The effect of corticosteroid administration on the complement-independent clearance of IgG-sensitized erythrocytes was examined in guinea pigs. 51Cr-labeled guinea pig erythrocytes coated with a known amount of high-avidity IgG antibody were injected into control and cortisone-treated C4-deficient guinea pigs, and cell survival was determined. In these animals with a genetically controlled total deficiency of the fourth complement component, cell-bound antibody does not activate the biologically active complement components; clearance is therefore complement independent. At low levels of sensitization, cortisone completely inhibited the splenic sequestration of IgG-coated red cells. As the amount of antibody coating the red cell was increased, higher cortisone doses were required to decrease the rate and magnitude of clearance. Eventually a level of erythrocyte sensitization was reached where cortisone did not significantly alter the clearance pattern compared to untreated controls. The cortisone effect was present by 3 days but required 5-7 days before it was maximal. No evidence was found to suggest that cortisone decreased the affinity of the antibody for the red cell membrane. Rather, the most likely explanation for these results is that cortisone affects the interaction between IgG on the erythrocyte surface and its receptor on splenic and hepatic macrophages. This experimental model of immune hemolytic anemia parallels those cases of warm-antibody-mediated disease in which complement plays no role. These findings suggest that a major clinical effect of cortisone therapy may be to decrease complement-independent erythrocyte clearance, thereby inducing a remission even in the presence of a positive antiglobulin test.

Corticosteroid preparations were first successfully used to treat patients with autoimmune hemolytic anemia over 20 yr ago, and they remain the most important therapeutic modality in warm-antibody (IgG) mediated hemolytic anemia. However, the mechanism by which they induced a remission is incompletely understood. Initially, it was postulated that corticosteroids decreased production of antierythrocyte antibody. The strongest evidence in support of this hypothesis was the clinical finding that corticosteroid therapy reduced the level of autoantibody in many patients. In addition,
metabolic studies of IgG synthesis and catabolism in man have demonstrated that cortisone can alter both the synthesis and degradation of IgG. A second suggestion was that cortisone decreases antigen-antibody interactions, thereby preventing the sensitization of erythrocytes. There is some clinical data to support this suggestion.

The third possibility considered was that corticosteroids inhibited the clearance of antibody-coated erythrocytes by fixed macrophages of the reticulo-endothelial system (RES). This belief was supported by two clinical observations. First, following corticosteroid therapy the onset of improvement was usually within a few days, and significant alterations in IgG synthesis and catabolism are reported to proceed more slowly. Second, some patients remained in complete remission at a time when their antiglobulin test was still positive. Several experimental findings also supported this possibility. Corticosteroids were shown to decrease the clearance of inert particles and heat-damaged erythrocytes by the RES. Furthermore, two studies demonstrated that erythrocytes coated with antibody and complement had decreased clearance when injected into steroid-treated animals. In both of the studies of antibody-sensitized erythrocytes, complement played a major role in clearance. Complement acts as an amplifier of clearance, and corticosteroids may decrease clearance of complement-coated cells in situations where antibody is of minor importance. However, in many cases of IgG-mediated hemolytic anemia, complement is not found on the erythrocyte surface.

We have recently described an experimental model of immune hemolytic anemia in the guinea pig which allows one to quantitatively determine the effect of corticosteroids on the clearance of IgG-coated cells in the absence of complement activation. This is made possible by the availability of guinea pigs which have a complete block in the classic complement pathway, the only pathway activated by antibody-sensitized erythrocytes in the guinea pig. Since in a large percentage of cases of warm-antibody-mediated hemolytic anemia, complement appears to play no role, this model may simulate the clinical situation. It was therefore of interest to determine in this experimental model the effect of corticosteroid therapy on the non-complement-dependent clearance of sensitized erythrocytes.

**MATERIALS AND METHODS**

The preparation and source of buffers, complement reagents, guinea pig erythrocytes, and rabbit anti-guinea pig erythrocyte antisera have been previously described, as have the methods of immunoglobulin purification and complement analysis.

**Animals**

C4-deficient (C4D) and "NIH multipurpose" guinea pigs (normal), weighing between 350 and 600 g, were utilized in this study. The C4D animals, a subline of the NIH multipurpose animals, have a genetically controlled, total deficiency of the fourth component of complement. The defect has been demonstrated to be inherited in a simple Mendelian recessive pattern, and C4D animals make monospecific antibody to C4 when normal guinea pig serum is employed as the antigen. No blood group differences have been identified among the NIH multipurpose or C4D animals. Chromium-tagged erythrocytes from NIH multipurpose donors survive normally (t½, 7.5 ± 1.0 days) when injected into C4D guinea pigs and vice versa. The biologic consequences of this deficiency have been previously studied in detail.
Cortisone

Cortisone acetate, 25 mg/ml (The Upjohn Co., Kalamazoo, Mich) was administered subcutaneously once daily. The doses employed were 5, 10, 20, and 100 mg/kg body weight. Previous studies with 5, 20, and 100 mg/kg/day have shown sustained 24-hr levels proportional to the dose, with the peak cortisol concentration being about 120, 290, and 1175 μg/100 ml, respectively. Control animals received 0.5-ml injections of sterile vehicle or 0.9% saline.

Clearance Studies

Clearance studies were performed 7-21 days following initiation of corticosteroid therapy except in kinetic experiments specifically designed to compare alteration in clearance at various times after starting therapy. This period was chosen because there were no detectable differences in the rate or extent of clearance over this time interval.

The technique employed in these studies has been previously described in detail,12,14 and only a brief outline will be included here. Normal guinea pig erythrocytes labeled with 51Cr were sensitized with highly purified, high-avidity rabbit IgG antibodies to produce a known number of complement-fixing sites as determined by the C1a fixation and transfer test. In previous studies employing radiolabeled antibody we have determined that about 2000 molecules of IgG antibody were necessary to form one complement-fixing site. After injection of 1.0 ml of 2.7 x 10^8 sensitized or control unsensitized cells into the hind foot vein, serial 0.1-ml bleedings from the retroorbital sinus were obtained to determine erythrocyte survival. The 0.1-ml samples were suspended in 1.0 ml of buffer containing 0.01 M EDTA and then counted in a gamma scintillation counter. At each level of sensitization, the red cell pellet and plasma were counted separately. Greater than 98% of the counts were always found in the erythrocyte pellet. C4-deficient guinea pig serum does not lyse the antibody-sensitized cells used in these studies. Cortisone-treated and control groups were always studied simultaneously. Groups of animals were sacrificed 24 hr following injection, and localization of sensitized erythrocytes was determined by counting the radiactivity of the whole organ. In all studies except those in which tissue localization was examined, erythrocyte survival was followed for 14 days. The clearance data were analyzed and plotted as previously described,12 and the means were compared utilizing the t test.

An attempt was made to determine whether hydrocortisone decreased the association of antibody to the cell surface, thereby decreasing clearance. Therefore, in one series of experiments, cells were sensitized in the presence of buffer containing 100 μg/ml hydrocortisone sodium succinate and washed prior to injection.

Effect of Cortisone Administration on Hematologic Parameters, Weight Gain, and Spleen and Liver Weights.

Groups of C4D guinea pigs receiving 20 or 100 mg of cortisone acetate were weighed every other day for 14 days. The animals were then sacrificed by removal of 10-15 cc of heart blood via a direct cardiac puncture with a 19-gauge small vein infusion set (Abbott Laboratories, North Chicago, Ill.) A 5-ml portion was placed in EDTA solution and the remaining blood allowed to clot for 2 hr at 22°C. The liver, spleen, and kidneys were weighed. The erythrocyte and leukocyte counts were performed utilizing an electronic particle counter Model S (Coulter Electronics, Inc., Hialeah, Fla.). Standard hematologic techniques were employed for the hematocrit and 100-cell differential leukocyte counts.

RESULTS

Clearance of IgG-sensitized Erythrocytes

At least 17 IgG C1-fixing sites (10,060 IgG molecules) per erythrocyte are required for accelerated clearance in C4D guinea pigs as opposed to 1.4 (2,012 IgG molecules) C1-fixing sites in normal animals. At 17 IgG C1-fixing sites in C4D animals, accelerated clearance was barely detectable over the first 2 hr, but by 24 hr approximately 40% of the erythrocytes were removed
from the circulation. These cells were found to be entirely trapped in the spleen. C4D guinea pigs treated once daily with 5, 10, 20, or 100 mg cortisone acetate/kg body wt had normal survival over the initial 2 hr, and less than 20% of the cells were removed within 24 hr. This percentage is within the 95% confidence limit previously determined in C4D or normal guinea pigs injected with radiolabeled but unsensitized erythrocytes.

Figure 1 compares the clearance curves in normal, C4D, and steroid-treated C4D guinea pigs following injection of erythrocytes coated with 34 IgG Cl-fixing sites. In complement-deficient animals, between 45% and 65% of these cells were removed in 24 hr while 25%–35% were cleared in cortisone-treated animals at all dosage levels except the 5-mg group. In this latter group the rate of clearance was slower than in untreated controls, but by 24 hr the percentage of cells cleared was within the range observed in untreated animals. Also illustrated by this figure is the remarkable ability of complement activation to augment clearance of the IgG-sensitized cells. The spleen was totally responsible for clearance of sensitized cells at these levels of IgG sensitization, and decreased splenic uptake accounted for the increased erythrocyte survival in cortisone-treated animals.

Figure 2 is a comparison of clearance curves at 80 IgG Cl-fixing sites in C4D and cortisone-treated C4D guinea pigs. There was a marked decrease in the rate and magnitude of clearance in cortisone-treated as compared to untreated deficient animals. Two C4D animals treated with 5 mg were identical to untreated controls. The increased survival of sensitized erythrocytes was primarily due to
decreased splenic uptake, although there was also a modest decrease in the hepatic clearance as well (Table 1). Not shown in Fig. 2 is clearance in normals. In this case the $\frac{t}{2}$ was less than 5 min, and less than 0.5% of the injected cells remained in the circulation after 2 hr.

When the level of sensitization was raised to 180 Cl-fixing sites per cell, the 100 mg/kg cortisone group still showed almost complete inhibition of clearance (Fig. 3). The 10 and 20 mg/kg groups showed a decrease in the initial rate of clearance; however, after 2 hr there was no difference in the per cent of cells remaining in the circulation ($p > 0.05$). In C4D animals at this level of sensitization the liver and spleen each sequestered approximately 40% of sensitized cells. In the 100-mg group, both splenic and hepatic clearance were decreased. As the level of sensitization was increased above 180 IgG Cl-fixing sites, the

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**Table 1. Organ Localization of $^{51}$Cr-labeled IgG-sensitized Erythrocytes in Normal, C4D, and Cortisone-treated C4D Guinea Pigs**

<table>
<thead>
<tr>
<th></th>
<th>Circulation</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidneys</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsensitized control (10)*</td>
<td>74.1 ± 5.8t</td>
<td>13.9 ± 5.1</td>
<td>5.6 ± 1.7</td>
<td>1.0 ± 0.7</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>80 IgG Cl-fixing sites</td>
<td>57.9 ± 4.1</td>
<td>41.8 ± 3.9</td>
<td>1.6 ± 0.3</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Normal (5)</td>
<td>20.3 ± 5.1</td>
<td>28.6 ± 7.3</td>
<td>49.1 ± 8.6</td>
<td>0.7 ± 0.3</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td>C4D (4)</td>
<td>57.7 ± 7.7</td>
<td>17.2 ± 3.7</td>
<td>23.1 ± 3.8</td>
<td>2.7 ± 0.8</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>Cortisone-treated C4D (6)</td>
<td>20.6 ± 5.1</td>
<td>28.6 ± 7.3</td>
<td>49.1 ± 8.6</td>
<td>0.7 ± 0.3</td>
<td>1.5 ± 0.9</td>
</tr>
</tbody>
</table>

*Number of animals in each group.

†Mean per cent ± 1 SE of radiolabeled cells 24 hr after injection. Data have not been corrected for radioactive blood within organs.
magnitude and rate of clearance in the 100-mg group gradually approached that seen with untreated deficient animals. At 500 IgG C1-fixing sites there was no difference between these two groups.

Experiments were also performed in which the clearance of cells sensitized with 80 IgG C1-fixing sites per cell in the presence of hydrocortisone was examined. These cells were rapidly cleared in vivo, and there was no evidence for decreased binding of these antibodies in the presence of cortisone.

**Effect of Cortisone Administration on Hematologic Parameters, Weight Gain, and Spleen and Liver Weights**

We have previously reported the effect of 20 and 100 mg/kg body wt cortisone acetate on these parameters in NIH multipurpose guinea pigs. The hematocrit, percentage weight gain, and splenic weight were not significantly different between control and treated groups, while the liver weight increased directly proportional to the dose. The results obtained with C4D animals, a subline of the NIH strain, were similar. The percent body weight gain was equivalent in control and treated groups, and, of special note for this study, the spleen weight was not significantly decreased in cortisone-treated complement-deficient animals as compared to either control C4D or normal guinea pigs. The guinea pig is categorized as a steroid-resistance species, and others have also shown no decrease in spleen weight in guinea pigs following cortisone administration. The white blood count was unchanged in treated animals, but significant alterations in the differential counts were noted. There was a greater than 50% reduction in the number of peripheral blood lymphocytes, while there was an equivalent increase in peripheral blood granulocytes.
Kinetic Analysis of the Cortisone Effect.

In one study using erythrocytes sensitized with 34 IgG C1-fixing sites, animals were studied after 2, 4, and 8 days of continuous cortisone administration (20 mg/kg body wt). Injections were staggered in order to study the deficient animals on the same day with the same preparation of sensitized cells. Two days of therapy produced no changes in the clearance pattern. At 4 days the rate of clearance was decreased, but in only 50% of the animals was the magnitude of clearance significantly less than controls. After 8 days of treatment, the full effect of corticosteroids was evident, and there was no alteration in clearance patterns with continued administration.

DISCUSSION

Utilizing a previously described model of immune hemolytic anemia and a unique strain of guinea pigs with a total deficiency of the fourth component of complement, we have been able to quantitatively examine the effect of corticosteroids on non-complement-mediated immune clearance of IgG-coated red cells. At low levels of IgG sensitization, erythrocytes were predominately sequestered by the spleen, and corticosteroids markedly inhibited this splenic function. IgG-coated red cells were removed from the circulation as if they had one-half to one-third the number of Cl-fixing sites per erythrocyte in cortisone-treated C4D compared to control animals. There was a dose-response relationship between the number of IgG molecules coating the cells and the inhibitory capacity of a given steroid dose. As the amount of antibody coating the red cell was increased, higher doses of cortisone were necessary to cause a significant inhibition of hepatic and splenic sequestration. Eventually a level was attained where the magnitude of clearance was not decreased even by 100/ mg/kg/day. At lower levels of sensitization, doses of cortisone which are comparable to those used in warm-antibody-mediated hemolytic anemia in man were effective at increasing survival.

These studies can be compared to studies in animals with intact complement function. First, far more antibody was required to cause clearance (Fig. 1). Second, cortisone treatment proved considerably more effective in preventing sequestration of antibody-sensitized cells in the absence of complement activation. If the quantity of antibody coating the red cell was chosen to produce comparable rates of clearance in normal and deficient animals, corticosteroids influenced both the magnitude and rates of clearance to a greater extent in the absence of complement function. Third, in both cases, as the amount of antibody coating the red cell was increased, the liver removed a progressively larger percentage of the cells. Lastly, cortisone inhibited both splenic and hepatic sequestering ability.

This model of immune hemolytic anemia was chosen for study because it closely parallels the situation in man. Steroids are most effective in warm-antibody-mediated hemolytic anemia. In this condition, complement compounds, specifically the key component C3, are often not found on the erythrocyte surface. Two molecules of IgG side by side are thought necessary to activate complement. Presumably, because the sparse distribution of Rh antigenic sites, antibody doublets cannot form on the erythrocyte membrane,
and therefore complement is not activated. In this model the rabbit anti-guinea pig erythrocyte antibody used can fix the first component of complement but does not activate the biologically important complement components because of the absence of C4. The alternate complement pathway is functional in C4D guinea pigs, but this pathway is not efficiently activated by antibody directed at erythrocyte surface membranes. Thus, in this experimental model as in the clinical situation, complement activation does not play an important role in clearance.

The molecular mechanism by which corticosteroids decrease immune clearance, thereby increasing survival of sensitized erythrocytes, is unknown. The clearance of erythrocytes coated solely with IgG is presumably mediated by the attachment of the IgG Fc fragment on the red cell membrane to the macrophage IgG receptor in the spleen. In cortisone-treated animals, IgG-sensitized erythrocytes were cleared as if they had fewer IgG molecules per cell. An attractive hypothesis which would explain all these observations is that corticosteroids decrease the number and/or avidity of IgG receptors on splenic macrophages. In this way there would be a decrease in erythrocyte–receptor interactions which, in turn, would lead to increased survival of sensitized erythrocytes. Regardless of the mechanisms involved, it is clear from these studies that corticosteroids decrease the sequestration of IgG-sensitized erythrocytes. This effect may be important in the induction of remissions in patients with warm-antibody hemolytic anemia and may provide an explanation for the finding of patients in remission with a persistently positive antiglobulin test.

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Complement-independent Clearance of IgG-sensitized Erythrocytes: Inhibition by Cortisone

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