Preparation of Agglutinating Antisera and Fluorescent-Antibody Conjugates Against *Pasteurella tularensis* in Equines

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The serological response in burros and horses to the viable LVS strain of *Pasteurella tularensis* was studied. High-titered agglutinating antisera and fluorescent-antibody conjugates were obtained in both groups of animals. Maximum titers were obtained in horses 14 to 21 days after the start of vaccination and in burros 21 to 28 days after the start of vaccination. The use of Woodhour's adjuvants or booster inoculations did not result in increased titers.

Bacterial antisera used in diagnostic laboratories have traditionally been produced in small laboratory animals. Although antisera prepared in these animals are very satisfactory, they are expensive to produce in large quantities. Large animals, particularly horses, have been used for preparing therapeutic and prophylactic bacterial antitoxins and antisera (5, 8). However, very little attention has been directed towards the use of large animals for producing bacterial diagnostic reagents. Bowmer (1) prepared in horses the international standards for *Clostridium botulinum* antitoxins which are used for the standardization of therapeutic and diagnostic antitoxins. Harrell et al. (4) prepared antisera in horses against the six types of *C. botulinum*. The latter antisera are used only as diagnostic reagents.

This paper describes the preparation of *Pasteurella tularensis* antisera in burros and horses. The effect of different primary vaccination schedules, the effect of vaccine with and without adjuvants, and the effect of booster doses of antigens given at intervals after the primary vaccination of the animals were studied.

**MATERIALS AND METHODS**

**Vaccine.** The attenuated LVS strain of *P. tularensis* was used for vaccination of the animals. Vaccines were prepared in essentially the manner described by Eigelsbach and Downs (3), except peptone cysteine broth was substituted for their liquid medium. Each batch of live vaccine was considered outdated after 30 days of storage at 5 C. In some experiments, the live vaccine was emulsified with an equal volume of adjuvant 65 described by Woodhour et al. (9). The number of viable cells in each lot of vaccine was determined by counting appropriate dilutions of organisms on glucose-cysteine-blood-agar plates.

**Evaluation of antisera.** Unless stated otherwise, the antigens used for evaluation of the *P. tularensis* antisera by the agglutination and fluorescent-antibody (FA) tests were live suspensions of the LVS strain in 0.85% sodium chloride. For the agglutination tests, the antigen was adjusted to the density of a no. 3 McFarland standard. The antigen-antisera mixture was incubated for 3 hr at 37 C, followed by overnight incubation at 5 C. The highest serum dilution giving a 2+ agglutination was considered the end point.

The FA titer was determined by the direct FA procedure, by using a BG-12 exciter filter and an OG-1 barrier filter. Conjugates were prepared by the following modifications of the method of Cherry et al. (2).

(i) The antiserum was diluted with 4 volumes of water, and the globulins were precipitated by adding an equal volume of saturated ammonium sulfate. (ii) The globulins were precipitated twice with ammonium sulfate. (iii) The globulins were conjugated with fluorescein isothiocyanate (FITC) at room temperature for 2 hr. Unreacted FITC was removed by dialysis against 0.02 M phosphate-buffered saline (pH 7.4). Prior to conjugation with FITC, the globulin fractions were checked for albumin by electrophoresis on cellulose acetate strips. Electrophoresis was carried out for 1 hr at room temperature with barbital buffer, ionicity 0.1 (pH 8.2). After electrophoresis, the strips were stained with Ponceau S and washed with 5% acetic acid. If albumin was detected, the globulin fraction was reprecipitated with ammonium sulfate.

The protein content of globulin fractions was determined before and after conjugation by the biuret test, by using a standard bovine serum albumin for establishing a standard curve. The FITC concentration of conjugated globulins was determined by optical den-
sity readings at 492 nm, with a standard curve established with fresh solutions of FITC suspended in phosphate-buffered saline (pH 7.4). All conjugates were adjusted to 10 mg of protein/ml. The fluorescence to protein ratio (F/P) of conjugates from different animals varied from 8 to 10 mg of FITC/mg of protein. The highest dilution that gave a 3+ to 4+ staining reaction was considered the end point.

Conjugates prepared from the final bleeding of the burros were evaluated for nonspecific staining by using KB tissue culture cells. The method described by Pittman et al. (7) was used, except that the amount of fluorescence was estimated visually rather than by a photometer. Slides containing the KB cells were reacted for 30 min at room temperature with serial twofold dilutions of the conjugate.

**Animals.** The horses used in this study were obtained from local suppliers. These animals weighed approximately 1,150 lb (520 kg) each. The burros were obtained through commercial channels from Mexico. These animals weighed approximately 350 lb (158 kg) each.

**Vaccination schedule.** The burros were inoculated subcutaneously (sc) with increasing numbers of live *P. tularensis* vaccine without adjuvant. The vaccination schedule is given in Table 1. The animals were test bled at weekly intervals and exsanguinated 44 days after the first inoculation.

Two different vaccination schedules were used for inoculation of the horses. In schedule I, three horses were inoculated intramuscularly (im) with $10^9$ cells in saline on day zero, followed by $10^9$ cells in saline im on day seven; two additional horses were inoculated by schedule I with the vaccine suspended in Woodhour's adjuvant (9). In schedule II, three horses were inoculated im with $10^9$ cells in saline followed by four im inoculations of $10^9$ cells in saline at seven-day intervals; three additional horses were inoculated by schedule II with the vaccine suspended in Woodhour's adjuvant. The im inoculations were equally distributed in the large muscles of the four legs.

The horses were given the first booster inoculations 100 days after the last primary inoculation. Several routes of inoculation and concentrations of cells were used. Five of the horses were given a second booster inoculation 268 to 338 days after the first one. The schedule of the booster doses is given in Table 2. Test bleedings were taken at weekly intervals during the primary vaccination series and at three or four day intervals after the booster inoculations.

| Day | No. of organisms inoculated subcutaneously$^a$ | Reciprocal agglutinin titer | Reciprocal FA titer |
|-----|-----------------------------------------------|------------------------------|---------------------|
| 0   | $10^4$                                        | NT$^b$                       | NT                  |
| 3   | $10^7$                                        | NT$^b$                       | NT                  |
| 7   | $10^8$                                        | NT$^b$                       | NT                  |
| 10  | $5 \times 10^8$                              | NT                           | NT                  |
| 14  | $5 \times 10^8$                              | 512                          | NT                  |
| 17  | $10^9$                                        | NT                           | NT                  |
| 21  | $10^9$                                        | 1,024                        | 80                  |
| 24  | $2 \times 10^9$                              | NT                           | NT                  |
| 28  | $3 \times 10^9$                              | 1,024                        | 160                 |
| 31  | $4 \times 10^9$                              | NT                           | NT                  |
| 35  | $2 \times 10^9$                              | 1,024                        | 160                 |
| 37  | $2 \times 10^9$                              | 1,024                        | 160                 |
| 42  | NT                                           | 1,024                        | 160                 |
| 44  | NT                                           | 1,024                        | 160                 |

$^a$ Viable LVS strain of *P. tularensis*.
$^b$ Not tested.

**Table 2. Vaccination schedule for the preparation in horses of *P. tularensis* antisera by using the viable LVS strain**

| Horse no. | Primary vaccination schedule | Adjuvants | Booster 1 | Booster 2 |
|-----------|-----------------------------|-----------|-----------|-----------|
|           |                             |           | Day       | iv        | im        | Day       | iv        | im         |
| 28        | I$^a$                       | None      | 119       | $2 \times 10^9$ | $2 \times 10^9$ | ND$^a$   |          |            |
| 29        | I                           | None      | 119       | $2 \times 10^9$ | $2 \times 10^9$ | ND       |          |            |
| 30        | I                           | None      | 119       | $2 \times 10^9$ | $2 \times 10^9$ | ND       |          |            |
| 31        | II$^c$                      | None      | 140       | $2 \times 10^9$ | $2 \times 10^9$ | 450      | $10^9$   | $2 \times 10^9$ |
| 32        | II                          | None      | 140       | $2 \times 10^9$ | $2 \times 10^9$ | 450      | $10^9$   | $2 \times 10^9$ |
| 33        | II                          | None      | 140       | $2 \times 10^9$ | $2 \times 10^9$ | 450      | $10^9$   | $2 \times 10^9$ |
| 36        | I                           | Yes       | 111       | $20 \times 10^9$ | $20 \times 10^9$ | Died     |          |            |
| 37        | I                           | Yes       | 111       | $20 \times 10^9$ | $20 \times 10^9$ | 450      | $10^9$   | $2 \times 10^9$ |
| 34        | II                          | Yes       | 112       | $20 \times 10^9$ | $20 \times 10^9$ | ND       |          |            |
| 40        | II                          | Yes       | 112       | $20 \times 10^9$ | $20 \times 10^9$ | 380      | $10^9$   | $2 \times 10^9$ |
| 41        | II                          | Yes       | 112       | $20 \times 10^9$ | $20 \times 10^9$ | Died     |          |            |

$^a$ Two im inoculations 7 days apart.
$^b$ These animals were not given a second booster inoculation.
$^c$ Five im inoculations 7 days apart.
RESULTS

P. tularensis antisera prepared in Mexican burros. The two Mexican burros inoculated with the viable LVS strain of P. tularensis gave essentially the same serological response. The agglutinin and FA titers of the serum from each test bleeding of one of the animals are given in Table 1. The maximum agglutinin titer of 1:1,024 was obtained 14 to 21 days after the start of vaccination and remained essentially the same during the remainder of the vaccination schedule, i.e., for 44 days. The FA titers reached their maximum of 1:160 approximately 28 days after the start of vaccination; these also remained essentially the same during the remainder of the vaccination schedule.

Both animals were exsanguinated 44 days after the start of vaccination. Samples of the final bleedings were evaluated by the agglutination and FA tests for cross-reactivity with heterologous organisms as well as homologous reactivity with the following strains of P. tularensis: LVS, Japan, P-38, and Turner. The cross-reactivity in both tests was very similar except that the Brucella species gave crosses in the agglutination test (1:32 to 1:128) but were negative in the FA tests. P. novicida reacted at a 1:32 dilution in the agglutination test and at a 1:20 dilution in the FA test. All four strains of P. tularensis gave essentially the same titers as reported above in the agglutination and FA tests.

Conjugates prepared from the final bleedings of the burros were also evaluated for nonspecific staining by using KB tissue-culture cells (7). Staining intensities varied from 4+ with undiluted conjugates to negative at a 1:32 dilution of the conjugates. Thus, although these conjugates were not evaluated on clinical material containing P. tularensis, the above data suggest that, if they were used at a dilution of 1:32 or higher, there should be no significant nonspecific staining of clinical specimens.

P. tularensis antisera prepared in horses. Eleven horses were inoculated with the live LVS strain by either schedule I or II (see above). The maximum agglutinin and FA titers were obtained on each animal 14 to 21 days after the start of vaccination (Table 3). The maximum agglutinin titers of individual animals varied from 1:256 to 1:1,024; the maximum FA titers varied from 1:80 to 1:360.

All of the animals were given booster inoculations, without adjuvant, 100 days after the primary inoculation; in addition, five of these animals were given a second booster inoculation without adjuvant 268 to 338 days after the first one (Table 2). No significant increase in agglutinin or FA titer over that obtained during the primary vaccination was noticed in any of the animals after the first or second booster dose (Table 3). Within 48 hr after the first booster inoculation, two of the animals died from an apparent toxemia resulting from the large numbers of organisms inoculated.

DISCUSSION

These studies show the feasibility of producing P. tularensis antisera in burros and horses. Good

| Horse no. | Primary vaccinationa | First booster | Second booster |
|-----------|----------------------|---------------|---------------|
|           | Day | Agglutinin | Day | FA | Day | Agglutinin | Day | FA | Day | Agglutinin | Day | FA |
| 28        | 14  | 1,024      | 14  | 320| 21  | 128        | 14  | 80 | ND  | ND | ND  | ND |
| 29        | 21  | 256        | 21  | 320| 4d  | 64         | 10  | 10 | ND  | ND | ND  | ND |
| 30        | 14  | 512        | 21  | 160| 4d  | 64         | 10  | 10 | ND  | ND | ND  | ND |
| 31        | 21  | 256        | 21  | 320| 4d  | 64         | 20  | ND | 21  | 256 | 14  | 40 |
| 32        | 21  | 256        | 21  | 160| 4d  | 32         | NT  |   | 21  | 256 | 7   | 20 |
| 33        | 21  | 256        | 21  | 80 | 4d  | 32         | 20  |    | 7   | 128 | 7   | 40 |
| 36        | 14  | 256        | 20  | 320| Died|             |     |   |     |     |     |    |
| 37        | 14  | 512        | 14  | 80 | 7   | 1,024      | 10  | 160| 7   | 512 | 14  | 40 |
| 34        | 14  | 1,024      | 20  | 80 | 10  | 256        | 10  | 160| ND  | ND | ND  | ND |
| 40        | 21  | 1,024      | 27  | 80 | 7   | 64         | 7   | 160| 7   | 256 | 7   | 20 |
| 41        | 14  | 1,024      | 14  | 80 | Died|             |     |   |     |     |     |    |

a See Table 2 for vaccination schedule.

b Reciprocal of highest dilution giving 2+ agglutination and 3+ to 4+ FA staining.

c These animals were not given a second booster inoculation.

d Maximum titer obtained in these animals was the same as the prebooster titer.
agglutinin and FA titers were obtained in both groups of animals after inoculation with the viable LVS strain of \textit{P. tularensis}.

Maximum agglutinin and FA titers were obtained 14 to 28 days after the start of vaccination in both groups of animals. Vaccination of the horses with two inoculations given 7 days apart (schedule I) was as effective as five inoculations given 7 days apart (schedule II). As might be expected, horses vaccinated by schedule II maintained their titer longer than did those animals inoculated by the shorter schedule I. The use of Woodhour's adjuvant did not appreciably affect the titers obtained by either vaccination schedule, since the range of titers was comparable in animals vaccinated with or without the use of adjuvant.

Very poor secondary responses were obtained in horses after both the first and second booster doses of antigen. Five of the animals did not show any increase in agglutinin or FA titer over the immediate prebooster titer. In the other four surviving horses, the agglutinin and FA titers were higher than those of the prebooster period, but not significantly higher than the titers obtained during the primary vaccination. Two of the five animals given booster inoculation of either $2 \times 10^{10}$ or $2 \times 10^{11}$ organisms intravenously (iv), and the same number im, died from what appeared to be a general toxemia. The use of these large inocula was predicated on the experience we had with horses 29 and 30. The latter two animals received one inoculation of $2 \times 10^{8}$ organisms im and $2 \times 10^{6}$ organisms iv, respectively. There was no increase in the agglutinin or FA titer over those of the prebooster period for either animal (Tables 2 and 3).

Nutter (6) recently reported on the vaccination of rabbits with the viable LVS strain of \textit{P. tularensis}. He did not report FA titers, but the agglutinin response in rabbits with the iv route of inoculation was comparable to those we obtained in equines with either the sc or im route of inoculation. Our results were also similar to those obtained by Nutter (6), in that a short vaccination schedule was the optimum method of vaccination and booster inoculations did not result in a higher antibody response than was obtained after the primary vaccination.

FA titers of 1:80 to 1:320 were obtained from all of the animals during or after the primary vaccination. These are comparable to the FA titers obtained by Yager et al. (10) in roosters after iv inoculation of the live SCHU S-4 strain.

From these results, it is apparent that either burros or horses can be used for the preparation of \textit{P. tularensis} FA or agglutinin antisera. When one considers a yield of 12 to 15 liters of antiserum from each average size horse and 2 to 3 liters from an average size burro, the advantages of using these animals for large-scale production are obvious. An additional advantage when using the live LVS strain is the short vaccination schedule. However, it is questionable whether equines can be used to prepare antisera against all bacteria. In our own laboratory, for example, we found that horses vaccinated with a killed \textit{P. pestis} antigen gave very poor quality FA antisera, although good agglutinin antisera were obtained. Other investigators have reported similar results after the vaccination of horses with \textit{P. pestis} (5).

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