In Vivo Instability of Red-Blood-Cell-Bound C3d and C4d

By Hugh Chaplin, Jr., Margaret E. Coleman, and Martha C. Monroe

Until now, there have been no measurements of the in vivo stability of red-blood-cell-bound C3d and C4d subfragments of the third and fourth components of human complement. We have recently described a radiolabeled antiantiglobulin method for measuring RBC-bound C3d and have demonstrated that small amounts of C3d are present on RBC of all normal subjects tested. In the present study, the method was applied to follow the increments above baseline of RBC-bound C3d and C4d produced by autotransfusing 3 normal volunteers with 160–200 ml of RBC strongly coated in vitro by C3d and C4d. Posttransfusion measurements were carried out over 21–34 days. Immediate and long-term in vivo survival of the transfused RBC was unimpaired by C3d and C4d coating. Of the bound C3d antigen, 85%-95% disappeared from circulating RBC in 5–8 days; the remainder disappeared more slowly, with half-times in the range of 8–29 days. C4d antigen disappeared substantially more slowly, describable by a single exponential function in 2 of the 3 subjects, with half-times in the range of 12–31 days. Recognition of the in vivo instability of RBC-bound C3d helps in interpreting steady-state and changing levels of RBC C3d coating in a variety of alloimmune and autoimmune disorders.

When red blood cells (RBC) are freshly coated in vitro by the third component of complement (C3), both the C3c and the C3d antigens are detectable on the cell membrane by appropriate monoclonal antiantiglobulin sera. Trypsinization of such cells results in proteolytic cleavage and loss from the cell membrane of a major subfragment bearing C3c antigen, leaving the cell coated with a minor fragment bearing C3d antigen. A similar loss of the C3c subfragment occurs simply by incubation in fresh human serum, indicating the presence in serum of proteolytic enzymes capable of cleaving the C3c-bearing fragment, leaving the RBC agglutinable only by anti-C3d sera. This cleavage occurs in vivo as attested to by the invariable demonstration of C3d, but not C3c, on the RBC of patients suffering from complement-mediated hemolytic anemia.

Mollison1 showed that a proportion of RBC coated by autologous C3 in vitro at low ionic strength were rapidly destroyed following transfusion, but that the remaining RBC survived normally for at least the succeeding 5 days. Atkinson and Frank2 confirmed these findings and demonstrated the pathophysiological significance of in vivo cleavage of C3c in mediating the "release" of a proportion of freshly C3-coated RBC sequestered by C3c-receptor-bearing macrophages (primarily Kupfer cells in the liver), allowing the C3d-coated cells to return to the circulation, where they appeared to survive normally over a subsequent 20–35 day period of observation. There are no C3d receptors on macrophages.3 Ross et al. have demonstrated receptors for C3d on subpopulations of normal lymphocytes.4,5 and Kurlander et al. have demonstrated an effect of RBC C3d coating on the cytotoxicity of B lymphocytes against IgG-coated RBC in vitro.6 There are many possible explanations for the apparent failure of potentially damaging C3d receptor-bearing cells to affect in vivo survival of C3d-coated RBC; important to any such considerations would be the amount and stability of C3d bound to the red cell membrane.

Until now, there have been no studies of the in vivo stability of RBC-bound C3d. The present study employed an in vitro radiolabeled antiantiglobulin method to measure the persistence of C3d on RBC coated in vitro and autotransfused into healthy volunteers. Similar measurements were made of RBC-bound C4d. 3Cr survival studies, carried out over 21–29 days, confirmed that the in vivo lifespan of the transfused cells was unaffected by the initially strong complement coating. Most of the bound C3d antigen disappeared from the transfused cells relatively rapidly; the disappearance of C4d antigen was substantially slower.

MATERIALS AND METHODS

Volunteers

Three lean healthy subjects volunteered for the autotransfusion studies. Subject 1 was a white male, 59 yr old, 6 ft tall, weighing 175 lb. Subject 2 was a white female, 25 yr old, 5 ft 6 in tall, weighing 140 lb. Subject 3 was a white male, 29 yr old, 6 ft tall, weighing 170 lb. Although it was unsuspected at the outset of the study, subject 3's plasma was noted to be somewhat yellow on occasion; subsequent studies confirmed intermittent mild unconjugated hyperbilirubinemia (1.8–2.4 mg/dl), entirely normal liver function tests, normal hemoglobin concentration, hematocrit, red blood cell indices and morphology, with 1.5% reticulocytes. Following a 48-hr fast (400 calories/24 hr), the unconjugated bilirubin doubled to 4.1 mg/dl. These findings, along with a mild shortening of RBC lifespan (3Cr

From the Department of Preventive Medicine and the Department of Medicine (Division of Hematology), Washington University School of Medicine, St. Louis, MO.

Supported in part by Grants CA-02918 and AI 15322-05 from the National Institutes of Health, Department of HHS.

Submitted January 17, 1983; accepted May 12, 1983.
T½ of 21 days, see Results) are consistent with the diagnosis of Gilbert’s syndrome. The investigation was approved by the Washington University Human Studies Committee, and informed consent was obtained.

**Antisera**

Rabbit anti-C3d and anti-C4d sera were prepared as previously described. Both sera were absorbed with human IgG at slight antigen excess. Anti-C3d was further absorbed with purified human C3c; anti-C4d was further absorbed with purified human C3d. Anti-γ serum was raised in rabbits hyperimmunized with purified human IgG; anti-light-chain reactivity was absorbed with purified anti-C3d and anti-C4d antibodies, and aliquots containing 100 μg IgG/ml were stored frozen at −80°C until used. Samples of ascites containing rat monoclonal anti-C3d and anti-C3g antibodies in 40% glycerol, 10 mM sodium azide, were kindly supplied by Dr. Peter J. Lachmann, Mechanisms in Tumour Immunity Unit, Cambridge, England. Preparation and properties of these antisera have been fully described. Rabbit anti-rat IgG purified by affinity chromatography from Sepharose-bound rabbit IgG. The purified antibody was iodinated by the chloramine T method (less than 1 atom 125I/mole IgG), and aliquots containing 100 μg IgG/ml were stored frozen at −80°C until used. Ten serial dilutions (1/2-1/1024) were freshly prepared in normal saline. Strong coating by C3d was verified by agglutination in serial dilutions of potent anti-C3d antiglobulin serum. In the case of the polyclonal anti-C4d and anti-C3d sera used in the present study, the cells were washed and incubated in 0.5 ml of 125I-labeled purified goat anti-rabbit IgG (1 μg/ml). The RBC are then separated from unbound antibody by sedimentation through 1.5 ml of undiluted N-butyl phthalate. RBC-bound labeled anti-rabbit IgG is then measured by counting the RBC button in a Beckman Gamma 4,000 counter. Values for each measurement represent the mean of triplicate measurements. Nonspecific binding of rabbit and goat sera are assayed in triplicate for each sample, substituting a 1/100 dilution of normal rabbit serum (NRS) for the anticomplement antiliglobulin serum. All values are corrected for nonspecific binding by subtraction of the cpm obtained with NRS. In addition, all values are corrected for radioactive decay and further corrected for variations in strength of individual “10%” RBC suspensions, based on duplicate measurements of hemoglobin concentration on each cell suspension. It has previously been shown that normal RBC have small amounts of C3d bound to their membranes and that the level of bound C3d varies within relatively narrow limits (SD ± 15%) for a given individual. Accordingly, baseline values for RBC-bound C4d and C3d were established for each normal volunteer based on the average of measurements made on 2-4 samples drawn at several-day intervals during the month prior to autotransfusion. Baseline values were subtracted from values obtained after autotransfusion, thereby giving the increments above baseline attributable to the strongly coated transfused RBC.

**Preliminary In Vivo Experiments**

All solutions, syrings, and glassware were sterile and pyrogen-free. Sterile precautions were observed for all manipulations. Approximately 2 ml of RBC were labeled with 15 μCi 51Cr, coated with C4b-C4bi and C3b-C3bi, washed, and trypsinized as described. The C4d,C3d-coated RBC were resuspended to a total volume of 10 ml, well mixed, and a weighed 8-ml aliquot was infused into the original donor. From the remaining RBC suspension, a 51Cr standard was prepared. Blood volume was estimated as 70 ml/kg body weight for male volunteers, 67.5 ml/kg body weight for females. Blood samples were drawn at 20 and 90 min and at 19 hr after infusion. One hundred percent “expected” immediate survival was calculated on the basis of the known volume of labeled RBC suspension infused and on the estimated total blood volume. On a separate occasion, the above study was repeated, omitting the LIS complement-coating steps but including trypsinization. On a third occasion, the original study was repeated but C4c and C3c were removed by 4-hr incubation in fresh autologous defibrinated serum, instead of by trypsinization, and blood sampling was continued at 2-4-day intervals for 17 days after infusion.

**Radiolabeled Antiglobulin Test**

Details of the method have recently been published. In brief, 0.05 ml of 10% washed RBC are incubated with 0.2 ml of rabbit antiglobulin serum (at a dilution providing antibody excess—1/100 in the case of the polyclonal anti-C4d and anti-C3d sera used in the present study). The cells are washed and incubated in 0.5 ml of 125I-labeled purified goat anti-rabbit IgG (1 μg/ml). The RBC are then separated from unbound antibody by sedimentation through 1.5 ml of undiluted N-butyl phthalate. RBC-bound labeled anti-rabbit IgG is then measured by counting the RBC button in a Beckman Gamma 4,000 counter. Values for each measurement represent the mean of triplicate measurements. Nonspecific binding of rabbit and goat sera are assayed in triplicate for each sample, substituting a 1/100 dilution of normal rabbit serum (NRS) for the anticomplement antiliglobulin serum. All values are corrected for nonspecific binding by subtraction of the cpm obtained with NRS. In addition, all values are corrected for radioactive decay and further corrected for variations in strength of individual “10%” RBC suspensions, based on duplicate measurements of hemoglobin concentration on each cell suspension. It has previously been shown that normal RBC have small amounts of C3d bound to their membranes and that the level of bound C3d varies within relatively narrow limits (SD ± 15%) for a given individual. Accordingly, baseline values for RBC-bound C4d and C3d were established for each normal volunteer based on the average of measurements made on 2-4 samples drawn at several-day intervals during the month prior to autotransfusion. Baseline values were subtracted from values obtained after autotransfusion, thereby giving the increments above baseline attributable to the strongly coated transfused RBC.

**Antigenic Agglutination Tests**

Ten serial dilutions (1/2-1/1024) were freshly prepared in normal saline. One drop of each dilution was mixed on a glass tile with one drop of 4×-washed 5% RBC suspension, gently rocked for 5 min at room temperature, and graded (+−++) by visual inspection. “Mixed field” reactions were verified by low power (100×) microscopy.

**Preparation of RBC Coated With C4d and C3d**

RBC coated by C4b-C4bi and C3b-C3bi were prepared by the low ionic strength sucrose (LIS) method, as described by Freedman and Mollison. One volume of whole blood in citrate phosphate dextrose adenine (CPD-A1) anticoagulant plus 25 volumes of 10% sucrose containing 0.15 M CaCl2 and 0.5 M MgCl2 were incubated with continuous gentle mixing for 5 min at 37°C. The suspension was incubated an additional 10 min at 37°C without mixing; the supernatant above the settled RBC was discarded and the coated RBC were then washed 4 times in saline. In preliminary experiments, the C4c and C3c fragments were removed by incubating the packed RBC in 4 volumes of 4 mM trypsin in 0.1 M phosphate-buffered saline, pH 7.7, for 10 min at 37°C. The cells were immediately washed 4 times in normal saline. Strong coating by C3d was verified by agglutination in serial dilutions of potent anti-C3d antiglobulin serum. In the majority of studies, C4c and C3c were removed by incubation of packed washed coated RBC in 2 volumes of fresh or fresh-frozen autologous defibrinated serum for 4 hr at 37°C. Removal of C4c and C3c was confirmed by failure of the RBC to agglutinate in serial dilutions of potent anti-C4c and anti-C3c antiglobulin sera.

**51Cr Labeling**

A quantity 15-65 μCi of 51Cr (as sodium chromate) was added to 5-15 ml of whole blood in CPD-A1 and incubated for 30 min at 37°C. The RBC were then washed 4 times in saline and resuspended to a hematocrit of 40% in fresh autologous CPD-A1 plasma prior to coating by C4 and C3.
IN VIVO INSTABILITY OF RBC-C3d AND C4d

- 20°C. The packed RBC were washed once with approximately 400 ml of sterile saline to remove potentially thrombogenic residual serum, resuspended in 200 ml of sterile saline, and reinfused promptly to the donor.

For the autotransfusion studies, the procedures described above for preparing RBC coated by C4d and C3d for the preliminary in vivo experiments were scaled up to permit sterile preparation of 160–200 ml of labeled strongly coated RBC. Approximately 450 ml of blood were drawn into CPD-A1 anticoagulant solution at approximately 8:00 a.m. A 15-ml aliquot was labeled with approximately 65 μCi of 51Cr; the labeled RBC were washed and returned to the CPD-A1 blood container. After complement coating and incubation with freshly thawed defibrinated autologous serum, the RBC were washed with 1 liter of saline in an IBM 2991 Model Blood Cell Processor (IBM Corporation, Princeton, NJ). The washed RBC were resuspended in approximately 200 ml of saline; the suspension was thoroughly mixed, and approximately 5 ml removed to prepare 36Cr and RBC-C3d standards and for characterization of complement coating by antiglobulin testing. The weight of the residual suspension was recorded, and the entire amount was infused into the original donor at a rate of approximately 5 ml/min, commencing at approximately 5:00 p.m. Recipients were closely observed during and for 90 min following infusion. No definite untoward symptoms or signs were observed. Subject 2 felt slightly chilly immediately on completion of transfusion and experienced a 0.4°C temperature elevation 50 min later, with return to normal in the subsequent half hour. For each subject, a 3-ml anticoagulated sample was obtained after infusion of 80–100 ml of suspension, centrifuged immediately, and inspected for any evidence of in vivo hemolysis before continuing. No hemolysis was observed. Blood samples (8 ml) were obtained immediately on completion of infusion and at 1.5, 4, 15, 23, and 39 hr. Thereafter, samples were obtained daily for 1 wk, every other day for 1 wk, and every 4–7 days for up to 2 additional weeks.

RESULTS

Preliminary In Vivo Studies

When approximately 1.5 ml of 51Cr-labeled trypsinized C4d, C3d-coated RBC were reinfused into the donor, markedly impaired in vivo survival was observed: 85% at 20 min, 55% at 90 min, less than 10% at 19 hr. A repeat study employing trypsinized RBC not previously coated by C4 and C3 gave closely similar results, indicating that trypsinization per se could account for the rapid in vivo RBC destruction. A third study on subject 1 employed 51Cr-labeled C4d, C3d-coated RBC prepared by 4-hr incubation in fresh autologous defibrinated serum. Based on the subject’s estimated total blood volume and the carefully measured volume of labeled RBC suspension infused, the average cpm of the 20-min and 90-min samples represented 102% of the expected survival for fresh autologous RBC. Sampling over the succeeding 17 days revealed entirely normal survival (T½=28–29 days). Thus, strong coating by C4d and C3d (comparable to that illustrated in Table 1) at the time of infusion did not per se result in any immediate or long-term impairment of in vivo RBC survival.

Table 1. Characterization of Cell Membrane Coating by C3, C4, and IgG on Serum-Treated RBC Autotransfused to Subject 1

<table>
<thead>
<tr>
<th>Specificity</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>1,024</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-C3c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-C3d</td>
<td>2</td>
<td>3</td>
<td>4+</td>
<td>4+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-C4c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-C4d</td>
<td>3+</td>
<td>2</td>
<td>1-</td>
<td>1+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Based on calculations employing the calibration curve published previously. RBC transfused in these experiments were coated by 4,500–7,250 mols C3d/cell.

Autotransfusion Studies

Based on preliminary measurements of RBC strongly coated with C3d and C4d by the method described here, it was estimated that 160–200 ml of coated RBC would need to be infused to produce a 4–6-fold increment above pretransfusion whole blood RBC-C4d, C3d values immediately after mixing in the recipient’s circulation. Antiglobulin agglutination studies on the coated RBC autotransfused to subject 1 are shown in Table 1. Negative reactions with anti-C4c and anti-C3c confirm that serum incubation had achieved effective cleavage of the major fragments containing C4c and C3c antigens. The cells were also nonreactive with a potent anti-γ reagent. 125I rabbit anti-rat IgG measurements following incubation of the serum treated RBC with rat monoclonal anti-C3d and anti-C3g antibodies demonstrated the presence of both the C3d and C3g antigens on the RBC transfused. Entirely similar results with rabbit and rat antibodies were obtained on the RBC autotransfused to subjects 2 and 3.

In light of the estimated blood volumes for each subject, the measured volumes administered, and the 51Cr-RBC and RBC-C3d standards prepared on aliquots of the RBC autotransfused, the percent of 100% “expected” survival was determined for the average of the immediate and 1.5-hr posttransfusion blood samples. The results are shown in Table 2. There is reasonably close agreement within the three pairs of results; values for the three subjects range close to a mean of 100%. These results confirm the 51Cr-RBC results on the previously described preliminary study carried out on subject 1.

Direct anti-C3d and anti-C4d antiglobulin agglutina-
Table 2. Percent of "Expected 100% Survival"* of RBC (51Cr) and of RBC-Bound C3d (25l Antiantiglobulin Test) 1.5 hr Following Autotransfusion

<table>
<thead>
<tr>
<th>Subject</th>
<th>RBC (51Cr)</th>
<th>RBC-Bound C3d</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Male)</td>
<td>111</td>
<td>114</td>
</tr>
<tr>
<td>2 (Female)</td>
<td>98</td>
<td>112</td>
</tr>
<tr>
<td>3 (Male)</td>
<td>89</td>
<td>91</td>
</tr>
</tbody>
</table>

*Based on blood volume estimated as 70.0 ml/kg for males, 67.5 ml/kg for females.

nation tests carried out on the immediate, 15-hr, and 39-hr posttransfusion samples from subject 3 are illustrated in Table 3. The presence of the strongly coated cells (constituting not more than 10% of the total RBC mass) is clearly demonstrable by the weak to moderate "mixed field" reactions observed. There was definite weakening of the anti-C3d reactions over the 39-hr interval; weakening was less striking for anti-C4d reactions. Entirely similar results were obtained for subjects 1 and 2.

Figure 1 illustrates the in vivo 51Cr-RBC survival results. Entirely normal T½ values were obtained for subjects 1 and 2. A mildly reduced T½ (21 days) was obtained for subject 3, who was subsequently demonstrated to have Gilbert's syndrome (see Materials and Methods), wherein modest shortening of RBC survival is found in 40%-50% of subjects.

Disappearance of the posttransfusion increments of RBC-C3d and RBC-C4d antigens are shown on semilogarithmic plots in Figure 2. Absolute values are illustrated by solid lines; values corrected for loss by RBC senescence are shown for the slower phase disappearances (where senescence had a greater effect on the slopes than during the rapid phases). Disappearance of C3d from normally surviving RBC was well described by two exponentials in all three subjects. The rapid phase, accounting for 85%-95% of the bound C3d, had T½ values in the range of 2.5-4 days. T½ values for the slow phase, corrected for RBC senescence, were 26 and 29 days in subjects 1 and 2, respectively, and 8 days in subject 3. By contrast, C4d antigen disappeared from surviving RBC at a single exponential rate for 2 of the 3 subjects, with senescence-corrected T½ values of 3 days for subject 2 and 14 days for subject 3. Disappearance of C4d antigen from surviving RBC in subject 1 was best described by 2 exponentials. The relatively rapid phase, accounting for approximately 50% of bound C4d, had a T½ of 3 days; the T½ for the slower phase (corrected for senescence) was 12 days. Absolute and senescence-corrected values for slopes (m) and correlation coefficients (r) for the three subjects are summarized in Table 4.

**DISCUSSION**

These studies confirm that RBC strongly coated by C4d,C3d have normal immediate and long-term in vivo survival, based on approximately 4 wk of observation. This was verified in two separate studies in subject 1 and in a single study in subject 2. The 21-day survival in subject 3, with Gilbert's syndrome, is typical of previously reported mildly decreased red cell survival in that condition.

The studies illustrated in Fig. 2 indicate that most
IN VIVO INSTABILITY OF RBC-C3d AND C4d

RBC-bound C3d antigen is relatively labile in vivo, with approximately 90% disappearing within 6–8 days. Approximately 10% is more tightly bound and disappears more slowly. C3d antigen disappearance is similar in subjects 1 and 2. Both the fast and slow phases are accelerated in the volunteer with Gilbert’s syndrome (subject 3); it is interesting to speculate whether this could be related to the undefined RBC defect responsible for mild shortening of in vivo RBC lifespan observed in that condition.

There are several clinical implications of the in vivo lability of RBC-C3d binding. When RBC coating by C3 occurs as a comparatively isolated event (e.g., complement-mediated drug-related immune hemolytic anemia, or sensitization by alloantibodies in transfused donor plasma), one should expect the C3d-positive direct antiglobulin reaction to become much weaker over the course of a few days after discontinuation of the drug, attributable to disappearance of RBC-bound C3d antigen and not to destruction of the coated RBC. We have recently observed an example of such quantitative RBC-C3d changes in a patient in the early recovery phase following bone marrow transplantation. As part of a separate study, the patient’s RBC-C3d was being followed at 2–3-day intervals (Fig. 3). Following 2 U of group O packed RBC (patient was group A), the patient’s already elevated RBC-C3d more than doubled. Over the succeeding week (days 3–10), the RBC-C3d returned to the pretransfusion value in the absence of hemolysis or blood loss. During this period, the hemoglobin concentration rose slightly despite uncorrected reticulocyte values no higher than 0.5%–1.5% and without transfusions. The changes in RBC-C3d are consistent with disappearance of 90% of acutely bound C3d antigen from normally surviving RBC over the 1-wk period. Direct anti-C3d antiglobulin tests were not performed.

Table 4. Slopes and Correlation Coefficients for Rates of Disappearance of RBC-Bound C3d and C4d Following Autotransfusion

<table>
<thead>
<tr>
<th>Subject</th>
<th>Log % Max cpm (Absolute Values)</th>
<th>Log % Max cpm (Corrected for RBC Senescence)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C3d</td>
<td>C4d</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>r</td>
</tr>
<tr>
<td></td>
<td>m</td>
<td>r</td>
</tr>
<tr>
<td>1 Fast</td>
<td>-.1807</td>
<td>-.9756</td>
</tr>
<tr>
<td>Slow</td>
<td>-.0270</td>
<td>-.9872</td>
</tr>
<tr>
<td>2 Fast</td>
<td>-.1508</td>
<td>-.9376</td>
</tr>
<tr>
<td>Slow</td>
<td>-.0279</td>
<td>-.9696</td>
</tr>
<tr>
<td>3 Fast</td>
<td>-.3647</td>
<td>-.9781</td>
</tr>
<tr>
<td>Slow</td>
<td>-.1412</td>
<td>-.9918</td>
</tr>
</tbody>
</table>

Fig. 2. Disappearance of the increments of RBC-bound C4d and C3d above baseline following autotransfusion of strongly C4d,C3d-coated cells. Values are expressed as percent of initial increment values (average for the immediate and 1.5-hr blood samples) and are based on counts per minute (cpm) of 125I-labeled goat anti-rabbit IgG bound after exposure of the RBC to hyperimmune rabbit anti-C4d and anti-C3d sera at antibody excess. Slopes were calculated by linear regression; solid lines represent absolute values, interrupted lines are calculated after correction for RBC senescence.
By contrast, persistence of a strongly positive anti-C3d antiglobulin reaction would indicate an ongoing complement-mediated process and would militate against the diagnosis of an isolated event as the cause.

Evans et al.\textsuperscript{15} pointed out a protective effect of RBC-C3d against further in vitro deposition of C3b by cold hemolytic antibodies. Despite this, strongly C3d-coated patient and transfused donor cells had a shortened in vivo survival in such patients.\textsuperscript{16} The rapid in vivo turnover of the majority of acutely bound C3d demonstrated in the present study helps to explain the dynamic state that exists in complement-mediated hemolytic anemias. The relative protection afforded by RBC-bound C3d is tempered by relatively rapid loss of C3d antigen, with renewal of the opportunity for new deposition of C3b and its attendant dangers.

Studies with rat monoclonal anti-C3g antibody demonstrated that the recently defined C3g antigen\textsuperscript{17} was also present on the serum-incubated C4d,C3d-coated RBC autotransfused. We have recently demonstrated both C3d and C3g on RBC from 9 consecutive patients with complement-mediated warm and cold antibody autoimmune hemolytic anemias.\textsuperscript{18} Thus, the RBC autotransfused were strictly analogous in this respect to RBC observed in patients with autoimmune hemolytic anemia. It would have been most interesting to have measured the stability of RBC-bound C3g antigen in the same manner as for C3d and C4d. Unfortunately, the level of cpm for the rabbit anti-rat IgG antiantiglobulin measurements of RBC-bound C3g was only 20% of the level of cpm obtained for RBC-bound C3d in the goat anti-rabbit IgG system. It was concluded, therefore, that the increments achieved above baseline would be too low for accurate measurement.

These experiments cast no light on the mechanisms by which C3d reactivity is lost from the membranes of normally surviving RBC. There is strong evidence that C3b is covalently bound to the carbohydrate moiety of cell membrane C3b receptors.\textsuperscript{19} It appears unlikely that disruption of the covalent bond would be unaccompanied by membrane damage and consequent impaired RBC survival. We are aware of no in vitro precedent for "blocking" of bound C3d by interaction with unspecified plasma constituent(s); however, in vitro studies at 37°C are generally short term and cannot be considered comparable to conditions obtaining in vivo. Because of the autotransfusion design of the present experiments, it was not possible to isolate the C3d-coated RBC at intervals following transfusion to look for evidence of morphological abnormalities or modifications of membrane chemical constituents. Care has been taken throughout this report to describe loss of C3d antigen reactivity rather than loss of the entire C3d-containing fragment. Chemical breakdown of much of the 30,000-40,000-dalton C3d fragment (including the sequences responsible for C3d antigenic reactivity) can be envisioned without requirement for disruption of the covalent bond linking C3d fragment to RBC membrane receptors. Analogous to the pinocytosis observed for anti-A-coated infant RBC,\textsuperscript{20} internalization of receptors bound to residual C3d fragment could play a role in the disappearance of detectable RBC-C3d from normally surviving cells. Investigation of these various possibilities should be possible by studying isologous donor RBC coated in vitro by labeled C3d and separable from recipient's RBC posttransfusion by differential agglutination.

Outside of the function of C4 as part of classical pathway C3 convertase, a role for RBC-bound fragments of C4 (in particular C4d) has not been defined. Clearly, freshly bound C4d is more stable than is C3d. There was more variability in the rate and pattern of C4d antigen disappearance among the three subjects than was observed for C3d. C4d antigen disappearance described a single exponential function in 2 of the 3 volunteers, but with very different T\textsubscript{1/2} values (14 versus 31 days). By contrast, in subject 1, 2 rates of disappearance were clearly evident, with the rapid
IN VIVO INSTABILITY OF RBC-C3d AND C4d

resulting in loss of half of the bound C4d antigen in 5 days. Whether the differences in rates of C4d and C3d antigen disappearance represent intrinsic differences characteristic of the three individual volunteers, or whether they reflect differences in C4d and C3d binding due to subtle variations in the in vitro preparation of the coated RBC, is unknown. It is interesting that the volunteer with Gilbert’s syndrome, whose RBC-C3d antigen disappeared so rapidly, did not show exceptionally rapid disappearance of C4d.

It is not known whether C4d and C3d antigens have similar patterns of disappearance from membranes of cells other than erythrocytes. The present findings suggest the value of broadened investigation of the in vivo stability of complement components on a variety of cells, on basement membranes, and on circulating and fixed immune complexes in normal subjects and in patients with a variety of alloimmune and autoimmune disorders.

ACKNOWLEDGMENT

References were made by the Division of Nuclear Medicine, Mallinkrodt Institute of Radiology, Washington University School of Medicine. We are also grateful to the Barnes Hospital Blood Bank for assistance in separating the sterile defibrinated serum and in washing the RBC for autotransfusion, and to Dr. Peter Lachmann, Mechanisms in Tumour Immunity Unit, Cambridge, England, for the gift of rat anti-C3d and anti-C3g monoclonal antibodies.

REFERENCES

In vivo instability of red-blood-cell-bound C3d and C4d

H Jr Chaplin, ME Coleman and MC Monroe