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Further Studies of Antigenic Structure of *Pasteurella pestis* in Gels.* (22260)

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The characterization of the antigens of *Pasteurella pestis* was begun by Schütze(1), who studied them using his serologic absorption and precipitin tests and identified a somatic thermostable and an envelope thermolabile antigen. The double diffusion method of Oudin(2) has provided another means of identifying additional antigens of *P. pestis*—10 by Ranson *et al.*(3), 8 by Burrows(4), 7 by Bhagavan *et al.*(5) and by us. Applying the Ouchterlony method(6) to living culture (7) revealed only 4 precipitation zones, but it was suspected that very small amounts of antigen might have remained undetected due to an excess of antibody in the antiserum used. It was learned that additional antigens do become evident if the culture is first concentrated by fractionation. Using the concentrated extract from *P. pestis* cells or the maximum growth of a shake culture supernatant as antigen in the Oudin test(2) has revealed 3 additional antigens.

Materials and methods. Strains. Those tested were one virulent strain, 195/P, isolated in a case of human plague in India, and 4 avirulent strains—E. V. 76(8,9), TRU(10) from the Java strain Tjiwidej R, TJS(11) and B1456-4(12) isolated by single colony picking from virulent strain B1456. The *P. pseudotuberculosis* strain was the avirulent strain 32, received from Dr. E. Thal, State Veterinary Medical Institute, Stockholm, Sweden. *Antisera.* The highly potent antiavirulent *P. pestis* gamma globulin was prepared commercially† from serum of rabbits hyperimmunized with living strain A1122. The *P. pseudotuberculosis* antiserum was prepared in this laboratory by hyperimmunizing rabbits with strain 32. *Agar.* A 1.6% Difco bacto-agar was prepared in normal saline and

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† By E. R. Squibb & Sons or Lederle Laboratories.

was sterilized by autoclaving at 15 lb pressure for 15 minutes. *Preparation of antigens.* Each strain was cultured in a casein hydrolysate mineral glucose medium(13) in a 1 liter wide-mouth Erlenmeyer flask containing 250 ml of the medium. The flask was fixed on a shaking machine kept in a 37°C incubation room. The culture was grown for 7 days and was divided according to the demands of the test.

The 5 methods of treating the cultures were (1) adding formalin to make a 1% solution and allowing the mixture to stand at room temperature for 48 hours, (2) adding toluene to make a 1% solution and allowing it to stand at room temperature for 48 hours, (3) placing it in a boiling water bath for 1 hour, (4) treating it with formalin as in (1) and then placing it in a boiling water bath for 1 hour and (5) treating it with toluene as in (2) and then placing it in a boiling water bath for 1 hour. Strains 195/P and B1456-4 were treated by all five methods, and the remaining 3 strains were treated only by the first two methods. The culture was then centrifuged in an angle rotor of a refrigerated centrifuge at 15000 rpm until all of the organisms had been deposited (approx. 30 minutes). The supernatant fluid was carefully removed and was ready for immediate use. *Preparation of tubes* (Oudin method). 1.6% agar in saline with 0.01% merthiolate was melted and maintained at 50°C. 0.7 ml of a 1:2 dilution of anti-A1122 gamma globulin was mixed with 0.3 ml of the melted agar. 0.5 ml of the mixture was delivered into a sterilized tube (8 mm x 80 mm). After the agar hardened, a second layer of 1 ml of 0.8% agar was carefully delivered over the bottom layer with a capillary pipette and allowed to become solid. 0.5 ml of the antigen preparation was delivered onto the top of the second agar layer, and a drop of toluene was added as preservative. The tubes were sealed with rubber stoppers and placed in the refrigerator overnight. The tubes were transferred to a 37°C incubator. Readings were made at the 3rd, 7th and 15th days.

Results. The visible precipitation zones appeared after 3 days of incubation when the antigens and antibodies had diffused into the plain agar layer. The number of zones depended on the antigen preparation used (Table I).

TABLE I. Number of Antigenic Components of *P. pestis* and *P. pseudotuberculosis* Revealed by the Oudin Test.

Antigen*	Treatment of antigens	No. of precipitation bands — Antiserum — *P. pestis* A1122 gamma globulin	No. of precipitation bands — Antiserum — *P. pseudotuberculosis* 32
195/P	Formalin	7	5
B1456-4		7	5
TJS		7	5
E.V. 76		7	5
TRU		7	5
195/P	Toluene	7	5
B1456-4		7	5
TJS		7	5
E.V. 76		7	5
TRU		7	5
195/P	Boiling	3	2
B1456-4		3	2
195/P	Toluene and boiling	3	2
B1456-4		3	2
195/P	Formalin and boiling	1	1
B1456-4		1	1

* 195/P is a virulent strain and the remaining 4 are avirulent.

With *P. pestis* antiserum, the chemically killed virulent and avirulent strains all contained 7 soluble antigens; 4 zones were intense and appeared earlier than the remaining 3. In the Oudin test 3 zones formed in the boiled preparation, 3 in the toluene-killed and boiled preparation and 1 in the formalin-killed and boiled preparation.

When *P. pseudotuberculosis* antiserum was used with the *P. pestis* strains, 5 zones appeared with the formalin or toluene treated, 2 with the boiled and the toluene-treated and boiled, and only 1 with the formalin-treated and boiled.

All of the thermostable antigens of *P. pestis* except the haptenized Fraction I were common to *P. pseudotuberculosis.*

Discussion. When the naturally occurring bacterial cells of *P. pestis* and *P. pseudotuberculosis* strains and their fractions were tested with anti-*P. pestis* gamma globulin by means of a modified Ouchterlony plate technic, 2

antigen-antibody precipitation reactions were common to both organisms, but Fraction I (envelope antigen) and II (toxin) reactions were specific for *P. pestis*(7). The very slight amount of Fraction I in strain 14 and TRU was not revealed, and this was to be expected because the antigens could not reach equivalence concentration.

The Oudin test revealed more antigenic components: All virulent and avirulent strains formed 7 precipitation zones, 5 of which were also common to *P. pseudotuberculosis*. Four of the corresponding antigens in the anti-*P. pestis* serum tube and 2 in the anti-*P. pseudotuberculosis* serum tube were distinctly precipitated. These 4 should therefore be considered major quantitatively. The others more faintly precipitated always appeared slowly.

Of the 7 antigens, 4 were thermolabile and 2, thermostable. The remaining antigen was the haptenized Fraction I. Of the antigens common also to *P. pseudotuberculosis*, 3 were thermolabile and 2, thermostable.

Schütze(1) pointed out that steaming the bacterial suspension in saline for a short time (¼ hour) haptenized the envelope antigen, but that it still precipitated in his precipitin test with its corresponding antibody. Steaming for 1 hour destroys it for *in vitro* reactions. But even after boiling the casein hydrolysate mineral glucose grown culture for 1 hour, the Fraction I (envelope antigen) antigen-antibody reaction zone still was sharply defined in the Oudin test. Boiling for 1 hour haptenizes Fraction I, but it will still form a precipitate with its corresponding antibody. This has been learned by boiling the highly purified Fraction I in different concentrations, using it as an external reactant.

Also in the complement-fixation test(14) using 4 combining units of the anti-Fraction I serum to react with the supernatant of the boiled and of the toluene-killed and boiled suspension, the reaction has been positive. The complement-fixation test was negative when the formalin-killed and boiled preparation was used.

The fact that 3 zones appeared in the boiled and in the toluene-killed and boiled antigens and that only 1 faint zone appeared in the formalin-killed and boiled antigen indicates that formalin affects the antigens and renders them thermolabile.

The technic of double diffusion in gels is of apparent value for comparative studies of heterologous, but closely related, organisms (7). More than 1 antigen is shared by *P. pestis* and *P. pseudotuberculosis*, and this may account for the cross immunity exhibited between these two species. The Oudin technic confirms that Fraction I is the specific antigen for *P. pestis*. Its toxin has cross-reacted with anti-*P. pseudotuberculosis* serum(15), but in a previous study(7) a slight amount of *P. pseudotuberculosis* toxin was not revealed with the anti-*P. pestis* gamma globulin. The *P. pestis* toxin prepared by the paper electrophoresis method in this laboratory has shown 3 bands with anti-*P. pseudotuberculosis* serum and 3 or 4 bands with anti-*P. pestis* gamma globulin. This might be considered a not highly purified toxin.

Virulent strains were not distinguished from avirulent strains by the test.

Summary. *Pasteurella pestis*, virulent or avirulent, contains 7 antigens demonstrable in the Oudin test. Five are common to *P. pseudotuberculosis;* 3 of these are thermolabile and 2 are thermostable. Of the 7 antigens of *P. pestis* 4 are thermolabile and 2 thermostable. The remaining antigen is haptenized Fraction I. Formalin affects all of the antigens except one, rendering them thermolabile; toluene does not affect them.

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Occurrence of Agglutinogens in Normoblasts.* (22261)

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It is well known that uptake of iron and hemoglobin synthesis occur in the normoblast. There is little information, however, as to the stage of development of the erythrocyte at which agglutinogens first appear. Bjorkman's(1) demonstration that normoblasts may be agglutinated by influenzal virus and a serum containing cold agglutinin suggests that receptors for agglutination make their appearance during this developmental period of the erythrocyte. In his experiments, the normoblasts were isolated from the peripheral blood of a patient with Di Guglielmo's erythro-leukemia; however, the cells were morphologically indistinguishable from myeloblasts. Inasmuch as neoplastic cells could have atypical immunologic characteristics, it seemed desirable to confirm these studies using normoblasts from a patient in which the cells were not of malignant origin.

An opportunity to study agglutinability of normoblasts arose in a patient with autoimmune hemolytic disease secondary to warm agglutinins. About a week following splenectomy, this patient had a hemolytic crisis at which time he released large numbers of nucleated red cells into the peripheral blood. During the period when the normoblast count exceeded 600 per 100 leukocytes, it was a simple matter to isolate them from the peripheral blood for agglutination experiments. A more detailed description of this case will be published elsewhere.

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Methods. To isolate the nucleated red cells, 1.0 ml of a 6% solution of dextran in 0.85% saline was added to 20 cc of the patient's freshly drawn blood in a large siliconized test tube. A 10% solution of sequestrene (disodium ethylenediamine tetracetate) was used as anticoagulant. The mixture was incubated at body temperature for 30 minutes. The supernatant plasma, rich in formed elements, was centrifuged at varying speeds, beginning at 600 rpm for 2 minutes. The residue was resuspended in saline and the supernatant plasma recentrifuged at progressively faster speeds. The resulting cell suspensions were checked for cellular content by means of the phase microscope. It was possible in this way to select test tubes which contained a reasonably pure suspension of normoblasts which were then washed 3 times in saline and prepared as a 4% suspension in 0.85% saline. Suspensions prepared in this way contained a minimal number of contaminating erythrocytes and lymphocytes, but no granulocytes or platelets were present. The normoblast suspension was brought in contact with equal portions of serial saline dilutions of serum from the same patient which contained a potent panhemagglutinin, active at 3°C and 37°C. The normoblast suspension was similarly added to serially diluted serum obtained from another patient with a high titer of cold hemagglutinins. The mixtures were incubated for 12 hours at 3°C and read macroscopically and under the phase microscope. The agglutinating effect of nor-