Immunization of Mice with Components of *Pasteurella multocida*¹

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Log-phase cells of *Pasteurella multocida* strain P-1059 were used to prepare isolated culture filtrate, cell wall, and cytoplasmic components. Culture filtrate was further separated by column chromatography. A portion of cytoplasm and culture filtrate was conjugated to ferritin by means of metaxylene disocyanate. Cell walls induced more protection in mice than the conjugated or unconjugated cytoplasm or culture filtrate. The cell walls caused edema and erythema when given intradermally in rabbits, whereas cytoplasm and culture filtrate produced dermal necrosis. The first of four chromatographically separated fractions of culture filtrate was possibly more immunogenic in mice than cell walls. This fraction was less reactive intradermally in rabbits than cell walls but more reactive than the other fractions.

*Pasteurella multocida* is known to cause sporadic outbreaks of disease in a wide variety of mammals and birds throughout the world. Various prophylactic measures have been tried including the use of vaccines, suspensions of attenuated or avirulent cells (10, 25, 32); bacterins, suspensions of cells killed by physical or chemical means (2, 12, 13, 15); chick embryo bacterins, cells grown in chick embryo, harvested, and killed chemically at the peak of growth (2, 5, 13); aggressins, inflammatory exudates (8, 31); particulate antigens, antigens found in culture filtrate after high-speed centrifugation (14); and other components of cells, various cell constituents such as capsule, cell wall, cytoplasm, and culture filtrate or fractions of these components (22, 32). Certain of these immunizing agents have been reported to give a high degree of protection in laboratory species upon challenge with fully virulent organisms. The purpose of the present studies was to determine which of the components of *P. multocida* are immunogenic in mice.

MATERIALS AND METHODS

**Source of organism.** A lyophilized culture of *P. multocida* strain P-1059 was obtained from the National Animal Disease Laboratory, Ames, Iowa, through the courtesy of Kenneth L. Heddeleston. This is an avian vaccine strain, highly virulent for young (16 week) turkeys and 45-week-old chickens (12). This strain was used for all antigen preparation and challenge.

**Cultivation of organism.** The lyophilized culture was reconstituted in Tryptose broth (Difco) and injected subcutaneously in each of two Beltsville white turkeys. The turkeys died in 40 hr, and their livers were collected aseptically. Small pieces of liver were stored at −70 C, and, when cultures were needed, a piece of liver was thawed and streaked on slants of corn meal infusion agar (referred to hereafter as infusion agar) and incubated for 14 hr at 37 C. Cultures were checked for purity by Gram stain and for proper strain identification by biochemical tests (12).

**Preparation of culture filtrate.** From freshly incubated slants, 10 more slants were streaked and incubated for 14 hr at 37 C. The growth was washed from these slants with cold distilled water and transferred to 20 Blake bottles containing infusion agar. The inocula were spread with the aid of glass beads and incubated for 14 hr at 37 C. After incubation, growth was loosened from the agar surfaces by adding water and rolling the beads about. Cell suspensions were filtered through a Büchner funnel containing Whatman no. 2 filter paper and fine glass wool. The cell suspension was centrifuged (GSA angle head rotor, Ivan Sorvall, Inc., Norwalk, Conn.) at 2,000 × g and 0 C for 30 min to remove coarse debris. The supernatant was again centrifuged at 10,400 × g for 1 hr. The latter supernatant was filtered through a type HA 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and was termed "culture filtrate."

**Rupture of organism.** The sediment obtained from the second centrifugation (above) was reconstituted

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in half of the original volume of water and centrifuged at 10,400 \( \times \) g for 30 min, and the process was repeated. The cells were then ruptured (28) in a Ribi cell fractionator (Ivan Sorvall, Inc., Norwalk, Conn.) at 20,000 psi and 5 to 10 C. Cooling of the needle valve was done by nitrogen gas. The percentage of rupture (by packed cell volume) was 81.6%. After rupture, the ruptured material was centrifuged at 7,710 \( \times \) g for 30 min, and the sediment was reconstituted in 30.0 ml of water and ruptured again. The ruptured material obtained the second time was processed as above, but this time the sediment was discarded and the supernatant was pooled with the supernatant collected after the first rupture.

**Preparation of cell wall and cytoplasm.** The pooled supernatant, obtained after rupture, was centrifuged (Spinco model L ultracentrifuge, Beckman Instruments, Inc., Palo Alto, Calif.) at 30,900 \( \times \) g and 0 C for 30 min to sediment cell walls and cell wall fragments. The sediment was reconstituted in water and ruptured again. The supernatant obtained after centrifugation at 30,900 \( \times \) g was centrifuged again at 105,000 \( \times \) g for 1 hr to remove cell wall fragments. The sediment obtained after the latter process was combined with the sediment which had been ruptured again and designated "cell walls." The supernatant obtained after centrifugation at 105,000 \( \times \) g was filtered through a type HA 0.45-\( \mu \)-m membrane filter (Millipore Corp.), and the filtrate was designated as "cytoplasm." Cell wall fragments were not detected in culture filtrate or in cytoplasm by electron microscopy. Cell wall preparations were of, course, much fragmented but there were no intact cells detectable by electron microscopy. Cell wall preparations were sterilized by treatment with 2 volumes of ether overnight at room temperature. On the next day, the ether was removed by bubbling nitrogen gas through the suspension. All preparations were tested for sterility by culture on blood-agar plates and in thioglycolate broth and by intraperitoneal inoculation in mice. All sterile preparations were then lyophilized, weighed, and stored in a desiccator until use.

**Preparation of conjugates.** A portion of culture filtrate and a portion of cytoplasm were conjugated with cadmium-free horse spleen ferritin (2 \( \times \) crystallized, Nutritional Biochemicals Corp., Cleveland, Ohio) by the technique described by Singer (30). Conjugates named accordingly were refrigerated until use.

To determine whether conjugation had occurred, conjugated as well as unconjugated samples were subjected to electrophoresis in acrylamide gel (E-C Apparatus Corp., Philadelphia, Pa.). The gel was stained with 0.25% amido black 10 B, cut into strips, and scanned with a recording densitometer (model-49, Photovolt Corp., New York, N.Y.). Some components in conjugated samples migrated at a different rate from components in unconjugated samples (Fig. 1).

**Preparation of other samples.** A portion of the cell wall preparation was treated with 0.25% sodium lauryl sulfate (SLS) overnight at room temperature. The mixture was dialyzed at 10 C against a continuous flow of distilled water for 4 days to remove traces of SLS; it was then refrigerated until use.

**Fractionation of culture filtrate by column chromatography.** Culture filtrate was fractionated (17) by using columns [61 by 2.5 cm; Technicon Chromatography Corp., Ardsley (Chauncey), N.Y.] of Sephadex G-50 fine, G-50 fine, and G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). Sephadex G-50 was the most satisfactory. Contents of tubes representing each of the peaks (Fig. 2) were pooled separately, lyophilized, weighed, and stored in a desiccator until use.

**Preparation of immunogens for inoculation.** Com-
TABLE 1. Protection of mice by components of Pasteurella multocida incorporated in incomplete Freund's adjuvant and water (4- to 5-week-old mice)

| Immunogens | Survivors/total challenged per dose of immunogen a |
|------------|--------------------------------------------------|
|            | 1.0 mg | 0.2 mg | 0.04 mg |
| Cell walls | 8/16 (50) | 4/15 (27) | 5/16 (31) |
| Cytoplasm | 5/15 (33) | 4/14 (28) | 0/13 (0) |
| Culture filtrate | 5/15 (33) | 1/15 (7) | 2/15 (13) |
| Culture filtrate conjugate | 3/14 (21) | 0/14 (0) | 1/14 (7) |
| Cell walls treated with SLS | 1/14 (7) | 1/15 (7) | 1/14 (7) |
| Cytoplasm conjugate | 2/6 (33) | 0/13 (0) | 0/16 (0) |

a Components were inoculated subcutaneously behind the ear of each mouse 45 days prior to challenge. Challenge dose was 104 live (by plate count) P. multocida. Mice surviving at least 21 days were killed, and spleens were cultured on blood-agar plates. No growth was detected after 5 days of incubation at 37 C.

b Of the controls, 15 of 15 died within 48 hr.

Values in parentheses are expressed as percentages.

Intradermal tests in rabbits. Intradermal toxicity of certain of the immunogens was determined in rabbits. The technique for preparation of dilutions and intradermal injection was as described by Larson et al. (18).

RESULTS

Immunization experiments in mice. Data on protection afforded by various components of P. multocida are presented in Table 1. The maximum protection against death was achieved by 1.0-mg doses of cell walls and amounted to 50% of the test animals. The 1.0-mg doses of the other preparations protected 7 to 33% of the mice. Whereas doses of 0.2 mg of cell walls and of cytoplasm protected 27 to 28% of the animals, only 7% or fewer were protected by similar doses of culture filtrate or of the conjugates. Doses of 0.04 mg of cell walls protected 31% of the animals, whereas 0.04-mg doses of the other preparations protected 0 to 13%. Little difference was evident in protection by the various components, and there was no clear relationship of dose to protection with any component. All control animals died within 48 hr after challenge. No growth was observed in spleen cultures from animals which survived 21 days.

Protection afforded by the four fractions of culture filtrate is summarized in Tables 2 and 3. In the first experiment (Table 2), only fraction 1 protected 3- to 4-week-old mice from lethal infection, whereas the mice which died survived an average of 6.4 days. Mice receiving fraction 4 and the mixture of fractions survived a little longer (1.6- and 1.4-day averages) than mice receiving fractions 2 (0.9-day average), 3 (1.1-day average), and controls which all died within 48 hr (0.8-day average).

The second experiment is summarized in Table 3. Maximum protection (63%) of 4- to 5-week-old mice against a homologous challenge was achieved by a 0.2-mg dose of fraction 1, whereas 1.0 mg of the same fraction was 50% effective and 0.04 mg was 13% effective. The 0.2-mg dose of fraction 4 was 38% effective, and the 1.0-mg dose of the mixture was 13% effective. All other inoculated mice died within 72 to 96 hr except one (a mouse given the 1.0-mg dose of the mixture) which died on the 12th day. The survival time of mice was prolonged as it was in the first experiment with fraction 1 and the mixture. Average survival of control mice was 1.1 days.

A preliminary study indicated that all of the culture filtrate fractions had traces of biuret (9)-positive material. However, only fraction 1 was obtained in a homogeneous state and the other fractions were not considered further. Fraction 1 appeared as a single component by electrophoresis on cellulose-acetate paper at pH 8.6 in...
TABLE 2. Protection of mice by fractions of culture filtrate of Pasteurella multocida incorporated in water alone (3- to 4-week-old mice)

| Immunogens and tube no. | Survivors/total challenged per dose of immunogen* |
|------------------------|-----------------------------------------------|
|                        | 1.0 mg | Avg no. of days until death | 0.2 mg | Avg no. of days until death | 0.04 mg | Avg no. of days until death |
| Fraction 1, no. 9, 10, 11 | 4/14 (29)* | 9.1 | 7/16 (44) | 9.4 | 0/16 (0) | 0.6 |
| Fraction 2, no. 19, 20, 21, 22 | 0/16 (0) | 0.6 | 0/16 (0) | 1.1 | 0/16 (0) | 0.8 |
| Fraction 3, no. 24, 25, 26 | 0/16 (0) | 0.7 | 0/15 (0) | 0.9 | 0/16 (0) | 1.7 |
| Fraction 4, no. 29, 30, 31 | 0/16 (0) | 2.1 | 0/15 (0) | 1.4 | 0/16 (0) | 1.3 |
| Mixture of all four fractions | 0/16 (0) | 1.7 | 0/16 (0) | 1.6 | 0/16 (0) | 0.9 |

* Fractions were inoculated subcutaneously in each mouse 21 days before challenge. Challenge dose was 133 live (by plate count) P. multocida. Mice surviving at least 21 days were killed and spleens were cultured on blood-agar plates. No growth was detected after 5 days of incubation at 37 C.

| Immunogens and tube no. | Survivors/total challenged per dose of immunogen* |
|------------------------|-----------------------------------------------|
|                        | 1.0 mg | Avg no. of days until death | 0.2 mg | Avg no. of days until death | 0.04 mg | Avg no. of days until death |
| Fraction 1, no. 9, 10, 11 | 4/8 (50)* | 10.9 | 5/8 (63) | 13.1 | 1/8 (13) | 4.4 |
| Fraction 2, no. 19, 20, 21, 22 | 0/8 (0) | 1.3 | 0/8 (0) | 1.5 | 0/8 (0) | 1.1 |
| Fraction 3, no. 24, 25, 26 | 0/8 (0) | 1.4 | 0/8 (0) | 1.3 | 0/8 (0) | 1.5 |
| Fraction 4, no. 29, 30, 31 | 1/8 (13) | 3.9 | 3/8 (38) | 8.6 | 0/8 (0) | 1.1 |
| Mixture of all four fractions | 0/8 (0) | 4.3 | 1/8 (13) | 3.8 | 0/8 (0) | 1.0 |

* Fractions were inoculated intraperitoneally in each mouse 21 days before challenge. Challenge dose was 107 live (by plate count) P. multocida. Mice surviving at least 21 days were killed and spleens were cultured on blood-agar plates. No growth was detected after 5 days of incubation at 37 C.

0.075-ionic strength Veronal buffer. The component stained with ponceau S and with glycoprotein stain. Fraction 1 also demonstrated a single immunodiffusion band by the Ouchterlony technique (24) when tested against rabbit antiserum developed against fraction 1. Fraction 1 accounted for 14.05% of 37.7 mg of culture filtrate recovered from a Sephadex column (40 mg applied). The fraction contained 3.13% protein (method of Lowry et al., 19) and about 63% total carbohydrate (4). Fraction 1 was further analyzed by two-dimensional thin-layer chromatography (26), and it was evident that 11 amino acids were present: lysine, arginine, glutamic acid, serine, glycine, aspartic acid, proline, alanine, threonine, valine, and either leucine or isoleucine. Only one terminal amino acid was detected (29) and it was either leucine or isoleucine. The concentration of 2-keto-3-deoxyoctulosonic acid (KDO; 1) in four separate lots of fraction 1 ranged from 0.09 to 0.11%. The concentration of heptose (23) in the same lots ranged from 0.01 to 0.10%.

Intradermal tests in rabbits. Sterile abscesses were produced in rabbit skin by inoculation of culture filtrate and cytoplasm and the skin lesion dose (SLD90; 16) was ≤0.39 μg. Only edema and erythema were produced by cell walls and the SLD90 was ≤0.35 μg.

When the individual fractions of culture filtrate were tested, an erythema and a little edematous swelling were detected, but no sterile abscesses were induced. Maximum activity occurred between 24 and 48 hr. The most active fraction was fraction 1 which had an SLD90 of 0.87 μg. The second and third most active were fraction 4 and the mixture of all four fractions which had...
SLD<sub>50</sub> values of 7.0 and 28.0 µg, respectively. Fractions 2 and 3 had SLD<sub>50</sub> values of 63.0 µg each.

DISCUSSION

An attempt was made to define the immunizing components of <i>P. multocida</i> strain P-1059 for mice. In assessing the significance of the findings, the purity of the preparations should be considered. Cell suspensions were freed from culture filtrate and washed twice. Therefore, cells were relatively free from culture medium ingredients before rupture. The final fragmented cell wall preparation appeared clean by electron microscopy and probably contained relatively little cytoplasm. The cytoplasm was centrifuged at a speed high enough so that no cell wall fragments detectable by electron microscopy remained in the final preparation. Similarly, there were no detectable cell walls or cell wall fragments in the culture filtrate. Hence, any cell wall material remaining in the cytoplasm or culture filtrate would have to have been solubilized or present as very small particles; this is a possibility. The actual bacterial products were thus only 20% of the culture filtrate and might well have contained some cytoplasm and solubilized cell wall material from autolyzed cells which presumably occur to a degree in such cultures. Results indicate that immunogenic material was found in cell wall, cytoplasm, and culture filtrate. On a weight basis, the protective potency of the three preparations was similar, although cell walls may have given slightly more protection than the other two preparations. The first culture filtrate component (fraction 1) isolated by column chromatography was the most immunogenic of the fractions. Fraction 1 was isolated as a single peak and was homogeneous by electrophoresis and immuno-diffusion.

Protection of mice from lethal infection by <i>P. multocida</i> components or fraction 1 was not directly related to dose. A similar lack of correlation of dose with response was seen in turkeys inoculated with the same cellular components (3) and with commercial bacterin produced with the same strain of <i>P. multocida</i>. The bacterin was not appreciably superior to the cellular components as an immunogen. A lack of correlation of dose with response was also seen in swine immunized with <i>Brucella suis</i> endotoxin containing preparations and challenged with live <i>B. suis</i> (6).

Immunosuppression conceivably could be caused by endotoxin in the cell walls (7) or by some constituent in the cytoplasm or culture filtrate similar to that described for the streptococci (11, 20). There is also a possibility that interaction among the constituents of <i>P. multocida</i> might have caused important determinant groups of an immunogen to be unavailable, because the SLD<sub>50</sub> of mixed fractions from the culture filtrate was 28 µg and that of fraction 1 was 0.87 µg. When one corrects the figures (7 µg of each fraction was present in 28 µg of mixture), the isolated fraction 1 is almost 10 times more active than the same material in the mixture. The culture filtrate caused dermal necrosis before gel fractionation, but neither the mixture of fractions nor the separated fractions caused detectable necrosis after gel fractionation. Three possible explanations are: (i) the separated necrotizing toxin was unstable after separation; (ii) an accessory factor, needed for expression of toxicity, was retained in the column; or (iii) quantitative relationships needed for toxicity were upset by the 1:1:1:1 ratio used in the mixture.

Fraction 1 was probably not the same as the immunogenic endotoxin (particulate antigen) described by Heddleston et al. (14) because it had very low levels of KDO and heptose. Fraction 1 did cause rabbit dermal erythema in doses as low as 0.39 µg, and this is the level of toxic activity one might expect from an endotoxin. However, on the basis of studies in other organisms (21), one would expect about 3 to 4% KDO and heptose for this level of activity. Fraction 1 probably had an amount of endotoxin too small to be detected by electrophoresis or immuno-diffusion.

The chemical data are compatible with the concept that fraction 1 contains a glycopeptide with about 63% carbohydrate and 30% protein. This is because a single electrophoretic component in the same location on cellulose-acetate strips was demonstrated with glycoprotein and protein stains and a single band was seen by immuno-diffusion. If a single peptide is present it contains 11 amino acids and is probably connected to the carbohydrate portion of the fraction through the carboxy-terminal end because there is an amino-terminal amino acid present. If conjugation of the peptide to ferritin involved the amino-terminal amino acid, proper expression of a determinant group may have been prevented. This could explain the poor protective performance of the conjugates. SLS treatment of cell walls also appeared to damage immunogenicity.

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