INHIBITION OF RED CELL SEQUESTRATION BY CORTISONE*

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More than a decade ago it was recognized that ACTH and cortisone ameliorate some hemolytic anemias in man (1–6). Nevertheless, the mechanisms responsible for their therapeutic effect have not been elucidated. It is generally believed that cortisone and its analogues benefit only those hemolytic anemias in which the red cells can be agglutinated by Coombs (antiglobulin) serum. Indeed, of the many published case reports (7, 8) there is but one well documented study (9) indicating that cortisone administration may benefit a "Coombs-negative" hemolytic disease, hereditary spherocytosis.

Agglutination of red cells by Coombs serum indicates the presence of protein, usually a globulin, attached to the cell surface. Although it has been demonstrated that red cells may be agglutinable in Coombs serum as a result of the non-immunologic adherence of protein (10–13), it is probable that the globulins attached to red cells in most cases of "Coombs-positive" hemolytic anemia are specific antibodies directed against either exogenous haptenes such as drugs (14, 15) or endogenous stromal antigens (16–19). This adherence of antibody to red cells may provoke hemolysis in vivo by causing complement fixation thereby resulting in direct cellular damage (20), or more often indirectly by causing red cell sequestration and destruction in the spleen or liver (21, 22). Theoretically, cortisone might suppress the injurious effects of antibodies on red cells in the following ways: (a) By decreasing the synthesis of antibody (23, 24), (b) by interfering with antigen-antibody union (25, 26), and (c) by inhibiting the consequences of this union (27–29). In the past each of these has been invoked to explain the salutary effects of steroid therapy in acquired hemolytic anemia.

Recently we have attempted to define the role of reticuloendothelial function in erythroclasia. Rats were injected with a variety of "reticuloendothelial-blocking" substances in an effort to modify the sequestration of red cells previously altered in vivo by sensitization with antiserum or by prolonged sterile

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incubation at 37°C. The destruction of these altered cells was compared in
groups of normal and treated animals. Of the agents studied, cortisone proved
the most interesting. A preliminary report of our findings has been published
(30).

Methods

The techniques employed have been described fully in a previous publication (31) and are
summarized below.

A. Sensitization of Red Cells.—Rat red cells obtained from freshly defibrinated blood were
labeled with Cr⁶¹ of high specific activity¹ in the form of Na₂Cr⁵¹O₄ (5 to 10 microcuries per
ml of defibrinated blood), centrifuged, and washed once in excess saline to remove unbound
chromium. To a 50 per cent saline suspension of labeled cells was added an equal volume of
an appropriate concentration of rabbit anti-rat-red cell antiserum (previously heated for
2 hours at 56°C). The sensitizing and agglutinating characteristics of this antiserum had been
determined previously. After incubation in antiserum for 1 hour at 37°C, the cells were sepa-
ratated by centrifugation, washed once in excess saline, and brought to a 40 per cent packed
red cell volume with fresh rat serum. In such cell preparations frank agglutination was de-
defined as red cell agglutination which persisted on suspension of the cells in a large (1:100)
volume of saline. Increased agglutinability was determined, in most instances by the Coombs
technique (32) employing rat antiserum, and in all instances with polyvinylpyrrolidone
(P.V.P.) (33).

B. Incubation of Red Cells at 37°C.—Rat blood was defibrinated using sterile precautions
and was incubated for 21 hours at 37°C; thereafter, the cells were labeled with Cr⁶¹, washed
in saline, and suspended as a 40 per cent red cell concentration in fresh rat serum as pre-
viously described.

C. Preparation of Radioactive Hemoglobin.—Red cells obtained from a normal donor rat
were labeled with Cr⁶¹, washed in saline, and lysed in distilled water. The hemolysate was
buffered (pH 7.6) and Seitz-filtered to remove the stroma.

D. Subjects.—The recipient animals were large (400 to 525 gm), Bartonella-free, white
male Sprague-Dawley rats of a Caesarian-derived strain. They were paired by weight, and
one of each pair received daily for 6 days an intramuscular injection of cortisone acetate,³
10 mg in 0.4 ml of physiologic saline. The paired control animal received 0.4 ml of saline
alone. Every rat was weighed daily at the time of injection.

E. Studies in Vivo.—In most experiments 4 animals were studied simultaneously—2
cortisone-treated rats with paired, normal controls. Occasionally accidental death occurred
during experimental procedures. This resulted in unequal numbers of animals within the
various groups studied and complicated somewhat statistical analysis of the data obtained.

By intravenous injection into a tail vein, the recipient animals received (a) 0.5 ml. of the
previously prepared 40 per cent suspension of Cr⁶¹-labeled sensitized or incubated red cells
or (b) 0.8 ml of the Cr⁶¹-labeled hemoglobin solution containing 2.5 mg of hemoglobin (an
amount calculated not to exceed the binding capacity of circulating haptoglobin).

After injection of the labeled material, tail puncture was performed distal to the site of
injection at specified time intervals. Two 0.1 ml blood samples were obtained and added
quantitatively to 1.0 ml of water and to 1.0 ml of heparinized saline respectively. The heparinized
calculated not to exceed the binding capacity of circulating haptoglobin.

³ Cortogen, Schering Corporation, Bloomfield, New Jersey.

¹ 300 to 500 mc per mg; Abbott Laboratories, North Chicago.
² Charles River Laboratories, Boston.
sacrificed by cervical dislocation approximately 3 hours after injection of the cells. The spleen, liver, lungs, left kidney, and right femur were dissected cleanly, rinsed in saline, and blotted gently with gauze sponges before being weighed. The Cr\textsuperscript{51} of whole blood, plasma, and of 2 gm or less samples of the dissected organs were measured in a well-type scintillation counter for a minimum of 10,000 counts.

F. Calculations.—The concentration of red cell Cr\textsuperscript{51} activity in whole blood immediately after injection was estimated from the following equation:

\[
(Cr^{51}_{wb})_0 = \frac{Cr^{51} \text{ inj}}{Bl \text{ vol}},
\]

where

\((Cr^{51}_{wb})_0\) represents the Cr\textsuperscript{51} activity in counts per minute per milliliter whole blood at time 0 after injection

\(Cr^{51} \text{ inj}\) represents total Cr\textsuperscript{51} activity injected in counts per minute

\(Bl \text{ vol}\) represents estimated blood volume

Measurements of total blood volume utilizing a Cr\textsuperscript{51}-labeled red cell dilution technique in 5 rats (similar in size and age to those studied) before and after 6 days of cortisone therapy revealed that, in untreated animals, total blood volume in milliliters averaged 4.46 per cent (± 0.25 per cent) of body weight in grams. After cortisone therapy the mean blood volume was increased slightly to 4.74 per cent (± 0.31 per cent) of body weight (0.1 > p > 0.05). Consequently, these two percentages were used to estimate total blood volume in control and cortisone-treated animals respectively.

The percentage of infused red cell Cr\textsuperscript{51} activity remaining in the circulation at a given time after injection was calculated from the following expression:

\[
\frac{(Cr^{51}_{wb})_x - (Cr^{51}Pb)_x}{(Cr^{51}_{wb})_0},
\]

where

\((Cr^{51}_{wb})_x\) represents the total Cr\textsuperscript{51} activity in counts per minute per ml whole blood at time x.

\((Cr^{51}Pb)_x\) represents the plasma Cr\textsuperscript{51} activity in counts per minute per ml blood at time x.

The Cr\textsuperscript{51} activities of the extirpated organs were calculated as (a) percentage of total Cr\textsuperscript{51} activity injected, and (b) percentage of total injected Cr\textsuperscript{51} activity contained per gram of organ.

As it had been shown previously (31) that the amount of antiserum employed to sensitize red cells determines the rate and site of their sequestration, it was presumed that different mechanisms were responsible for cellular trapping and destruction dependent upon the intensity of immunologic injury produced. We wished to observe the effect of steroid administration on each of these destructive mechanisms; consequently, the sequestration of red cells sensitized to varying degrees was studied in several small groups of normal and cortisone-treated animals. In order to allow adequate statistical evaluation of the resultant data, the technique of analysis of variance (34, 35) was utilized. In contrast, the incubated red cells and hemoglobin solutions were prepared in a standardized fashion resulting in uniformity of the infusates. Sufficiently large groups of treated and control recipient animals were studied to allow statistical evaluation of the data obtained by means of the "t test" (34).

RESULTS

A. Observations in Vitro

1. Sensitized Red Cells.—The addition of various quantities of rabbit antiserum to rat red cells (Table I) resulted in (a) sensitization of red cells to
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Coombs serum and to polyvinylpyrrolidone (P.V.P.) without agglutination at relatively small concentrations of antiserum; (b) red cell agglutination at moderate concentrations of antiserum; and (c) traces of hemolysis, occurring only in the presence of a large volume of fresh rat serum as a source of complement, at relatively large concentrations of antiserum. When still larger quantities of antiserum (ratio antiserum/red cell > 1.0) were employed in an attempt to produce more intense red cell sensitization, frank intravascular hemolysis ensued on subsequent injection of these cells into the recipients. This was indicated by unusually high Cr$^{51}$ activities in the plasma and kidneys. Since here the sequestration of labeled red cells was obscured by a concomitant release of labeled hemoglobin, these data will not be considered.

2. Incubated Red Cells.—Sterile incubation of rat blood for 21 hours at 37°C resulted in brownish discoloration of the red cells caused by methemoglobin formation. On subsequent manipulation of the cells prior to injection some hemolysis invariably occurred.

3. Cr$^{51}$-Labeled Hemoglobin.—The absence of cellular particles and of hemoglobin crystals was verified microscopically before injection.

B. Observations in Vivo

1. Sensitized Red Cells.—A previous publication describes the kinetics of immune red cell destruction in normal rats (31). The rate of disappearance of such red cells from the circulation was found to be directly related to the quantity of sensitizing antiserum employed. At concentrations of antisera producing little or no agglutination in vitro (ratios of antiserum to packed red cells of 0.17 to 0.33), red cell destruction was relatively slow and incomplete with splenic sequestration predominating. With higher ratios of antiserum to red cells (0.50 to 1.00) hepatic sequestration became prominent while the efficiency of splenic trapping diminished. Only at frankly lytic concentrations of antiserum (ratios of antiserum to red cells of greater than 1.00) did significant (>1 per cent) Cr$^{51}$ activity accumulate in the kidneys of the recipient animals.
No significant uptake of Cr\textsuperscript{51} activity by the lungs or femurs of the recipients could be detected at any level of red cell sensitization.

In cortisone-treated rats the disappearance of sensitized red cells, at each level of red cell sensitization studied, was slower than that in control animals. Because of the complex character of the survival curves, no attempt was made to analyze rate constants. However, the data relating to red cell survival 3 hours after injection of cells (Table II, Fig. 1) were analyzed statistically and the

**TABLE II**

*The Effect of Cortisone on the Sequestration of Sensitized Red Cells*

<table>
<thead>
<tr>
<th>Antiserum (ml) No. animals Studied</th>
<th>Circulating red cell Cr\textsuperscript{51} activity per cent injected</th>
<th>Hepatic Cr\textsuperscript{51} activity at 3 hrs., per cent injected</th>
<th>Splenic Cr\textsuperscript{51} activity at 3 hrs., per cent injected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Cortisone</td>
<td>Normal</td>
</tr>
<tr>
<td>0.00</td>
<td>3</td>
<td>3</td>
<td>93.0 ±9.1</td>
</tr>
<tr>
<td>0.17</td>
<td>2</td>
<td>4</td>
<td>60.2 ±1.6</td>
</tr>
<tr>
<td>0.33</td>
<td>2</td>
<td>2</td>
<td>45.0 ±6.6</td>
</tr>
<tr>
<td>0.50</td>
<td>5</td>
<td>7</td>
<td>42.6 ±13.1</td>
</tr>
<tr>
<td>1.00</td>
<td>3</td>
<td>2</td>
<td>12.8 ±0.6</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>18</td>
<td>100.0 ±0.0</td>
</tr>
</tbody>
</table>

*† Plus or minus 1 standard deviation.

differences observed were found to be significant at the 0.1 per cent level \( p < 0.001 \). In contrast, the survival of unsensitized red cells (ratio of antiserum to red cells = 0) in normal and cortisone-treated recipients at 3 hours was identical (93 per cent). Although this figure is somewhat lower than anticipated, it supports the validity of the relative percentages employed to estimate blood volume in the two groups of animals (i.e., 4.46 and 4.74 per cent).

The livers of the cortisone-treated rats were heavier, paler in color, and somewhat more friable than their normal counterparts. On microscopic examination of ultrathin sections,\footnote{We are indebted to Dr. Leon Weiss, presently in the Department of Anatomy, Johns Hopkins University School of Medicine, for the preparation of these sections. The tissues were fixed in osmium tetroxide, sectioned to a thickness of 2 microns, and stained with either hematoxylin and eosin or with PAS.} the liver cells were so distended with glycogen that the
sinusoids appeared reduced in number and diameter. The hepatic Cr\textsuperscript{51} content (total and per gram of liver weight) of the cortisone-treated animals was significantly diminished (\( p < 0.001 \)) compared with that of the controls (Table II, Fig. 2). However, no diminution in hepatic Cr\textsuperscript{51} content could be demonstrated when steroid-treated animals received normal red cells. This suggests that steroid administration produced no diminution in the intrahepatic vascular space.

Fig. 1. The survival of Cr\textsuperscript{51}-labeled, antibody-treated red cells 3 hours after injection into normal (\( \bullet----\bullet \)) and into cortisone-treated (\( ○----○ \)) rats. Each point represents the mean of several observations with one standard deviation shown.

Although the spleens of the cortisone-treated rats were uniformly small, they differed histologically from normal only in their reduced content of lymphoid tissue with no apparent disturbance of their normal sinusoidal structure. No statistical differences in the splenic accumulation of sensitized red cells could be demonstrated between the two groups of animals (Table II, Fig. 3). In contrast to the control animals where, at a given level of red cell sensitization, there was little variation in splenic sequestration (total and per gram of spleen) from animal to animal, these values in the treated rats varied considerably. At each level of antiserum employed, the spleens of the cortisonized animals actually contained, on the average, greater Cr\textsuperscript{51} activity per gram of tissue than

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Fig. 2. The hepatic sequestration of Cr\textsuperscript{51}-labeled, antibody-treated red cells in normal (●—●) and in cortisone-treated (○—○) rats. Each point represents the mean of several observations with one standard deviation shown.
Fig. 3. The splenic sequestration of $^{51}$Cr-labeled, antibody-treated red cells in normal (●-●) and cortisone-treated (○-○) rats. Each point represents the mean of several observations with one standard deviation shown.
did the controls. This trend was more marked at greater degrees of erythrocyte sensitization.

In none of the steroid-treated animals was there significant accumulation (>1 per cent of injected counts) of Cr$^{51}$ activity in the lungs, kidney, or femur.

A study was made of the duration of steroid therapy necessary to inhibit hepatic sequestration of sensitized red cells. Studies identical with those already described were performed in 32 rats, 16 pretreated daily with cortisone acetate for periods of 1 hour to 4 days, and 16 untreated controls. Each recipient received the same volume of chromated red cells sensitized with a fixed quantity of antiserum (ratio antiserum to red cells = 0.5) and the hepatic accumulation of Cr$^{51}$ activity 3 hours thereafter was measured. The results (which include the data from 6 days of cortisone treatment depicted in Table II), expressed as the mean percentage of the hepatic Cr$^{51}$ activity found in the control animals, indicate that no inhibition could be demonstrated before the 3rd day of cortisone administration (Fig. 4). Statistical analysis of these data by the method of rank sum tests (34) yield the following measures of significance ($p$) for the various treatment periods: at 2 days 0.557; at 3 days 0.171; at 4 days 0.014, and at 6 days 0.003.
2. Incubated Red Cells.—The organ uptake of Cr$^{51}$ from labeled red cells that had been incubated for 21 hours at 37°C was determined 3 hours after injection in two groups of rats: (a) one group of 6 animals that had received cortisone acetate for 6 days as previously described; another group of 7 animals that had received parenteral injections of saline in equivalent volume. It was found (Table III) that the percentage of injected Cr$^{51}$ activity recovered in the livers of the control animals (42.0 ± 10.7 per cent) greatly exceeded that found in the steroid-treated group (19.6 ± 11.6 per cent)—0.005 > p > 0.001. In contrast, the spleens of the two groups of animals took up the same amount of Cr$^{51}$-labeled cells (controls: 33.2 ± 7.2 per cent vs. cortisone-treated: 33.3 ± 5.7 per cent)—p > 0.9.

3. Hemoglobin.—The organ distribution of Cr$^{51}$-labeled hemoglobin was studied in 12 rats; 6 had received cortisone acetate intramuscularly for 6 days as previously described and 6 had received saline. The results, depicted in Table IV, indicate that the hepatic sequestration of hemoglobin was unimpaired in cortisonized rats. Only minor differences were noted in the Cr$^{51}$ uptakes of other organs in the two groups of animals.
DISCUSSION

Several investigators have studied the effects of adrenocortical steroids on experimentally induced hemolytic anemias in animals (36–40). In most instances heterologous antisera, presumably containing hemolysins, were injected directly into control and steroid-treated animals and no significant amelioration of the hemolytic processes was observed. These reports have led to the conclusion that, in acquired hemolytic anemia, adrenal steroids must act primarily to suppress antibody synthesis. Certain objections to this conclusion may be raised: (a) Steroid-induced remissions in acquired hemolytic anemias may occur so rapidly (within a few days) that it is difficult to accept suppression of antibody synthesis as the primary effect of such therapy; (b) in man, suppression of antibody synthesis by cortisone has not yet been conclusively demonstrated (41–46), and (c) often no appreciable diminution in red cell sensitization (as measured by the antiglobulin titer) occurs early in cortisone-induced remission (47). On the other hand, these objections are not necessarily insurmountable. For example, the numerous reports indicating that administration of adrenocortical steroids diminishes antibody production in a variety of species make it likely that this also occurs in man. Further, the observed effects of a modest reduction in antibody synthesis in a situation involving rapid consumption of antibody (as may be the case in immunohemolytic anemias) might be sudden and dramatic. Finally, the “measurement” of antibody adsorbed to red cells by the antiglobulin titration method is theoretically unsound, and the results obtained by measuring the protein which can be eluted from red cells may be difficult to interpret (48).

The present studies were initiated not to disprove the possibility that cortisone may inhibit antibody synthesis but to establish whether cortisone affects the sequestration of red cells altered in vitro in a standardized fashion.

The data here presented indicate that cortisone retards the sequestration of antibody-sensitized and of incubated red cells by the liver. The mechanism of this effect is unknown at present. It cannot derive from a specific action of steroids on immune processes since the sequestration of cells injured by non-immunologic techniques was similarly inhibited.

Among the possible explanations to be considered are:

1. Modification of Intrahepatic Blood Flow.—It has long been recognized that administration of adrenocortical steroids to animals causes prompt accumulation of glycogen in liver cells (49). Ingle et al. (50), studying the time-response effect of cortisone (in doses comparable to those utilized in this study) on liver glycogen in the rat, found that glycogen accumulates rapidly within the first few days, reaches peak levels by 5 days, and slowly diminishes thereafter. In the rat, as in other species (51), this accumulation of glycogen causes attenuation and distortion of hepatic sinusoids. Conceivably this could result in diminished
hepatic blood flow. Indeed, if this were not the case, the maintenance of normal volume flow would require either an increased flow rate through constricted channels or the opening of intrahepatic shunts. In either event the perfusing red cells might be subjected less to intrahepatic stasis and to the action of phagocytic Kupffer cells lining the hepatic sinusoids. As yet, however, there is insufficient information regarding the effects of cortisone on hepatic blood flow to warrant further discussion.

2. Inhibition of Phagocytosis.—There are similarities between the sequestration of altered red cells and the clearance of colloidal substances (foreign particles, bacteria, denatured proteins, etc.) from the blood by the reticuloendothelial system (12, 52). Thus, it has been demonstrated that stable colloids (33), lightly opsonized bacteria (54), and red cells lightly sensitized with antibody (31) are sequestered relatively slowly, principally by the spleen; unstable colloids, more intensely opsonized bacteria, and more heavily sensitized red cells are sequestered relatively rapidly, primarily by the liver. It has been reported that adrenocortical steroids impede phagocytosis in general (55–58) and phagocytosis by Kupffer cells in particular (59), although other investigators have denied such effects (60–64). Consequently, basic disagreement still exists whether steroids affect the clearance from the blood of intravenously injected bacteria or colloids (65–68). To date, there has not been enough information obtained in vivo to distinguish between possible local vascular effects of adrenocortical steroids and their alleged action as inhibitors of phagocytosis. For example, Nicol and Bilbey (58) observed that cortisone administration slowed the clearance of intravenously administered colloidal carbon in mice. This was accompanied, histologically, by diminished accumulation of carbon within the liver. These observations, which are quite analogous to ours, may be interpreted as demonstrating either direct inhibition of phagocytosis by cortisone or indirect interference with reticuloendothelial function secondary to cortisone-induced vascular changes within the liver. Recently Packer et al. (69) have presented convincing evidence gathered in vitro that steroids impair erythropagocytosis by leukocytes. In the present study, however, histologic examination of ultrathin sections of liver from normal rats obtained at various times after infusion of sensitized red cells revealed principally intrasinusoidal clumping of cells with very little evidence of phagocytosis as the red cells were being sequestered. Consequently it is unlikely that the inhibitory effect of cortisone upon hepatic sequestration is due to erythropagocytosis. Furthermore, it would be difficult to explain why sequestration was impaired only in the liver if owing to the suppression of a general mechanism (i.e., phagocytosis).

3. Impairment of Hemoglobin Capture by the Liver.—It is conceivable that, after infusion of altered red cells into cortisone-treated rats, hepatic sequestration and red cell destruction proceed normally but that abnormal amounts of the Cr<sup>51</sup>-labeled hemoglobin released escape from the liver. This possibility was
excluded by the studies showing that the livers of cortisonized rats took up Cr¹⁹⁵-labeled hemoglobin normally.

In several respects, the experimental findings reported here are in accord with past clinical experience with steroids in acquired hemolytic states. (a) There is usually a latent period of a few days' duration between the onset of therapy and hematologic improvement. (b) A number of patients have been reported (72) who failed to benefit from splenectomy but who subsequently were improved by steroids. This indicates that any effect cortisone may have on the spleen is not essential to its therapeutic action. Indeed, the efficacy of steroid therapy tends to correlate inversely with the results of splenectomy in given patients (72). (c) Although few appropriate studies have been conducted in man, in at least two reported cases (in reference 21, case 10; and in reference 73, case 23) cortisone-induced remissions have been associated primarily with a reduction in the hepatic, rather than the splenic, uptake of autogenous, Cr¹⁹⁵-labeled red cells.

It is hoped that further studies in animals and man will clarify the fundamental action of cortisone in inhibiting red cell sequestration and so direct future approaches to therapy.

SUMMARY

The effect of cortisone on the sequestration of (a) antibody-coated red cells and (b) incubated red cells was studied in rats. Cortisone administration inhibited the hepatic sequestration of red cells altered by non-immune as well as by immune injury. There was a latent period of 2 days between the institution of cortisone therapy and its first manifest effect on hepatic sequestration.

The splenic sequestration of altered red cells was not inhibited by cortisone, and there was no inhibition of the sequestration of hemoglobin by either liver or spleen.

It is suggested that steroids may inhibit hepatic sequestration through hemodynamic effects.

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BIBLIOGRAPHY


*Previous reports that cortisone (70) or prednisolone (71) had no suppressive effect on experimental hemolysis by isoantibodies in man are presumably explained by the fact that the steroids were administered for an insufficient length of time (48 hours or less) to affect sequestration. Although short-term administration of cortisone did not affect the extent of hemolysis or leukopenia, it did block the febrile reaction to immune hemolysis (70, 71).


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