

COMPLEMENT-DEPENDENT RELEASE OF IMMUNE COMPLEXES FROM THE LYMPHOCYTE MEMBRANE*

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Mouse B lymphocytes can bind soluble immune complexes by means of two operationally distinct membrane receptors, one specific for the Fc portion of the antibody molecules aggregated by the antigen (1, 2), and the other specific for complement molecules, presumably C3-derived (3). Membrane receptors with similar properties have been found on macrophages, granulocytes, monocytes, and platelets (4-7). There is suggestive evidence that these receptors for complement and immunoglobulin play a primary role in the initiation of phagocytosis (8-13) and, in some circumstances, in the triggering of the release of vasoactive amines from platelets (14, 15) and of lysosomal enzymes from granulocytes (16, 17). The functions of the receptors on lymphocytes are less clear. The receptors may promote the follicular localization of antigen in lymphoid organs (18, 19), be instrumental in the induction of the cytotoxicity of B lymphocytes for antibody-coated target cells (20-22), or perhaps, as recently proposed, play a role in the initiation of the immune response (23).¹

Soluble immune complexes, prepared with ¹²⁵I-labeled bovine serum albumin (BSA),² antiserum to BSA, and mouse complement bind to lymphocytes primarily through the complement receptor. The complexes are seen in autoradiographs to accumulate at one pole of the cells, and are neither shed nor interiorized if the cells are suspended in tissue culture medium for several hours at 37°C (24). We now report a new finding: the membrane-bound complexes can be released from the cells by means of a complement-dependent mechanism, involving the alternate, or properdin, pathway (25-29) of complement utilization.

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¹ Dukor, P., G. Schumann, R. H. Gisler, M. Dierich, W. König, U. Hadding, and D. Bitter-Suermann. 1973. Complement-derived B cell activation by cobra venom factor and other mitogens? Manuscript submitted for publication.

² Abbreviations used in this paper: BSA, bovine serum albumin; C3ina, C3 inactivator; C3PA, C3 proactivator; CVF, cobra venom factor; EA, antibody-sensitized erythrocytes; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid; KAF, conglutinin activating factor; PBS, phosphate-buffered saline.

Materials and Methods

Materials.—CF1 mice were obtained from Carworth Laboratory Animals, Jamaica, N. Y. The inbred strains, CBA/J and AKR, were purchased from Jackson Laboratories, Bar Harbor, Maine. The AKR mice are C5-deficient.

Tissue culture medium RPMI-1640 and phosphate-buffered saline (PBS, Dulbecco) were obtained from Grand Island Biological Co., Grand Island, N. Y. Each was adjusted to pH 7.4. Cobra venom factor (CVF) was obtained from Cordis Laboratories, Miami, Fla. Crystallized BSA was obtained from Pentex Biochemicals, Kankakee, Ill.

Lyophilized whole guinea pig serum was purchased from Cordis Laboratories. C4-deficient guinea pig serum was given to us by Dr. Ira Green. A unique, genetically C3-deficient human serum (30), having normal C3-inactivator activity, was a gift from Doctors F. Rosen and C. Alper.

A pool of mouse antiserum to BSA was obtained by injecting CBA mice in the hind footpads with 250 μ g of BSA in complete Freund's adjuvant (0.06 ml per mouse), and bleeding 4 wk later. All sera were stored at -70°C .

Purified human C3 (2 mg/ml) and C3b (1 mg/ml) were gifts from Dr. V. Bokisch. The C3b was isolated from trypsin-treated C3. Both solutions were dialyzed against PBS before use. Sheep erythrocytes sensitized with antibody (EA) were prepared as in (4).

Labeling of BSA with ^{125}I .—Carrier-free Na ^{125}I in 0.1 M NaOH, 325 mCi/ml, was obtained from New England Nuclear, Boston, Mass. The BSA was iodinated by the method of Hunter and Greenwood (3, 31). After iodination the protein solution was extensively dialyzed against saline and centrifuged at 20,000 g for 30 min. The final preparation had an activity of about 2×10^6 cpm/ μ g protein, and was stored at -70°C .

Complex-Coated Lymphocytes.—Soluble immune complexes were prepared as described before (3) by mixing 5 ml of ^{125}I BSA, 5 μ g/ml, with 1 ml of mouse anti-BSA serum, diluted 1:2. The proportion between antigen and antibody for optimal binding was found by preliminary titration to be close to equivalence. After an incubation of 30 min at 37°C , 0.25 ml of fresh mouse serum (as a complement source) was added, and the complexes incubated a further 30 min at 37°C . The complexes were then mixed with washed mouse lymph node cells, 0.5 ml of complexes per milliliter of cell suspension containing 5×10^7 cells/ml, and incubated for 40 min at room temperature. The complex-coated cells were then washed by centrifugation to remove unbound complexes, and resuspended to a concentration of 10^8 cells/ml. Under these conditions, the cells bind about 30% of the ^{125}I -labeled BSA incorporated into the complexes.

Release of Cell-Bound Complexes.—Aliquots of 0.05 ml of the complex-coated cell suspension were added to 12×75 mm tubes (5×10^6 cells per tube). To each tube was added 0.2 ml of a dilution of fresh mouse or guinea pig serum, or medium alone as a negative control. The guinea pig serum was first absorbed with CF1 mouse spleen cells at 0°C . 5 ml of cold serum were added to a pellet of washed cells prepared from the spleens of three mice and the absorption allowed to proceed for 30 min in an ice bath. All dilutions were made either in RPMI medium or PBS at pH 7.4. (It was found that buffers containing Tris, Veronal, or Hepes inhibited the release activity).

So that the complex release in each tube would begin simultaneously, the cells and sera were precooled in ice, since the release is completely inhibited at 0°C . The tubes were then incubated for the desired time at 37°C , with periodic shaking. Then the tubes were placed into an ice bath, and 2 ml of ice-cold medium added to each. The tubes were mixed on a vortex mixer, centrifuged, and the radioactivity of the pellets and supernatants measured in a gamma counter.

Autoradiography.—Mouse lymph node cells coated with ^{125}I -labeled immune complexes, or the same cells after complex release by serum, were pelleted by centrifugation, and suspended in a few drops of 1% fetal calf serum in RPMI. The cells were centrifuged onto microscope

slides by means of a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). The slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), exposed for 4 wk, developed with Kodak D-19 developer, and stained with MacNeal tetra-chrome. Cells showing 10 or more grains were counted as positive.

⁵¹Cr Labeling of Mouse Lymphocytes.—⁵¹Cr as Na₂ ⁵¹CrO₄, 1 mCi/ml in saline, was obtained from New England Nuclear. The mouse lymph node cells were labeled with ⁵¹Cr, at the same time that they were binding the preformed immune complexes, by adding sufficient Na₂ ⁵¹CrO₄ to the mixture to get a final concentration of 100 μCi/ml. The cell suspension was incubated at 37°C for 30 min and washed by centrifugation. The ⁵¹Cr activity of the washed, labeled cells was about 10⁵ cpm/10⁶ cells.

Purification of Factor B.—Factor B (or C3PA) was obtained from guinea pig serum as described in (32). The purification was monitored by using a rabbit antiserum specific for guinea pig factor B, which was a gift from Doctors V. Brade and M. Mayer. Briefly, the procedure consisted of (a) fractionation of a crude pseudoglobulin fraction on DEAE-cellulose and (b) passage of the active fractions from the previous step on CM-cellulose and then on Sephadex QAE-A50. A good resolution of peaks in the latter step was obtained. In the peak which had factor B activity, one major and two minor bands were detected by disk electrophoresis in acrylamide gel. The identity of the bands was established by longitudinally slicing the gel after the run and (a) staining half with amido-schwartz, (b) assaying the other half, imbedded in an agarose plate, by double diffusion against rabbit antisera specific for factor B and for whole guinea pig serum. The major band reacted with anti-factor B serum, and one of the minor bands was identified as immunoglobulin.

RESULTS

Release of Cell-Bound Complexes by Serum. Kinetics and Effect of Dilution.—Mouse lymph node lymphocytes were incubated with [¹²⁵I]BSA, mouse antiserum to BSA and fresh mouse serum as a source of complement, as described in the Methods section. After this treatment, 20–30% of the cells (B lymphocytes) bind the complexes. The cells were subsequently washed by centrifugation and reincubated at 37°C in fresh mouse serum. About 80% of the labeled BSA was released from the cells in 20 min. Thereafter little additional release occurred (Fig. 1). Autoradiographs of the lymphocytes after serum treatment showed that a substantial portion of the remaining complexes was trapped

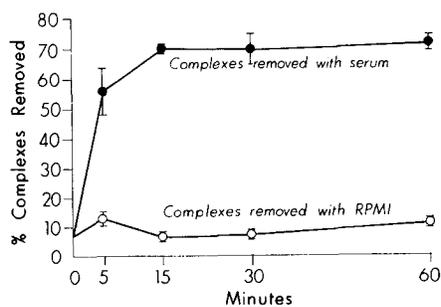


Fig. 1. Release of immune complexes from surface of CF1 lymph node cells by undiluted CF1 mouse serum at 37°C, after the standard release procedure described in the Methods section. Each point represents the mean of three tubes. The vertical bars show standard errors.

within small clumps of cells. However, a few heavily-labeled cells (less than 1% of the total) were still present. The remaining cell-associated complexes, which could not be removed by further addition of serum, had not been ingested by the cells, since they could be removed by subsequent treatment with excess BSA, as described in reference (24).

The release activity, which is also found in human, rat, and guinea pig serum (Fig. 2), is very sensitive to dilution. It is slowed down and incomplete when cells are incubated in less than about 10% of serum. The activity diminishes at temperatures below 37°C, and is completely inhibited at 0°C.

Condition of Released Complexes and of Cells after Release.—The serum-mediated release activity is not the result of antigen-antibody dissociation or

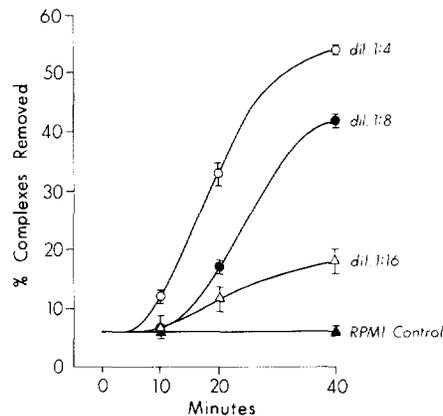


FIG. 2. Effect of serum dilution upon kinetics of immune complex release. The complexes were released from CF1 mouse lymph node cells at 37°C by guinea pig serum diluted in RPMI. The guinea pig serum was previously absorbed with CF1 spleen cells at 0°C. Each point represents the mean of two tubes. The vertical bars show standard errors.

extensive enzymatic degradation of the antigen because the ^{125}I BSA which is released can be precipitated by 50% saturated ammonium sulfate, and is completely eluted within the void volume when passed through a column of Sephadex G-200. However, an important change in the complexes is evident, as they will no longer bind to fresh lymphocytes. The lack of binding is not caused by the generation of an inhibitor of binding, unless the inhibitor has a molecular weight in excess of 200,000 daltons, since partially purified removed complexes which are eluted in the void volume of a Sephadex G-200 column are entirely inactive.

We have considered the possibility that the complex release is simply the result of cell damage mediated by complement; this does not appear to be the case because release is not accompanied by cell damage measurable by ^{51}Cr release or by Trypan blue exclusion (Table I). Furthermore, complexes are not

released from the membrane when the cells are disrupted by freezing and thawing three times.

Complement Dependence of Complex Release.—The release activity is totally inhibited in the presence of 0.01 M Na₃H EDTA or by heating the serum at 50°C for 30 min. These results suggested that complement might be involved in this process, and this was confirmed by subsequent findings. The release is abolished by pretreatment of the serum with insoluble immune complexes such as sheep erythrocytes coated with anti-erythrocyte antibodies, or BSA-anti-BSA precipitates prepared at equivalence.

One key component of serum involved in the release activity is C3, because (a) treatment of serum with CVF abolished its activity. 1/2 ml aliquots of CF1 mouse serum, diluted 1:4, were mixed with 0.05 ml of threefold serial dilutions

TABLE I
Absence of Lymphocyte Damage Measurable by ⁵¹Cr Release after Immune Complex Removal with Serum

Experiment no.	Treatment of ⁵¹ Cr-labeled, complex-coated mouse lymphocytes*	⁵¹ Cr released (±SE)	Complexes released (±SE)
		%	%
1	CF1 mouse serum, dil. 1:2	14.3 ± 0.6	73.4 ± 3.2
	RPMI	13.8 ± 0.4	7.2 ± 0.4
	Freeze and thaw 3×	78.8 ± 0.5	12.0 ± 0.6
2	Guinea pig serum, dil. 1:2	12.8 ± 0.7	50.8 ± 1.2
	RPMI	12.2 ± 1.4	8.2 ± 0.3

* The cells were incubated with serum or RPMI for 30 min at 37°C. The guinea pig serum was previously absorbed with mouse spleen cells at 0°C.

of CVF ranging from 1:10 to 1:2,430. The mixtures were incubated at 37°C for 30 min, and kept in an ice bath until assayed. A 50% inhibition of release was obtained with a CVF dilution of 1:550 and the activity was completely abolished by CVF at 1:90. (b) A unique genetically C3-deficient human serum is devoid of activity, but it can be made active by the addition of purified human C3 (Fig. 3). However, purified human C3b fragments, obtained by trypsin hydrolysis of C3, could not activate the C3-deficient serum. By themselves, C3, C3b, or a mixture of C3 and C3b were totally inactive. Components further in the complement sequence than C3 probably are not necessary because the release activity is observed in C5-deficient mouse serum.

The release activity can be generated through the alternate pathway of complement fixation. Serum from C4-deficient guinea pigs is active, and release requires Mg⁺⁺ but not Ca⁺⁺ ions. Sufficient EGTA to chelate both Ca⁺⁺ and Mg⁺⁺ ions completely inhibits the release; this can be totally restored by adding excess Mg⁺⁺. Fig. 4 shows the results of kinetic studies designed to further

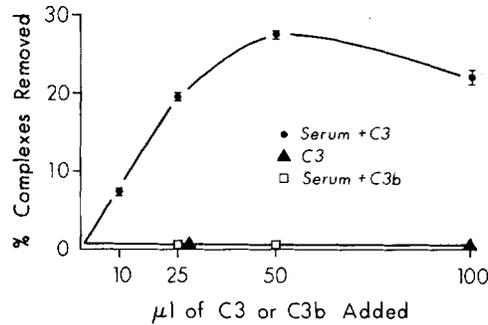


FIG. 3. Activation of genetically C3-deficient human serum for immune complex release by addition of purified human C3. To tubes containing 5×10^6 complex-coated CF1 mouse lymph node cells, kept in an ice bath, were added 50 μ l of serum, various amounts of purified C3 or C3b, and sufficient PBS to give a total volume of 0.25 ml. The release of complexes was allowed to proceed for 30 min at 37°C. The original concentrations of the purified C3 and C3b were 2 mg/ml and 1 mg/ml, respectively. Each point represents the mean of two tubes. The vertical bars show standard errors.

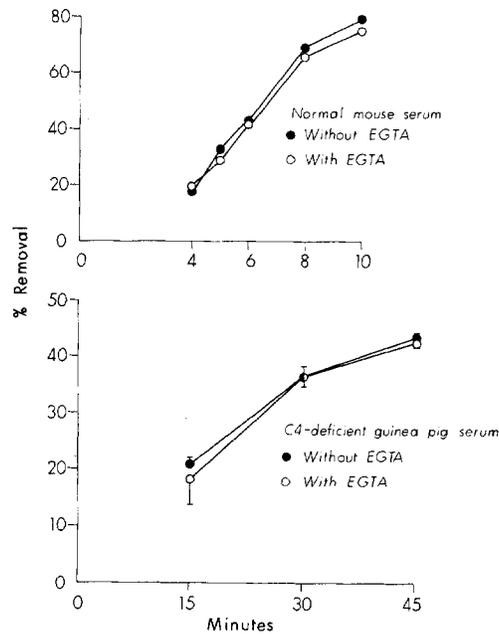


FIG. 4. Time-course of immune complex release from CF1 mouse lymph node cells, using normal CF1 mouse serum (top) or C4-deficient guinea pig serum (bottom), in the presence or absence of Ca^{++} . The mouse serum was diluted 1:5 in PBS. The guinea pig serum was diluted 1:4, and was previously absorbed with CF1 mouse spleen cells at 0°C. Sufficient EGTA was added to a portion of each serum to chelate all of the Ca^{++} present (as determined in control experiments involving lysis of EA). MgCl was added to all sera to ensure an excess of Mg^{++} . Final concentrations of EGTA and MgCl in the mouse serum were 2 mM and 2.5 mM, respectively, and in the guinea pig serum, 1.5 mM and 2.0 mM. For the guinea pig serum each point represents the mean of two tubes. The vertical bars show standard errors.

clarify this point. In the presence of excess Mg^{++} , the rate of release of complexes with C4-deficient guinea pig serum or with mouse serum was not reduced in the presence of sufficient EGTA to chelate all of the Ca^{++} . We have also found that a concentration of Ca^{++} ions in excess of 2 mM inhibits the release. Another indication of the participation of the alternate pathway in the complex release is that purified factor B completely restores the activity of heat-inactivated C4-deficient guinea pig serum (Fig. 5). Normal serum behaved differently from C4-deficient serum in this experiment; a higher

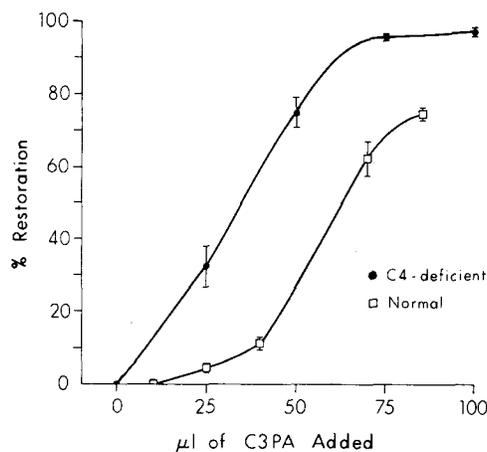


FIG. 5. Restoration with purified factor B (C3 proactivator) of the release activity of heat-inactivated (50°C, 30 min) normal or C4-deficient guinea pig sera. The sera were previously absorbed with CF1 mouse spleen cells. To tubes containing 5×10^6 complex-coated CF1 mouse lymph node cells, kept in an ice bath, were added 50 μ l of normal serum or 75 μ l of C4-deficient serum, various amounts of purified guinea pig factor B ($OD_{280 \text{ nm}}, 1 \text{ cm} = 1.16$), and sufficient PBS to give a total of 0.25 ml. The complex removal was allowed to proceed for 45 min at 37°C. The heat-inactivated sera had no release activity, as compared with PBS controls. The unheated normal serum gave 32.5% specific (PBS control subtracted) release; the unheated C4-deficient serum gave 39.6% specific release. The amount of factor B added is plotted as microliters per 75 μ l of serum. Each point represents the mean of two tubes. The vertical bars show standard errors. C4-deficient serum, ●—●; normal serum, □—□.

concentration of factor B was necessary to restore release activity to heat-inactivated normal serum (Fig. 5). One possible explanation for this difference is that in normal serum the classical complement pathway is simultaneously triggered by the complex-coated cells and inhibits the release activity of the alternate pathway, perhaps by competing for C3.

Experiments were also performed to determine whether the release activity behaved in the manner of a stable enzyme, that is, without being consumed or modified during the reaction. Complexes were removed from lymphocytes with guinea pig serum or mouse serum at 37°C for 30 min. The supernatants, which contained the inactive removed complexes, were then added to tubes containing

fresh, complex-coated lymphocytes. No further removal of complexes was observed. A sample of guinea pig serum which was simultaneously incubated at 37°C retained its full potency. Thus, this experiment suggests that if a removal enzyme is generated, it becomes inactive in a matter of minutes at 37°C, and/or a substrate which is necessary for removal is used in the reaction.

DISCUSSION

The main new findings of this paper are that (a) certain membrane-bound immune complexes can be released by serum and (b) the complement system plays a central role in this phenomenon. The present data were obtained in a convenient *in vitro* experimental model, in which the cells bearing the complexes were lymphocytes.

We have also obtained evidence that a similar mechanism probably operates *in vivo*.³ When soluble immune complexes were injected intravenously into mice, they were rapidly picked up by circulating cells (mainly platelets) and within minutes released into the plasma. In contrast, complexes which had been previously released *in vitro* from the membranes of lymphocytes did not bind to the platelets. These findings suggest that the regulation by complement of the interaction between immune complexes and cell membranes may be operative for other cell types (such as mouse platelets) which have C3 receptors on their surfaces.

It is interesting that C3, which presumably is the link connecting the complexes to the plasma membrane, also appears to play a key role in their liberation from the cell surface. Such a dual role for C3 is not novel; for example, the activation of the alternate pathway by C3b, itself a product of complement utilization, has been described (33, 34). The release of complexes involves the alternate pathway of complement fixation, but its mechanism is not yet understood at the molecular level. The following possible mechanisms of complex release should be considered.

(A) C3 conversion products, generated when fresh serum is added to the complex-coated cells, could compete with the complexes for the C3 receptors on the cell membrane. This cannot be the sole explanation for our findings because, in addition to being released, the complexes simultaneously lose their affinity for the cell membrane of lymphoid cells *in vitro*, as well as their capacity for binding to mouse platelets *in vivo*. Moreover, (a) Purified human C3b or C3 conversion products generated in mouse or guinea pig serum by treatment with EA, immune precipitates, or C566 are entirely ineffective for complex release. (b) EAC1423 prepared with human complement components, and known to have C3b on their surfaces, do not bind to mouse lymphocytes (35); that is, mouse lymphocytes have no binding sites for human C3b, and yet human serum is effective for complex release. These findings render unlikely a competi-

³ Miller, G., and V. Nussenzweig. Manuscript in preparation.

tive mechanism for release, unless one postulates unique binding properties for nascent C3 conversion products.

(B) An enzymatic activity, generated through the alternate pathway, might modify the complex-bound C3 and inactivate its binding site for the cell membrane. At least three serum enzymes are known to modify C3 or C3 products. Two of them, $\overline{C42}$ and C3 activator, are short-lived at 37°C and are known to act upon native C3 (36, 37). The third enzyme, C3 inactivator (or KAF) (38–40), which acts upon C3b and generates two other fragments, C3c and C3d (41), can be excluded as being responsible for the release of complexes for the following reasons: (a) C3ina is not generated by complement activation, but is preformed in serum. (b) C3ina is neither thermolabile nor dependent upon ions for its activity. (c) A C3-deficient serum which does not remove complexes possesses normal C3ina activity (30). (d) We have recently found that B lymphocytes, in contrast to granulocytes, have receptors not only for C3b but also for the complement fragment (C3d) generated by the activity of C3ina on C3b.⁴ In other words, C3ina should be ineffective for the release of immune complexes bound to lymphocytes by means of C3b because the product which is generated through the activity of C3ina on C3b retains its affinity for the cell surface.

(C) Products generated through the alternate pathway might block the expression of the binding sites on the immune complexes for the cell membrane. There is a precedent for this hypothesis in the observations of Blum (42) and of Koethe et al. (43), who noticed that the activation of the complement sequence through the alternate pathway with zymosan or CVF generates products which block the lysis of hemolytic cellular intermediates obtained by the classical complement sequence. According to this hypothesis, when the antigen-antibody-complement complexes are prepared, the classical pathway is activated and a C component with binding activity for B lymphocytes is incorporated into the complexes. Later, after the incubation of complex-coated cells in a high concentration of fresh serum, products of the alternate pathway modify or cover the C3 binding sites on the complexes, and these are released and simultaneously deactivated. Whatever the explanation for the complex release, this system provides a convenient assay for the functioning of the alternate complement pathway in the mouse and in other mammalian species.

Our findings indicate that complement can regulate the interaction between immune complexes and the membranes of leukocytes. In these experiments, binding and release of complexes have been artificially, though conveniently separated. In a physiological situation, in which complement is constantly available, its interaction with antigen-antibody complexes may sequentially produce intermediates whose binding affinities for cell membranes first increase and then decrease within a short time. In this context it would be interesting to

⁴ Eden, A., G. Miller, and V. Nussenzweig. Manuscript submitted for publication.

verify whether a transient contact between these immune complexes and the cell membrane can trigger effector mechanisms such as the release of vasoactive amines from platelets. Finally, our findings raise the interesting possibility that alterations in the complement system leading to a defective release mechanism may be implicated in the pathogenesis of immune complex diseases.

SUMMARY

Soluble antigen-antibody-complement complexes bound to mouse B lymphocytes are rapidly released from the cell membrane in the presence of normal serum from several mammalian species. The release is not the result of antigen-antibody dissociation or extensive degradation of the complexes. However, the released complexes have been altered because they will no longer bind to fresh lymphocytes. The release is not the result of lymphocyte damage mediated by complement. It is complement-dependent, and is generated either preferentially or exclusively via the alternate pathway, since it occurs in C4-deficient serum, is Mg^{++} but not Ca^{++} dependent, and requires C3 proactivator. C3 inactivator is not involved. The release activity of the serum, once generated, is unstable at 37°C. The release of complexes from the lymphocyte membrane by serum provides a convenient assay for the functioning of the alternate pathway in the mouse and in other species.

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