that attained by the freeze–thaw procedure.

Summary. The sensitivity of the passive hemagglutination reaction was increased considerably by subjecting stabilized rabbit erythrocytes to periodate oxidation, repeated freezing and thawing, or to low pH prior to coating with the antigen BSA. Cells were stabilized by pyruvic aldehyde followed by formaldehyde and then coated with antigen at low pH.

1. Hirata, A. A. and Brandriss, M. W., J. Immunol. **100**, 641 (1968).
2. Hirata, A. A. and Stall, W. T., Proc. Soc. Exptl. Biol. Med. **130**, 343 (1969).

Received Feb. 26, 1969. P.S.E.B.M., 1969, Vol. 131.

Demonstration of Blood Group Substance A Bound to *Pasteurella pestis* (33993)

ANTHONY J. LUZZIO
(Introduced by F. DeVenuto)
U. S. Army Medical Research Laboratory, Fort Knox, Kentucky 40121

Recently it was reported that high titer anti-A agglutinins and hemolysins occurred in human blood Group O donors immunized against plague with *Pasteurella pestis* vaccine. It was suggested that this result was due to the presence of blood Group A antigen in the vaccine injected (1). In view of the significance of this observation relative to obvious potential problems inherent in a relationship between *P. pestis* vaccine and blood group antigens, it is important to determine the source and extent of this antigenic relationship.

In the present study, sera from rabbits that were given primary immunizing doses of human Group A red cell stromata were quantitatively assayed for hemolysins against human A, B, and O red cells at periodic intervals following injection. The animals were then placed into four subgroups and reinjected with: human type A red cell stromata, intact commercial *P. pestis* vaccine containing trace amounts of beef heart extracts and porcine peptones and peptides (vaccine I), washed *P. pestis* bacilli from vaccine I, and vaccine I cell free suspending medium. At periodic intervals following injection, the serum from each rabbit was assayed to determine the secondary immune response for hemolysins against human A, B, and O red cells elicited by each preparation.

In a second experiment one group of rabbits was initially injected with human type A red cell stromata, while a second group was initially injected with washed *P. pestis* bacilli harvested from a commercial vaccine containing trace amounts of beef heart extract and soya peptones and peptides (vaccine II). Each of these two groups was subsequently divided into two subgroups, and each of the subgroups was then reinjected with human A red cell stromata, or washed *P. pestis* bacilli (vaccine II), respectively.

The data from these experiments showed that hemolysins against all human red cell types are elicited in a typical primary and secondary immune response when rabbits are immunized with human A red cell stromata. Washed *P. pestis* bacilli stimulated anti-A hemolysins only when they were prepared from a vaccine containing porcine peptones and peptides in addition to beef heart extract (vaccine I). It is concluded that *P. pestis,* cultured or maintained in an environment containing meat extracts or meat peptones and peptides, binds blood group A specific substance, from these sources, on its cell surface or incorporates it as an integral part of the cell.

Materials and Methods. Preparation of antigens, P. pestis *vaccine. P. pestis* vaccine I was a commercial preparation (Cutter Laboratories) containing 2×10^9 formalin killed *P pestis* bacilli/ml of suspending medium which

consisted of: sodium chloride (injection, USP) trace amounts of beef heart extract, agar, bovine and porcine peptones, and formalin and phenol in preservative concentrations of 0.01 and 0.5%, respectively.

Vaccine II was a commercial preparation (Cutter Laboratories) containing 2×10^9 formalin killes *P. pestis* bacilli/ml of suspension. The suspending medium consisted of: sodium chloride (injection, USP) and trace amounts of: agar, beef heart extract, yeast extract, and peptones and peptides of soya and casein. Fromalin and phenol were added in preservative concentrations of 0.02 and 0.5%, respectively.

Vaccine suspending medium. Vaccine I was centrifuged in a refrigerated International Centrifuge (model PR-2) at 3000 rpm for 30 min, after which the cell free supernatant was decanted and retained for injection.

Washed P. pestis *bacilli. P. pestis* bacilli remaining after the removal of the supernatant, following centrifuging, were washed five times with 0.15 *M* phosphate-buffered saline, pH 7.4. After the final wash the cells were resuspended in the same buffer containing 0.01% Merthiolate so that 1.0 ml contained 2×10^9 bacilli.

Human red blood cells. Human blood, obtained from the Blood Transfusion Division, US Army Medical Research Laboratory, Fort Knox, Kentucky, was used within 21 days after collection. On the day used the red cells were separated from the blood serum by centrifuging and decanting and repeatedly washed with cold Veronal buffer, pH 7.4, (2) until a colorless supernatant resulted. The packed red cells were resuspended in Veronal buffer and standardized at 500 mμ with a Coleman Jr., spectrophotometer by measuring the hemoglobin liberated by distilled HOH lysis. The optical density (OD) of the clear lysate was 0.71 ± 0.05 which corresponded to a count of $4.8 \pm 0.85 \times 10^9$ red cells.

Preparation of type A red cell antigen. Human type A red cell stromata were prepared by the stepwise lysis of the red cells in decreasing concentrations of phosphate-buffered saline (2). After removal of all pink color by repeated washings, the stromata were resuspended in 0.15 *M* phosphate buffered saline, pH 7.4, counted with a hemocytometer, and further diluted with buffer so that 1.0 ml contained 6×10^7 or 8.14×10^9 cells/ml. The stromata were not autoclaved to inactivate isophile antigens.

Immunizations. Twelve male rabbits weighing between 2.5 and 3.0 kg were given a single primary intravenous injection of 6×10^7 human type A, red cell stromata in 1.0 ml of phosphate-buffered saline. Subsequently, the animals were divided into 4 subgroups, and the subgroups were reinjected intravenously with one of the following preparations: 1.0 ml of whole *P. pestis* vaccine I, 1.0 ml of saline containing 0.01% Merthiolate and 2×10^9 washed *P. pestis* bacilli from vaccine I, 1.0 ml of cell free vaccine suspending medium, or 1.0 ml of human type A, red cell stromata (6×10^7 cells), respectively.

In a second experiment using 20 male rabbits weighing between 2.5 and 3.0 kg, 10 were initially injected with human type A red cell stromata, and 10 were injected with washed *P. pestis* bacilli harvested from vaccine II. To enhance the possible demonstration of blood group substances in washed bacilli from vaccine II a more intensified schedule of immunization was used. The immunization schedule consisted of five primary injections of red cell stromata, in single doses of 8.14×10^9 cells, or washed *P. pestis* bacilli, in single doses of 2×10^9 cells. The injections were made 1 day apart with a 5-day lapse between the third and fourth injections.

The two groups were subsequently divided into two subgroups each. Those initially injected with type A red cell stromata were reinjected with three single doses (2×10^9 cells) of washed *P. pestis* bacilli, or three single doses (8.14×10^9 cells) of human type A red cells stromata. Similarly, those initially injected with washed *P. pestis* bacilli were also reinjected with human type A red cell stromata or washed *P. pestis* bacilli.

All animals were bled before, and at periodic intervals after injection. The blood was allowed to clot at room temperature, and the

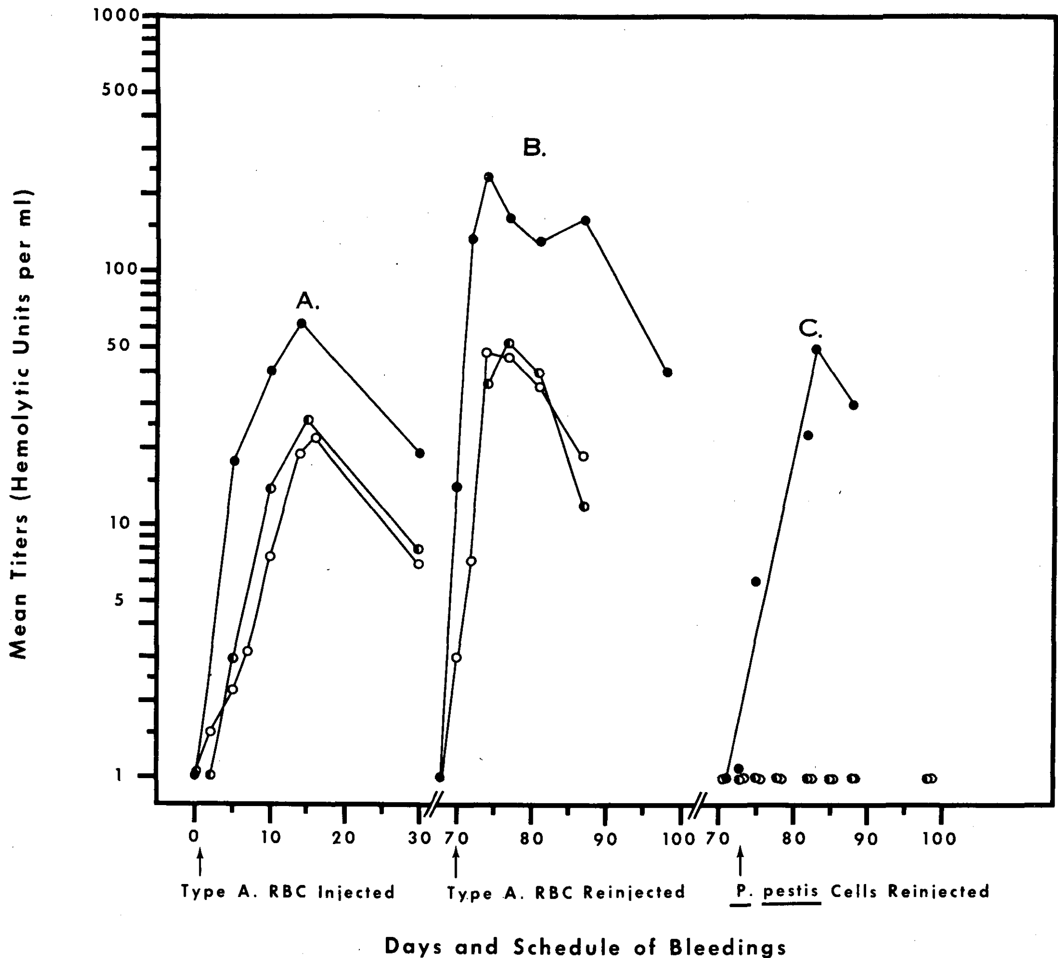

FIG. 1. Anti-A (●); anti-B (◒); and anti-O (○) hemolysin response in rabbits following injection with: (A) human type A red cell stromata; (B) reinjection with human type A red cell stromata, and (C) reinjection with washed *P. pestis* bacilli from vaccine I.

serum was separated by centrifuging and decanting. All sera were stored at —15°, and were thawed and inactivated at 56° for 30 min on the day of analysis.

Hemolysin titrations. A detailed description of the method used for the titration of hemolysin was given in (4). The hemolytic activity of serum tested against human type A, B, and O red cells was measured in 50% hemolytic units, which is defined as the amount of serum which lysed exactly half of the cells in a standardized 2-ml suspension of human red cells in the presence of four 50% units of guinea pig complement. The standard suspension of red cells contained 4.9×10^8 red cells/2 ml which when packed by centrifuging and lysed with 5 ml of distilled water gave a reading of 0.71 ± 0.05 OD on a model A Coleman junior spectrophotometer at 500 mμ.

The curves for hemolysin production are graphed on a semilogarithmic plot of titer against time, and the titers are shown as the number of 50% units/ml of serum. Complement was titrated with standardized human type A, red cells and a standard hemolysin of known titer.

Results. The data in Fig. 1 show that rabbits injected with single primary and secondary immunizing doses of human type A, red cell stromata produced hemolysins against human type A, B, and O, red cells in typical

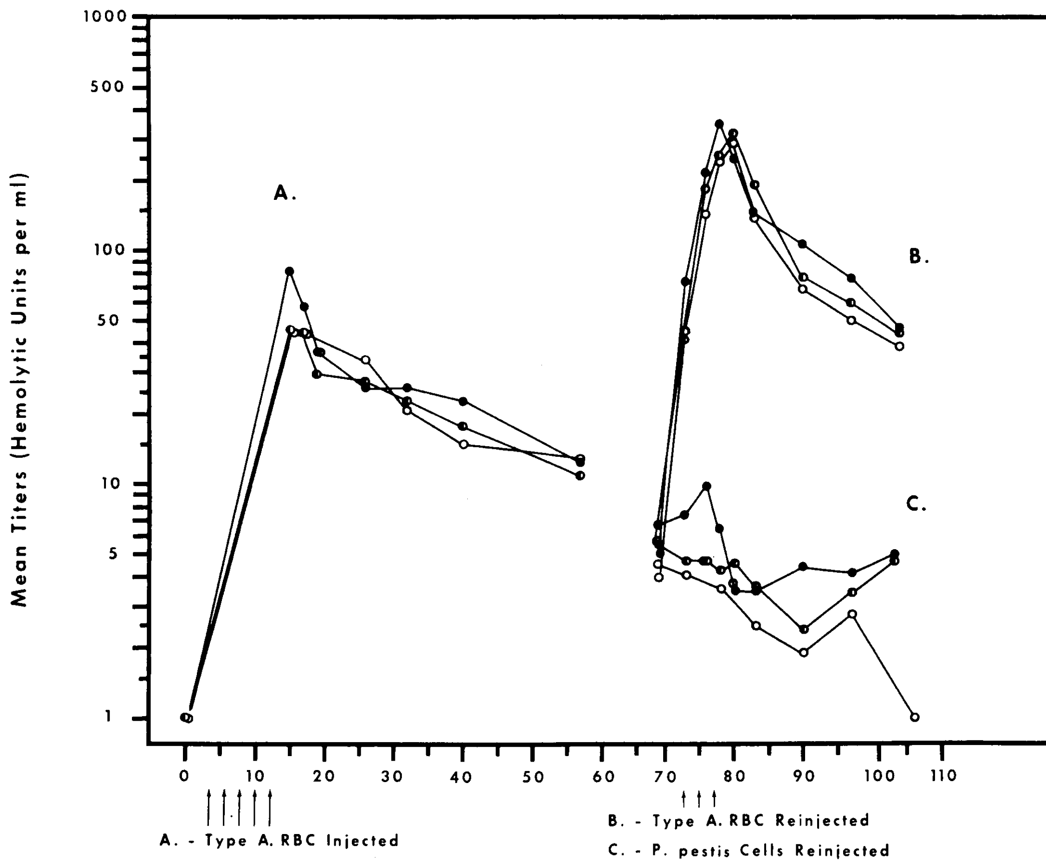

FIG. 2. Anti-A (●), anti-B (◒), and anti-O (○) hemolysin response in rabbits following multiple injections of: (A) human type A red cell stromata, (B) reinjection with human type A red cell stromata, and (C) reinjection with washed *P. pestis* from vaccine II.

primary and secondary immune responses. In both immune responses the homologous anti-A hemolysin occurred in greater concentration than anti-B or anti-O hemolysins. Figure 1 also shows that in those animals first sensitized with human type A red cell stromata and then reinjected with washed *P. pestis* from vaccine I, a secondary response resulted which elicited hemolysins specific only for human type A red cells.

Data not recorded here showed that while washed *P. pestis* bacilli from vaccine I stimulated significant amounts of anti-A hemolysin, in rabbits first sensitized with human type A red cell stromata, whole vaccine I, cell washings, or cell-free vaccine suspending medium did not produce antibody against any of the red cells.

Rabbits that received multiple primary and secondary immunizing injections of human type A red cell stromata produced mean peak titers of 82, 46, and 46 units of anti-A, B, and O hemolysins, respectively, during the primary response (Fig. 2A). Student's t test, applied to the data, showed that anti-A hemolysin was significantly higher than anti-B or O ($p < .05$), and that a significant difference between anti-A, B, and O hemolysins did not occur during the secondary immune response (Fig. 2B). When compared to the

group of rabbits that received a single immunizing injection (Fig. 1), the multiply injected group (Fig. 2) produced more antibody following primary and secondary stimulation with human type A red cell stromata. These results are in agreement with previously reported findings that, within certain ranges, an increase in antigenic stimulation induces greater antibody hemolysin production within a shorter time (5).

A secondary rise in anti-A hemolysin titer did not occur in rabbits reinjected with three doses of washed *P. pestis* cells from vaccine II after primary stimulation with human type A red cell stromata (Fig. 2C). At peak anti-A titer, anti-A, B, and O hemolysin titers were 10, 5, and 4 units, respectively. The differences between titers were not statistically significant when Student's t test was applied to the data ($p > .10$). The data in Figs. 2A and C suggest that these are residual titers from the primary immunization with human type A red cell stromata.

Data not recorded here showed that rabbits given five primary injections of washed *P. pestis* from vaccine II in single doses of 2.0×10^9 cells, did not elicit an immune hemolysin response against any of the red cell types before or after three reinjections with the same antigen. A primary type immune response was produced in rabbits after reinjection with human type A red cell stromata subsequent to primary stimulation with *P. pestis*.

Discussion. Studies involving group O military blood donor personnel showed that anti-A hemolysin and agglutinin antibody is markedly increased following multiple injections of plague vaccine (1). The data reported here confirms this finding for rabbits, and indicate that blood group A specificity is imparted to *P. pestis* by blood group substance contaminating sources present in the medium used to cultivate the organisms. This conclusion is drawn from the finding that repeatedly washed *P. pestis* prepared from a vaccine containing peptones and peptides of animal origin (vaccine I) elicited anti-A hemolysins in rabbits, whereas *P. pestis* prepared from an environment of peptones and peptides of plant origin (vaccine II) did not. It is interesting that whole vaccine I or its cell free suspending medium did not show the marked anti-A immune response elicited by the washed bacilli harvested from the same vaccine. There is no ready explanation for this discrepancy, except for the possibility that antigenic groups on the cell surface could have been masked by media products easily removed by cell washing. It appears that, in rabbits, the blood group specific substance expresses itself antigenically only when a concentrated amount binds to the cell surface, and that in soluble form it is either weakly antigenic or present in subminimal antigenic concentrations.

It is generally accepted that blood group substances present in meat digests stem from the excretion of these products into tissues via the gastric and intestinal mucosa and other secretory organs where they have origin [see review by Kabat, (6)]. Stock (7) pointed out that bacteria cultured on media containing peptones may be contaminated with significant amounts of blood group substances from this source. The presence of group A, B, and O antigens has been reported in a number of animal parasites cultivated or isolated from animal tissues (8, 9), and Pettenkoffer and Bickerich (10) reported that *P. pestis* grown on media free of blood group contaminating substances had no influence on the anti-A response following injection into rabbits. The present report supports these findings, and provides additional evidence which suggests that blood group substances are firmly fixed to the bacterial cell, by a simple or more complex binding mechanism. Thus, the status of the blood group substances changes from that of a mere contaminant in the environment, to that of being an integral part of the bacterial cell. It becomes increasingly evident that the role of peptides, peptones, and other meat digests must be considered when they are present in an environment used to culture organisms that are to be utilized for the study of antigenic relationships between certain organisms and blood group antigens or for antigenic

preparations to stimulate specific immune responses.

Summary. Primary and secondary immune responses occurred in rabbits following injection of type A human red cell stromata. Hemolytic activity against human B and O red cells also occurred, but not to the same extent seen for the type A red cells. Washed *P. pestis* bacilli stimulated a secondary, specific, anti-A hemolysin response only when they were harvested from a vaccine containing porcine peptones and peptides, in addition to beef heart extract. The data clearly show that *P. pestis* bacilli grown in media containing meat products contain an antigen similar to human Group A angien. Furthermore, the results suggest that Group A antigen, from these contaminating sources, is bound or incorporated in the bacterial cell surface.

1. Camp, R., Jr. and Shields, C. E., Military Med. 132, 426 (1967).

2. Kabat, E. A. and Mayer, M. M., "Experimental Immunochemistry," Thomas, Springfield, Illinois (1961).

3. Jaraslow, B. N. and Taliaferro, W. H., J. Infect. Diseases 98, 75 (1956).

4. Taliaferro, W. H. and Taliaferro, L. G., J. Infect. Diseases 87, 37 (1950).

5. Taliaferro, W. H. and Taliaferro, L. G., J. Infect. Diseases 113, 155 (1963).

6. Kabat, E. A., "Blood Group Substances." Academic Press, New York (1956).

7. Stock, A. H., J. Bacteriol. 38, 511 (1939).

8. Oliver-Gonzales, J. and Torregrosa, M. V., J. Infect. Diseases 74, 173 (1944).

9. Oliver-Gonzales, J., Proc. Soc. Exptl. Biol. Med. 84, 520 (1953).

10. Pettenkoffer, H. J. and Bickerich, R., Zentr. Bakteriol. Parasitenk., Abt. I. Orig. 179, 433 (1960).

Received Feb. 28, 1969. P.S.E.B.M., 1969, Vol. 131.

Antigenicity of Psittacosis Vaccines Killed by Ionizing Radiation (33994)

GEORGE G. WRIGHT, NORMAN S. SWACK,[1] AND JOHN R. MITZEL

Medical Sciences Laboratory, Fort Detrick, Frederick, Maryland 21701

Nonviable psittacosis vaccines killed by the action of formaldehyde have been derived from preparations of the agent propagated in tissues of animals, in embryonated eggs, or in tissue culture (1–3). Preparations of high initial infectivity yielded vaccines that provoked significant protection against intraperitoneal challenge in mice and other animals. Protection was less effective against respiratory challenge, although vaccines propagated in human diploid cell cultures were evidently more effective than earlier preparations (3). Further increase in antigenicity would be desirable to increase the level and duration of immunity, or to obviate the requirement for adjuvants or for multiple-dose immunization. Recent studies showed that vaccines for a variety of agents killed by ionizing radiation retain greater antigenicity than vaccines killed by chemicals or heat (4–6). Observations on the applicability of this method to preparation of psittacosis vaccines are presented below.

Materials and Methods. Tissue culture. Four- to 6-day-old monolayers of the human diploid cell strain WI-38 (7) were used for propagation of agent for preparation of vaccines. Cultures were used between passages 20 and 30.

Agent strain. The Borg or Louisiana strain of *Chlamydia psittaci* was obtained from the Virus and Rickettsia Division, Fort Detrick, as the fourth yolk-sac passage. The sixth yolk-sac passage was used both for seed inoculum and challenge of mice.

Suspensions of Borg agent. Cell monolayers were inoculated with Borg agent and after incubation for 2 hr at 37° were washed with balanced salt solution (BSS) (8). Maintenance medium was then added, consisting of basal medium (9) prepared with BSS and

[1] Present address: Virology Laboratory, Veterans Administration Hospital, West Haven, Connecticut 06516.