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Immune complex alterations occur on the human red blood cell membrane

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Summary. Experimental antigen–antibody complexes (Ag–Ab) were incubated at 37° with human red blood cells (RBC) suspended in autologous normal serum and the reaction stopped after progressively increasing times. Bound antigen–antibody–complement complexes (Ag–Ab–C) were eluted from C3b receptors and the eluted Ag–Ab–C re-incubated with different blood cell types suspended in serum, or centrifuged (along with unbound Ag–Ab–C found in the serum) through 20–50% sucrose gradients. Ag–Ab–C recovered from C3b receptors shortly after initial binding to RBC bound efficiently to other RBC, polymorphonuclear and mononuclear cells, and sedimented rapidly. Ag–Ab–C simultaneously present in the serum sedimented with a similar velocity. Ag–Ab–C recovered at a subsequent time during RBC interaction bound less well to each blood cell type, and sedimented less rapidly. Decreased amounts of rapidly sedimenting Ag–Ab–C were present in the serum. Ag–Ab–C recovered from C3b receptors at a still later time in the course of RBC interaction bound poorly to each cell type, and sedimented slowly. Increased amounts of slowly sedimenting Ag–Ab–C were found in the serum. These findings indicate that alterations in properties of immune complexes can occur while they are associated with C3b receptors on RBC membrane in solid phase.

INTRODUCTION

Immune complexes (IC), once formed, react with complement and interact with cells. Although aspects of complement activation (reviewed in Goers & Porter, 1978; Muller-Eberhard & Schreiber, 1979) and cellular receptor interactions (reviewed in Bianco, 1977; Ross, 1980) have been studied in detail, limited attention has focused on the manner in which these processes cooperate in the handling of IC in whole blood.

In previous studies using experimental IC (Medof & Oger, 1982; Hekmatpanah *et al.*, 1982), we have found that, following addition of antigen–antibody complexes (Ag–Ab) to normal human serum and autologous unseparated blood cells, red blood cells (RBC) compete with other blood cell types for antigen–antibody–complement complexes (Ag–Ab–C). Interaction of IC with RBC occurs transiently (Medof, Prince & Oger, 1982e), but IC present in serum after RBC interaction differ from IC in serum prior to RBC interaction with respect to their binding and physical properties.

Abbreviations: IC, immune complexes; Ag–Ab, antigen-antibody complexes; RBC, red blood cells; Ag–Ab–C, antigen–antibody–complement complexes; PMN, polymorphonuclear cells; MNC, mononuclear cells; SΔ30, human serum heated at 56° for 30 min or (SΔ120) for 120 min; C3b/C4b inactivator (I); H, β1H; PBS, 0·005 M phosphate-buffered isotonic saline, pH 7·4; HBSS, Ca^{++}-Mg^{++}-free Hanks's balanced salt solution; RPMI/OA, RPMI medium containing 0·2% ovalbumin; BSA, bovine serum albumin; TMV, tobacco mosaic virus.

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In the present investigation, we examined the relationship between RBC interaction and change in properties of IC in the above experimental system. We isolated Ag–Ab–C from C3b receptors on RBC at different times and compared the recovered Ag–Ab–C with respect to their ability to bind to other RBC, polymorphonuclear cells (PMN) and mononuclear cells (MNC), and with respect to their sedimentation profiles following centrifugation through 20–50% sucrose gradients. The results of our studies provide evidence that alterations in the properties of IC occur in solid phase, while IC are associated with C3b receptors on RBC membrane.

MATERIALS AND METHODS

Serum, reagents and cells

Blood samples were drawn from healthy volunteers into uncoated tubes for serum and heparinized tubes for cells and immediately placed on ice. Serum was separated at 0°. Portions were heated at 56° for 30 min (SΔ30) or for 120 min (SΔ120). C3b/C4b inactivator (I) was obtained from Cordis Laboratories (Miami, FL). The concentration was determined by functional assay (Pangburn, Schrieber & Muller-Eberhard, 1977) with added β1H (H) (Whaley & Ruddy, 1976).

RBC were separated as previously described (Medof & Oger, 1982). PMN and MNC were separated by dilution of heparinized blood with an equal volume of Ca^{++}-Mg^{++}-free Hanks's balanced salt solution (HBSS), centrifugation through a discontinuous Ficoll–Hypaque gradient (prepared by overlayering Ficoll–Hypaque [$d = 1{\cdot}078$] on Ficoll–Hypaque [$d = 1{\cdot}119$] (Cutts, 1970), and collection of cells at the interfaces. Unseparated blood cells were prepared by centrifugation of heparinized blood at 1500 *g* and decantation of the plasma. Separated RBC, PMN and MNC, and unseparated cells were washed three times with HBSS, once with RPMI medium containing 0·2% ovalbumin (RPMI/OA), resuspended in RPMI/OA, and kept on ice. Separated RBC contained < 1 PMN or MNC per 10^5 RBC; PMN, < 1% MNC; and MNC, some platelets but < 1% PMN (Medof & Oger, 1982).

Preparation of Ag–Ab

Ag–Ab were prepared from ^{125}I-labelled bovine serum albumin (BSA) and guinea-pig antiserum to BSA as previously described (Medof & Oger, 1982). After incubation, the reaction mixture was diluted four-fold with ice-cold RPMI/OA and transferred to an ice bath. Ag–Ab prepared in this way (four-fold antibody excess) showed no precipitate (even after storage for 3 days at 4° and centrifugation) and gave maximal percentage binding to blood cells after reaction with serum (Medof & Oger, 1982; Medof *et al.*, 1982e).

Reaction of Ag–Ab with serum and cells

Separated RBC (or unseparated blood cells in preliminary studies) were resuspended to original blood volume in RPMI/OA, divided into 500 μl aliquots, and pelleted. After removal of supernatant, cell pellets were resuspended in 250 μl of autologous serum and the suspensions kept on ice. Seventy-five microlitres of Ag–Ab (diluted in RPMI/OA as described above) were added and the mixtures placed at 37°. The reactions were stopped at 2, 4 or 7 min (and other times in preliminary studies) by addition of 375 μl of ice-cold RPMI/OA and transfer to an ice bath. Cells were pelleted by centrifugation at 1500 *g* for 10 min at 0°, supernatants transferred to ice, and the pellets washed three times with 1 ml of ice-cold RPMI/OA. Pellets were counted in a gamma counter to determine bound radiolabel.

Dissociation of bound Ag–Ab–C from RBC

Ag–Ab–C-coated RBC pellets were resuspended in 250 μl of SΔ30 or serum concentration of I in RPMI/OA and mixtures placed at 37°. After 30 min, 250 μl of ice-cold RPMI/OA was added, the cells pelleted at 0°, and supernatants containing dissociated Ag–Ab–C separated and placed on ice.

Reaction of dissociated Ag–Ab–C with serum and fresh cells

After aliquoting to obtain appropriate cell numbers, separated RBC, PMN, or MNC were pelleted, resuspended in 250 μl of autologous serum, and placed on ice. One hundred microlitre samples of supernatant containing dissociated Ag–Ab–C, recovered after 2, 4 or 7 min, were added and the mixtures placed at 37°. The reactions were stopped after increasing times of incubation, and the cells pelleted, washed, and counted as described above.

Ultracentrifugal analysis of Ag–Ab–C in serum and dissociated from RBC

Four hundred microlitre samples of supernatants containing Ag–Ab–C dissociated from RBC after 2, 4 or 7 min, or of serum fractions containing unbound Ag–Ab–C separated at the same time points, were applied to sucrose gradients made 20–50% in 0·005 M

phosphate-buffered saline (PBS), pH 7·4. Gradients were centrifuged at 4° in a Beckman SW40 rotor run in an L5-75 ultracentrifuge with the $\omega^2 t$ integrator preset to $8{\cdot}10 \times 10^{10}$ rad^2/sec (~equivalent to 2·75 hr at 30,000 r.p.m.) as previously described (Medof *et al.*, 1982e). Six-drop (0·25 ml) fractions were collected from top to bottom using a Beckman Densiflow apparatus. ^{125}I-BSA, ^{125}I-human IgM, and tobacco mosaic virus (TMV) were employed as 4 S, 19 S, and 198 S markers. Samples of undiluted Ag–Ab, Ag–Ab–C prepared by incubating one volume of (undiluted) Ag–Ab at 37° for 30 min with an equal volume of serum diluted 16-fold in RPMI/OA, and IC-containing serum isolated 20 min following incubation of Ag–Ab at 37° with RBC suspended in undiluted serum (Medof *et al.*, 1982e) were used in control studies.

RESULTS

Isolation of Ag–Ab–C from RBC

Preliminary experiments were performed to select times for analysis of Ag–Ab–C bound to RBC and determine conditions for dissociation from C3b receptors. Kinetics of interaction of Ag–Ab–C with RBC following incubation of Ag–Ab at 37° with separated human RBC suspended in autologous normal serum is shown in Fig. 1A, together with that following identical incubation, but employing unseparated human blood cells. The results of control studies using ^{125}I-BSA in place of Ag–Ab, SΔ30 in place of fresh human serum, or equal numbers of sheep RBC (known not to bear C3b receptors) in place of human cells are also shown. Binding of Ag–Ab–C following incubation of Ag–Ab with human RBC suspended in serum occurred within 1 min, reached a maximum at 4 min, and then declined, such that < 10% of Ag–Ab–C remained bound after 12 min. Binding to separated human RBC and to unseparated human blood cells varied with time in an identical fashion, and no binding occurred in the absence of antibody, complement, or C3b receptors.

The reaction between Ag–Ab, serum, and RBC was stopped at 4 min. After centrifugation and washing, Ag–Ab–C-coated RBC were resuspended in SΔ30 or purified I. The mixture was placed at 37°, the reaction stopped after progressively increasing times, and percentage dissociation determined. The results of two experiments using SΔ30 are shown in Fig. 1B. Greater than 90% of bound Ag–Ab–C dissociated into the supernatant within 16 min. Similar results were obtained using purified I. Less than 5% dissociation occurred after 32 min in the presence of SΔ120 or RPMI/OA.

Two minutes of interaction of Ag–Ab with serum and RBC (while binding was increasing), 4 min (at peak binding), and 7 min (while binding was decreasing) were therefore selected as appropriate times to study RBC-bound Ag–Ab–C, and incubation for 30 min at 37° in SΔ30 or I taken as adequate conditions for dissociation.

Binding properties of Ag–Ab–C bound to RBC at different times

Ag–Ab–C recovered from RBC receptors at 2, 4 and 7 min were compared with respect to their ability to react further with complement and blood cells. In this set of experiments, upon initial incubation of Ag–Ab with RBC suspended in serum as described above, 20% of added counts were bound at 2 min, 46% were bound at 4 min, and 33% remained bound at 7 min. Following incubation of Ag–Ab–C-coated RBC in SΔ30 or I, > 95% of RBC-bound counts were recovered in supernatants at all three time points. Samples of supernatants containing Ag–Ab–C dissociated from RBC at each time point were added to purified RBC, PMN, or MNC suspended in fresh serum. The mixtures were placed at 37°, the reactions stopped after increasing times, and percentage binding determined. The results using SΔ30 are shown in Fig. 2, panels A, B and C, respectively. Maximum percentage binding to each cell type progressively decreased, and time required for maximum binding to each cell type progressively increased as a function of duration of time of Ag–Ab–C on the RBC membrane prior to dissociation. Comparable results were obtained using I.

Physical properties of Ag–Ab–C bound to RBC (and in serum) at different times

Ag–Ab–C recovered from RBC receptors at 2, 4, and 7 min were compared with respect to their size. Additionally, Ag–Ab–C recovered from RBC at each time point were compared to Ag–Ab–C simultaneously present in the serum. In this set of experiments, upon initial incubation of Ag–Ab with RBC suspended in serum, 21% of added counts were bound at 2 min, 41% were bound at 4 min, and 20% remained bound at 7 min. Following incubation of Ag–Ab–C-coated RBC in SΔ30 (employed in these studies), > 97% of RBC-

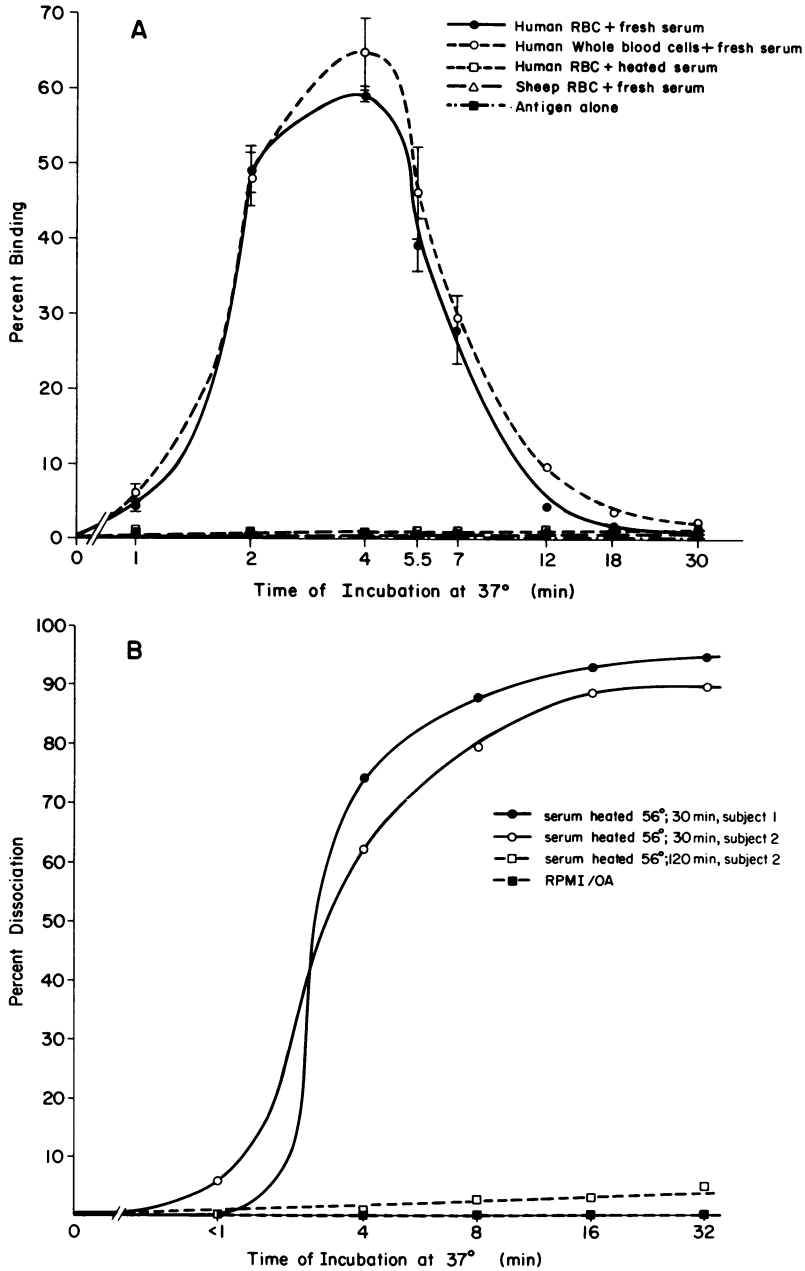

Figure 1. Panel A: Kinetics of binding of IC following incubation of Ag–Ab at 37° with human RBC suspended in autologous serum. Kinetics of binding following incubation of Ag–Ab with unseparated human blood cells and results of control studies in which antibody was omitted. SΔ30 substituted for fresh serum, or (equal numbers of) sheep RBC substituted for human blood cells are shown. Panel B: Kinetics of dissociation of IC following incubation of Ag–Ab–C-coated RBC at 37° in SΔ30. Results of two representative experiments (employing serum and RBC from two different individuals) and control studies in which SΔ120 or RPMI/OA was substituted for SΔ30 are shown.

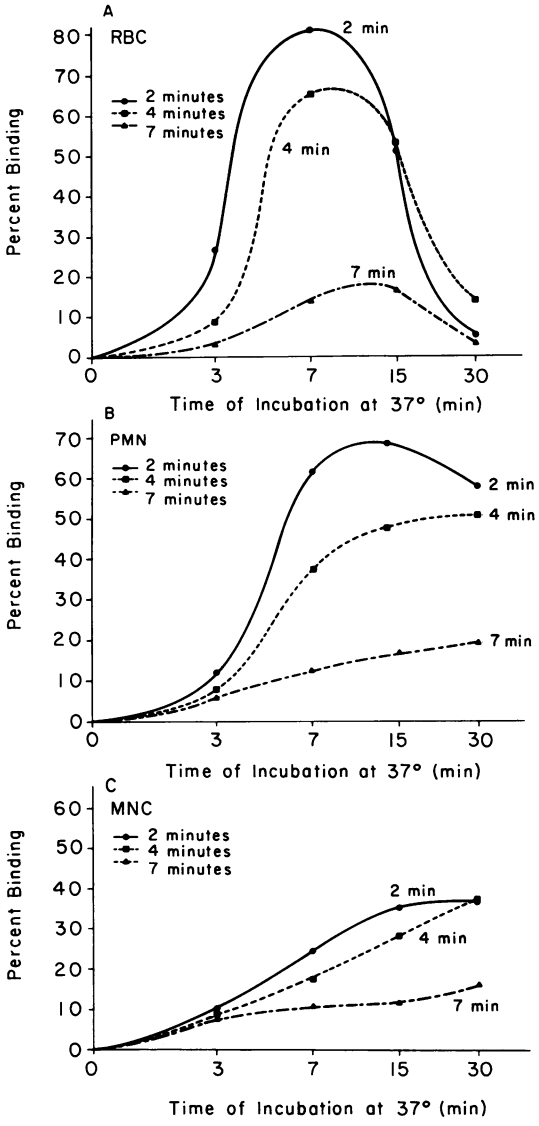

Figure 2. Kinetics of binding of Ag–Ab–C isolated from the RBC membrane after 2, 4, or 7 min (of incubation of Ag–Ab with serum and RBC) following incubation at 37° with (2×10^9) RBC (panel A), (1×10^7) PMN (panel B), or (1×10^7) MNC (panel C) suspended in serum.

bound counts were recovered in all cases. Supernatants containing Ag–Ab–C dissociated from RBC at each time point, and sera containing unbound Ag–Ab–C at the respective time points, were applied to 20–50% sucrose gradients and sedimentation profiles of Ag–Ab–C determined. The results are shown in Fig. 3. Ag–Ab–C recovered from RBC receptors at 2 min (panel A) were heterogeneous and sedimented with a mean velocity of ~175 S. Ag–Ab–C present in the serum sedimented with a broader profile. Ag–Ab–C recovered from RBC receptors at 4 min (panel B) sedimented with a mean velocity of ~100 S. Decreased proportions of rapidly sedimenting Ag–Ab–C were present in the serum. Ag–Ab–C recovered from RBC receptors at 7 min sedimented with a mean velocity of ~50 S and appeared more homogeneous. Increased proportions of Ag–Ab–C with similarly reduced sedimentation velocity were present in the serum. Greater than 85% of the total counts initially added to RBC were accounted for, and < 1% found at the tube bottoms in any case.

Control studies were performed to exclude an effect of the dissociation process on Ag–Ab–C size. Ag–Ab–C prepared with serum diluted 16-fold were bound to RBC and, after washing, the RBC-bearing Ag–Ab–C incubated at 37° with SΔ30 or I under conditions identical to those above. Supernatants containing dissociated Ag–Ab–C were applied to sucrose gradients along with samples of original Ag–Ab and unreacted Ag–Ab–C, and after centrifugation their sedimentation profiles compared. The results are shown in Fig. 4. Ag–Ab and unreacted Ag–Ab–C sedimented with a broad profile. Ag–Ab–C recovered from RBC receptors following incubation with SΔ30 or I were not reduced in size, whereas IC present in serum (20 min) following incubation of Ag–Ab at 37° with RBC in undiluted serum (shown as control) were substantially reduced in size.

DISCUSSION

Previous studies (Medof *et al.*, 1982e) using the experimental system employed in this investigation have shown that IC present in the serum following interaction with RBC in human blood are altered with respect to their binding and physical properties. In the present investigation, the relationship between RBC interaction and change in properties of IC was examined. Ag–Ab–C were dissociated from washed RBC isolated after progressively increasing times during reaction between Ag–Ab, serum, and RBC, and the dissociated Ag–Ab–C compared. Progressive decreases in the ability of the recovered Ag–Ab–C to bind to blood cells in the presence of serum were observed as were progressive reductions in their size.

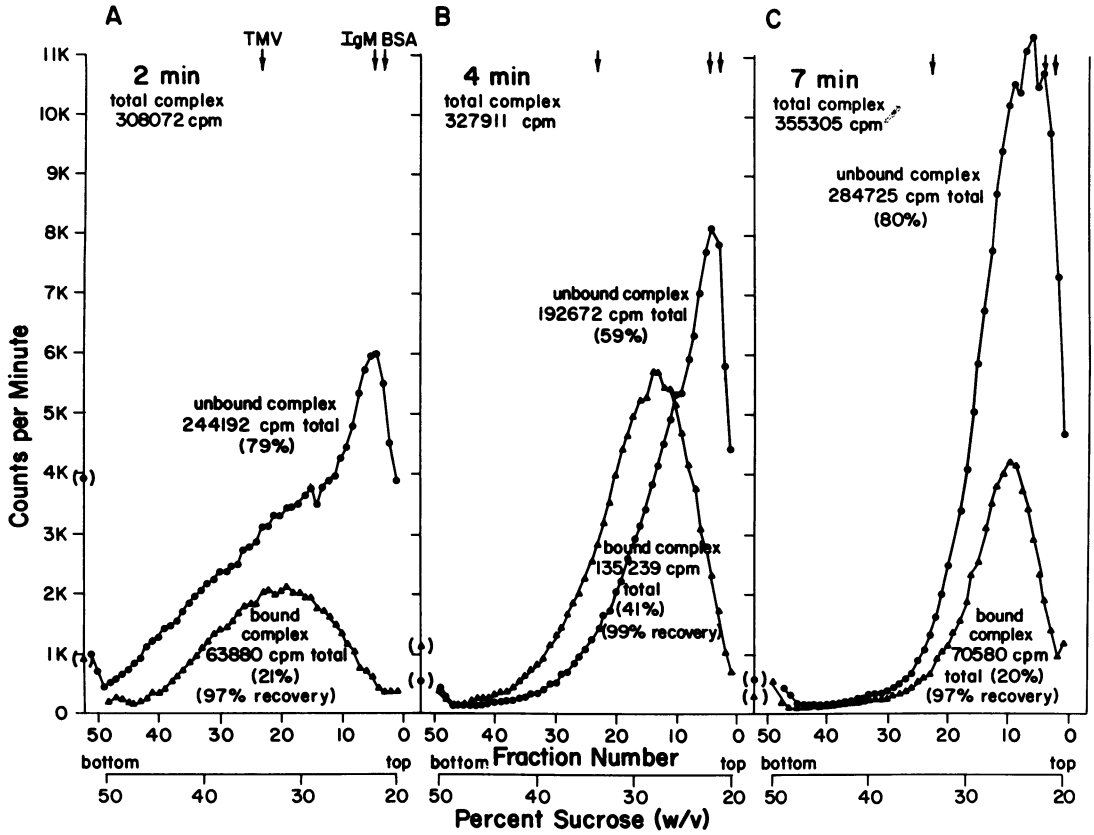

Figure 3. Sedimentation profiles of Ag–Ab–C isolated from the RBC membrane and present in the serum after 2 min (panel A), 4 min (panel B), or 7 min (panel C) (of incubation of Ag–Ab with serum and RBC). The positions of 4 S BSA, 19 S IgM, and 198 S TMV markers are shown and the proportions of bound and free Ag–Ab–C and percentage recovery of Ag–Ab–C bound to RBC at each time point given.

These findings indicate that, in the above experimental system, alterations in the properties of IC occur while IC are associated with RBC membrane in solid phase.

Ag–Ab–C were dissociated from C3b receptors on RBC using SΔ30 or purified I, and SΔ120 was used as a control. Other studies (Medof, Prince & Mold, 1982c) have shown that I is the factor in SΔ30 mediating dissociation, that SΔ120 lacks I activity, and that I-mediated dissociation occurs independently of H. Ag–Ab–C recovered from RBC at 2 min gave high binding to all cell types, and ultracentrifugal control experiments showed that dissociation with SΔ30 or I did not significantly change IC size. These findings, and the fact that dissociation at each time point was performed in an identical fashion, argue that the dissociation procedure did not lead to the alterations in properties of IC observed.

Several lines of evidence suggest that more than one process is involved in the alterations of IC during association with C3b receptors on RBC demonstrated in this study. Other studies with the same IC (Medof, Prince & Mold, 1982d; Medof *et al.*, 1982c) have shown that binding to RBC is not prevented by preincubation of IC with I and H, that during subsequent release of IC from RBC by I, C3b in IC is degraded, and that RBC binding is required for this I-mediated breakdown. Recent experiments with purified C3b receptor (CR1; Medof *et al*, 1982b) have shown that the action of RBC on degradation of IC-C3b is mediated by this glycoprotein alone. The studies described in the companion paper (Medof & Prince, 1983) have revealed that, concurrently with CR1-dependent C3b degradation by I, additional C3b is accumulated via the alternative pathway. Repetitive C3b breakdown could lead to progressive loss of ability of IC to react further with complement and

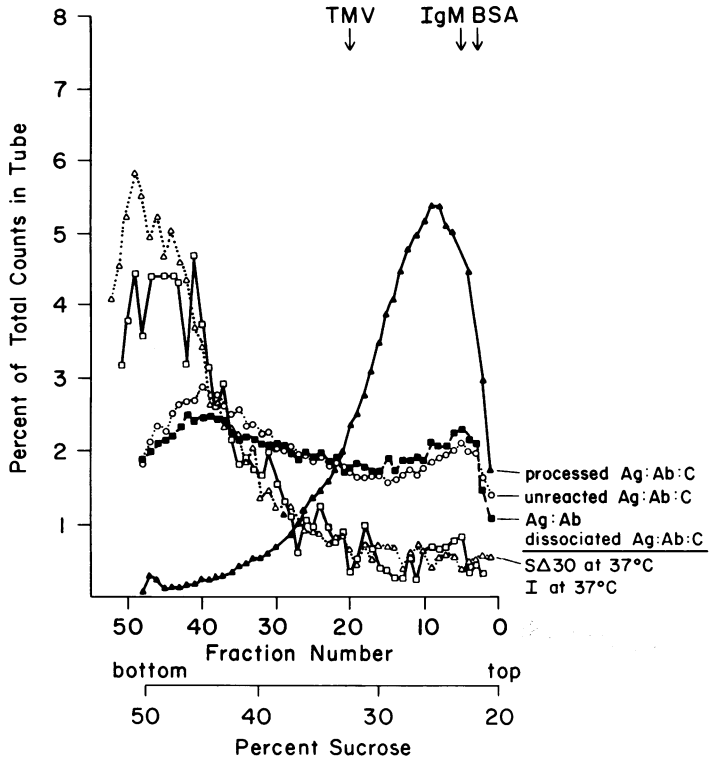

Figure 4. Sedimentation profiles (in 20–50% sucrose gradients) of Ag–Ab–C prior to RBC interaction (unreacted Ag–Ab–C) and following dissociation from RBC (dissociated Ag–Ab–C) in SΔ30 or in I at 37°. Sedimentation profiles of Ag–Ab alone (Ag–Ab) and Ag–Ab–C following release from RBC in undiluted serum at 37° (processed Ag–Ab–C) are shown for comparison. The positions of 4 S BSA, 19 S IgM, and 198 S TMV markers are indicated.

C3b receptors, while concurrent uptake (bonding) of additional C3b (Law & Levine, 1977; Gadd & Reid, 1982; Medof *et al.*, 1982a) could lead to progressive disruption of antigen/antibody lattice as described in association with the phenomenon of complex release activity (Miller & Nussenzweig, 1975; Takahashi *et al.*, 1976).

Association of IC with the RBC membrane (in solid phase) might not only facilitate processing of IC by complement but could serve additional purposes. Such association would keep IC out of the fluid phase while they are biologically active and large, and thus might reduce the potential for induction of inflammation or deposition in tissues.

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