Stable Reagent for the Detection of Antibody to the Specific Fraction I Antigen of Yersinia pestis

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A stable hemagglutinating antigen for detection of fraction I (FR-I) antibody of Yersinia pestis (Pasteurella pestis) is described. The antigen was prepared by sensitizing tanned, pyruvaldehyde-treated sheep erythrocytes (PAT SRBC) with FR-I antigen. Preliminary standardization by titration of each lot of FR-I was required to minimize the effect of molecular heterogeneity of specific FR-I antigen and to eliminate nonspecific reactions caused by the presence of a minor antigenic contaminant. In tests with sera from rabbits, dogs, and humans, FR-I PAT SRBC were as reactive as the previously employed standard antigen, FR-I-sensitized tanned erythrocytes. Fluid suspensions of FR-I PAT SRBC stored at 4 C for 3 months, or lyophilized preparations stored at ambient temperature for 6 months, showed no loss in antigenic activity.

The hemagglutination (HA) test, with the use of fraction I (FR-I)-sensitized tanned erythrocytes, is the most reliable simple technique for demonstration of plague antibody, provided that trained personnel and reliable reagents are available. With the current interest in the ecology and epidemiology of plague, use of this serological procedure has increased considerably (1, 5, 9, 10). However, many problems arise in connection with standardization, utilization, and interpretation of HA tests (11). Reports on attachment of proteins to tanned aldehyde-treated cells (7, 8) and on the HA activity of such treated cells (6) led us to investigate application of these techniques to the serology of plague. A stable HA antigen was developed by sensitizing pyruvaldehyde-treated sheep erythrocytes with purified FR-I of Yersinia pestis (Pasteurella pestis). Details for preparation of this antigen and experimental data on its specificity, sensitivity, and stability are described in this paper.

MATERIALS AND METHODS

Diluents. All reagent solutions were prepared in sterile, nonpyrogenic water for injection (USP). Sodium chloride for injection (USP) was employed in all saline diluents; saline containing 1% absorbed, inactivated rabbit serum constituted the washing fluid for FR-I-sensitized erythrocytes and the diluent for serum and antigen in HA titrations. Sodium azide at a final concentration of 0.1% was used as a preservative in suspending menstrua for storage of treated or sensitized erythrocytes. Buffering capability was supplied by Sorenson's 0.067 M phosphate buffer; except for preparation of the pyruvic aldehyde reactant, all buffer solutions were at pH 6.0.

Tanned, pyruvaldehyde-treated erythrocytes (PAT SRBC). Sheep red blood cells (SRBC), collected in Alsever's solution and stored at 5 C, were washed three times with 50 volumes of saline. One volume of 50% suspension was added with constant mixing to the following reactant solution: 1.5 volumes of 25% pyruvic aldehyde (40% aqueous solution, Aldrich Chemical Co., Milwaukee, Wis.) in 7.0 volumes of 1% sodium carbonate and 0.7 volume of pH 8.0 buffer, with final adjustment of the mixture to pH 7.0, if required. After reaction overnight at 4 C, the suspension was centrifuged at 1,000 × g for 5 min, and the treated SRBC were washed three times as described above. Washed, packed PAT SRBC were resuspended in a solution composed of 5.0 volumes of pH 6.0 buffer and 1.0 volume of 0.05% tannic acid (USP, powdered, Mallinckrodt Chemical Works, St. Louis, Mo.). After incubation at 25 C for

1 A portion of this work will be included in a dissertation to be presented by Dan C. Cavanaugh to the Graduate Faculty of the Microbiology Department, University of Maryland, College Park.
15 min, the treated PAT SRBC were sedimented and washed as before.
A 50% suspension of PAT SRBC prepared in 0.1% sodium azide-buffer diluent was stored at 4 C until used for serum absorption or processed to provide control antigen for HA titrations. A 10% suspension of PAT SRBC in the same diluent was reserved for FR-I sensitization studies.

FR-I-sensitized PAT SRBC. Lots of FR-I antigen from strains A1122, PKR 159, and 195/P of Y. pestis were prepared from acetone-killed and dried cells according to methods of Baker et al. (2). Stock solutions of FR-I dissolved to a concentration of 2.5 mg/ml in phosphate buffer were stored at -20 C.
Each stock solution of FR-I antigen was titrated by serial twofold dilution in buffer. Portions of 10% PAT SRBC suspension were sedimented, and each portion was resuspended to its original volume in one of the dilutions of FR-I antigen. After incubation for 30 min at 37 C and three washes with 50 volumes of 1% serum-saline diluent, sensitized PAT SRBC were adjusted to 50% suspension with sodium azide diluent and stored at 4 C.
For use in serum titrations, these suspensions and corresponding preparations of unsensitized PAT SRBC were sedimented, washed once, and adjusted to 0.5% suspension in 1% serum-saline diluent.
Before lyophilization, sodium azide diluent in 50% suspensions of sensitized or unsensitized PAT SRBC was replaced with an equal volume of absorbed, inactivated normal rabbit serum. Samples of 1.5 ml were dispensed in lyophilic ampoules, shell-frozen in an acetone-dry ice bath, and dried on a Thermovac freeze dryer. Dried antigens were resuspended in 150 ml of 0.1% sodium azide-saline and allowed to rehydrate for 48 hr prior to use.

Standard HA antigen. Tanned SRBC were sensitized with FR-I antigen as described by Chen and Meyer (3). Nonsensitized, tanned SRBC were used as control for the standard antigen.

Sera. Serum samples from humans immunized with plague vaccine, USP (Cutter Laboratories), and from dogs and rabbits infected with Y. pestis were stored at 4 C; sodium azide at a final concentration of 0.2% was employed as preservative. All sera were absorbed and inactivated by incubating a mixture of 9 volumes of serum with 1 volume of a 50% suspension of nonsensitized PAT SRBC for 30 min at 56 C. After centrifugation to remove SRBC, sera were ready for use.

HA titrations. HA titrations were performed in U-bottom plates with microtitration equipment. A sufficient number of replicate twofold dilutions of each test serum were prepared in a final volume of 0.05 ml to provide paired sets of dilutions for each antigen preparation. In each paired set, 0.05-ml amounts of FR-I sensitized SRBC were added to one dilution series, and corresponding amounts of nonsensitized SRBC were added to the other dilution series. Titrations were incubated overnight at 25 C and observed for agglutination. The end point of each titration was defined as the highest serum dilution that demonstrated 4+ agglutination, i.e., the bottom of the well was covered uniformly with erythrocytes.

As a control for specificity of each antigen preparation, a rabbit antiserum for FR-I and a human serum that reacted with a protein contaminant of FR-I preparations, but not with FR-I, were titrated in the same manner.

RESULTS
Each of three lots of FR-I contained a minor, nonspecific, antigenic contaminant that was readily absorbed on the surface of PAT SRBC (Fig. 1). The relative avidity of contaminant and FR-I for sites on PAT SRBC was not determined, but at high concentrations of FR-I preparations the contaminant was responsible for nonspecific agglutination with some sera. However, by titrating each lot of antigen, it was possible to select a concentration that reacted to titer with specific FR-I antisera but did not react with antibody for the nonspecific component in the control human serum. In the absence of careful titration, there was no assurance that HA titer was specific for FR-I antigen. For example, serum from one individual who had no known contact with Y. pestis had an HA titer of 1:2,048 for PAT SRBC sensitized with an excess of FR-I but did not agglutinate PAT SRBC sensitized with the optimal concentration of the same preparation. This nonspecific antigenic activity was not an artifact of the PAT sensitization procedure because it could be demonstrated with standard HA antigen preparations.

![Fig. 1. Hemagglutination titers for fraction I and nonspecific antibodies in replicate serum titrations tested with tanned pyruvaldehyde-treated erythrocytes after sensitization with various concentrations of three antigen preparations.](http://aem.asm.org/)

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Essentially no difference in HA antibody titer was noted when sera from rabbits, dogs, and humans were titrated with PAT antigens or standard HA antigen (Table 1). Titers obtained in 1969 and 1970 with FR-I sensitized tanned SRBC were in remarkable agreement with each other, as well as with titers obtained with PAT SRBC antigens. Reactions with lyophilized antigen in this series of titrations indicate the stability of the lyophilized product; these titrations were conducted with antigen that apparently was unaltered by shipment around the world, including a transit time of 6 weeks at ambient tropical temperatures.

As further evidence for the stability of PAT antigens, no appreciable difference in titers for individual sera or in geometric mean titers was observed when 25 sera were tested with freshly prepared standard HA antigen or with stored PAT antigens (Table 2). Prior to use, PAT antigens were stored either in liquid suspension for 3 months at 4°C or in the lyophilized state for 6 months at room temperature (25°C). Differences between antigen did not exceed the variability observed between repeated tests with a single antigen.

After prolonged storage, liquid suspensions of sensitized PAT SRBC occasionally demonstrated reactivity with standard control serum. These antigen preparations could be "revived" by sedimenting the cells and resuspending them in fresh serum-saline diluent.

**DISCUSSION**

Experimental results reported in this paper demonstrate that a stable HA antigen for detecting specific FR-I antigen can be prepared by utilizing SRBC stabilized with pyruvaldehyde and tannic acid as the carrier for FR-I antigen of *Y. pestis*. Reactivity of this antigen was unchanged by storage for at least 3 months in the fluid state at 5°C or for at least 6 months in the lyophilized state at 25°C. Decreased activity that was occasionally observed after storage in the fluid state could be readily eliminated by resuspending the antigen in fresh diluent, suggesting that a small amount of antigen was released from binding sites on PAT SRBC and acted as a competitive inhibitor for the reaction between antibody and bound antigen.

Our findings are in agreement with those of Ling (7, 8) and Jennings (6), who reported that the PAT technique was superior to Formalin or tannic acid techniques for preparation of antigen-sensitized erythrocytes. Ease of preparation, reproducibility, and storage stability of the product are important criteria for selecting a satisfactory procedure. In contrast to the short shelf life of tanned erythrocyte antigens and the difficulties of preparation and repro-

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### Table 1. Specific hemagglutinating (HA) activity of tanned erythrocytes (standard antigen) and tanned, pyruvaldehyde-treated erythrocytes (PAT) after sensitization with fraction I antigen of *Y. pestis*

| Serum and source | Reciprocal HA titer | Reciprocal HA titer | Reciprocal HA titer |
|------------------|---------------------|---------------------|---------------------|
|                  | Standard, 1969      | Standard, 1970      | PAT wet, 1970       | PAT lyophilized, 1970 |
| 1. Rabbit        | 2,048               | 2,048               | 2,048               | 2,048 |
| 2. Rabbit        | 1,024               | 2,048               | 1,024               | 1,024 |
| 3. Human         | Negative            | Negative            | Negative            | Negative |
| 4. Human         | 512                 | 256                 | 256                 | 256 |
| 5. Human         | 8                   | 8                   | 8                   | 8 |
| 6. Human         | 512                 | 256                 | 512                 | 512 |
| 7. Dog           | 16                  | 32                  | 16                  | 32 |
| 8. Dog           | 256                 | 256                 | 256                 | 512 |
| 9. Dog           | 2,048               | 1,024               | 2,048               | 2,048 |

### Table 2. Comparison of hemagglutinin (HA) titers in tests with freshly sensitized, tanned erythrocytes (standard antigen) and with stored preparations of sensitized, tanned pyruvaldehyde-treated erythrocytes (PAT)

| Serum no. | Reciprocal HA titer |
|-----------|---------------------|
|           | Standard | PAT | Lyophilized PAT |
| 1         | 256      | 256 | 256 |
| 2         | 1,024    | 512 | 256 |
| 3         | 64       | 64  | 128 |
| 4         | 1,024    | 1,024 | 1,024 |
| 5         | 128      | 128 | 128 |
| 6         | 512      | 1,024 | 1,024 |
| 7         | 256      | 256 | 128 |
| 8         | 512      | 512 | 256 |
| 9         | 128      | 256 | 256 |
| 10        | 128      | 256 | 256 |
| 11        | 128      | 256 | 256 |
| 12        | 128      | 128 | 128 |
| 13        | 128      | 256 | 256 |
| 14        | 256      | 256 | 256 |
| 15        | 256      | 128 | 256 |
| 16        | 64       | 128 | 64 |
| 17        | 64       | 128 | 128 |
| 18        | 64       | 64  | 64 |
| 19        | 128      | 128 | 128 |
| 20        | 256      | 256 | 256 |
| 21        | 512      | 512 | 512 |
| 22        | 1,024    | 1,024 | 1,024 |
| 23        | 128      | 256 | 256 |
| 24        | 256      | 512 | 512 |
| 25        | 128      | 256 | 256 |
| GMTa       | 200      | 242 | 223 |

* GMT = Geometric mean titer.
ducibility with Formalin-treated erythrocyte antigens, PAT antigens, when properly standardized, are remarkably stable and reproducible. Furthermore, in serum titrations the close agreement between standardized PAT antigens, fluid or lyophilized, and the standard tanned HA antigen indicate that the preparations measure the same antibody activity.

The importance of standardizing each lot of FR-I by titration cannot be overemphasized. Variations in molecular heterogeneity of FR-I (4) and in amount of antigenic contaminant could affect the reactivity of sensitized cells. Molecular heterogeneity probably was responsible for the observed differences in concentration (micrograms per milliter) required for preparation of FR-I reactive cells. Of greater interest, however, is the demonstration of a minor protein contaminant in all FR-I preparations. High concentrations of FR-I containing appreciable amounts of this contaminant occasionally reacted nonspecifically with sera from individuals who had no known contact with Y. pestis. While we have not succeeded in isolating or eliminating this contaminant from FR-I, it was possible by careful titration to select a concentration of FR-I wherein the effect of contaminant was minimized but there was sufficient FR-I for sensitizing treated SRBC.

Differences between various lots of FR-I dictate a necessity for preparing and standardizing HA reagents for plague serology in a centralized facility under rigorously controlled conditions. The stability of FR-I PAT SRBC would permit such production. Certified reagents could be dispensed to smaller laboratories as required, and the presence of sodium azide preservative in dilution fluid would eliminate the need for refrigeration during transit. Furthermore, since sodium azide preservative in test serum does not interfere with detection of FR-I antibody, extensive serum surveys can be performed at laboratories far removed from collection areas.

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