Isolation of Antigens of *Pasteurella pestis*

I. Lipopolysaccharide-Protein Complex and R and S Antigens

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ABSTRACT

LARRABEE, ALLAN R. (Fort Detrick, Frederick, Md.), JOHN D. MARSHALL, AND DAN CROZIER. Isolation of antigens of *Pasteurella pestis*. I. Lipopolysaccharide-protein complex and R and S antigens. *J. Bacteriol.* **90**:116-119, 1965.—*Pasteurella pestis* contains at least 18 different antigens, 2 of which will protect experimental animals from challenge infection. A specific polysaccharide isolated and described as a hapten was isolated as a complete antigen. Two additional antigens were isolated from *P. pestis*. The preparation of antisera directed against these three antigens and the content of protein, lipid, and carbohydrate of each preparation were studied. None of the preparations will protect mice from challenge infection with virulent *P. pestis*. A basis for naming the new antigens which does not conflict with previously published designations is presented.

Studies published by Lawton, Fukui, and Surgalla (1960) showed that *Pasteurella pestis* contains at least 18 different antigens. Two of these antigens, F₁ (Baker et al., 1952) and V (Burrows and Bacon, 1956) are immunogenic. Burrows (1963) reviewed the world literature as it pertains to knowledge of the antigenic properties of *P. pestis*.

Davies (1956) described a specific polysaccharide of *P. pestis* which was a nonantigenic lipopolysaccharide material differing from the gram-negative endotoxins by virtue of its non-toxicity. He prepared specific antiserum by conjugating the hapten with the protein component of the O somatic antigenic complex of *Shigella dysenteriae*.

The work reported here concerns the isolation and purification of a protein-lipopolysaccharide complex from *P. pestis* which is antigenic and is serologically related to the specific polysaccharide of Davies; the isolation and partial purification of two additional antigens are also reported. The designation of the two new antigens, R and S, will be discussed.

MATERIALS AND METHODS

Ouchterlony double-diffusion test. The gel diffusion test was performed according to the method of Lawton, Fukui, and Surgalla (1960). Reactions were read at 16, 24, 72, and 144 hr. The gel diffu-

sion test as read at 24 hr will be referred to as the standard test.

Dialysis and centrifugation. All antigen samples were dialyzed 16 to 18 hr against 14 liters of 0.01 M potassium phosphate buffer (pH 7.2) at 7°C or until the dialysate was free of salt. Precipitates were collected in a Lourdes centrifuge at a relative centrifugal force of 18,000 to 25,000 × *g* for 10 to 15 min. Protein measurements were based on the biuret reaction.

Hanging curtain electrophoresis. A Beckman model CP instrument was equilibrated with 0.01 M sodium acetate buffer (pH 4.7). The main reservoir buffer feed to the curtain was adjusted to a value of 15 ml/12 hr. A sample containing 15 mg of protein per ml was applied to the center tab of the curtain at a rate of 0.25 ml/hr. A current of 500 v and 13 to 16 ma resulted in optimal separation. Use of a Beckman model CPF-2 fraction collector allowed continuous runs of 2 to 3 days. Fraction collector tubes were numbered from 1 (cathode side) to 32 (anode side).

Antisera for standard test. Polyvalent antiplague rabbit serum prepared by E. R. Squibb and Sons, New York, N.Y. (lot #9170-1) was used in the standard test to assay for the presence of *P. pestis* antigens and served as a basis for assaying an antigen during fractionation procedures. The use of monovalent serum replaced the polyvalent as the former became available.

Preparation of acetone powder. Acetone-killed and dried cells (AKD) of *P. pestis* strain EV 76 were prepared according to the method of Baker et al. (1952), except that Casman medium (Difco) was substituted for beef heart infusion agar. Cultures were incubated for 24 hr at 25 and 37°C and were harvested in 0.85% sodium chloride solution.

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Acetone powder extract. In a typical experiment, 150 g of AKD cells were extracted in 1,500 ml of 0.05 M tris(hydroxymethyl)aminomethane chloride buffer (pH 7.8). After standing overnight, the supernatant fluid was collected by centrifugation. The residue was suspended in 800 ml of 0.05 M potassium phosphate buffer (pH 6.8) and subjected to sonic oscillation with a Branson 20-kc model LS 75 sonifier (Heat Systems, Great Neck, N.Y.) for 5 min at 10 amp. The suspension was centrifuged after standing overnight. The combined extracts totaled 2,000 ml and contained 43 g of protein. Further treatment of the residue will be described in a later paragraph. The combined extracts were subjected to ammonium sulfate precipitation while the pH was maintained between a value of 7 and 8 by the addition of ammonium hydroxide. Proteins that precipitated between 47 and 55% and between 70 and 100% of saturation were dissolved in a small volume of 0.01 M potassium phosphate buffer (pH 7.2) and were dialyzed. Each of these two ammonium sulfate fractions and a thiocyanate extract of the original residue yielded a partially purified antigen preparation when treated as described.

Preparation of antisera. An adjuvant solution of 4% sodium alginate-0.67% calcium gluconate (Colab Laboratories, Inc., Chicago Heights, Ill.) was used to dilute an equal volume of a solution of 0.50 mg of antigen before each injection. White rabbits were immunized with two injections 14 days apart. Antisera were collected by cardiac puncture 14 days after the second immunization.

Chemical analysis. The carbohydrate content was estimated both by the Molisch reaction and by the tryptophan test (Kabat and Mayer, 1961). Lipid ester was determined by the method of Snyder and Stephens (1959). Density-gradient centrifugation was by the procedure of Martin and Ames (1961).

Protection tests. Bagg strain white mice were injected intramuscularly with two equal graded doses of antigen in adjuvant 1 week apart. At 3 weeks after the second immunization, experimental mice and control mice were challenged by the intraperitoneal route with approximately 1,000 LD₅₀ of *P. pestis* strain 195/P.

Preparation of lipopolysaccharide-protein complex. Heat treatment of the 47 to 55% ammonium sulfate fraction for 30 min at 15 psi pressure (121 C) caused 80% of the protein to precipitate irreversibly. This procedure reduced the number of precipitin lines in the standard test from six to two. The antigen producing the heavier of these two lines was not retarded by gel filtration chromatography on a Sephadex-200 (Pharmacia, Sweden) column equilibrated with 0.01 M phosphate buffer (pH 7.2) and eluted with the same buffer. The material emerging with the void volume is designated as lipopolysaccharide-protein complex.

Preparation of antigen R. The addition of 53 mg of protamine sulfate to 484 mg of 70 to 100% fraction protein in 22 ml of 0.10 M phosphate buf-

fer (pH 7.2) resulted in the formation of a stringy precipitate. The supernatant fluid was collected by centrifugation and dialyzed against 0.01 M sodium acetate buffer (pH 4.7). The solution obtained was subjected to hanging curtain electrophoresis. Fractions showing a common precipitin line in the standard test were pooled and pervaporated to a workable volume. The material appearing in tubes 16 to 20 is designated as antigen R, and another found in tubes 23 to 26 was shown to be identical to the lipopolysaccharide-protein complex.

Preparation of antigen S. The AKD cell residues from several different preparations were pooled and suspended in 2,500 ml of 1 M potassium thiocyanate and were subjected to sonic oscillation. Insoluble material was removed by centrifugation. After dialysis to remove the thiocyanate, the supernatant fluid was autoclaved for 15 min at 15 psi (121 C). The precipitate was removed by centrifugation. The supernatant fluid was raised to pH 11 with ammonium hydroxide, and ammonium sulfate was added to 90% of saturation. The precipitate which formed was dissolved in 0.01 M phosphate buffer (pH 7.2) and was processed by gel filtration chromatography on Sephadex-200. Material that emerged with the void volume showed a single precipitin line and was designated as antigen S.

RESULTS

Rabbits injected with lipopolysaccharide-protein complex and antigens R and S consistently produced antisera directed against these three preparations. The three antisera obtained showed a single precipitin line at 24 hr in the standard test when reacted against homologous antigen or the acetone powder extract. However, when reacted against viable whole-cell suspensions of *P. pestis* or *P. pseudotuberculosis*, a second precipitin line occasionally appeared at 72 hr with antisera prepared against antigen R (Table 1). Concentration of antigen R to 100 units failed to elicit a second line.

The lipopolysaccharide-protein complex and antigens R and S were shown to be different from antigens D, E, F, I, K, L, Q, T, V, and W by the use of authentic samples of these antigens and their homologous antisera. Antiserum directed against the specific polysaccharide preparation of Davies forms a precipitin line with the lipopolysaccharide-protein complex in the standard test. Antisera prepared by immunization with the lipopolysaccharide protein complex formed a single line when tested against phenol-extracted material prepared according to the method of Davies (1956). When both antigens were placed in adjacent wells, the precipitin lines joined to form a line of identity. When the antilipopolysaccharide-protein complex serum was tested

TABLE 1. *Antigens detected in Pasteurella pestis and P. pseudotuberculosis grown at 28 and 37 C*

Strain	Lipopoly-saccharide-protein complex		R		S	
	28	37	28	37	28	37
<i>P. pestis</i>						
EV 76	+	±	-	+	+	-
195/P	+	±	±	+	+	-
M1	+	±	±	+	+	-
M23	+	+	-	+	+	+
DF1	+	+	-	+	+	+
PKR159	+	±	±	+	+	+
A12	+	±	±	+	-	+
A1122	+	±	-	+	-	+
Tjiwidej	+	±	-	+	+	-
Alexander OX	+	±	-	+	+	-
<i>P. pseudotuberculosis</i> *						
1 I	-	-	+	+	+	+
16 II	-	-	±	+	+	+
43 III	-	-	-	+	+	+
32 IV	-	-	-	+	+	+
25 V	-	-	-	+	+	+
43R	+	+	+	+	+	+
25R	+	+	+	+	+	+
1R	+	+	+	+	+	+

* Strain numbers are in arabic; type designations are in roman numerals. R indicates an untypable mutant from parent numbered culture.

† A second line appeared after incubation for 72 to 144 hr. Symbols: + = positive in 24 hr; ± = weak or late reaction; - = negative.

TABLE 2. *Analysis of antigen preparations*

Antigen	Carbohydrate (as glucose)			Lipid ester* (as tristearin)
	Molisch test	Tryptophan test	Nucleic acid	
Lipopolysaccharide-protein complex . . .	7.8†	15†	0.6†	70†
R	0.5	1.8	0.7	1.9
S	1.4	4.3	2.1	12

* An amount of 1 mg of tristearin equals 3.365 meq.

† Numbers indicate milligrams per 10 mg of protein.

against whole cells of *P. pestis* and *P. pseudotuberculosis* strains, the 28 C cultures of *P. pestis* reacted more strongly, giving a single heavy line at 24 hr, whereas the 37 C cultures gave a weak line after incubation for 72 to 144 hr. Serologi-

cally untypable strains of *P. pseudotuberculosis* gave a single line in 24 hr, whereas type-specific strains failed to react in 144 hr (Table 1). The high lipid and carbohydrate content of this material compared with the other preparations is shown in Table 2.

Some difficulty was encountered in attempts to measure the molecular weight of these antigens. If human hemoglobin (66,700) and beef liver catalase (250,000) are used as standards, antigen R shows a molecular weight of 106,000. Inability of lipopolysaccharide-protein complex to be retained on Sephadex-200 would indicate a molecular weight in excess of 200,000; yet, owing to its high lipid content, this material floats on a 5 to 20% sucrose gradient after 17 hr of centrifugation. Certain serum lipoproteins behave similarly (Lindgren et al., 1962). Behavior of antigen S on Sephadex-200 would also indicate a molecular weight in excess of 200,000. Values obtained for this antigen varied from 342,000 to 560,000.

Graded doses of the lipopolysaccharide-protein complex or antigens R and S, ranging from 1 µg to 1 mg in adjuvant, were nonimmunogenic for mice challenged with 1,000 LD₅₀ of *P. pestis* 195/P.

DISCUSSION

Davies (1956), using a hot phenol extraction method, isolated the specific polysaccharide of *P. pestis*. In a later work, Davies (1958), using the same technique, showed that the common antigen of *P. pseudotuberculosis* was antigenically related to the specific polysaccharide of *P. pestis*. In both instances, the isolated compounds were nonantigenic lipopolysaccharide haptens which could be rendered antigenic by conjugation with *S. dysenteriae* lipoprotein complex. Because of the low solubility and the nonantigenicity of the hapten, he discussed the advantage of isolating it with some protein attached. The lipopolysaccharide protein reported here fulfills this requirement. Identity reactions have been obtained between the hapten and the complex when tested against antiserum prepared against isolated lipopolysaccharide complex and antiserum prepared against the conjugated hapten.

The weak and delayed reaction occurring when tests were carried out against *P. pestis* whole-cell antigens grown at 37 C was interpreted as being masked by the presence of large quantities of F, V, and W antigens, which are produced at this temperature and not at 28 C.

The occurrence of a reaction between untypable, rough *P. pseudotuberculosis* whole-cell antigens and lipopolysaccharide-protein complex antisera confirm the close antigenic relationship between these systems. Failure of typable smooth

strains of *P. pseudotuberculosis* to react is due to the masking of the common antigen by one or more of the other 9 type-specific lipopolysaccharide antigens.

Lawton, Fukui, and Surgalla (1960) reported the detection of 18 antigens in *P. pestis* and *P. pseudotuberculosis* by gel diffusion based on the production of a single-line reaction between antisera and antigens. When they compared their system with the more complex 10-lined analysis of Crumpton and Davies (1956), they were able to equate four reactions, F, I, V, and W. With the kind collaboration of W. D. Lawton, the lipopolysaccharide-protein complex and antigens R and S were tested against all available standards. These preparations did not react with D, E, F, I, K, L, Q, T, V, or W. Test systems for B, C, G, H, J, M, N, and O were based on cross-absorption techniques and were not available for testing. The antigens reported in this study represent two new isolated antigen-antibody systems of the *P. pestis*-*P. pseudotuberculosis* complex available for testing.

The presence of a second precipitin line appearing in 72 hr when anti-R serum was reacted against whole-cell preparations represents an antigen which constitutes less than 1% of the purified material, but which occurs in higher concentration in whole-cell suspensions of strains other than EV 76.

Since the antigens are not analytically pure, the carbohydrate and lipid values should not be interpreted as absolute values. However, Table 2 does show the high lipid content of the lipopolysaccharide-protein complex, which is similar to Davies' specific polysaccharide hapten.

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