Destruction of Red Blood Cells. VII. Apparent Autosensitization to Dog Red Blood Cells.* (17450)

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In an occasional case of atypical hemolytic anemia a serum agglutinin active against the patient's own red blood cells has been found.[1] This may be merely the result of the presence of a serum substance with entirely fortuitous properties resembling in activity the seemingly nonspecific agglutination of red blood cells by antiserum developed in rabbits against Type 14 pneumococcus[2] or by certain viruses.[3] There are, however, two possible strictly immunological explanations for the presence of such an autoagglutinin: 1. The antibody (autoagglutinin) could be formed in response to some antigen in the red blood cell, or 2. the antibody could be formed for some other antigen but might only incidentally possess the capacity to react with a receptor in the blood cell.

That an animal can produce antibody for components of tissues of the same species has been amply demonstrated.[4] Hektoen and Schulhof,[5] after injecting rabbits intravenously with solutions of rabbit thyroglobulin, demonstrated serum precipitins for rabbit thyroglobulin in a titer as high as 1:10,000. Such sera did not react with the thyroglobulin of beef, dog, human, monkey, rat, sheep or swine. Several workers[6,7] have shown that formalized rabbit serum is antigenic for rabbits. Furthermore, rabbit brain extracts (containing lipoids)[8] and rabbit fibrinogen[9] stimulate the formation in rabbits of complement-fixing antibodies and precipitins. Schwentker and Comploier[10] and the Caveltis[11] have demonstrated the formation of complement-fixing antibody for homologous renal tissue.

Such observations suggested that an animal might be sensitized experimentally to its own red blood cells. If such sensitization were demonstrated it would support the explanation that in certain instances of acquired hemolytic anemia in man the serum autoagglutinins may be formed in response to an antigen in red blood cells. Therefore, the following studies were made.

Materials and Methods. Four female mongrel dogs each weighing approximately 25 lb were maintained on a diet of horse meat and commercial preparations of dog food supplemented with 1.2 g of ferrous sulfate a day. Red blood cells, hemoglobin concentration, hematocrit, icterus index, and reticulocyte and white blood cells in oxalated samples of venous blood were determined by conventional methods on the average of every 10 days. Repeated determinations of osmotic and mechanical fragility of the red blood cells were made by modifications of technics previously described.[12,13] Serum complement titrations, complement fixation, and cold agglutinin and warm agglutinin titers were followed periodic-

* This investigation was aided in part by a grant from the John and Mary R. Markle Foundation.

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ally according to standard technics.[14,15] Serum from rabbits previously injected repeatedly with normal dog serum was heated at 56°C for 30 minutes and employed in a manner similar to that described by Coombs *et al.*[16] Thus, equal aliquots of saline suspensions of washed dog cells were incubated for one hour at 37°C with the anti-dog serum rabbit serum, centrifuged at about 300 rpm for 2-3 minutes, resuspended by gentle agitation and examined microscopically under a cover slip for agglutination.

Procedures and Results. Several methods were employed. The red blood cells from 10 cc of defibrinated blood were frozen, thawed and then incubated in 4 cc of streptococcal toxin containing 400,000 skin test doses for 24 hours at 37°C. The suspension was then injected intraperitoneally into the dog from which the cells had been obtained. Fourteen such injections were made in 2 dogs at 2 day intervals. During the period of injection and for the 18 following days no significant alterations of results in the laboratory tests listed above were found.

In the study of 2 other dogs red blood cells from 10 cc of defibrinated blood were incubated 24 hours at 37°C after being inoculated with 6 to 24 hour broth culture of hemolytic *Staphylococcus aureus* (Costello strain). The blood culture was then filtered through a Seitz filter and frozen for 24-48 hours before being used. After thawing, the filtrate was injected intraperitoneally into the dog from which the cells had been obtained. To 1 dog 10 such injections were given and to another dog 11 injections at 48-72 hour intervals. During the period of injection and the following 17 days no significant alterations of results in the laboratory tests listed above occurred. One dog showed fluctuations in mechanical fragility of its red blood cells from 3.2 to 50% unassociated with other changes in the results of laboratory tests listed. As marked fluctuations in mechanical fragility of apparently normal dog cells sporadically occur[17] such an isolated observation was not considered indicative of autosensitization.

Seventeen to 18 days after the previous experiments each of the 4 dogs was given a single subcutaneous injection of a modified Freund antigen[18] consisting of a mixture of 1 cc of its own washed, packed red blood cells, 1 cc of mineral oil‡, 0.2 cc of a lanolin-like substance§ and 1 mg of heat killed *Mycobacteria tuberculosis* (H37Rv strain).‖ Over a period of 6 weeks following the injection no changes were noted in osmotic fragility or complement titer and no development of cold or of warm agglutinins or of serum complement-fixing antibodies occurred. The washed red blood cells did not clump in anti-dog serum rabbit serum. The mechanical fragility of the cells from 2 of the dogs fluctuated in an unpredictable manner. However, in light of previous experience[17] this was not considered indicative of autosensitization.

Six weeks after the injection of the above described modified Freund antigen a second similar preparation was made with the red blood cells of each dog. The mixture was then heated at 57°C for 2½ hours, cooled, added to 2 cc of fresh pig serum and injected immediately subcutaneously. Again there were no changes in 3 of the dogs as tested by the laboratory procedures listed above. However, in 8 observations made from the 25th to the 35th post-injection day the washed red blood cells from the fourth dog showed definite agglutination when suspended in anti-dog serum rabbit serum. On the 36th, 37th, 38th and on subsequent post-injection days the cells were found non-agglutinable by such a procedure. During the period in

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[15] Stats, D., and Wasserman, L. R., *Medicine*, 1943, **22**, 363.

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[18] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

‡ The brand of mineral oil used was Bayol F, supplied by the Standard Oil Company of New Jersey.

§ The lanolin-like substance was Falba, the trade name for a preparation of Pfalz and Bauer, Inc., New York.

‖ Supplied through the courtesy of William Steenken, Jr.

which the washed red blood cells were agglutinated by anti-dog serum no anemia or increase in reticulocytes or icterus index appeared. The osmotic fragility of the red blood cells remained normal. No cold or warm agglutinins were demonstrable. The complement titer of the serum (1:32) did not fall; and no serum complement-fixing antibody for the dog's own red blood cells was demonstrable. The mechanical fragility of the red blood cells ranged from 3.1 to 6.1%; values considered by Shen[17] to be normal for dog red blood cells.

Summary and Conclusions. Several attempts were made to sensitize 4 dogs to their own red blood cells. Three dogs showed no definite evidence of autosensitization. Although 2 of these 3 dogs showed occasional marked fluctuation in mechanical fragility unaccompanied by other changes during these studies this was considered to be due to technical difficulties. However, for a short period in one dog, 25 days after the injection of a modified Freund antigen, there was agglutination of washed red blood cells when they were suspended in anti-dog serum rabbit serum. During this period of apparent autosensitization there was no anemia, reticulocytosis or jaundice. The red blood cells showed no alteration in osmotic or mechanical fragility. There was no fall in complement titer. Cold and warm agglutinins did not appear. There was no evidence of a serum complement-fixing antibody for the dog's own red blood cells. Such apparent autosensitization supports the explanation that in certain instances of acquired hemolytic anemia in man serum autoagglutinins may be formed in response to an antigen in red blood cells.

Received October 10, 1949. P.S.E.B.M., 1949, **72**.

The Appearance of Acetylcholine During Normal Labor. (17451)

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In our earlier studies on the acetylcholine (Ach) of the human placenta,[1] blood from pregnant women was not available for investigation. In consideration of the potent hydrolytic action of the blood cholinesterase, it was inferred that Ach of human placenta does not appear to reach the maternal circulation in any noticeable quantity.

Opportunity to restudy this problem has not presented itself until lately. On the basis of an analysis of 288 blood samples of 152 cases during pregnancy and at different stages of labor, our previous impression has to be modified.

Four cc of blood were obtained each time and poured into 2 volumes of 95% pure alcohol. The mixture was thoroughly ground, diluted with 2 volumes of saline and centrifuged. The supernatant fluid was measured, and usually 0.5 cc or less was used for assay. Alcohol-free extract was also prepared by evaporating 5 cc to dryness in room (23-30°) and re-taking up with 5 cc of frog Ringer solution. The extracts were assayed on the toad's rectus test which depends on the principle of equal potentiation of Ach standard and the unknown by eserine. So if the matched ratio is identical before and after eserine, it is taken as Ach. Otherwise, Ach is considered to be nil.

Table I gives results showing a gradual appearance of Ach toward the second trimester of gestation, an apparent increase of Ach with the onset of labor, and a disappearance beginning 48 hours after labor.

The blood of 10 non-pregnant women and

[1] Chang, H. C., and Wong, A., *Chin. J. Physiol.*, 1933, **7**, 151.