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REPORT NO. 798

DEMONSTRATION OF BLOOD GROUP SUBSTANCE A BOUND TO PASTEURELLA PESTIS

(Final Report)

by

Anthony J. Luzzio, Ph.D.

5 November 1968

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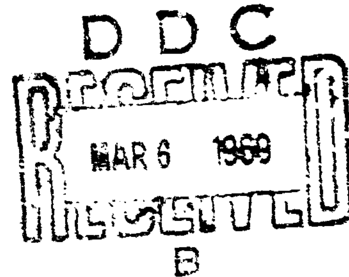

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DEMONSTRATION OF BLOOD GROUP SUBSTANCE A BOUND TO *PASTEURELLA PESTIS*

(Final Report)

by

Anthony J. Luzzio, Ph. D.

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US ARMY MEDICAL RESEARCH LABORATORY
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Pasteurella pestis and Human Blood Group
Cross-Reacting Antigens
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ABSTRACT

DEMONSTRATION OF BLOOD GROUP SUBSTANCE A BOUND TO PASTEURELLA PESTIS

OBJECTIVE

To determine the source, occurrence and location of human blood group antigens in P. pestis vaccines, and to quantitate the hemolytic potency of rabbit anti-P. pestis sera for human type A, B and O red cells.

METHODS

Hemolysins for human red cells were quantitatively determined in the sera of rabbits initially immunized with human type A red cell stromata and then reinjected with various gross fractions prepared from P. pestis vaccines. Serum samples were assayed at periodic intervals following injection, and primary and anamnestic hemolysin response curves were established for rabbits after initial injection with human A red cell stromata, and then after reinjection with P. pestis whole vaccine or various fractions prepared from the vaccine.

SUMMARY

Repeatedly washed P. pestis bacilli prepared from a vaccine containing peptides and peptones of animal origin elicited human anti-A red cell hemolysins in rabbits, whereas similarly treated P. pestis from a vaccine containing peptones and peptides of plant origin did not. Whole vaccine and its various subfractions did not stimulate antibody production for any of the human red cell types when injected into rabbits.

CONCLUSIONS

Human blood group A specificity is imparted to P. pestis by blood group contaminating substances present in the media used to cultivate the organism. It appears that, in rabbits, the specific substance expresses itself antigenically only when a concentrated amount is bound to the cell, and that in soluble form it is weakly antigenic or present in subminimal antigenic concentrations.

DEMONSTRATION OF BLOOD GROUP SUBSTANCE A BOUND TO *PASTEURELLA PESTIS*

INTRODUCTION

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 this report, 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 divided 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, and 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 x 10^9 formalin killed P. pestis bacilli per 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 0.05 percent, respectively.

Vaccine II was a commercial preparation (Cutter Laboratories) containing 2 x 10^9 formalin killed P. pestis bacilli per 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. Formalin and phenol were added in preservative concentrations of 0.04 and 0.5 percent, respectively.

Vaccine suspending medium. Vaccine I was centrifuged in a refrigerated International Centrifuge (Model PR-2) at 3000 rpm for 30 minutes, 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 percent merthiolate so that 1.0 ml contained 2 x 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 (2) until a colorless supernatant resulted. The packed red cells were resuspended in 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 ± 0.85 x 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 (3). After removal

of all pink color by repeated washings, the stromata were resuspended in full strength physiological-isotonic phosphate buffered saline, counted with a hemacytometer, and further diluted with buffer so that 1.0 ml contained 6×10^7 or 8.14×10^9 cells per 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 four subgroups, and the subgroups were reinjected intravenously with one of the following preparations: 1.0 ml whole P. pestis vaccine I, 1.0 ml saline containing 0.01 percent merthiolate and 2×10^9 washed P. pestis bacilli from vaccine I, 1.0 ml cell-free vaccine suspending medium, or 1.0 ml 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 one day apart with a five 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 cell 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 serum was separated by centrifuging and decanting. All sera were stored at -15°C, and were thawed and inactivated at 56°C for 30 minutes on the day of analysis.

Hemolysin titrations. A detailed description of the method used for the titration of hemolysin may be found in (4). The hemolytic activity of serum tested against human type A, B and O red cells was measured in 50 percent 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 percent 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 x 0.05 OD on a Model A Coleman Jr. 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 percent units per ml of serum.

Complement was titrated with standardized human type A, red cells and a standard hemolysis of known titer.

RESULTS

The data in Figure 1 (next page) 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 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, page 6) produced more antibody following primary and secondary stimulation with human type A red cell stromata. These results are in agreement

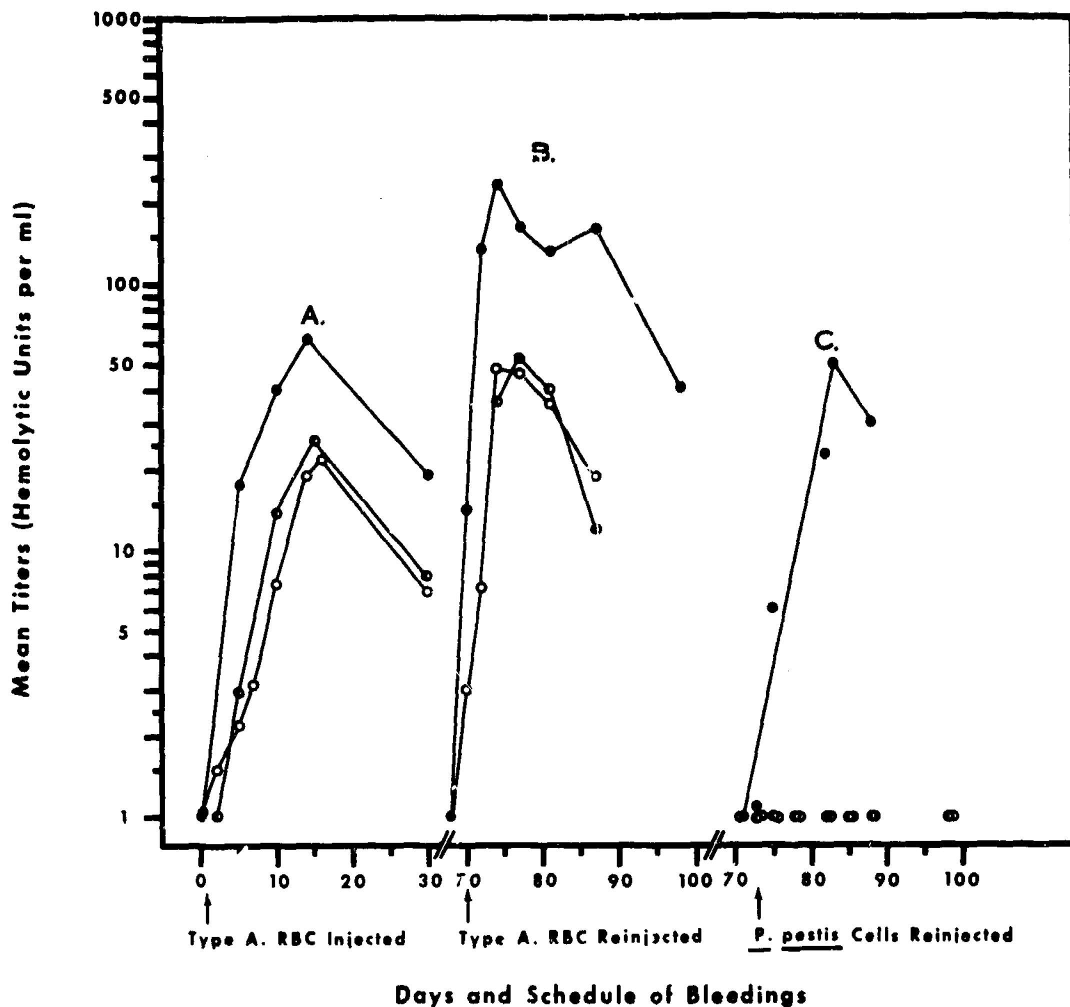

Fig. 1. Anti-A (solid circles), anti-B (semisolid circles), and anti-O (open circles) hemolysin response in rabbits following injection with: A. human type A red cell stromata, B. reinjection with human type A red cell stromata, and C. reinjection with washed P. pestis bacilli from vaccine I.

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).

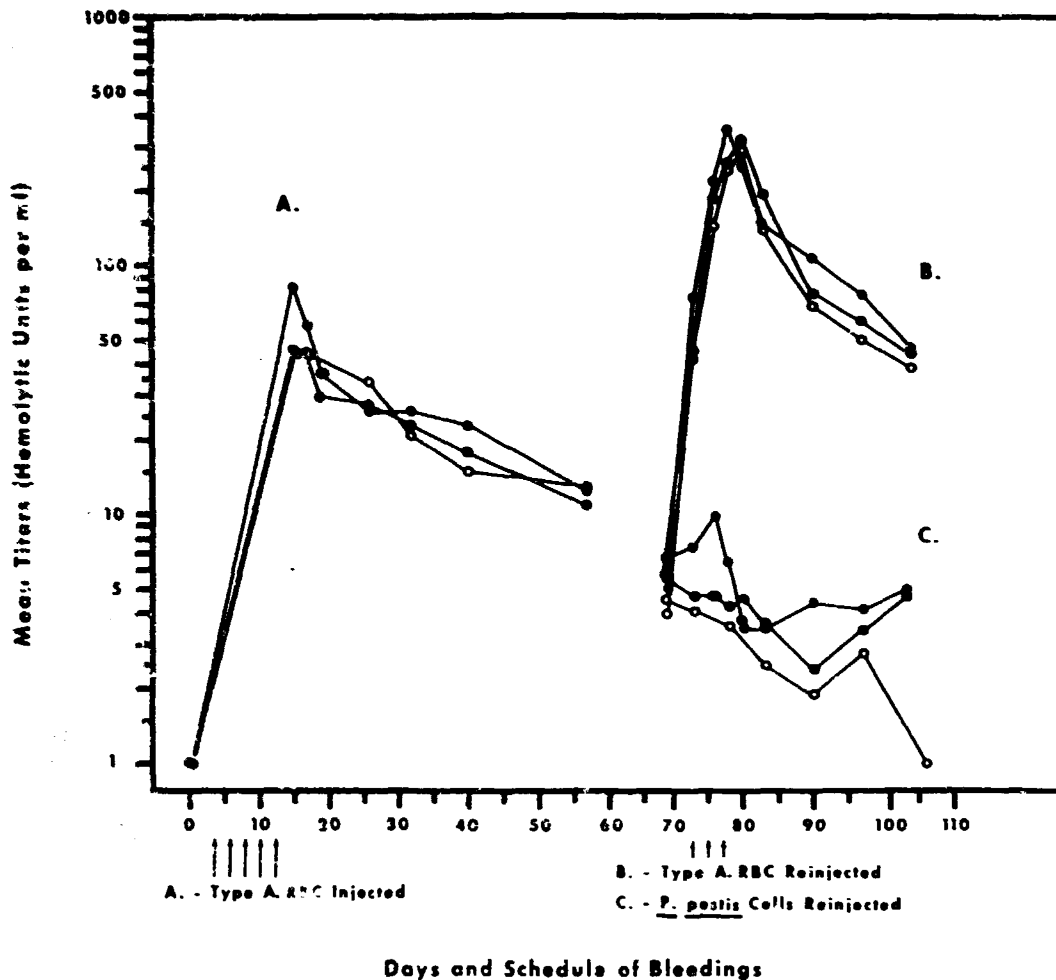

Fig. 2. Anti-A (solid circles), anti-B (semisolid circles), and anti-O (open circles) hemolysin response in rabbits following multiple injections of: A. human type A red cell stromata, B. reinjection with human type A red cell stromata, and C. reinjection with washed P. pestis from vaccine II.

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 Figures 2A and 2C 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 confirm 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 of its cell-free suspending medium did not show the marked anti-A immune response elicited by the washed bacilli harvested from the same vaccine. 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 gastic 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.

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13. ABSTRACT

Washed P. pestis bacilli prepared from a vaccine containing peptides and peptones of animal origin elicited human anti-A red cell hemolysins in rabbits, whereas similarly treated P. pestis from a vaccine containing peptones and peptides of plant origin did not. Whole vaccine and its various subfractions did not stimulate antibody production for any of the human red cell types when injected into rabbits. (U)

Apparently, human blood group A specificity is imparted to P. pestis by blood group contaminating sources present in the media used to cultivate the organism. The evidence shows that, in rabbits, the specific substance expresses itself antigenically only when a concentrated amount is bound to the cell, and that in soluble form it is weakly antigenic or present in subminimal antigenic concentrations. (U)

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