears to be overwhelmed by high-dose MAd1*pt*4 infection; death occurs before a systemic response in the form of splenomegaly, leukocytosis, and increase leukocytes in the peritoneum can develop. The treatment of mice with BPV could provide the mouse with a systemic response at the time of virus inoculation; however, one must consider that clinical signs of systemic response might not be involved in the resistant state. We have observed that acellular fractions of *B. pertussis* have decreased ability to induce splenomegaly and leukocytosis, yet retain resistance-inducing activity (R. S.

Stinson, J. D. Lee, L. Williamson, and A. Winters, Abstr. 19th Natl. Meet. Reticuloendothel. Soc. 1982, abstr. no. 56, p. 70). The mechanism of BPV-induced resistance markedly decreased virus particles from the inoculum or subsequent multiplication in peritoneal lavage cells obtained 3 days after infection (or both).

Study of the molecules or complex of molecules in *B. pertussis* that are responsible for induction of the virus-resistant state, and investigation of their mechanism of immunomodulation might lead to new methods for prevention and treatment of virus infections.

ACKNOWLEDGMENTS

This work was initiated at the Department of Medical Microbiology and Immunology, University of South Florida, Tampa. Support was provided by University of Alabama Research Committee projects no. 1116 and 1143, Public Health Service Biomedical research support grants NIH 5 S07 RR05749-05 and PHS 2 S07 RR07151-06 from the National Institutes of Health, Public Health Service Grant AI 15108-01 from the National Institutes of Allergy and Infectious Disease, and Office of Naval Research contract N00014-83-K-0597.

LITERATURE CITED

1. Ayme, G., M. Caroff, R. Chaby, N. Haeffner-Cavaillon, A. LeDur, M. Moreau, M. Muset, M.-C. Mynard, M. Roumiantzeff, D. Schulz, and L. Szabo. 1980. Biological activities of fragments derived from *Bordetella pertussis* endotoxin: isolation of a nontoxic, Shwartzman-negative lipid A possessing high adjuvant properties. Infect. Immun. 27:739–745.
2. Balachandran, N., S. Bacchetti, and W. E. Rawls. 1982. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. Infect. Immun. 37:1132–1137.
3. Chaby, R., G. Ayme, M. Caroff, R. Donikian, N. Haeffner-Cavaillon, A. Le Dur, M. Moreau, M.-C. Mynard, M. Roumiantzeff, and L. Szabo. 1979. Structural features and separation of some of the biological activities of the *Bordetella pertussis* endotoxin by chemical fractionation, p. 185–190. *In* C. R. Manclark and J. C. Hill (ed.), International symposium on pertussis. Department of Health, Education, and Welfare publication no. (NIH)79-1830. U.S. Government Printing Office, Washington, D.C.
4. Fish, F., J. L. Cowell, and C. R. Manclark. 1984. Proliferative response of immune mouse T-lymphocytes to the lymphocytosis-promoting factor of *Bordetella pertussis*. Infect. Immun. 44:1–6.
5. Fishel, C. W., D. G. Halkias, T. W. Klein, and A. Szentivanyi. 1976. Characterization of cells present in peritoneal fluids of mice injected intraperitoneally with *Bordetella pertussis*. Infect. Immun. 13:263–272.
6. Friedman, J. J. 1955. Circulating and tissue hematocrits of normal unanesthetized mice. Am. J. Physiol. 196:420–422.
7. Hewlett, E. L., C. O. Roberts, J. Wolff, and C. R. Manclark. 1983. Biphasic effect of pertussis vaccine on serum insulin in mice. Infect. Immun. 41:137–144.
8. Ho, M.-K., A. S. Kong, and S. I. Morse. 1980. The *in vitro* effects of *Bordetella pertussis* lymphocytosis-promoting factor on murine lymphocytes. V. Modulation of T cell proliferation by helper and suppressor lymphocytes. J. Immunol. 124: 362–369.
9. Jerne, N. K., C. Henry, A. A. Nordin, H. Fuji, A. M. C. Koros, and I. Lefkovits. 1976. Antibody synthesis *in vitro*. Methods Immunol. Immunochem. 5:303–374.
10. Kasuga, T., Y. Nakase, K. Ukishima, and K. Takatsu. 1954. Studies on *Haemophilus pertussis*. V. Relation between the phase of bacilli and the progress of the whooping-cough. Kitasato Arch. Exp. Med. 27:57–62.
11. Kees, U., and R. V. Blanden. 1976. A single genetic element in *H-2K* affects mouse T-cell antiviral function in poxvirus infection. J. Exp. Med. 143:450–455.
12. Kendrick, P. L., G. Eldering, M. K. Dixon, and J. Misner. 1947. Mouse protection tests in the study of pertussis vaccine: a comparative series using the intracerebral route for challenge. Am. J. Public Health 37:803–810.
13. Kirchner, H., M. T. Scott, H. M. Hirt, and K. Munk. 1978. Protection of mice against viral infection by *Corynebacterium parvum* and *Bordetella pertussis*. J. Gen. Virol. 41:97–104.
14. Klein, T., W. Benjamin, A. Winters, and H. Friedman. 1981. Pertussis induced immunomodulation, p. 275–292. *In* H. Friedman, T. W. Klein, and A. Szentivanyi (ed.), Immunomodulation by bacteria and their products. Plenum Publishing Corp., New York.
15. LeFrancois, L. 1984. Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action in vivo. J. Virol. 51:208–214.
16. Mathews, J. H., and J. T. Roehrig. 1982. Determination of the protective epitopes on the glycoproteins of Venezuelan equine encephalomyelitis virus by passive transfer of monoclonal antibodies. J. Immunol. 129:2763–2767.
17. Morse, S. I. 1976. Biologically active components and properties of *Bordetella pertussis*. Adv. Appl. Microbiol. 20:2–25.
18. Munoz, J. J., H. Arai, R. K. Bergman, and P. L. Sadowski. 1981. Biological activities of crystalline pertussigen from *Bordetella pertussis*. Infect. Immun. 33:820–826.
19. Munoz, J. J., and R. K. Bergman. 1977. *Bordetella pertussis*, immunological and other biological activities, vol. 4. Marcel Dekker, Inc., New York.
20. Munoz, J. J., and R. K. Bergman. 1979. Biological activities of *Bordetella pertussis*, p. 143–150. *In* C. R. Manclark and J. C. Hill (ed.), International symposium on pertussis. Department of Health, Education, and Welfare publication no. (NIH)79-1830. U.S. Government Printing Office, Washington, D.C.
21. Parfentjev, I. A. 1955. Bacterial allergy increases susceptibility to influenza virus in mice. Proc. Soc. Exp. Biol. Med. 90:373–375.
22. Sato, Y., J. L. Cowell, H. Sato, D. G. Burstyn, C. R. Manclark. 1983. Separation and purification of the hemagglutinins from *Bordetella pertussis*. Infect. Immun. 41:313–320.
23. Sugimoto, M., Y. Nakanishi, M. Otokawa, N. Uchida, T. Yasuda, H. Sato, and Y. Sato. 1983. Effect of *Bordetella pertussis* leukocytosis(lymphocytosis)-promoting factor (LPF) on the physical lymphoepithelial-cell association studied with the use of an *in vitro* model of mouse thymus. J. Immunol. 130:2767–2774.
24. Winters, A. L., and H. K. Brown. 1980. Duodenal lesions associated with adenovirus infection in athymic "nude" mice. Proc. Soc. Exp. Biol. Med. 164:280–286.
25. Winters, A. L., H. K. Brown, and J. K. Carlson. 1981. Interstitial pneumonia induced by a plaque-type variant of mouse adenovirus. Proc. Soc. Exp. Biol. Med. 167:359–364.

DISTRIBUTION LIST

Immunological Defense Program

Annual, Final and Technical Reports (one copy each except as noted)

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

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

Dr. Edward A. Havell
Trudeau Institute
P.O. Box 59
Saranac Lake, NY 12983

Dr. Arthur G. Johnson
Department of Medical
Microbiology and Immunology
University of Minnesota
School of Medicine
2205 East 5th Street
Duluth, MN 55812

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

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

Dr. Ernest D. Marquez
Bioassay Systems Corporation
225 Wildwood Avenue
Woburn, MA 01801

Dr. Robert I. Mishell
Department of Microbiology
and Immunology
University 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

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

Annual, Final and Technical Reports (one copy each except as noted)

Dr. Jeannine A. Majde, Code 441CB
Scientific Officer, Immunology Program
Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217

Administrator (2 copies) (Enclose DTIC Form 50)
Defense Technical Information Center
Building 5, Cameron Station
Alexandria, VA 22314

Annual and Final Reports Only (one copy each)

Commanding Officer
Naval Medical Command
Washington, DC 20372

Commanding Officer
Naval Medical Research & Development Command
National Naval Medical Center
Bethesda, MD 20814

Director, Infectious Diseases Program Center
Naval Medical Research Institute
National Naval Medical Center
Bethesda, MD 20814

Commander
Chemical and Biological Sciences Division
Army Research Office, P.O. Box 12211
Research Triangle Park, NC 27709

Commander
U.S. Army Research and Development Command
Attn: SGRD-PLA
Fort Detrick
Frederick, MD 21701

Commander
USAMRIID
Fort Detrick
Frederick, MD 21701

Directorate of Life Sciences
Air Force Office of Scientific Research
Bolling Air Force Base
Washington, DC 20332

Administrative Contracting Officer
ONR Resident Representative
(address varies - obtain from Business Office)

Final and Technical Reports Only

Director, Naval Research Laboratory (6 copies)
Attn: Technical Information Division, Code 2627
Washington, DC 20375