Dynamics of Interaction Between Complement-fixing Antibody/dsDNA Immune Complexes and Erythrocytes

In Vitro Studies and Potential General Applications to Clinical Immune Complex Testing

Ronald P. Taylor, Carol Horgan, Michele Hooper, and Joseph Burge
Departments of Biochemistry and Internal Medicine, University of Virginia, Charlottesville, Virginia 22908

Abstract

Soluble antibody/^{3}H-double-stranded PM2 DNA (dsDNA) immune complexes were briefly opsonized with complement and then allowed to bind to human erythrocytes (via complement receptors). The cells were washed and subsequently a volume of autologous blood in a variety of media was added, and the release of the bound immune complexes from the erythrocytes was studied as a function of temperature and time. After 1–2 h, the majority of the bound immune complexes were not released into the serum during blood clotting at either 37°C or room temperature, but there was a considerably greater release of the immune complexes into the plasma of blood that was anticoagulated with EDTA. Similar results were obtained using various conditions of opsonization and also using complexes that contained lower molecular weight dsDNA. Thus, the kinetics of release of these antibody/dsDNA immune complexes differed substantially from the kinetics of release of antibody/bovine serum albumin complexes that was reported by others. Studies using the solution phase C1q immune complex binding assay confirmed that in approximately half of the SLE samples that were positive for immune complexes, there was a significantly higher level of detectable immune complexes in plasma vs. serum. Freshly drawn erythrocytes from some SLE patients exhibiting this plasma/serum discrepancy had IgG antigen on their surface that was released by incubation in EDTA plasma. Thus, the higher levels of immune complexes observed in EDTA plasma vs. serum using the C1q assay may often reflect the existence of immune complexes circulating in vivo bound to erythrocytes.

Introduction

There is increasing evidence that indicates that erythrocytes (RBCs)[1] may play a key role in the normal removal from the circulation of potentially pathogenic complement-fixing immune complexes (1–7). It is now well established that the principal means by which RBCs bind such immune complexes is through their immune adherence receptors (CR_1, specific for C3b and C4b) (6–13). Once bound to RBCs, the immune complexes can be removed from the RBC by passage through the liver (without RBC destruction) (7), or be released from the RBC through the action of serum factors such as C3b inactivator (I) (14, 15). Several recent studies have demonstrated that in certain patients with immune complex-mediated diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis there is a reduction in the number of these receptors on their RBCs (2, 4, 5), and we have presented evidence for a decrease in the complement-mediated binding of antibody/double-stranded DNA (dsDNA) immune complexes to the RBCs of certain patients with these diseases (16). It has been further suggested that immune complex-mediated tissue damage can result in these particular patients if the normal RBC-mediated clearance mechanism for removal of immune complexes is impaired.

In view of the recognized importance of complement-fixing antibody/dsDNA immune complexes in the pathogenesis of SLE (17–23) we have focused our studies on this system. While investigating the mechanism by which antibody/dsDNA immune complexes are released from normal vs. SLE RBCs, we were surprised to observe that significantly fewer immune complexes were released from normal or SLE RBCs in clotted blood compared with EDTA-chelated blood. This implies that there could be a discrepancy between apparent immune complex levels measured in plasma vs. serum, which could have a significant bearing on the detection of immune complexes in the circulation of patients with SLE (or other diseases). We report here the in vitro quantitation and analysis of this phenomenon, and furthermore, we also provide direct evidence that this observation is in fact relevant to a clinical immune complex assay (the C1q solution phase assay) in SLE patients.

Methods

Blood samples. Blood was collected from normal healthy volunteers or SLE patients (16) at the University of Virginia School of Medicine after protocols approved by an Internal Human Subjects Investigation Committee.

Preparation of immune complexes. Soluble DNA/anti-DNA immune complexes (DA) were prepared by incubating a solution of ^{3}H-labeled PM2 dsDNA (0.5–1.0 μg/ml) with an equal volume of high anti-dsDNA titer SLE plasma MA (a 1–5-fold dilution) for 1 h at 37°C (24). In some cases the DNA was sonicated on ice for four periods of 15 s each to generate double-stranded fragments of 5×10^5 mol wt (25). Soluble, complement-opsonized DNA/anti-DNA immune complexes (C-DA) were prepared by incubating 1 vol of a solution of DA with half a volume of a threefold dilution of normal human serum complement (NHSC). The mixture was incubated at 37°C for 5–10 min to optimize complement fixation and then layered onto sucrose

Dr. Hooper is a Fellow of the Arthritis Foundation.

Address reprint requests to Dr. Taylor, Department of Biochemistry, or to Dr. Burge, Department of Medicine.

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1. *Abbreviations used in this paper:* dsDNA, double-stranded DNA; NHSC, fresh normal human serum used as a complement source; GVB^{+2} buffer, gelatin-veronal buffer containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$; AHG, heat-aggregated human IgG; RBCs, human erythrocytes; I, C3b inactivator; C-DA, soluble, complement-opsonized DNA/anti-DNA immune complexes isolated by sucrose gradient ultracentrifugation; DA, soluble DNA/anti-DNA immune complexes; SLE, systemic lupus erythematosus.

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density gradients that were isokinetic for DNA (24). The gradients were centrifuged for 105 min at 28,000 rpm at 4°C (SW 28 rotor). The material that spun to the bottom of the gradient (≥ ~200S) was dissolved in borate saline (0.15 M NaCl, 0.03 M borate, pH 7.8) and frozen until use. This material (C-DA) was diluted in gelatin-veronal buffer containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ (GVB^{+2} buffer) (8) for use in the binding assays (see below). In a few cases, other SLE sera or plasma with high anti-dsDNA titers were used, and similar results to those reported for MA were obtained (see below).

In ~½ of our experiments, antibody/^{3}H-dsDNA complexes were prepared and opsonized with NHSC and bound to RBCs after our standard binding assay (9). In this case the samples were not "purified" by the sucrose gradient ultracentrifugation procedure described above. We will refer to these complexes as "whole serum" complexes, since these complexes remained in the serum milieu. Both "purified" C-DA and "whole serum" antibody/^{3}H-dsDNA complexes were prepared under conditions such that 80–95% of the input counts bound to the RBCs of normal individuals under the standard conditions of the assay. In those experiments in which SLE patient RBCs with lowered binding were examined (see below), the binding of the input immune complex was in the range of ~35–75%.

The sera of five different individuals were used at various times as separate sources of NHSC in these experiments, and in no case did we observe any effect in either the binding or release experiments (see below) attributable to the interaction between the serum of one individual and the RBCs of another. This is presumably because under the standard conditions of the erythrocyte linked complement fixation assay (9, 16) hemagglutination reactions were precluded due to the relatively low concentrations of reactants.

Binding and release studies. In a typical experiment, multiple aliquots of 100 μl each of a dilution of C-DA were incubated for 20 min at 37°C with 100 μl of a 25% suspension of freshly drawn and washed RBCs in glass centrifuge tubes. The samples were then centrifuged at 1,500 *g* for 3 min to pellet the RBCs, and the percentage of radiolabel bound to the cells was determined by counting 100 μl of the supernatant. The remaining supernatant was discarded, the cells were then gently washed with 1 ml of GVB^{+2} buffer, and the number of counts released in the entire wash was determined. Subsequently, 1 ml of the "releasing medium" (see below) was added to the RBC pellet that contained the bound antibody/^{3}H-dsDNA immune complex, the sample was gently mixed, and then incubated for 1 h at 37°C or 2 h at room temperature (24°C). Subsequently, the sample was again centrifuged and the number of counts released after the final incubation was determined by counting 400 μl of the supernatant. All determinations were run in duplicate.

When "whole serum" immune complexes were used, 100 μl of the antibody/^{3}H-dsDNA immune complex were incubated for 20 min at 37°C with 100 μl of a 25% solution of RBCs and 50 μl of a fourfold dilution of NHSC. After the sample was centrifuged, 125 μl of supernatant was counted to determine the percentage counts bound, and then the same release procedure described above for C-DA was used.

Preparation of "releasing media". All blood donors were bled twice on the day experiments were conducted. The first time they were bled, aliquots of their whole blood were stored in either Alsevier's solution (one part blood to two parts Alsevier's) or "lavender top" blood collection tubes (Vacutainer; Beckton-Dickinson & Co., Rutherford, NJ) (containing EDTA) or "blue top" blood collection tubes (containing sodium citrate) to prevent coagulation. A sample of blood from the Alsevier's solution was washed and used to supply RBCs for the initial binding assay (9). When the release experiment was started (~2½ h after the first bleeding) the donor was bled again, and duplicate 1-ml aliquots of freshly drawn blood (not anticoagulated) were added immediately to the RBC pellets that contained the bound antibody/^{3}H-dsDNA complexes. After mixing, the samples were allowed to clot at either 37°C for 1 h or ~2 h at room temperature. Parallel studies were also conducted using 1-ml aliquots of the anticoagulated blood samples that were previously collected. Blood samples from different donors were never mixed; the pelleted RBCs from a given donor were only mixed with blood from the same individual. These experiments were specifically designed to closely simulate the typical procedure used in a hospital clinic for blood collection and processing. This is the reason whole blood was used as the "releasing medium." In a few experiments, fresh plasma or serum (not containing any RBCs) was used as a "releasing medium," and the same general trends were observed as for whole blood (see below).

Calculations and analysis. A number of other "releasing media" were also examined to determine their effect on the bound immune complexes. For purposes of clarity, we present in Table I an example of the typical conditions and calculations used in the procedure. The

Table I. Typical Raw Data and Analysis of the Binding of Complement-fixing Antibody/^{3}H-dsPM2 DNA Immune Complexes to RBCs and Their Subsequent Release Upon Addition of Different Media and Further Incubation for 1 H at 37°C

	Initial binding step		Washing step		Release step	
"Releasing medium"	Supernatant, dpm	% Bound*	Supernatant, dpm	% Released‡	Supernatant, dpm	% Released§
GVB^{+2}	118	95	120	3	79	4
Fresh whole blood (allowed to clot)	137	94	116	3	450	19
Blood anticoagulated with Alsevier's	160	93	128	3	505	31
Whole blood (anticoagulated with EDTA)	146	94	125	3	1,637	92
Whole blood (anticoagulated with sodium citrate)	119	95	ND‖	ND‖	782	32
DNase	136	94	129	3	1,763	(100)¶

* All samples were run under identical conditions until the release step, when different "releasing media" were added. The total input of ^{3}H-PM2 dsDNA was 4,694 dpm. As 50% of the supernatant was counted, then % bound = (1 − supernatant, dpm/2,347) × 100. ‡ Based on an average of 4,412 dpm bound (94% × 4,694), the % release = (wash supernatant, dpm/4,412) × 100. The average wash supernatant was 124 dpm. § The total dpm capable of being released was defined by the value obtained for the DNase sample, 1,763 dpm. Thus, % release = (release supernatant, dpm/1,763) × 100. The maximum number of dpm that could potentially be found in 400 μl of supernatant (out of a total of 1 ml) = (4,412 − 124) × 40% = 1,715 dpm, in good agreement with the experimentally defined total of 1,763 dpm for the DNase sample. The samples that were clotted or anticoagulated with sodium citrate were corrected for excluded volume effects (see experimental section). ‖ ND, not done. ¶ Defined as 100 and not calculated.

"total counts" that could be released was determined experimentally by using DNase (0.05 mg/ml in 0.15 M NaCl, 0.01 M phosphate, and 0.01 M $MgCl_2$, pH 7.4 [11]) to release the ^{3}H-DNA that was bound to the RBCs. The number of ^{3}H-counts released by this experimental procedure was within 10% (or better) of the expected number of counts that could be released (see calculations in Table I), and was used as a basis for calculating the percentage of radioactivity released by the other "releasing media." In the case of the whole blood samples (allowed to clot), and the samples anticoagulated with sodium citrate, a correction must also be made for the "excluded" volume in the solution that is unavailable to the ^{3}H-dsDNA. This problem was solved experimentally by simply counting aliquots of known amounts of ^{3}H-dsDNA previously added to the various milieu (The nonspecific binding of ^{3}H-dsDNA in these systems were always <10%.). The appropriate corrections (only required for the clotted samples and those anticoagulated with sodium citrate) that compensated for the excluded volume effect have been made in all cases. The reproducibility in duplicate points in a typical experiment averaged ~ ±10% or better of the reported value. The means and standard deviations for each experimental release protocol were calculated, and where appropriate the differences between the results of different procedures were analyzed for significance by the *t* test.

C1q solution phase assay. We used the standard C1q assay (26) as we have previously reported (27) to measure immune complexes in plasma or serum samples from SLE patients. Plasmas were obtained after allowing the blood samples (anticoagulated with 0.01 M EDTA) to stand for 1 h at 37°C. Standard curves using heat-aggregated human IgG (AHG) at multiple dilutions in phosphate-buffered saline (PBS) between 3 and 800 μg/ml were run each day. Typically, a concentration of 800 μg/ml gave 60% ^{125}I-C1q binding, 50 μg/ml gave 21% binding, and 12 μg/ml gave 15% binding. The lower limit of sensitivity in the assay ranged between 5- and 10-μg/ml equivalents of AHG. The background binding by NHSC was always <7%. Results for serum and plasma samples of SLE patients are expressed in terms of the equivalent concentration of AHG that would give rise to the C1q binding observed. In a number of control studies, EDTA was added to serum samples (after clotting) to a final concentration of 0.01 M, and we found that this relatively high EDTA concentration did not significantly affect the apparent immune complex levels detected in the C1q assay (see below).

Determination of human IgG bound to RBCs of SLE patients. Specifically purified rabbit anti-human IgG (28) was radiolabeled with ^{125}I by the iodogen method (29). In order to reduce nonspecific binding to human RBCs, an aliquot of a solution of this ^{125}I-labeled protein was first preadsorbed against an equal volume of a 50% suspension of freshly drawn and washed RBCs. After centrifugation at 2,000 *g* for 5 min, the supernatant containing the preadsorbed ^{125}I-labeled anti-human IgG was used in a calibrated assay to measure human IgG bound to RBCs. The calibration was performed as follows (30). Known amounts of trace-labeled AHG were allowed to bind to 100 μl of a 25% suspension of washed human RBCs in the presence of a complement source. The cells were then centrifuged and washed three times and the preabsorbed ^{125}I-labeled rabbit anti-IgG was added. After incubations at both 37°C and 4°C, the samples were centrifuged, washed three times, and the net uptake of the ^{125}I anti-IgG was determined. Under the conditions of these experiments, ~10% of the input aggregated human IgG bound to the RBCs. However, if the incubation of aggregated human IgG, RBCs, and complement was performed in the presence of 0.01 M EDTA (to prevent complement activation (8)), the binding of the aggregated human IgG was reduced to <1%. Several lines of evidence indicated the binding of the ^{125}I anti-human IgG to RBCs was specific for human IgG bound to the RBCs. First, the binding to washed normal human RBCs that were not reacted with AHG (or were reacted with AHG in the presence of 0.01 M EDTA) averaged <2% in the calibration experiments. In addition, the binding was saturable. That is, when a sufficiently large amount of AHG was bound to the RBCs via complement, the subsequent binding of the ^{125}I anti-human IgG leveled off at values between 20 and 40%, depending upon the preparation used. A more complete description of this experiment will be published elsewhere (30).

Whole blood samples from SLE patients were obtained either in tubes that contained sodium citrate (held on ice) or in tubes that contained EDTA, and the RBCs in these samples were obtained as follows. The citrate tubes were centrifuged immediately and washed six times in cold isotonic saline. Blood samples in the EDTA tubes were kept at 37°C for 1–3 h and then centrifuged, and they also were washed six times in cold isotonic saline. In each case, the buffy coat that contained white cells was removed during the washing procedures. Subsequently, triplet aliquots of the washed RBCs (100 μl of a 25% suspension) were reacted with known amounts of the preadsorbed ^{125}I anti-human IgG for 45 min at 37°C and 45 min at 4°C. The samples were then centrifuged, washed three times, and the uptake of the ^{125}I label was determined. The calibration experiments were used to convert these results into nanograms of human IgG bound. Finally, probably due to the differences in the wash procedures in the calibration studies and those with the whole blood samples, we noted small differences in the background binding of the ^{125}I anti-IgG for the RBCs blood of normal individuals in the two procedures (typically 1.5% vs. 2.2%, respectively). For this reason, the "background" level of binding to RBCs from normals is compared with the values that were obtained for the SLE RBCs under the same conditions.

Results

Release of DNA/anti-DNA immune complexes from RBCs by various "releasing media". As shown in Fig. 1, the majority of C-DA previously bound to RBCs from a number of different donors was not released upon further incubation in most of the media examined. In particular, it can be seen that during blood clotting at 37°C, <⅓ of the bound material was released, whereas in contrast, there was considerably more release in the samples that were incubated in blood anticoagulated with EDTA. Blood anticoagulated with citrate or with Alsevier's solution induced release at levels intermediate between clotted blood and EDTA blood.

On the other hand, "whole serum" immune complexes were considerably more labile, because in addition to the release of some bound counts during the wash (typically 30%), >50% of the remaining bound counts were released during incubation in blood samples that were anticoagulated with either EDTA, citrate, or Alsevier's solution (Fig. 2). It should also be noted that during blood clotting at 37°C, only ~25% of these complexes were released, and if the blood was allowed to clot at room temperature (24°C) then the amount released averaged <10% (Fig. 2).

C-DA that was prepared with sonicated dsDNA lead to similar trends (Fig. 3). The complexes were usually released with high efficiency in blood anticoagulated with EDTA, but if the blood was allowed to clot during the incubation there was less release at 37°C, and even a smaller fraction was released during clotting at room temperature. (Fig. 3).

The differences between samples allowed to clot vs. anticoagulated samples with respect to the amount of immune complex released from the RBCs were also affected by kinetic considerations. In fact, the percentage of immune complex released in the anticoagulated samples at 24°C depended upon the amount of time the samples were allowed to stand before they were centrifuged (Table II).

Investigation of possible mechanisms. The rather high degree of release seen in the blood obtained from lavender top tubes (anticoagulated with EDTA) (Figs. 1–3) was not due to an artifact introduced by the EDTA. In some experiments,

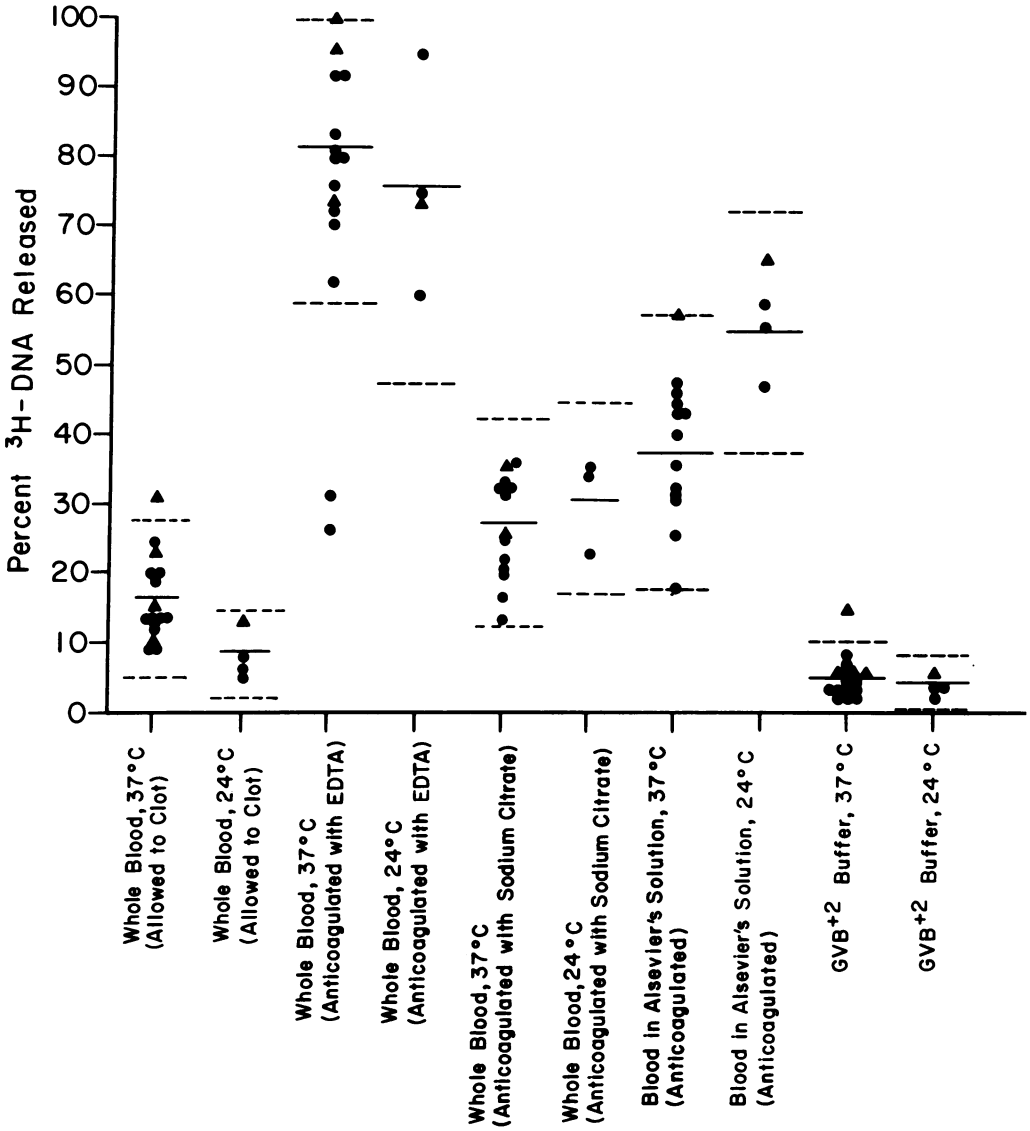

Figure 1. The release of bound C-DA (the ^{3}H-DNA was monitored) from RBCs in a number of "releasing media" at either 37°C (1 h) or 24°C (2 h). The RBCs from a total of 15 normal individuals were examined with C-DA that was prepared from ^{3}H-dsPM2 DNA and either plasma MA (●) or plasma CA (▲), which also had a high titer for dsDNA. The solid horizontal lines represent the mean, and 2 SD above and below the mean are represented by the horizontal dashed lines. In a few instances these latter values were <0% or >100%, and the dashed lines were therefore omitted for clarity. Two of the normals were considerably more than 2 SD below the mean for the samples anticoagulated with EDTA at 37°C and were not included in the calculations of the mean and standard deviations. The differences between blood allowed to clot at 37°C vs. the anticoagulated blood samples at 37°C were highly significant ($P < 0.001$). The same level of significance was also found in Figs. 2–4 when the results for the anticoagulated blood samples were compared with the results for those blood samples allowed to clot.

EDTA was added to the GVB^{+2} "releasing medium" to a final concentration of ~0.01 M, and the degree of release of the bound immune complexes was not significantly different from that seen in GVB^{+2} buffer alone. Alsevier's solution alone (not containing any blood) gave results similar to those observed for GVB^{+2} buffer.

We have also investigated whether the antibody/DNA immune complexes could be released more rapidly in the absence of the additional RBCs in the "releasing media." When a release study was conducted at 37°C for 1 h by adding 1 ml of fresh normal human serum alone to C-DA bound to RBCs, 40% of the counts were released, and 49% were released when 1 ml of fresh plasma alone (containing EDTA) was used to effect release. The companion experiment with 1 ml of whole blood gave 16% release (sample allowed to clot) and 75% release in whole blood samples that were anticoagulated with EDTA. In another experiment, we used either fresh normal human serum alone or heat inactivated normal human serum as the "releasing media" for bound, "whole serum" immune complexes. After 1 h at 37°C, 52% of the bound counts were released in the fresh serum and 60% were released in the heat-inactivated serum (where further complement opsonization was precluded). Thus, even in whole plasma or serum (i.e., no "extra" RBCs), at 37°C the release of bound antibody/PM2 dsDNA immune complexes from erythrocytes was not a rapid reaction. In contrast, immune complexes that contained globular protein antigens are reported to be released much more rapidly (10).

In a few cases, we have attempted to simulate even more closely the in vivo conditions for antibody/dsDNA immune complex formation and subsequent binding to RBCs. For example, we independently added small aliquots of antibody (plasma MA) and ^{3}H-dsPM2 DNA (the ratio corresponded to antibody excess, based on previous calibration studies) to either a 45% suspension of RBCs in fresh autologous serum or plasma (anticoagulated with citrate) or to the blood preparations in Alsevier's. The immune complex must have formed and fixed complement very rapidly under these conditions (37°C) because within 5 min, >90% of the ^{3}H-DNA was bound to the RBCs. Even after 40 min of incubation at 37°C, more

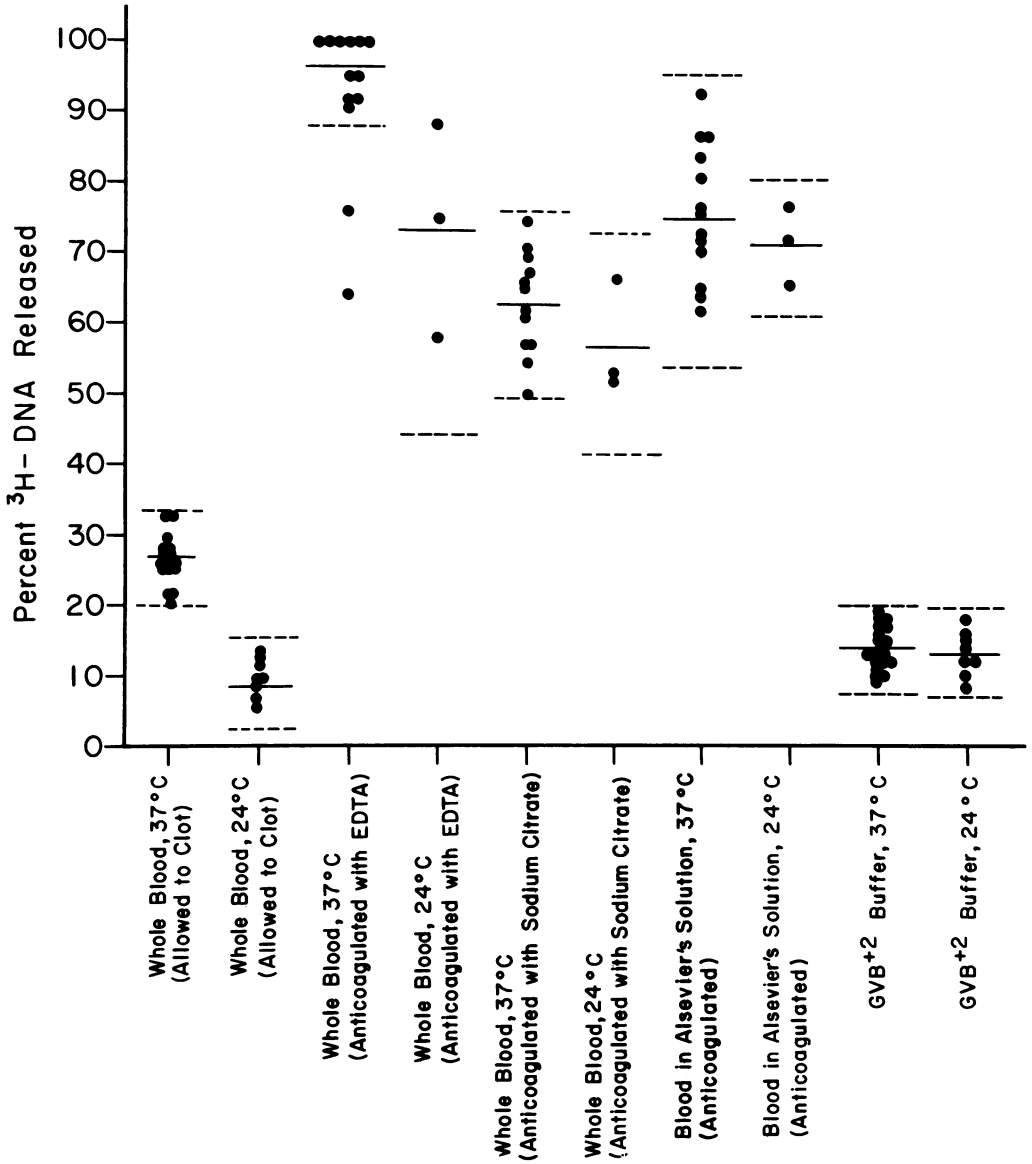

Figure 2. The release of "whole serum" antibody/^{3}H-dsPM2 immune complexes from RBCs of 10 normal individuals under conditions similar to those in Fig. 1. Although the initial binding of these complexes was also 80–95%, generally ~30% of the bound counts were lost in the first GVB^{+2} wash (see experimental section). The means and standard deviations were calculated and plotted as in Fig. 1.

than half of the ^{3}H-DNA remained bound to the RBCs. In a control experiment, heat-inactivated normal human serum was substituted for MA, and no binding of the ^{3}H-dsDNA to the RBCs could be detected.

Preliminary characterization of the released immune complexes. Antibody/^{3}H-dsDNA immune complexes that were released from the clotted RBCs could be precipitated (≥85%) in 50% saturated ammonium sulfate, and in addition still had *S* values of at least 200*S* because they were pelleted when subjected to the sucrose gradient ultracentrifugation procedure described above for the initial preparation of C-DA. However, the released complexes showed <10% rebinding to fresh RBCs under standard assay conditions. Finally, if these released immune complexes were added to a sample of fresh whole blood that was allowed to clot, 35±5% of the complexes were found in the centrifuged RBC clot.

Influence of reduced immune complex binding capacity of SLE RBCs on the release reaction. We have previously reported that certain SLE patients have RBCs with a reduced capacity to bind complement-fixing antibody/dsDNA immune complexes (16). Therefore, we have also examined the blood of a few selected SLE patients (some with "normal" RBCs and some with RBCs with a reduced binding capacity) to determine if those immune complexes that were bound to their RBCs were released more efficiently than was the case for individuals whose RBCs had higher binding capacity. Although only a relatively small number of SLE patients with RBCs with a reduced binding capacity were included, the results suggest that after normalizing for a reduced initial binding of the immune complex, the release reaction from SLE RBCs is quantitatively similar to what we have observed for RBCs of "normal" individuals (Fig. 4).

Comparison of SLE plasmas vs. sera using the solution phase C1q immune complex binding assay. We next performed the solution phase C1q binding assay (26) on a number of SLE plasmas and sera to determine if our results might have general applicability to the question of immune complex determinations in plasma vs. serum. About one-half of the

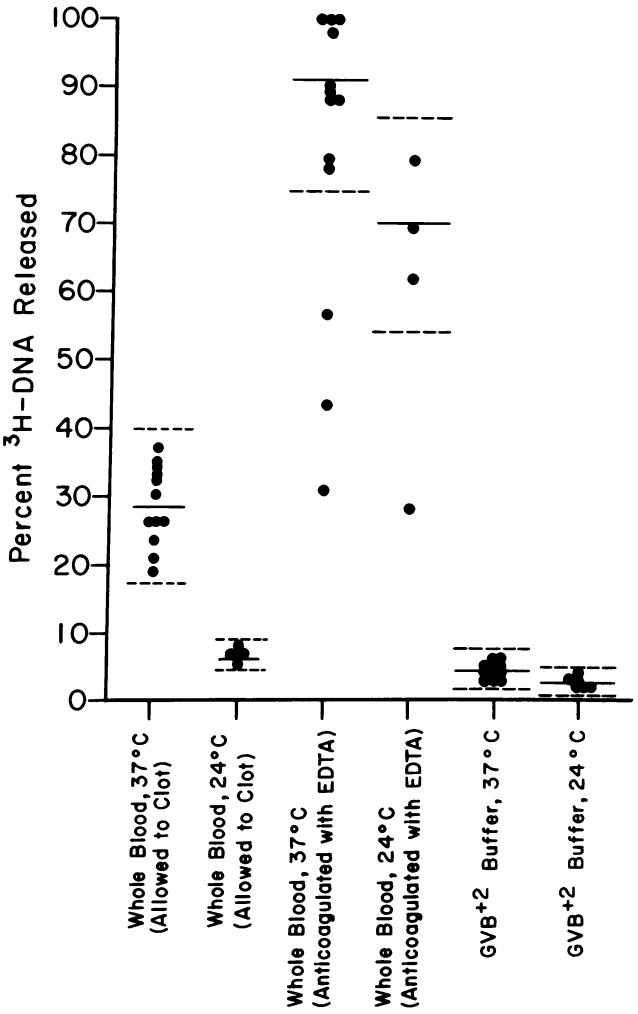

Figure 3. The release of bound C-DA from RBCs from 10 normal individuals for immune complexes prepared from plasma MA and sonicated ^{3}H-dsPM2 DNA. Similar conditions as in Fig. 1. Less than 10% of the bound counts were lost in the first GVB^{+2} wash. The means and standard deviations were determined and plotted as in Fig. 1.

patients we examined had significant levels of immune complexes (>10 μg/ml equivalents of AHG) in either plasma or serum, and the results for these individuals are seen in Table III. In about half of these positive samples we detected a significantly higher immune complex concentration in the plasma samples (group I of Table III). The increases were not an artifact due to the presence of EDTA in the plasma samples. Serum samples that were treated with comparable amounts of EDTA actually had either the same or lowered levels of detectable immune complexes. For example, the concentration of immune complexes in a serum sample of patient Du (see Table III) was reduced to 40-μg/ml equivalents of AHG when it was treated with EDTA. Finally, we note that in one individual (group III) the immune complex level actually was higher in serum than plasma, and we have no explanation for this finding. However, we do note that the relative increase for plasma vs. serum in group I tended to be much larger than the increase in serum vs. plasma in group III.

Examination of SLE RBCs for human IgG. We used ^{125}I anti-human IgG to assay for human IgG on RBCs from 19 consecutive SLE clinic patients. Their blood was treated in one of two ways: it was drawn in citrate, and washed immediately, or it was allowed to incubate in EDTA at 37°C for a few hours before washing. This study was also done in concert with the solution phase ^{125}I-C1q immune complex assay on the SLE plasma vs. serum samples.

We found that 5 out of 19 of the SLE patients had RBCs (drawn in citrate and washed immediately) that bound the ^{125}I anti-human IgG at levels significantly higher than the levels seen for normal individuals (Table IV). In addition, we found that the amount of ^{125}I-label bound by their RBCs was higher in samples isolated immediately from citrate compared with the level seen in RBCs that were isolated after a few hours of incubation at 37°C in EDTA. All five of these SLE patients had demonstrable immune complexes in the C1q assay, and four out of five had higher immune complex levels in their plasma vs. serum. Finally, of the original group of 19 patients, four other patients had increased immune complex levels in their plasma vs. serum, but their RBCs bound the ^{125}I anti-human IgG in the same range as normals. It should be noted that a quantitative comparison of the results of the two assays in Table IV with respect to absolute amounts of IgG is impossible, because they are based on different calibration standards. For example, the results of the C1q assay are expressed in terms of microgram per milligram equivalents of heat aggregated IgG and not absolute amounts of IgG in an immune complex.

Discussion

The results of this study indicate that most of the complement-fixing antibody/dsDNA immune complexes bound to RBCs

Table II. Percentage Release from RBCs of Bound, "Whole Serum" Complement-fixing Antibody/^{3}H-dsPM2 DNA Immune Complexes As a Function of Time at 24°C*

	5 Min		15 Min		30 Min		2 H	
Releasing medium	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
GVB^{+2}	17	12	14	13	15	12	11	16
Whole blood (anticoagulated with EDTA)	27	55	38	70	45	91	58	88
Whole blood (anticoagulated with sodium citrate)	19	18	24	46	40	50	53	67

* The RBCs of two different normal individuals were used in experiments 1 and 2, respectively. After 2, h the percentage release in the samples that were allowed to clot was 18 and 17, for experiments 1 and 2, respectively.

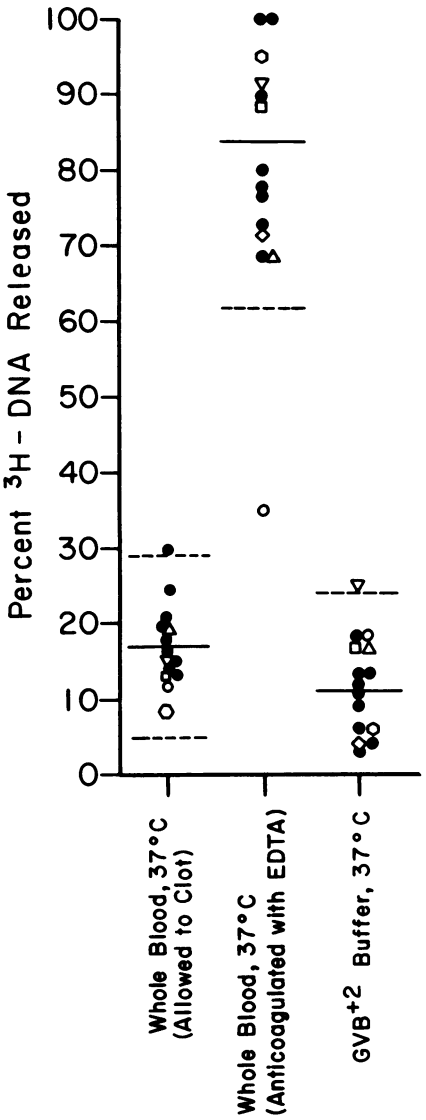

Figure 4. The release of bound C-DA (the same complex as in Fig. 1) from the RBCs of patients with SLE. The RBCs from nine patients with "normal" RBCs (●) and six patients with RBCs with lowered binding capacity for C-DA (denoted individually by ○, □, △, ▽, ◇, and o) are plotted. The results for the whole blood samples from SLE patients were not significantly different ($P > 0.50$) from those for the RBCs of normal individuals for comparable experiments. For example, the percentage release for the normal individuals at 37°C in the samples allowed to clot was 15.9±6.0 SD (Fig. 1). The comparable study for the SLE patients gave a percentage release of 17.3±6.0 SD.

are not released into the serum during blood clotting. This lack of release is even more pronounced at room temperature than at 37°C. The generality of these observations is first demonstrated by the fact that they are obtained for immune complexes that are opsonized under different conditions (Figs. 1 and 2). Secondly, similar results are seen for immune complexes containing lower molecular weight dsDNA (Fig. 3). Finally, the low degree of release during clotting is demonstrable for the blood of both normal individuals and SLE patients, whose RBCs had reduced immune complex binding capacity (Fig. 4). Although it has been suggested that in studies directed

*Table III. Immune Complex Levels in Plasma and Serum Samples in SLE Patients Determined by the Solution Phase ^{125}I-C1q Assay**

Patient	Plasma	Serum	Patient	Plasma	Serum
Group I‡					
Ba	38±17	<5	Ha	370±30	205±20
Co	450±70	240±30	He	30±15	<5
Du	200±40	80±30	Ho	270±25	115±5
Ev	105±20	49±8	Mr	85±20	20±3
Fe	91±30	26±6	Ms	170±30	<5
Ge	42±10	8±6	Mu	800±200	72±6
Gi	800±300	300±70	Wd	170±15	105±22
Group II§					
Be	230±30	220±20	Gu	140±45	170±80
Br	110±20	120±14	Gy	310±80	145±80
Ga	50±3	35±9	Ki	275±30	155±45
Co	400±100	300±60	Mc	280±40	350±80
Cs	260±60	185±20	Me	72±14	63±7
Fr	61±20	63±20			
Group III‡					
Ch	62±10	120±10			

* All results are reported in equivalents (μg/ml) of AHG. The means and standard deviations are reported.
‡ Difference between plasma and serum are significant at $P < 0.05$ or better.
§ Differences between plasma and serum are not significant ($P > 0.05$).

toward detecting circulating immune complexes the blood should be allowed to clot at 37°C (31), an examination of the literature indicates that in most instances (with the exception of cryoprecipitation studies [32–36]) blood from SLE patients is clotted at 23–25°C (usually room temperature [37–40]).

If our results can be extrapolated to the in vivo situation, they suggest that a fraction of complement-fixing antibody/dsDNA immune complexes (and possibly other immune complexes as well) could be bound to circulating RBCs and not

Table IV. Binding of ^{125}I Anti-Human IgG by RBCs from SLE Patients and Immune Complex Levels in Their Plasma vs. Serum

	^{125}I Anti-human IgG bound (counts)*		Immune complex levels‡	
	Blood drawn in citrate and RBCs washed immediately	Blood drawn in EDTA and incubated for 1–3 h at 37°C before RBC wash	Plasma	Serum
Patient				
Bo	22,200±1,300	15,900±800	500±60	22±12
Bu	24,700±200	22,300±100	50±9	12±7
Ev	22,000±200	13,000±150	12±2	<5
Gu	27,000±400	12,000±300	200±10	210±40
Jo	20,700±200	10,200±300	30±8	12±5
Normals§	14,900±2,300	14,400±1,500		

* 23,000 counts bound corresponds to ~10 ng aggregated human IgG bound per $2.5 \cdot 10^8$ RBCs, or ~160 IgG/RBC. 12,000 counts bound corresponds to ~16 IgG/RBC. A total of 600,000 counts were used per assay.
‡ All results are reported in equivalents (μg/ml) of AHG. The means and standard deviations are reported.
§ The average for five different individuals is reported.

released or detected in the serum under standard conditions. Similar consideration of the conditions of time and temperature apply to the potential release of immune complexes from RBCs and their detection in plasma (Table II), and the data clearly indicate that a considerably higher immune complex level would be expected in plasma vs. serum for the usual conditions of blood handling. Our observation of higher immune complex levels in many SLE plasmas vs. sera using the C1q assay (Table III) suggests that the "release" data we have obtained for the antibody/dsDNA system does have general implications for other immune complex systems as well. Finally, our finding that in certain patients these differences in plasma vs. serum also correlated with higher levels of IgG on RBCs processed immediately (Table IV) (compared with those incubated in EDTA to allow release) provides further evidence supporting these hypotheses.

Possible mechanisms for these observations. Based on previous studies of Medof et al. (14, 15) on a different immune complex system, it is most reasonable that the release reaction involves the degradation of C3b on the immune complexes to fragmentation products (C3d,g [41]) that do not bind with high affinity to RBCs. The question then arises as to why this mechanism does not operate with high efficiency in clotting blood. It is possible that in the samples that were allowed to clot, a fraction of the complexes were released, but because they were still large they were trapped in the clot. However, we have noted that the released immune complexes remained relatively large (>200*S*). In addition, at most, ~⅓ of the "released" immune complexes added back to whole blood appeared in the separated RBC clot. Even assuming that no fresh complement was fixed to allow rebinding to the RBCs (see below), this suggests that a physical trapping mechanism can only account in part for our observations.

Another event which could decrease the release of the bound immune complexes would include their further opsonization (and acquisition of fresh C3b) by fresh complement in the "releasing media." In the samples that were anticoagulated with EDTA, no complement fixation could occur (9), but there was still complement activity in the whole blood samples and those treated with sodium citrate or diluted into Alsevier's solution. This could also explain the enhanced release activity of the EDTA blood. We do note, however, that even when heat-inactivated serum was used as a "releasing medium," only 60% of the bound counts were released after 1 h at 37°C, compared with 52% release with fresh serum (see results "Investigation of possible mechanisms").

Finally, one other possible mechanism must be considered. It is known that DNA can be released in serum during blood clotting (42). This extra DNA could theoretically affect the antibody/DNA ratio in the immune complexes and possibly decrease apparent release. However, we have demonstrated previously (9) that any additional DNA would compete with the ^{3}H-labeled DNA for anti-DNA antibodies, and this would have the net effect of increasing the release of complexes during blood clotting; in fact, we observe a decrease in their release.

In summary, a number of potential factors probably act in concert to account for the observed differences in the release reaction in different media. In any case, the important point of the present study is simply that regardless of the mechanism, RBC-bound immune complexes are released rather inefficiently during the clotting process.

Implication for immune complex determinations in plasma vs. serum. We observed a significant discrepancy between immune complex levels measured in plasma vs. serum using the C1q assay. We believe that this result can be explained in terms of the in vivo binding of immune complexes to RBCs, their in vitro liberation into plasma, but their lack of release into serum during clotting. This discrepancy would be most pronounced in those situations in which a large fraction of the immune complexes in the circulation are bound to RBCs. In fact, as we have noted (Table IV), in a few cases we correlated these C1q assay differences in individual SLE patients with the observation of significant quantities of IgG (greater than normals) bound to their RBCs that were released when the cells were incubated at 37°C in EDTA. We elected to assay for RBC-bound IgG rather than complement because the latter can attach to RBCs by mechanisms other than fixation to antibodies (43). The possibility of simply detecting IgG autoantibodies to RBCs might confuse interpretation of our results. However, Inada et al. (44) have reported that, for the majority of their SLE patients, positive results in the direct Coomb's test for IgG were not due to autoantibodies directed against RBCs. In addition, autoantibodies would not be expected to show the release phenomenon characteristic of immune complexes. Thus, it is likely that much of the IgG we detected was contained in immune complexes that were bound to SLE RBCs. Evidence that SLE RBCs sometimes carry immune complexes in vivo has been published previously by Inada et al. (45, 46).

Finally, we note that not all individuals who showed the plasma vs. serum discrepancy in the C1q binding assay showed a corresponding difference in the RBC bound IgG assay. There are clearly many possible explanations for this observation. These include: (*a*) artifacts associated with the potential release of immune complexes through multiple washes; (*b*) differences in the inherent sensitivity of the C1q assay (for immune complexes in plasma or serum) vs. the anti-IgG assay (for IgG bound to RBCs); and (*c*) the possibility that some of the complexes detected in the C1q assay had relatively small amounts of IgG but contained other immunoglobulins.

It might be expected, based on in vitro studies with bovine serum albumin (BSA)/anti-BSA immune complexes (10), that immune complexes would be rapidly released from RBCs in vivo via interaction with I (10, 14, 15). However, we have noted that this release reaction is not nearly so rapid for the antibody/^{3}H-PM2 dsDNA system in vitro (see Results, "Investigation of possible mechanisms"), and we have no information on how efficiently the process would operate on different immune complexes in vivo. We also emphasize that dsDNA/IgG anti-dsDNA immune complexes form moderately large, soluble immune complexes that fix complement quite efficiently, and they generally do not show any evidence for cross linking (30, 47). The physical chemistry of these immune complexes is quite different from that observed for immune complexes containing globular protein antigens. This may provide an explanation for the significant differences in the release kinetics between the dsDNA/human anti-dsDNA system we have examined and those reported for the BSA/guinea pig anti-BSA system (10, 14, 15).

The clearance mechanism reported by Cornacoff et al. (7) would also tend to reduce the level of RBC-bound immune complexes. However, there is no information currently available regarding the efficiency of this mechanism for the specific

clearance of antibody/dsDNA immune complexes, or most other immune complexes, as well.

In certain disease states, other phenomena could influence the distribution of immune complexes that are "free" or bound to RBCs. Among these are: (*a*) saturation of the reticuloendothelial system (48); (*b*) decreased RBC binding capacity for immune complexes due to a lowered number of C3b receptors (2, 4, 5); (*c*) decreased levels of complement proteins in SLE patients (49); and (*d*) the specific physical and immunological properties (size, stoichiometry, ability to fix complement and bind to RBCs, etc.) of the different immune complexes likely to be found in each disease. Considerations such as these may account for our observation that only about half the SLE patients positive for immune complexes had higher levels in plasma vs. serum.

In summary, our results with DA suggest that under specific circumstances (where a significant fraction of circulating immune complexes is bound to RBCs), immune complex levels observed in serum samples may not adequately reflect total circulating immune complexes. Under these circumstances, a simple modification of the sample processing in preparation for the C1q binding assay may allow more complete immune complex measurement. That is, use of EDTA-blood incubated at 37°C for 1 h would allow release (and subsequent detection) of the RBC-bound fraction of the immune complexes. Using this technique we observed significantly higher immune complex levels in plasma vs. serum in about half of a random sample of SLE outpatients. Our results also suggest that in the future, a complete understanding of the fate and detection of complement-fixing immune complexes in humans will require a detailed analysis of the interaction of these complexes with RBCs in the circulation.

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