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Hemolytic Anemia in Rabbits Following Injection of Bacterial Endotoxin. (23788)

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Alterations in the numbers of circulating white cells and platelets after injections of Gram negative bacterial endotoxins are well known(1,2). Corresponding changes in the red cells have not been as clearly defined. Braude *et al.* found variable effects of *E. coli* endotoxin on the blood volume and hematocrit of rabbits, with little change in the circulating red cell mass, although leukopenia and neutropenia occurred(3). Similarly, Kopp noted little change in the hemoglobin levels of patients receiving typhoid-paratyphoid (TAB) vaccine for production of therapeutic fever(4). Cartwright *et al.*(5) found that injecting 5 doses of typhoid vaccine intramuscularly into dogs did not induce the hypoferremia and anemia that occurs during infection. Willison observed that various bacterial exotoxins depressed the reticulocyte response to a constant blood loss anemia in rabbits but the effect of bacterial endotoxins was not investigated(6). It will be shown here that rabbits may develop a mild hemolytic anemia after a single injection of endotoxin and that they invariably develop such anemia after multiple injections of endotoxin derived from typhoid bacilli.

Materials and methods. Male albino buck rabbits, weighing 5-6 lb at the beginning of the experiments, were used. *Endotoxin.* Trypsin treated *S. typhi* (Ty2) endotoxin was prepared as previously described(7). Briefly, an overnight culture† was killed with chloroform, digested with trypsin, precipitated with acetone and extracted by repeated alcohol-ether precipitations from aqueous solution. The final solution, in physiological saline, was heated at 56° for 30 minutes in a sterile vial and stored at −25°C. The LD_{50} of this endotoxin injected intravenously into 5-6 lb rabbits varied between 0.01-0.1 ml of an undiluted solution containing 10 mg per ml of endotoxin by dry weight. *Hematological studies.* Whole blood and plasma hemoglobin concentrations, reticulocyte counts, and mechanical and osmotic fragility of red cells were determined by the usual methods(8). The hemoglobin levels of apparently normal rabbits were found to vary from 10.5 to 14 g %, and the reticulocyte counts from 0.6 to 3.0%. *Labelled red cells.* Five ml of blood was drawn by cardiac puncture and the red cells labelled with 40-70 microcuries of radioactive sodium chromate ($Na_2Cr^{51}O_4$) according

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to the method of Jandl *et al.*(9). Aliquots of blood (0.02-0.1 ml) were hemolyzed in 3 ml of distilled water and the radioactivity determined in a well-type scintillation counter. The blood specimen obtained 24 hours after injection of the labelled cells was considered to have 100% radioactivity and subsequent values were plotted semilogarithmically without correcting for elution of Cr^{51}(10). The half survival time (T ½) of the labelled red cells was determined graphically, and was found to average 14.5 days in normal rabbits. The value obtained by Donahue *et al.* was 12 days(10). The "PVP" (polyvinyl pyrrolidone) test was used to detect sensitization of red cells: 5% PVP (K-44) solution buffered at pH 7.4 was used(11). The Coombs test was performed on saline washed red cells using antisera obtained from rats that had received multiple injections of rabbit serum.

Results. Effect of a single intravenous injection of endotoxin. Each of 6 rabbits was given a single dose of endotoxin intravenously: 0.1 ml undiluted was given to 2 and 0.1 ml of a 1:8 dilution to the other 4. Varying degrees of mild reticulocytosis not exceeding 5% with corresponding decreases in the hemoglobin concentrations of 1-2 g % were observed from the 4th to the 15th day after the injections of endotoxin in one of the rabbits that received undiluted and in 2 of those that received diluted endotoxin. In the other 3 rabbits, no such changes were noted. Red cell survival time was studied before and after injection of endotoxin in the 2 rabbits receiving 0.1 ml of undiluted endotoxin. The T ½ was normal for both rabbits (15.5 and 14.5 days respectively) prior to injection of endotoxin. However, 9 days after the endotoxin had been given the red cells of one rabbit disappeared at a rate corresponding to a T ½ of 9.5 days. The decreased survival of the red cells occurred during the period when the reticulocytosis and lowered hemoglobin levels were observed. No changes in the survival time of the red cells were observed in the other rabbit, and there were no significant changes in hemoglobin and reticulocyte levels.

Effect of multiple injections of endotoxin. Three intravenous injections of 0.05 ml of a 1:8 dilution of endotoxin were given during 5 days to each of 5 rabbits. Two of the 5 rabbits developed minimal reticulocytosis 12 days after the first injection. In another experiment, 5 rabbits each received a total of 9 injections of 0.05 ml of a 1:8 dilution of endotoxin over a period of 2 weeks; 4 of these 5 developed mild reticulocytosis between the 10th and 20th days after the first injection and there were small corresponding decreases in the hemoglobin levels. Finally, large doses of endotoxin were given daily for about 3 weeks to 4 "immune" rabbits which had received multiple doses of endotoxin in the past, but had not received endotoxin during the preceding month or two. The reticulocyte and hemoglobin levels in such animals are invariably normal after one month's rest from injections. Two of these "immune" animals received multiple injections of 0.5-0.1 ml of a 1:4 dilution of endotoxin and the other two received daily 0.05-0.30 ml of undiluted endotoxin in increasing doses. The observations on one rabbit of each group are shown in Fig. 1 and 2. In all 4 animals, reticulocytosis first appeared 7-10 days after injections of endotoxin were begun and maximal reticulocyte counts of 8-12% occurred subsequently. Hemoglobin concentrations were decreased in all the animals; the maximal change being a reduction from 12 to 8 g %. That increased destruction of red cells occurred as a result of repeated injections of endotoxin is shown in Fig. 2. The red cell survival studies in this rabbit showed that the T ½ fell to 7 days, and indicated that the accelerated destruction

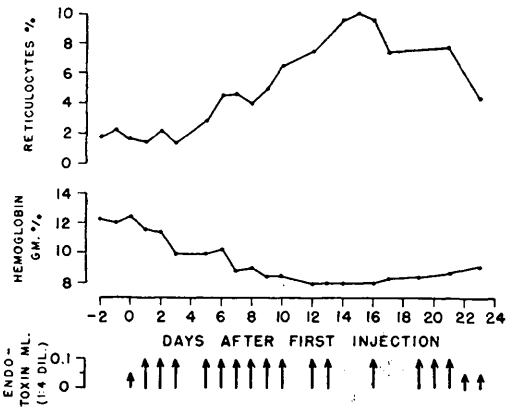

FIG. 1. Effect of multiple injections of endotoxin on reticulocyte counts and hemoglobin concentrations in blood of a rabbit.

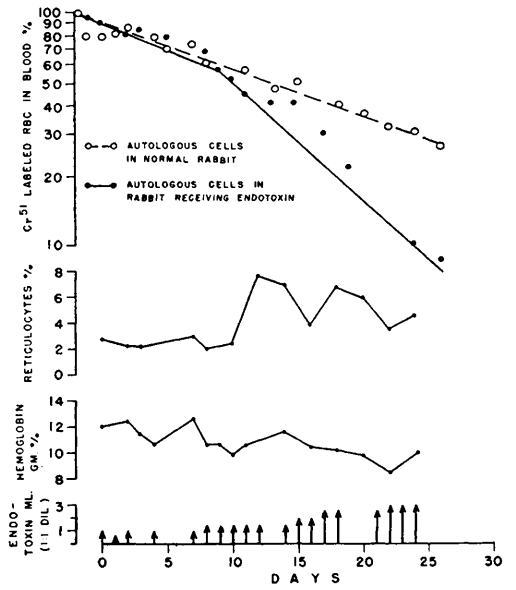

FIG. 2. Effect of multiple injections of endotoxin on red cell survival, reticulocyte counts and hemoglobin concentrations in blood of a rabbit.

of the red cells began about 8 days after the first injection of endotoxin. In all 4 "immune" animals the hemolytic process was self-limiting; despite continued injections of endotoxin the reticulocyte counts decreased and hemoglobin levels increased during the third or fourth week after the first injection of endotoxin.

Investigations of mechanisms of hemolysis following injection of endotoxin. 1. *In vitro* agglutinating effects of endotoxin were investigated by adding a solution containing 10 mg/ml of endotoxin to an equal volume of a 10% suspension of washed or unwashed red cells in autologous plasma from normal animals. No gross or microscopic agglutination and no hemolysis were observed after one-half hour's incubation at 37°C. Similarly, addition of endotoxin to washed or unwashed red cells in autologous serum from hyperimmune animals did not cause agglutination. Agglutination of red cells in the presence of endotoxin could be produced only if the red cells were allowed to incubate with endotoxin before antibody was added.

2. In order to determine whether administration of endotoxin to a hyperimmune animal would induce hemolysis *in vivo*, a hyperimmune rabbit was given 1.0 mg of endotoxin intravenously. Within the next hour the hemoglobin concentration of the blood increased by 2.3 g % and returned to pre-treatment values during the next 24 hours. There was neither microscopic autoagglutination of the red cells nor evidence of sensitization of the red cells, as determined by the PVP test. A second hyperimmune rabbit was given 100 mg of endotoxin intracardially. As in the previous animal, the hemoglobin concentration of the blood rose transiently by 1.2 g % over pre-treatment values during the hour after injection, and the plasma hemoglobin rose from 18.9 mg % to 56.7 mg % during the 15 minutes after the injection of this large dose of endotoxin. The animal died 2½ hours after the injection.

3. When red cells were obtained at various times during the course of the hemolytic response to the injection of endotoxin into immune rabbits, these cells showed no abnormality of osmotic or mechanical fragility. Such red cells did not agglutinate in the presence either of PVP or of antisera to rabbit serum (Coombs' test), indicating that these cells were probably not sensitized with autologous serum proteins.

4. Since the hemolytic response occurred a week or more after the injection of endotoxin and was more profound in animals that had received multiple doses of endotoxin, the effect of passive transfer of antibody to endotoxin was studied. Each of 2 rabbits was given 10 ml of serum from a rabbit that had received multiple injections of endotoxin. The antiserum agglutinated endotoxin-coated red cells to a titer of 1:8192(12). A third rabbit received 10 ml of serum from an untreated donor. Each animal was then given 0.05 ml of a 1:8 dilution of endotoxin. Only one of the animals that received immune serum showed a transient reticulocytosis to 4.5%, and this occurred 10 days after the injection of endotoxin. Such a response is consistent with that of normal animals receiving a single dose of endotoxin. The passive transfer of antibodies to endotoxin did not appear to augment or accelerate the hemolytic process that occurs after an injection of endotoxin.

5. A rabbit was made anemic by giving re-

peated doses of endotoxin. The red cells of the anemic rabbit, when transfused into a normal recipient rabbit, had a normal survival time ($T\ ½ = 14$ days).

6. Splenomegaly was found uniformly in the animals that had received multiple doses of endotoxin. Uninjected rabbits of the size used in these experiments have a mean splenic weight of about 0.4 g per kg body weight. whereas the animals that received repeated doses of endotoxin had a mean splenic weight of 1.6 g per kg body weight. The role of the spleen in the pathogenesis of the hemolytic anemia herein described was investigated by giving a non-immune. splenectomized rabbit 3 injections of endotoxin. The animal developed transient reticulocytosis and a decreased hemoglobin concentration in the blood beginning on the 12th day after injection of endotoxin. Thus. splenectomy did not abolish the hemolytic response to the injection of endotoxin.

Discussion. Hemolytic anemias may be accompanied by abnormalities in the red cells or in the host. The hemolytic anemia that follows the injection of endotoxin is probably not related to an abnormality of the red cell. This is supported by the absence of sensitization *in vitro,* the normal mechanical and osmotic fragilities, and particularly the normal survival in normal recipients of cells from anemic animals. The absence of sensitization makes it unlikely that the hemolytic anemia is due to attachment *in vivo* of endotoxin to red cells. followed by agglutination and hemolysis in the presence of anti-endotoxin antibody(13). However, the possibility cannot be excluded that cells coated with endotoxin *in vivo* were removed rapidly from the circulation of immune animals and hence were not detectable peripherally. Such an occurrence might explain the hemoglobinemia observed in the immune animal shortly after injection of a large dose of endotoxin.

The role of antibody to endotoxin in the production of the hemolytic response is not clear. The hemolytic response occurs during the second week after the injection of single or multiple doses of endotoxin, suggesting that antibody may be involved. On the other hand, the presence of antibody alone is insufficient to induce hemolysis since hyperimmune animals with high titers of antibody may not be anemic, and since partial recovery from the hemolytic anemia may occur while the injections of endotoxin are still being given. Furthermore, the passive transfer of antibody to normal recipients did not accelerate or augment the reticulocyte response to the subsequent injection of a single dose of endotoxin.

It appears likely from the foregoing evidence that the mild hemolytic anemia occurring after injection of endotoxin may be secondary to an alteration in the host tissues. The latent period of a week or more that is required for development of anemia after injection of endotoxin may represent a lag period during which mechanisms that increase red cell destruction are activated. Since the anemia is more apparent after repeated doses of endotoxin, at a time when the spleen is enlarged, the analogy to experimental hemolytic anemias such as those produced by injections of methyl cellulose in rats is suggested(14). However, the anemia herein described was not well correlated with the size of the spleen. The largest spleen (2.5 g/kg body weight) was found in a hyperimmune rabbit that was no longer anemic. Furthermore, splenectomy did not appear to eliminate the hemolytic response to endotoxin.

Since injection of endotoxin is followed by functional changes in the reticulo-endothelial system(2) it is conceivable that unusual activation of this system accounts for the hemolytic anemia. Repeated injection of endotoxin in rabbits produces increased granulocytic hemopoiesis and increased numbers of phagocytes in the spleen, lymph nodes and bone marrow(15). One of the functional consequences of such stimulation may be an increased capacity for random red cell destruction.

A similar hemolytic anemia occurs in mice after injection of zymosan(16) and there are indications that reticuloendothelial stimulation may account for the observation here also.

Anemia is a frequent finding in pyelonephritis and in bacteremias of various etiologies. These anemias are occasionally hemoly-

tic(17). It is conceivable that the release of endotoxin may play a role in the pathogenesis of hemolytic anemias during infection.

Summary. 1. A mild hemolytic anemia secondary to intravenous injection of bacterial endotoxin into rabbits is described. The anemia is characterized by reticulocytosis, decreased hemoglobin levels in the blood, and decreased survival of red blood cells. 2. Single injections of endotoxin occasionally produce a mild hemolytic response in rabbits during second week after injection. Repeated injections of endotoxin lead to more striking hemolytic changes which also reach their peak during second week after first injection. The hemolytic process tends to abate despite repeated administration of endotoxin. 3. Evidence is presented to indicate that the hemolytic response is not due to demonstrable changes in the red cells of the animals receiving endotoxin. There is no change in osmotic or mechanical fragility of the red cells, no sensitization to appropriate anti-rabbit-protein antisera or to polyvinyl pyrrolidone, and no evidence of hemagglutination *in vivo* during the hemolytic episode. In addition, there is no decrease in the life span of red cells when these are taken from an anemic rabbit and transfused into a normal one. 4. Presence of antibody does not seem to play a critical role in development of hemolytic anemia, and the presence of the spleen is not essential for the hemolytic response to endotoxin. 5. It is suggested that the hemolytic response is most likely due to an as yet unexplained activation of the reticulo-endothelial system.

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