Antigens of *Pasteurella tularensis*:
Preparative Procedures

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Ether-water (EW) extraction of *Pasteurella tularensis* produced better antigens than five other chemical procedures. EW extracts produced from stationary-phase, liquid-grown, saline suspensions of strain SCHU S4 cells regularly induced agglutinin and precipitin formation in rabbits. Mice, guinea pigs, and monkeys also responded to EW extracts but with lower antibody levels. The use of strains of lower virulence, acetone-dried cells, organisms grown on a solid medium, and abbreviated extraction conditions all resulted in extracts with a diminished antigenicity, but logarithmic-phase and stationary-phase cells yielded equivalent EW extracts. The use of adjuvant, hyperimmunization, and large doses of antigen increased the precipitin responses of rabbits without appreciably altering the agglutinin response. By the appropriate combination of centrifugal fractionation of EW extracts, use of adjuvant, and vaccination schedule, rabbit antisera with either predominantly agglutinating or precipitating activities were obtained.

A comparative study of antigens extracted from *Pasteurella tularensis* by various chemical procedures has not been reported. Viable and killed whole-cell vaccines usually have been employed for producing antisera in animals (2, 4, 6). A few studies on antigenic extracts have been reported, but the emphasis was on immunogenicity (5, 7) or their utility as a serological reagent for assessing preformed antibody (1, 2, 6, 12).

The objective of this study was to determine the efficacy of several chemical procedures for preparing antigenic extracts of *P. tularensis* measured by their ability to induce antibody formation.

MATERIALS AND METHODS

*P. tularensis*. Six strains ranging from fully virulent to avirulent were used. The strains, in decreasing order of virulence, were SCHU S4, 503, 425, LVS, Depue, and 38A. LVS and 503 were of Eurasian origin, and all others were of North American derivation.

Cultural conditions. Stock cultures were prepared in a modified casein hydrolysate medium (MCPH) similar to that described by Mills et al. (Bacteriol. Proc., p. 37, 1949). *P. tularensis* was grown on one of two media prior to extraction. Glucose-cysteine blood-agar (GCBA) was prepared by adding 2% packed human erythrocytes to rehydrated glucose-cysteine-agar (BBL). Cultures were initiated by spreading 0.1 ml of MCPH culture on the surface of GCBA in 100-mm petri dishes. Cultures were incubated for 48 hr at 37°C, and the organisms were collected in and washed twice by centrifugation (8,400 × g) in 0.85% NaCl.

Peptone-cysteine-broth (PCB) was also employed for the growth of *P. tularensis* (8). Cultures were initiated by inoculating 2-liter Erlenmeyer flasks, each containing 300 ml of PCB with 5% volume of MCPH culture and incubating at 37°C for 6 hr (log-phase) or 16 hr (stationary-phase) with agitation. Organisms harvested by centrifugation (8,400 × g) were washed twice with 0.85% NaCl, and the moist weight was determined before further treatment.

Acetone-dried *P. tularensis*. Saline-washed cells were suspended and washed three times in acetone, and the packed dry cells were stored in a desiccator.

Trichloroacetic acid-extraction techniques. A cold trichloroacetic acid-extraction technique recommended by Staub (9) was employed initially and found to be ineffective for producing extracts antigenic for rabbits. A second trichloroacetic acid technique was also used (3); the second technique was a more rigorous extraction at a slightly elevated temperature.

Hot-water extraction. A 1% suspension of cells in distilled water was heated to 80°C for 30 min, and the cells were packed by centrifugation. The packed cells were resuspended in distilled water, and the extraction was repeated one time; the two supernatant solutions were pooled (3).

Phenol-water extraction. The technique recommended by Tauber and Garson (10) was applied to *P. tularensis*. This technique uses a 50% solution of
phenol in water with the temperature maintained below 42 C.

**Pyridine-formic acid extraction.** Ten per cent (w/v) suspensions of saline-washed bacteria were prepared in an equimolar mixture of pyridine and formic acid. The extractions were performed by a technique described by Nowotny (3) except that the glycolipids were not precipitated by methanol.

**Hexadecyltrimethylammonium bromide extraction.** Saline-washed organisms were suspended in a 0.5% solution of this detergent. The extraction was performed by the technique of Nowotny referred to above.

**EW extraction.** The ether-water (EW) extraction technique developed by Ormsbee, Bell, and Larson (5) was employed with some variations. In the basic method, a saline suspension (10%, w/v) of organisms was treated with an excess of ethyl ether (2.5 volumes) overnight at room temperature; the saline solution was used as antigen after removal of ether by dialysis against water and removal of particulate debris by centrifugation at 8,400 × g. A variation employed was to reduce the extraction time to 3 hr and the ether to 0.2 volume.

**Sero logical techniques.** The techniques of bacterial agglutination and agar-gel precipitation have been reported (4).

**Animals.** New Zealand White rabbits of both sexes (1.8 to 2.5 kg), rhesus monkeys (Macaca mulatta) of both sexes (3.2 to 4.7 kg), male Hartley strain guinea pigs (325 to 375 g), and female Fort Detrick mice (18 to 20 g) were used.

**Administration of antigens.** Except where noted, antigens were administered by the intravenous (iv) route and secondary injection of the antigens was performed 28 days after primary administration. Rabbits received doses adjusted to contain 1 mg of anthrone-reactive carbohydrate (CHO, reference 11). All other animals received 0.5 mg of CHO per kg of body weight. When Freund's adjuvant was used, equal volumes of the antigen and adjuvant were mixed, and the dose was divided into two equal portions and administered subcutaneously (sc) in the inguinal region.

### RESULTS

Comparative effectiveness of extraction techniques. The agglutinin responses of rabbits to six different antigens extracted from PCB-grown cells are presented in Table 1. The second trichloroacetic acid-extraction (3) and EW techniques produced antigens that induced the two highest mean titers, reciprocal primary response titers of 832 and 896, respectively; all other antigens induced agglutinin titers less than one-half the above values, and the phenol-water technique resulted in the least antigenic preparation. In no case was the secondary response to any of the antigens significantly different from the primary response. Maximal titers to both the trichloroacetic acid-extraction (3) and EW antigens were observed consistently 7 days after either primary or secondary administration of antigen.

Precipitin response data, obtained by double diffusion in agar, are presented in Table 2. Only the EW antigen regularly induced the formation of precipitins in rabbits, and usually more than one precipitating antigen-antibody system was detected. The other five antigens varied in their ability to induce precipitin formation; only one of the rabbits inoculated with phenol-water extracts and none of those inoculated with pyridine-formic acid extracts produced precipitating antibodies.

**EW extraction of various strains of P. tularensis.** The survey of extraction technique was performed with strain SCHU S4. Results of a study of EW extraction with three samples from each of five additional strains are presented in Table 3. None of these strains was appreciably different from SCHU S4 in supplying extracts capable of inducing agglutinin formation in the rabbit. Precipitin responses were less consistent with extracts of these strains; some sera failed to produce

### Table 1. Agglutinin response of rabbits to chemically prepared Pasteurella tularensis SCHU S4 antigens

| Preparative technique                  | No. of prepna | Primary response | Secondary response |
|----------------------------------------|---------------|-----------------|--------------------|
|                                        |               | Mean      | Range        | Mean   | Range        |
| Nowotny-trichloroacetic acid           | 5             | 832<sup>b</sup> | 80-2,560 | 768    | 320-2,560 |
| Hot water                              | 5             | 384       | 160-1,280 | 544    | 320-640    |
| Ether-water                            | 5             | 896       | 640-1,280 | 1,024  | 640-2,560 |
| Phenol-water                           | 5             | <10       | <10-20    | <10    | <10-10     |
| Pyridine-formic acid                   | 5             | 16        | <10-160   | 18     | <10-320    |
| Hexadecyltrimethylammonium bromide     | 5             | 136       | 20-320    | 240    | 20-1,280   |

<sup>a</sup> Two rabbits per preparation.

<sup>b</sup> Reciprocal maximum agglutinin titer.
TABLE 2. Precipitin response of rabbits to chemically prepared Pasteurella tularensis
SCHU S4 antigens

| Preparative technique          | No. of bands | No. of bands |
|-------------------------------|--------------|--------------|
|                               | Primary      | Secondary     |
|                               | response     | response     |
| Nowotny-tri-chloroacetic acid| 5            | 1-0 (0.7) b   | 0-1 (0.9)    |
| Hot water                     | 5            | 0-1 (0.2)    | 0-2 (0.9)    |
| Ether-water                   | 5            | 0-1 (0.1)    | 0-0          |
| Phenol-water                  | 5            | 0-1 (0.3)    | 0-2 (1.0)    |
| Pyridine-formic acid          | 5            | 0-0          |              |
| Hexadecyltrimethylammonium bromide | 5              | 0-1 (0.3)    | 0-2 (1.0)    |

a Two rabbits per preparation.
b Range individual rabbits (arithmetic mean).

TABLE 3. Antibody response of rabbits to ether-water extracts of five strains of Pasteurella tularensis

| Strain | Mean agglutinin titer | Maximum no. of precipitin bands |
|--------|-----------------------|---------------------------------|
|        | Primary response | Secondary response | Primary response | Secondary response |
| 503    | 832 a              | 480                          | 0-3 b           | 0-2                 |
| 425    | 512                 | 896                          | 0               | 2                    |
| LVS    | 640                 | 832                          | 0-2             | 0-3                 |
| Depue  | 576                 | 676                          | 0-2             | 0-4                 |
| 38A    | 640                 | 640                          | 0-1             | 0-3                 |

a Reciprocal maximum titer.
b Range of individual rabbits.

e A mean of two rabbits per preparation.

Antibody responses of various animals to EW extracts. The agglutinin and precipitin responses of monkeys, guinea pigs, and mice to an EW extract of strain SCHU S4 are presented in Table 4. The agglutinin responses were lower in all three species than observed in rabbits; mean values were approximately fourfold lower. Additionally, the precipitin responses were less extensive, and some monkeys and guinea pigs failed to produce reactive sera. Guinea pigs proved least useful; five of nine died of anaphylactic shock after revaccination.

TABLE 4. Antibody response of animals to ether-water extracts of Pasteurella tularensis
SCHU S4

| Animal   | Mean agglutinin titer | Maximum no. of precipitin bands |
|----------|-----------------------|---------------------------------|
|          | Primary response | Secondary response | Primary response | Secondary response |
| Monkey   | 256 a             | 176                          | 0-2 b           | 0-2                 |
| Guinea pig | 240             | 224                          | 0-1             | 0-1                 |
| Mouse    | 224               | 192                          | 1 c             | 1 c                 |

a Reciprocal maximum titer.
b Range of individual animals.
c Pooled sample.

e A mean of two rabbits per preparation.

TABLE 5. Effect of variations in ether-water extraction technique on antigenicity for rabbits

| Variation            | Mean agglutinin titer | Maximum no. of precipitin bands |
|----------------------|-----------------------|---------------------------------|
|                      | Primary response | Secondary response | Primary response | Secondary response |
| Acetone-dried cells  | 512 a             | 1,024                        | 0-1 b           | 2-3                 |
| GCBA grown cells     | 640                 | 768                          | 1               | 1-3                 |
| Logarithmic-phase cells | 1,088            | 1,088                        | 1-3             | 2-3                 |
| 3-Hr extraction, 3/5 volume ether | 1,536           | 704                          | 1-2             | 2                   |

a Reciprocal maximum agglutinin titer.
b Range individual rabbits.
c Glucose-cysteine-blood-agar.
TABLE 6. Effect of variations in administration technique on antibody response of rabbits

| Variation                                | Mean agglutinin titer | Maximum no. of precipitin bands |
|------------------------------------------|-----------------------|---------------------------------|
|                                          | Primary | Secondary | Primary | Secondary |
| Extract in Freund’s adjuvant             |         |           |         |           |
| 10.0 mg of CHO                          | 896<sup>a</sup> | 768       | 2-4<sup>b</sup> | 4-5       |
| 0.1 mg of CHO                           | 1,152   | 2,560     | 2-3     | 4-5       |
| Hyperimmune                             | 576     | 704       | 1       | 1-2       |
| 34,000 × g Pellet                       | 1,280   | 1,280     | 4       | 4         |
| 34,000 × g Supernatant fluid in Freund’s adjuvant | 352     | 832       | 1-3     | 1-3       |
|                                          | 36      | 32        | 2-4     | 4-5       |

<sup>a</sup> Reciprocal maximum agglutinin titer.
<sup>b</sup> Range of individual rabbits.
<sup>c</sup> CHO, anthrone-reactive carbohydrate.

uses a large excess of ether and overnight extraction. The results of a marked reduction in both the amount of ether and the extraction time are shown. The primary agglutinin response was improved somewhat, but the precipitin response was decreased; the usual array of three precipitins was not observed.

Based on these studies, the extraction procedure used routinely employed moist saline-washed cells of the virulent SCHU S4 strain harvested after 16 hr of growth in PCB and extracted overnight with 2.5 volumes of ether.

**Administration techniques.** Table 6 presents the results of some variations in procedures of administering antigen to rabbits. Incorporation of the extract into complete Freund’s adjuvant and sc administration had no effect on the usual agglutinin responses but did potentiate precipitin formation, especially during the secondary response.

When the standard 1.0-mg dose of CHO was increased 10-fold, the antibody responses during the secondary phase were increased, but this gain was offset by high mortality; 50% of the animals died after the second injection. A 10-fold reduction to 0.1 mg had no marked effect on the agglutinin response, but the precipitin response was reduced. The optimum dose of extract had been fortuitously selected at the outset of the study.

A common procedure for increasing antibody responses is hyperimmunization, and, accordingly, 1.0-mg doses were administered iv at weekly intervals for 8 weeks. The agglutinin titers never appreciably exceeded those usually observed after a single injection. The precipitin response was increased and was generally equivalent to that observed after use of adjuvant.

By centrifuging the EW extract at 34,000 × g, cell wall and soluble fractions were collected. Preliminary trials indicated that when these antigens were separately administered to rabbits, the resulting antisera had different serological activities. The antibody response to the 34,000 × g pellet was predominantly agglutinins. Some animals gave the usual number of precipitin bands, but these were markedly reduced in intensity. When the 34,000 × g supernatant liquid was incorporated into Freund’s adjuvant, the agglutinin response was markedly inhibited. The precipitins were, however, numerically equivalent to the array obtained when whole extract was mixed with adjuvant, but some differences in reactivity were observed (Fig. 1). Therefore, two types of antisera can be easily prepared; antiserum collected 7 days after primary administration of the 34,000 × g pellet is predominantly an agglutinating serum, whereas that collected 7 days after reinjection with the 34,000 × g supernatant fluid in adjuvant is a precipitating serum.

**DISCUSSION**

These studies established that EW extraction of *P. tularensis* provides the most useful antigens for antibody production and delineated the conditions that can be varied to suit particular requirements. This procedure was shown to be applicable to at least six strains of the organism, regardless of degree of virulence and geographic origin, and four species of laboratory animals.

**FIG. 1. Agar-gel precipitin reactions.** Center well, whole EW extract. Top well, antiserum from rabbit injected with whole EW extract in adjuvant. Lower left well, antiserum from rabbit injected with 34,000 × g pellet. Lower right well, antiserum from rabbit injected with 34,000 × g supernatant liquid in adjuvant.
were shown to respond with some level of antibody production. The best combination of antigens from strain SCHU S4 administered to rabbits.

These studies have an interesting sidelight, further evidence of the lack of a classic endotoxin in this bacterium. Lipopolysaccharide preparations from *P. tularensis* have been shown to possess some, but not all, of the various activities associated with other bacterial endotoxins (M. L. Guss, Bacteriol. Proc., p. 89, 1970). All six of the extraction techniques have been used for preparation of endotoxins from members of the Enterobacteriaceae. Although the standard 1.0-mg dose of antigenic CHO employed in these studies is far in excess of the median lethal dose of endotoxins for rabbits, no deaths were observed that could be attributed to this activity. Therefore, these preparations failed to exhibit an important characteristic of endotoxin, namely, lethal toxicity for the rabbit.

The observation that the major serological activities of antisera can be preselected by the appropriate combination of centrifugal fractionation, use of adjuvant, and injection schedule may provide a method for investigating the role of various antigens in infection immunity. The earlier work of Ormsbee, Bell, and Larson (5) amply documented the efficacy of EW extracts for producing immunogenic vaccines; EW extraction, in addition, offers a method for investigating the role various subcellular components play in the induction of immunity. It also provides a good crude antigenic mixture for the preparation of purified antigens and their related monospecific antisera.

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