AD-A126 203 ROLE OF COMPLEMENT IN BLOOD PRESERVATION AND BLOOD BANKING(U) MASSACHUSETTS UNIV MEDICAL SCHOOL WORCESTER I O SZYMANSKI FEB 83 DAMD17-79-C-9055 1/1

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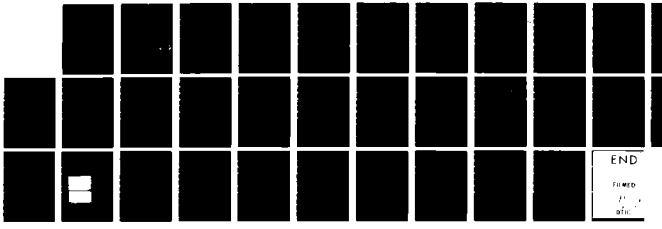

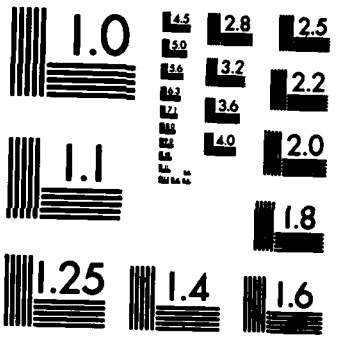

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ROLE OF COMPLEMENT IN BLOOD PRESERVATION AND BLOOD BANKING

Final Report

Irma O. Szymanski, M.D.

February, 1981

Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-79-C-9055

University of Massachusetts
Worcester, MA 01605

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A126 203
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) ROLE OF COMPLEMENT IN BLOOD PRESERVATION AND BLOOD BANKING	5. TYPE OF REPORT & PERIOD COVERED Final- 1 May 1979 - 31 December 80
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Irma O. Szymanski, M.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-79-C-9055
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Massachusetts Medical School Worcester, MA 01605	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62772A.3S162772A814.00.047
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21701	12. REPORT DATE February 1981
	13. NUMBER OF PAGES 32
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Cell-bound C3: Immunochemical quantitation of
on fresh human erythrocytes
on stored human erythrocytes
Quantitation of Soluble C3: effect of conversion of BIC to BIA
Measurement of C3 Storage Lesion

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

We are describing here in detail a new method to quantitate cell-bound complement in accurate molecular terms. The method is extremely sensitive so that it is possible to quantitate the presence of even ten molecules per cell and it can be utilized to measure complement on various blood cells in-vivo and in-vitro.

Utilizing this method we quantitated C3d molecules on fresh RBC and C3b molecules on both stored and quality control RBC (EC43). On the average, 94 C3d molecules and zero C3b molecules were detected on fresh RBC. RBC that had been stored

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20. Abstract (continued)

at 4°C for 21 days had acquired about 64 C3b molecules per cell. The number of C3b molecules on heavily sensitized EC43 varied between 2700 to 3000. Other investigators, using radioactive antibodies, detected about 550 C3d molecules on fresh RBC and about 200,000 C3b molecules on EC43. These differences in results apparently reflect methodological bias and could be explained the following way: Since non-monoclonal antibodies were used, they probably consisted of a mixture of antibodies directed to various epitopes on the antigen molecule so that the ratio between radiolabeled antibody and antigen molecules exceeded one. This would favor high results in the radioactive method. In contrast, our results were not calculated on the basis of antibody-antigen ratio but were compared to particle-bound C3 standard. The new immunochemical method will be applied for quantitation of C3 on various populations of stored RBC to determine whether RBC aged in-vivo are more susceptible to complement-induced storage lesion than young RBC.

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SUMMARY

Studies done during the contract period May 1, 1979 to December 31, 1980 are reported. Part of the studies were already reported in the Annual report and will not be repeated here.

We are describing here in detail a new method to quantitate cell-bound complement in accurate molecular terms. The method is extremely sensitive so that it is possible to quantitate the presence of even ten molecules per cell and it can be utilized to measure complement on various blood cells in vivo and in vitro.

Utilizing this method we quantitated C3d molecules on fresh RBC and C3b molecules on both stored and quality control RBC (EC43). On the average, 94 C3d molecules and zero C3b molecules were detected on fresh RBC. RBC that had been stored at 4°C for 21 days had acquired about 64 C3b molecules per cell. The number of C3b molecules on heavily sensitized EC43 varied between 2700 to 3000. Other investigators, using radioactive antibodies, detected about 550 C3d molecules on fresh RBC and about 200,000 C3b molecules on EC43. These differences in results apparently reflect methodological bias and could be explained the following way: Since non-monoclonal antibodies were used, they probably consisted of a mixture of antibodies directed to various epitopes on the antigen molecule so that the ratio between radiolabeled antibody and antigen molecules exceeded one. This would favor high results in the radioactive method. In contrast, our results were not calculated on the basis of antibody-antigen ratio but were compared to particle-bound C3 standard. The new immunochemical method will be applied for quantitation of C3 on various populations of stored RBC to determine whether RBC aged in-vivo are more susceptible to complement-induced storage lesion than young RBC.

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STATEMENT OF THE PROBLEM

We have previously observed that fragments of human complement (in a state of either C3b or C3b[i]) accumulate to red blood cell (RBC) membrane during blood storage at 4°C.[1] We have proposed that RBC-bound C3 might mediate the destruction of irreversably damaged stored RBC. To clarify this process further, we wanted to quantitate the number of C3 molecules bound to RBC during storage at 4°C. Since the existing methods were neither accurate nor sensitive enough, our work during the contract year was directed toward developing a new method to quantitate RBC-bound C3. The problem with the currently used assays is that known cell-bound C3 standards are not available to permit calibration of the results obtained by different methods.

Our method to quantitate cell-bound C3 is based on the principles of antiglobulin (anti-C3) consumption. The ability of complement-sensitized cells to consume a fraction of a standard amount of anti-C3 is proportional to the number of bound C3 molecules per cell and to the total number of cells used in the test. With the aid of known standards it is possible to draw conclusions about the quantity of C3 on unknown RBC. The strength of anti-C3 is measured in the AutoAnalyzer by determining the strength of agglutination of EC43 caused by the antiserum.

In order to standardize the test system, it was necessary to evaluate thoroughly the three reagents used in the test: anti-complement antibodies, EC43, and the bound C3 standard.

In our annual report we described our studies on:

1) The mechanism responsible for complement uptake by RBC in low ionic strength solutions (LIS), and characterization of other related phenomena occurring under these conditions to aid us in the preparation of EC43 in a reproducible manner, and,

2) Quality control of the anti-complement antisera by preparing test RBC that are coated only with single, specific complement components and storing them in frozen state for prolonged periods before use.

We are now reporting on our new method as it has evolved.

BACKGROUND

All methods to quantitate either soluble or cell-bound C3 depend on the ability of C3 to react with anti-C3 antibodies. Two different antibodies are available: anti-C3c which reacts with C3b, the major fragment of C3 and anti-C3d which detects C3d, the small terminal binding site of C3. Soluble C3 is usually measured with the aid of anti-C3c (nephelometric assays[2] or radial immunodiffusion[3] method). Complement bound to cells in-vivo can be quantitated only with the aid of method anti-C3d antibody because C3b is not stable in-vivo.[4] However, when complement binding occurs in-vitro, also C3b can be detected. The anti-C3c antibody is readily available commercially, but pure

anti-C3d antibodies are more difficult to obtain and they are also more expensive. The published methods have not always revealed which specific anti-C3 was used to quantitate cell-bound complement.

As said previously, the quantitation of cell-bound C3 requires that anti-C3 antibody combines with its antigen. This interaction is measured in various ways to yield quantitative information about bound C3. Three general approaches have been described:

1. Measurement of residual anti-C3 antibody after incubation of known amount of anti-C3 with the C3 antigen to determine the quantity of antibody consumed.[5,6]

2. Measurement of bound anti-C3 directly by Cla fixation and transfer test.[8,9]

3. Measurement of bound C3 indirectly by using radiolabeled antibodies and analyzing the results by Scatchards analysis.[9,10,11]

The difficulty in all these methods relates to the fact that there are no calibrated standards for cell-bound C3 (neither for C3d nor C3b molecules). Anti-C3 consumption methods, however, employ accurate, easy-to-obtain soluble C3 standards. The problem in these tests has been pointed out by Borsos and Leonard[5] who noticed that the dose-response curves for cell-bound and soluble C3 are different. Therefore, comparison of unknown cell samples to soluble standard curve yields variable results for the cell-bound C3.

Measurement of the cell-bound C3 by the Cla fixation and transfer test yields number of C1 binding sites and does not directly relate to the number of bound C3 molecules although correlation exists between these two parameters.

Measurement of the number of bound C3 molecules on the basis of Scatchards analysis[12] of radiolabeled antibodies assumes one to one relationship between antigen and antibody. These calculations ignore the probability that each C3 molecule probably has many epitopes for antibody binding.

In our work, we decided to use the anti-C3 consumption technic and prepare calibrated cell-bound standards to be able to measure the results accurately. In addition, it was also necessary to determine the specificity of our anti-complement antibodies and prepare test RBC in a reproducible fashion. The progress on the anti-complement antibodies and test RBC was reported previously in the Annual Report. Calibration of the cell-bound standard was more difficult than we could have foreseen.

In our initial attempt, we wanted to prepare RBC standards (EC3). These cells had several problems: C3b affixed to RBC in-vitro tended to disappear during liquid and frozen storage of these cells. In addition, RBC also had an unknown amount of C3d on their membrane, thus excluding them as sensitive standards.

Since Zymosan activates the alternative complement pathway, we decided to prepare C3 sensitized zymosan particles and quantitate the zymosan-bound C3 indirectly by measuring the C3 concentration in serum before and after exposure to zymosan. The C3 concentration could not be measured by the radial immuno-

diffusion method[3] because the method is relatively insensitive. Our automated method revealed unusual data: serum appeared to contain more C3 after than was present before zymosan exposure. Therefore, we planned to radiolabel purified C3 and thus quantitate the proportion of plasma C3 attaching to zymosan particles. Utilizing the lactoperoxidase method,[13] we were able to label the C3 molecules. However, we were unable to demonstrate significant uptake of radiolabeled C3 by zymosan particles, indicating that either inactivation of the complement molecule had occurred during labeling procedure, or that a proportionally small amount of C3 attached to zymosan, an amount outside the accuracy of radioactive counts.

From the foregoing, it appeared that a) only a small fraction of serum C3 bound to zymosan particles, and that b) during plasma-zymosan interaction, changes occurred in soluble C3 molecules so that they reacted more avidly with the anti-C3 antibody. A detailed study of the interaction between anti-C3c antibody and various forms of soluble C3 (native or activated) and zymosan-bound C3 was carried out. These data revealed marked differences in the reactivity of the soluble, native C3 and converted C3 with the anti-C3c antibody. However, the reactivity of the converted C3 with anti-C3c antibody was identical to that of zymosan-bound C3 (ZC3). Therefore, utilizing various commercial C3 plasma standards in their converted form, we were able to calculate the number of C3 molecules on our zymosan standards. We also observed that the reactivity of the zymosan standards was similar to that of RBC-bound C3, permitting us to determine the number of C3 molecules on red blood cells.

APPROACH TO THE PROBLEM

Comparison of the Reactivity of Anti-C3c with ZC3 and with both native and converted soluble C3

In these tests we compared the ability of various concentrations of both soluble C3 and ZC3 to neutralize a standard amount of anti-C3c. Soluble C3 was tested in:

a) native serum
b) serum incubated at 37°C for 24 hours
c) serum previously incubated with zymosan
d) serum previously incubated with RBC in low ionic strength (LIS)

We also measured the quantity of C3 in variously treated plasmas by radial immunodiffusion method[3] and determined the electrophoretic mobility of C3 in these samples.

Calibration of zymosan-bound C3 (ZC3) standard

The amount of C3 per volume of ZC3 was calibrated by comparing its reactivity to several known commercial, soluble C3 standards.

Comparison of the reactivity of both anti-C3c and anti-C3d antibodies with ZC3 and various RBC sensitized with C3

In these tests we compared the ability of various concentrations of ZC3 and of cell-bound C3 to neutralize a standard amount of anti-C3c or anti-C3d.

The following C3-sensitized RBC were tested:

a) fresh normal RBC
b) stored normal RBC
c) EC43

Measurement of RBC-bound C3

In these tests we determined the amount of C3d and C3b bound normal fresh RBC, to stored RBC, and to quality control RBC (EC43).

METHODS

PERFORMANCE OF THE ANTI-C3 CONSUMPTION TEST USING THE AUTOANALYZER

In this section we are presenting the method to quantitate cell-bound C3 in a format of a standard operating procedure.

I. GENERAL PRINCIPLES

The anti-C3 consumption test measures cell-bound or fluid-phase C3 quantitatively. It proceeds in two main phases, first being the "neutralization" or "antibody consumption" phase, during which period test samples and standards are combined with antibody (i.e., anti-C3 or anti-C3d) and incubated overnight at 4°C so that part of the antibody is neutralized. In the second phase the amount of antibody remaining is quantitated in the AutoAnalyzer by determining its ability to agglutinate EC43 (red blood cells sensitized heavily with C3).

Accurate determinations require that (1) the final or "target" dilution of the antibody after it has been combined with RBC suspensions or serum dilutions in phase One must not vary from one sample to another; (2) an accurate standard curve must be generated with tests each day; (3) test results must fall in the range defined by the standard curve; (4) the molecular form of the C3 protein must be the same in the test samples as it is in the standard used.

It should be noted that C3 is a complex molecule which, by the very nature of its biological function, fragments into various subunits. The state of the molecule determines the rate at which it combines with anti-C3 antibodies. Care must be taken, therefore, to ensure that the standard and the unknown are equally reactive before any quantitative conclusions can be reliably drawn.

II. PREPARATION OF REAGENT RBC (EC43) FOR THE AUTOANALYZER TESTS[14]

These cells, prepared daily, are heavily sensitized with complement by exposing red cells and plasma to low ionic strength.

IIA. Reagents

1. RBC, glycerolized 10 ml aliquots in -80°C freezer. Deglycerolize.[15]
2. FFP - 4 ml aliquots in -80°C freezer. Thaw.

3. 5% mannitol, pH 6.0 - Dissolve 50g mannitol in H_2O in a one liter volumetric flask, add 10 ml of buffer (6), add H_2O to one liter.
4. Saline - 0.9g NaCl, 0.1g NaN_3 plus H_2O to 100 ml.
5. Ficoll - 0.5g ficoll-400 per 100 ml saline.
6. 0.1 M $NaPO_4$ buffer, pH 6.0
   A. 0.2 M 2.839g Na_2HPO_4 plus H_2O 100 ml
   B. 0.2 2.760g Na H_2PO_4 H_2O plus H_2O to 100 ml
   Combine 12.3 ml A. with 87.7 B., then dilute with H_2O to 200 ml.

IIB. Steps

1. Divide deglycerolized RBC into two equal aliquots in 50 ml conical centrifuge tubes. Centrifuge at 3500 rpm for 3 minutes. Remove supernatant.
2. Add half of one FFP aliquot to each tube.
3. Pre-warm mannitol to 37°C.
4. To each tube, add a volume of mannitol that equals 10 times the combined RBC and FFP volume.
5. Incubate for 12 minutes at 37°C.
6. Centrifuge at 3500 rpm for 3 minutes, remove supernatant fluid, and wash 4 times with saline.
7. Suspend RBC to 10% in ficoll for use in AutoAnalyzer.

III. PREPARATION OF ANTI-C3 AND ANTI-C3d[1]

Before antisera can be used in the AutoAnalyzer antiglobulin tests, some special preparations are required to ensure specificity and sensitivity.

IIIA. Removal of Heteroagglutinins

1. Reagents

   a) 10% bromelin - combine 1 gm of bromelin with 10 ml saline, mix with stirring rod, incubate for about 30 minutes at 4°C. Centrifuge 10 minutes at 3500 rpm, harvest supernatant fluid.
   b) 0.5% albumin - contains 0.5g bovine albumin per 100 ml saline.
   c) 1/5 antiserum - one volume of antiserum plus 4 volumes 0.5% albumin.
   d) Bromelin-modified RBC - wash RBC of group O, A and B three times with saline, pool, and suspend to 50% Hct in saline. Add 1/5 volume to 10% bromelin, incubate 15 minutes at 37°. Wash 4 times with saline, centrifuge 5 minutes at 3500 rpm. Remove as much of the supernatant fluid as possible.

2. Procedure

   a) Combine one volume bromelin-modified RBC with 10 volumes of 1/5 diluted antiserum. Incubate for one hour at room temperature on rotator, cycling at approximately 100 rpm.
   b) Centrifuge at 3500 rpm for 5 minutes and remove supernatant antiserum for use.

This absorption is usually sufficient to remove heteroagglutinins from anti-C3 and anti-C3d.

IIIB. Tests for Specificity and Sensitivity

1. Manual titrations

a) Prepare serial dilutions of antiserum samples from before and after absorption (these will start at 1/5. Thus, dilutions will be 1/10, 1/20, 1/40, etc).
b) Test antiserum titer with normal and bromelin-modified RBC and with EC43 (RBC suspended to 2% in saline).
c) Absorbed serum should be non-reactive with normal and bromelin-modified cells, and should show high titered reactivity with EC43.

2. AutoAnalyzer Titrations[1]

These tests are used to verify specificity and to select the final dilution of antiserum needed for quantitative neutralization studies.

A. Reagents

1) 1% PVP - 1 gm PVP K-90 plus 100 ml saline plus 4 drops Tween-20.
2) Saline - with 2 drops Tween-20 per liter.
3) 1% Triton - 100 ml triton X-100 and 1 gm sodium azide dilution to 1 liter with H_2O.
4) RBC - 10% RBC suspensions in ficoll, made with fresh RBC, RBC stored at 4°C at least on week, bromelin-modified RBC, and EC43 prepared as above.
5) 0.5% albumin - prepared as above.

B. Steps

1. Prepare serial dilutions of absorbed and unabsorbed antiserum in 0.5% albumin.
   Anti-C3 1/5m, 1/2m, 1/1m, 1/500,000, 1/200,000, 1/100,000, 1/50,000 for EC43.
   Anti-C3 1/100,000, 1/50,000, 1/20,000, 1/10,000, 1/5,000, 1/2,000 for fresh and stored RBC.
   Anti-C3 1/10,000, 1/5,000, 1/2,000, 1/500, 1/200, 1/100 for bromelin-modified RBC.
   Anti-C3d 1/50,000, 1/20,000, 1/10,000, 1/5,000, 1/2,000, 1/1,000, 1/500 for EC43.
   Anti-C3d 1/5,000, 1/2,000, 1/1,000, 1/500, 1/200 1/100, 1/50 for fresh, stored and bromelin-modified RBC.

2. Test in AutoAnalyzer, sampling antiserum dilutions, other reagents running continuously.

IIIC. Interpretation

Absorbed serum should not agglutinate bromelin-modified RBC. Normal fresh RBC should be agglutinated by anti-C3d and not by anti-C3c. Stored RBC should be agglutinated by anti-C3 and anti-C3d.

EC43 agglutination is used to select the "target" dilution of antibody used in neutralization studies. The target is the highest dilution of antibody which agglutinates EC43 strongly. This ensures that small amounts of antibody neutralization will be measureable. For anti-C3, this dilution is usually 1/200,000; for anti-C3d, 1/5,000.

3. Storage

Anti-C3d - 0.5 ml aliquots of 1/5 dilution are stored at -80°.
Anti-C3 - 0.5 ml aliquots of 1/5 dilution are stored at -80°.
Because anti-C3 is used at greater dilution than anti-C3d, aliquots of this antibody are further diluted to 1/50 and stored in 0.5 ml aliquots at -80°C.
Aliquots are thawed when needed, and may be refrozen at -20°C without deterioration.

IV. PREPARATION OF ZYMOSAN STANDARDS, ZC3

Zymosan, a yeast cell wall polysaccharide, activates complement by the alternative (Properdin) pathway[16,17] upon exposure to serum and becomes heavily sensitized with C3. Unlike EC43, it can be frozen and thawed without changes in C3 levels. It therefore makes a suitable standard for particle-bound C3 determinations.

IVA. Reagents

1. Zymosan - powdered zymosan A from Sigma Chem. Co, stored in refrigerator.
2. FFP - sample plasma aliquots used to make "EC43", thawed at 37°.
3. 0.5% albumin - as above.
4. 0.9% NaCl - as above.

IVB. Steps[18]

1. Mix 2 gm of zymosan with 10-20 ml saline in test tube.
2. Place in boiling waterbath for 30 minutes.
3. Wash 3 times with saline in 50 ml conical centrifuge tube. After last wash, remove all supernatant to pack zymosan.
4. Add 44 ml (11 thawed aliquots) of FFP, mix well.
5. Incubate for 15 minutes at 37°C. Centrifuge at 3500 rpm for 5 minutes. Remove supernatant.
6. Wash 3 times with saline and resuspend to the original volume of zymosan plus plasma with saline.
7. Divide into aliquots of 0.25 ml and store frozen at -80°C.

IVC. Diluting for Standard Curve

A series of twenty data points evenly spaced along the X-axis is generated by the following protocol. First, a 1/200 dilution is made by diluting 1.25ml of ZC3 (from frozen aliquots) to 250 ml with 0.5% albumin. Then:

Tube	ml 1/200	ZC3 + ml Alb.	rel. Units	Tube	ml 1/200	ZC3 + Alb.	rel. units
1	20	0	20	11	10	10	10
2	19	1	19	12	9	11	9
3	18	2	18	13	8	12	8
4	17	3	17	14	7	13	7
5	16	4	16	15	6	14	6
6	15	5	15	16	5	15	5
7	14	6	14	17	4	16	4
8	13	7	13	18	3	17	3
9	12	8	12	19	2	18	2
10	11	9	11	20	1	19	1

These dilutions are aliquoted into 0.5 ml amounts and frozen at -80°C in sets of tubes 1-20.

V. NEUTRALIZATION PHASE OF THE ANTIGLOBULIN CONSUMPTION TEST (AGCT)

In this part of the test, dilutions of test samples and standards are combined with antibody and incubated to allow the antibody to be consumed by the antigen. The procedures listed below for preparing red blood cell suspension for the AGCT are used for 2 point determinations. If more data points are required, a series of twenty dilutions can be prepared by following the pipetting protocol given in section 4. below. In this case, the starting suspension for E stored or E fresh is 20%, and for EC43 start with 1%.

VA. Reagents

1. Antibody Stock Dilutions

a) 50 ul anti-C3c 1/50 to 100 ml in volumetric flask with 0.5% albumin to make 1/100,000 stock.
b) 200 ul anti-C3d 1/5 to 100 ml in volumetric flask with 0.5% albumin to make 1/2500 dilution .

2. Fresh or Stored RBC suspensions

a) Wash 1 ml RBC 4 times with saline; suspend to approx. 10% with 0.5% albumin in 15 ml graduated tube.
b) Do CBC in Coulter counter, adjust volume of supernatant to make Hct. = exactly 10%.
c) Pipet 2 ml of 10% suspension to a 12 x 75 mm test tube, add 857 ul albumin to make 7% suspension.

3. EC43 Suspensions

a) Wash approximately 0.5 ml EC43 and suspend to exactly 10% in 0.5% albumin, obtaining CBC as above.
b) Dilute to 1% suspension (e.g., 1 ml 10% EC43 + 9 ml albumin).
c) Add 0.2 ml 1% EC43 to 0.8 ml albumin to make 0.2% suspension, and 0.25 ml 1% EC43 to 0.75 ml albumin to make 0.25% suspension.

4. Serum or Plasma

a) Incubate at 37° for 24 hours (test won't work without this step±)
b) Pipet 50 ul serum and combine with 9.95 cc albumin to make 1/200 dilution.
c) Pipet 0.5 ml 1/200, q s to 50 ml in volumetric flask to make 1/20000 stock dilution, the correct starting point for normal serum. Make a series of 20 dilutions:

Tube	ml serum 1/20,000	ml albumin	rel. units
1	2	0	20
2	1.9	0.1	19
3	1.8	0.2	18
4	1.7	0.3	17
5	1.6	0.4	16
6	1.5	0.5	15
.	.	.	.
.	.	.	.
20	u.1	1.9	1

5. ZC3
Thaw one set of 20 dilutions prepared as above.

VB. NEUTRALIZATION PROCEDURE

1. Label 12 x 75 mm tube for each dilution of ZC3, cells, or serum to be tested.
2. Add 0.5 ml of antigen dilution to appropriate tube, except fresh or stored cells, which use 0.7 ml.
3. Make 4 control tubes containing 0.5 ml of albumin.
4. Except for fresh or stored cells, add 0.5 ml of antibody stock dilution to each tube, including controls. For 10% cells, add 0.63 ml of antibody; for 7%, add 0.65 ml (these volumes equal the volume of supernatant albumin in each tube, thus achieving the desired target dilution of anti-C3, a 1/2 dilution of the stock).
5. Cover all tubes and place in test tube rack. Place rack on rotator in 4°C room, laying nearly on its side so that tubes are inclined at about 10° above horizontal.
6. Incubate overnight at 4°C with rotator cycling at about 100 rpm.
7. Centrifuge all tubes containing particles (cells or ZC3) for 15 minutes at 3500 rpm.
8. Transfer liquid from all tubes to appropriately label sample cups for testing in the AutoAnalyzer.

VI. AUTOANALYZER TESTING

In this phase, the amount of anti-C3 remaining after neutralization is measured as a function of the ability of the test samples to agglutinate EC43 in the AutoAnalyzer. The more C3 present in the sample during incubation, the less anti-C3 will remain, resulting in decreased agglutination.

VIA. Reagents

1. 10% EC43, prepared as above.
2. 0.9% NaCl, 0.1% NaN_3, prepared as above, with 2 drops Tween-20 per liter.
3. 1% PVP: dissolve 1 gm PVP K-90 in 100 ml saline, add 4 drops Tween-20.
4. 1% triton - 100 ml triton X-100 and 1 gm NaN_3 diluted to one liter with H_2O.
5. 0.5% albumin, prepared as above.
6. H_2O with 2 drops Tween-20 per 100 ml.

VIB. Procedure

1. AutoAnalyzer is warmed up, cleaned and calibrated, with saline and triton running in their respective lines.
2. Both EC43 mixed constantly on magnetic stirrer, and PVP are aspirated into the AutoAnalyzer. These reagents run constantly throughout the test.
3. After about 2 minutes, sampler is activated. Sampler cam runs at 30/hr., sample: wash ratio=7:1. Cups are arranged in sampler to make reading results as accurate as possible. This entails organizing dilutions of antigen so that they are tested from most neutralized to least neutralized, and placing two consecutive cups containing only 0.5% albumin at the beginning of the run and in between separate series of dilutions.
4. When hemoglobin from EC43 arrives in the colorimeter flow cell, standard calibration is adjusted to read an optical density (O.D.) of between 80 and 90 at 550 nm.
5. After run is completed, red blood cell and PVP lines are placed in H_2O with tween, and hemolyzed samples (supernatants from fresh and stored RBC) are tested again. The O.D. of these samples is corrected by subtracting the value obtained in the second run from that obtained in the first run.

VII. CALCULATION OF RESULTS

Two main types of calculations are carried out, depending on whether results are to be expressed as C3 concentration (mg/dl) or as number of C3 molecules per cell. The first type is used to quantitate soluble C3 and to determine the concentration of C3 in the zymosan standards. The second type is for determining the average number of C3 molecules per cell in a given RBC sample.

VIIA. Measurement of C3 Concentration in ZC3 by Comparison to Known Serum Standard (or vice versa)

1. Find the final dilution of ZC3 assigned the relative unit value of 1. NOTE: One unit refers to the target dilution of ZC3. If ZC3 was diluted 1/4000 prior to combining with anti-C3, ZC3 target dilution is 1/8000. This is Unknown sample.
2. Find the final dilution of serum standard assigned relative unit value 1, usually 1/800,000. This is Known sample.

3. Calculate proportionality of dilutions (PD) between one R.U. known/unknown, e.g., 1/8000,000:1/8000 = $\frac{8000}{800,000} = \frac{1}{100}$
4. Plot results on s linear graph. X-axis = R.U., Y-axis = O.D.
5. Calculate ratio of R.U. of known/unknown for the various data points of unknown.
6. Calculate mean ratio (MR).
7. The concentration of C3 in unknown sample = PD x MR x C3 concentration in standard. For example: $\frac{1}{100}$ x 1.212 x 103 mg/dl = 1.248 mg/dl

VIIB. Calculation of the number of C3 molecules per cell by comparison to ZC3

1. Calculate the number of molecules in 1 R.U. of ZC3.
e.g., since ZC3 contains 1.248 mg C3/dl or 1.248×10^{-2} mg/ml, one RU/ml contains $1.248 \times 10^{-2} \times 1/8000$ mg/ml = 1.56×10^{-6} mg/ml; This is converted to number of molecules with the aid of Avogadro's number so that 1 RU/ml contains

$$\frac{1.56 \times 10^{-6} \text{ mg/ml}}{1.8 \times 10^{8} \text{ (M.W. of C3 in mg)}} \times (6.02 \times 10^{23}) = 5.22 \times 10^{9} \text{ molecules in 1 R.U. ZC3}$$

2. Calculate the number of RBC in 1 R.U. This number is expressed as number of RBC/ml target dilution of antibody.
Example: If 700 ul of 10% RBC had a RBC count of 1.02×10^{8} RBC/ml, and was combined with 630 ul antibody stock dilution, the sample contains 0.70 m. x 1.02×10^{8} RBC/ml = 7.14×10^{7} RBC. Since the total fluid volume is 1.26ml, there are 5.67×10^{7} RBC/ml fluid volume. Usually the sample containing the smallest amount of RBC is assigned a relative unit value of one.
3. Plot results on graph as in previous section.
4. Calculate ratio of R.U. of known/unknown for the various data points of unknown.
5. Calculate the mean ratio (M.R.) e.g., 1.212
6. The number of C3 molecules/cell =

$$\frac{\text{M.R. x } \Delta\text{C3 molecules in 1 R.U. ZC3}}{\Delta\text{RBC in 1. R.U.}}$$

$$\frac{1.212 \times 5.22 \times 10^{9}}{5.67 \times 10^{7}} = 111.6 \text{ molecules/cell}$$

RESULTS

Studies with Soluble C3

Figure 1 shows the results of a typical experiment in which we measured the amount of anti-C3c antibody remaining in samples that had been incubated with various dilutions of both native and treated serum. These data show that native serum was relatively inefficient in neutralizing the anti-C3c, and that the efficacy increased progressively following a brief exposure of the serum to a) RBC and low ionic strength solutions, b) to zymosan, and c) following a 24-hour incubation of serum at 37°C.

Immunoelectrophoretic studies showed that following incubation of serum at 37°C for 24 hours, part of C3 had converted to the electrophoretically rapidly migrating variety β1A (Figure 2). However, no consistent changes were detected in the concentration of C3 in sera following incubation of serum at 37°C when C3 concentration was determined by the radial immunodiffusion method (Table 1).

Figure 3 shows the comparison of the relative efficiency of ZC3 and incubated normal human sera (N=5) to neutralize a standard dilution of anti-C3c antibody. In these tests we assigned a unit value of one to zymosan diluted 3000 times, whereas the unit values for normal, incubated sera varied from 1/236,000 to 1/388,000. These data show that the dose-response curves of ZC3 were identical to those of normal incubated sera.

A detailed experiment was performed in which we tested the efficacy of multiple dilutions of ZC3 and of one normal, incubated serum to neutralize a standard amount of anti-C3c antibody. It is clear from the results shown in Figure 4 that the dose-response curves of both ZC3 and incubated plasma are identical, making it possible to standardize the ZC3 accurately with various commercial calibrator C3 sera.

Calibration of ZC3

Table 2 shows the results of the calibration of ZC3. Nine separate experiments were done in which the zymosan standard was tested against four different commercial serum C3 standards. On the average, the ZC3 contained 1.248 mg ± 0.058 C3 per dl. By comparison to ZC3 standard we determined C3 concentration of five incubated normal sera. These results (Table 1) compare well with those of the RID method.

Studies with Cell-bound C3 using anti-C3c

Figure 5 shows the results of a typical experiment in which the remainder of anti-C3c antibody activity was measured following exposure of the antiserum to various amounts of ZC3 and stored RBC. The data show that although the dose-response curves of ZC3 and stored RBC were similar, they were not quite identical. However, the results calculated at either end of the curve differed only slightly from each other, so that this method could be used to quantitate C3 on stored RBC. In contrast, the dose response curves of ZC3 and EC43 were identical (Figure 6). The freshly collected RBC did not consume anti-C3c antibody at all.

Studies with Cell-bound C3 using anti-C3d

Figure 7 shows the results of a typical experiment in which the remainder of anti-C3d antibody activity was measured following exposure of the antiserum to various amounts of ZC3, fresh and stored RBC and of EC43. The data show that the dose-response curves of all these materials were identical indicating that cell-bound C3d molecules can be quantitated by comparing the reactions of these cells with anti-C3d antibody to those of ZC3.

Quantitation of Cell-bound C3d using anti-C3d

The mean number of RBC-bound C3 molecules on normal RBC was 94 ± 30 (n= 12). RBC stored at 4°C between six and 26 days (mean = 10.8 days, n=16) had

117 ± 27.5 (N=16) molecules per RBC. On the average, it appeared that RBC stored for 10 days had acquired 23 additional C3d molecules on their membranes due to accumulation of C3b. Since these differences between fresh and stored RBC were relatively small, it was necessary to employ anti-C3c antibody to discern more clearly the differences between the number of C3 molecules bound to fresh and stored red blood cells.

Quantitation of C3 on stored and quality control RBC

RBC stored for 20-22 days at 4°C had an average of 64.7 ± 20 (N=12) molecules on their membrane. EC43, on the other hand had between 2700 to 3000 molecules of C3b on their membrane.

DISCUSSION

Complement system is involved in clearing microorganisms from body, thus exercising a beneficial effect.[19,20] When the target of complement action is human RBC, harmful effects such as hemolytic anemia may arise.[21,22] Deposition of C3 to RBC during 4°C storage may also play a role in cell destruction in vivo.[1] To evaluate more precisely the complement sensitization of RBC during storage, it was necessary to quantitate the number of C3 molecules affixed to stored RBC. Various immunochemical methods have been developed to measure cell-bound C3.[5,7,9] Accurate quantitation, however, is difficult due to unavailability of carefully calibrated cell-bound C3 standards. For that reason, a new method was developed in our laboratory using the principle of antiglobulin consumption also exploited by Borsos and Leonard[5]. However, we quantitated the anti-C3c antibody with the AutoAnalyzer rather than with a hemolytic assay, and we relied on particle-bound rather than soluble C3 standards.

As pointed out by Borsos and Leonard,[5] cell-bound C3 reacted with anti-C3 more avidly than did soluble C3, indicating that particle-bound C3 standards are required to measure C3 on cells. However, the reactivity of soluble C3 with anti-C3 was also variable and increased drastically following fragmentation of native C3 (β1C) to its converted form (β1A). Although C3 was not completely converted during incubation of serum at 37°C for 24 hours, the AutoAnalyzer studies showed that C3 in this serum reacted as well as C3 in its totally converted form. It has been previously recognized that clinical quantitation of serum C3 is affected by conversion of C3,[23-25] particularly since C3 in commercial standards is in the form β1A. Apparently the observed variability depends on the quality of antiserum used.[23] In our hands, changes in C3 concentration were not detected by radial immunodiffusion method following conversion of C3, probably due to the specificity of the particular antiserum incorporated into the commercial kits used by us. Using automated antiglobulin consumption test we observed that the reactivity of serum C3 increased following exposure to zymosan and that about 1% of serum C3 became zymosan-bound. Furthermore, the reactivity of ZC3 was identical to converted soluble C3 in incubated serum and in commercial standards, permitting calibration of ZC3 against the commercial standards with anti-C3c antibody. We assumed that ZC3 had equal numbers of both C3b and C3d molecules. If, however, conversion of C3b molecules to C3d had taken place during the brief exposure of zymosan to serum, the numbers of C3d molecules on ZC3 would be higher that those of C3b.

It was possible to measure RBC-bound C3 on the basis of ZC3 standards because both reacted identically with both anti-C3c and anti-C3d antibodies. C3 on stored RBC reacted somewhat differently with anti-C3c than ZC3, possibly indicating that C3 on stored RBC was not in form C3b.

In recent years it has been recognized that normal RBC have small quantities of both C3d[10,26-28] and C4d on their membranes.[10,28] We detected an average of 94 C3d molecules on normal RBC, whereas Freedman and Massay, employing a radioactive method, measured an average of 557 molecules.[10] Since their results depended on reactions between radiolabeled goat anti-rabbit IgG and cell-bound rabbit anti-human C3d, the differences between our results and theirs could be explained if several anti-rabbit IgG molecules reacted with each bound IgG (anti-C3d) molecule.

Utilizing anti-complement consumption technics,[5] Fisher et al. quantitated the number of C3 on RBC exposed to anti-Le[a] and fresh serum.[6] Depending on the volume of serum used, RBC became sensitized with between 115 to 23,850 molecules of C3. Freedman and Massey[9] and Chaplin et al.[11] determined that EC43 prepared by the low ionic strength method had about 200,000 C3 molecules per cell. In contrast, we detected only between 2,700 to 3,000 C3 molecules on EC43. These discrepancies are due to methodological differences and might indicate that the ratio between radiolabelled antibody and cell-bound antigen is larger than one. Since we used calibrated particle-bound C3 standards, we believe our estimates to be more realistic.

Our method was applied to measure the number of C3 molecules acquired by RBC during 4°C liquid storage of blood. On the average, RBC stored for 21 days had 64 C3b (or C3bi) molecules per cell, whereas the freshly collected RBC had none. It is important to determine whether a certain population of cells (perhaps the older RBC) do contain higher than average amounts of C3, rendering them highly susceptible to phagocytosis. Since the number of C3 molecules on stored RBC is relatively low, it is not surprising that acquisition of C3 during blood storage has been difficult to detect by routine methods. It is also likely that the structural state of these molecules is more critical to their biological behavior than the exact number of cell-bound C3 molecules.

We have presented a new sensitive immunochemical method to quantitate RBC-bound C3 in molecular terms, and have applied this technic to quantitate fragments of C3 bound to fresh normal and stored RBC as well as onto heavily complement-sensitized RBC. Further applications of this test include measurement of C3 on old and young RBC as well as on other blood cells.

CONCLUSIONS AND RECOMMENDATIONS

A new method to quantitate cell-bound C3 in molecular terms was described. Utilizing this method we quantitated the number of C3d molecules on fresh RBC and number of C3b molecules on both stored and quality control RBC (EC43). On the average, 94 C3d molecules were detected on fresh RBC, while no C3b molecules could be seen. RBC that had been stored at 4°C for 21 days had acquired about 64 C3b molecules per cell. The number of C3b molecules on heavily sensitized EC43 varied between 2700 to 3000. Other investigators, using radioactive antibodies, detected about 550 C3d molecules on fresh RBC and about 200,000 C3b molecules on EC43. These differences in results reflect methodological bias

and we believe they could be explained the following way: Since non-monoclonal antibodies were used, they probably consisted of a mixture of antibodies directed to various epitopes on the antigen molecule so that the ratio between antibody and antigen molecules exceeded one. This would favor high results in the radioactive method whereas our results would be unaffected since they were controlled with a particle-bound C3 standard.

Since it appears that using this method it is now possible to obtain accurate measurements on cell-bound C3. Further data must be collected to define accurately a storage-induced lesion on RBC. It is particularly important to evaluate whether cells of different in vivo age become sensitized with complement to various degrees during blood storage and whether the heavier sensitized RBC are phagocytized preferentially upon transfusion in-vivo.

Utilizing this method it is now possible to quantitate complement sensitization of various blood cells in-vivo and in-vitro.

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Table 1: Calculation of C3 concentration in native normal serum and incubated normal serum by the radial immunodiffusion (RID) method and in the incubated normal serum by an automated (AA) method.

C3 CONCENTRATION mg/dl

	Normal Serum	Incubated Serum	
	RID	RID	AA
B.C.	143	143	168
P.P.	123	135	135
R.D.	82	89	98
S.H.	110	110	105
S.	125	125	120

Table 2: Standardization of ZC3 against various commercial calibrator C3 sera.

Commercial Standard Company	C3, (mg/dl)	ZC3, (mg/dl)
Atlantic Antibodies (Westbrook, ME)	103	1.23
	103	1.15
	103	1.26
Meloy (Springfield, VA)	40	1.25
	40	1.21
	123	1.30
	123	1.36
	248	1.24
	248	1.23
		Mean 1.248
		S.D. ± 0.058

Figure 1

Neutralization of anti-C3c (target 1/200,000) with various quantities of differently treated normal serum. The ability of anti-C3c to agglutinate EC43 after the neutralization procedure is shown as a function of the relative amount of serum used (1 relative unit = 1/200,000). The lower the optical density value, the higher the antibody activity in the sample.

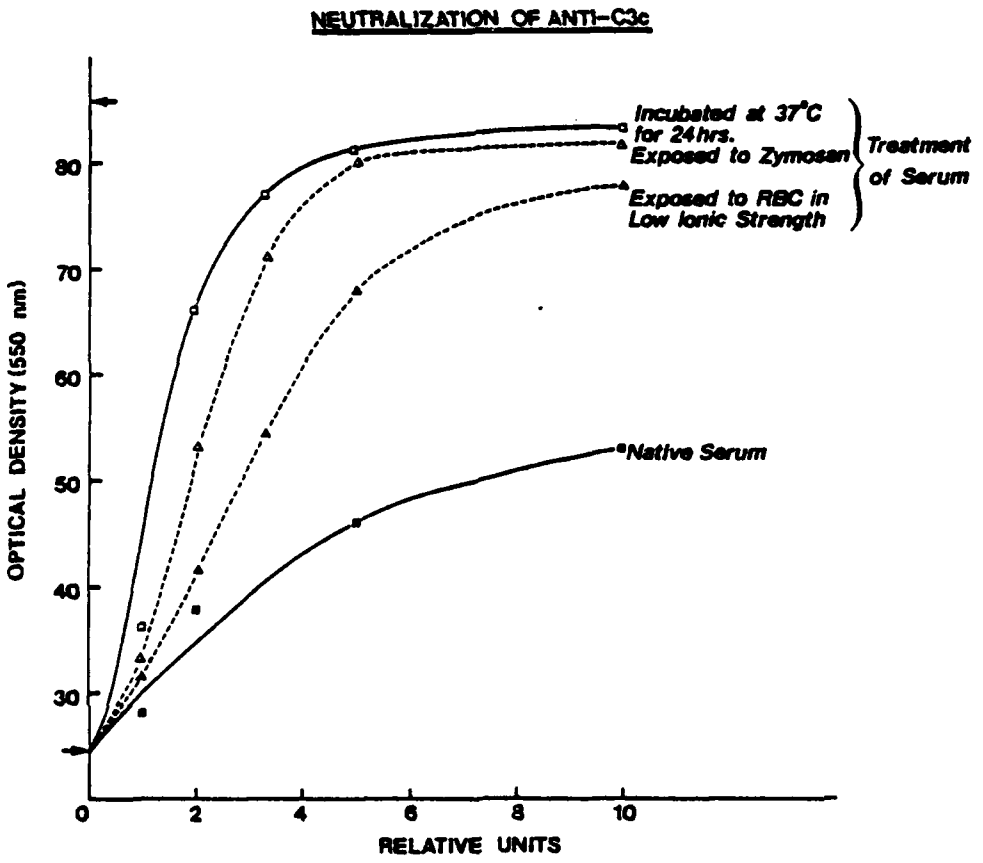
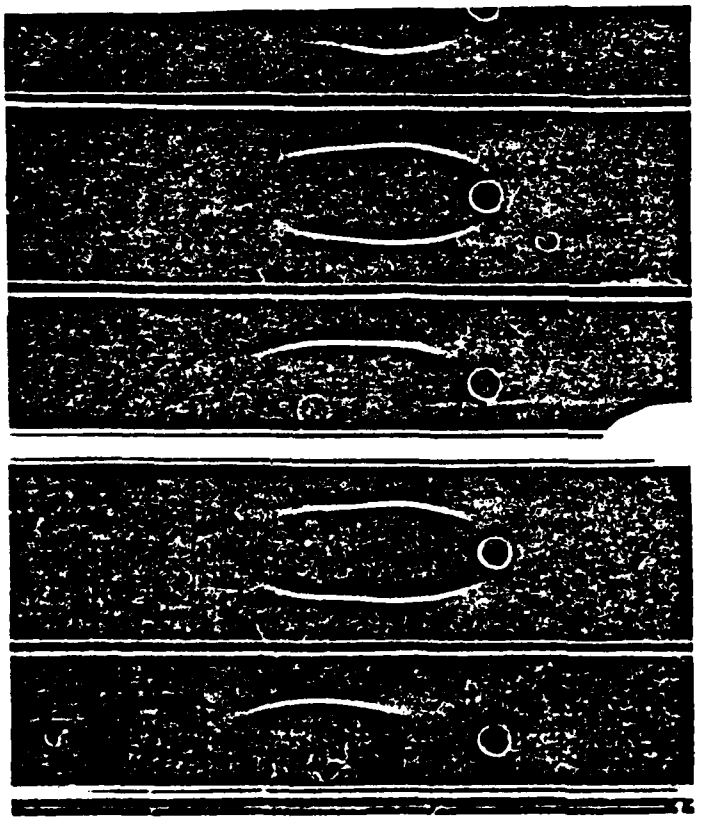


Figure 2

Electrophoretic studies of serum C3 with anti-C3c. C3 is present in both nonconverted and converted form in both pooled serum and in serum incubated for 24 hrs. at 37°C. C3 in fresh serum is present in nonconverted form, whereas C3 is totally converted following incubation at 37°C for 48 hours.



Serum,
incubated 1 hour 56°C

Pooled serum

Serum,
incubated 24 hours 37°C

Pooled serum

Serum,
incubated 48 hours 37°C

Figure 3

Neutralization of anti-C3c (target 1/200,000) with various dilutions of five different normal sera, previously incubated at 37°C for 24 hours, and with complement sensitized zymosan standards (ZC3). The ability of anti-C3c to agglutinate EC43 after the neutralization procedure is shown as a function of the relative amount of serum (1 RU varied for each different sera) and EC3.

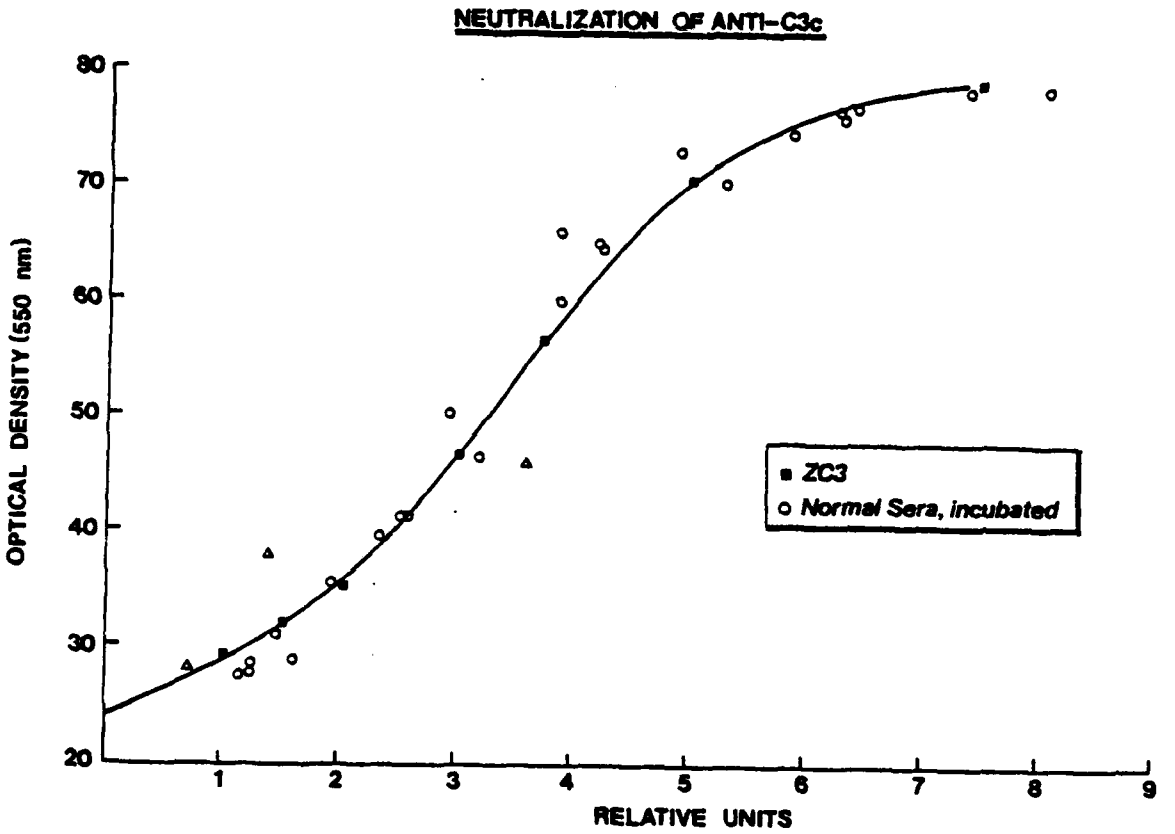


Figure 4

Neutralization of anti-C3c (target 1/200,000) with various dilutions of a normal serum, previously incubated at 37°C for 24 hours, and with complement sensitized zymosan particles (ZC3). The ability of anti-C3c to agglutinate EC43 after the neutralization procedure is shown as a function of the relative amount of serum (1 RU = 1/1,080,000) and of ZC3 (1 U = 1/8000).

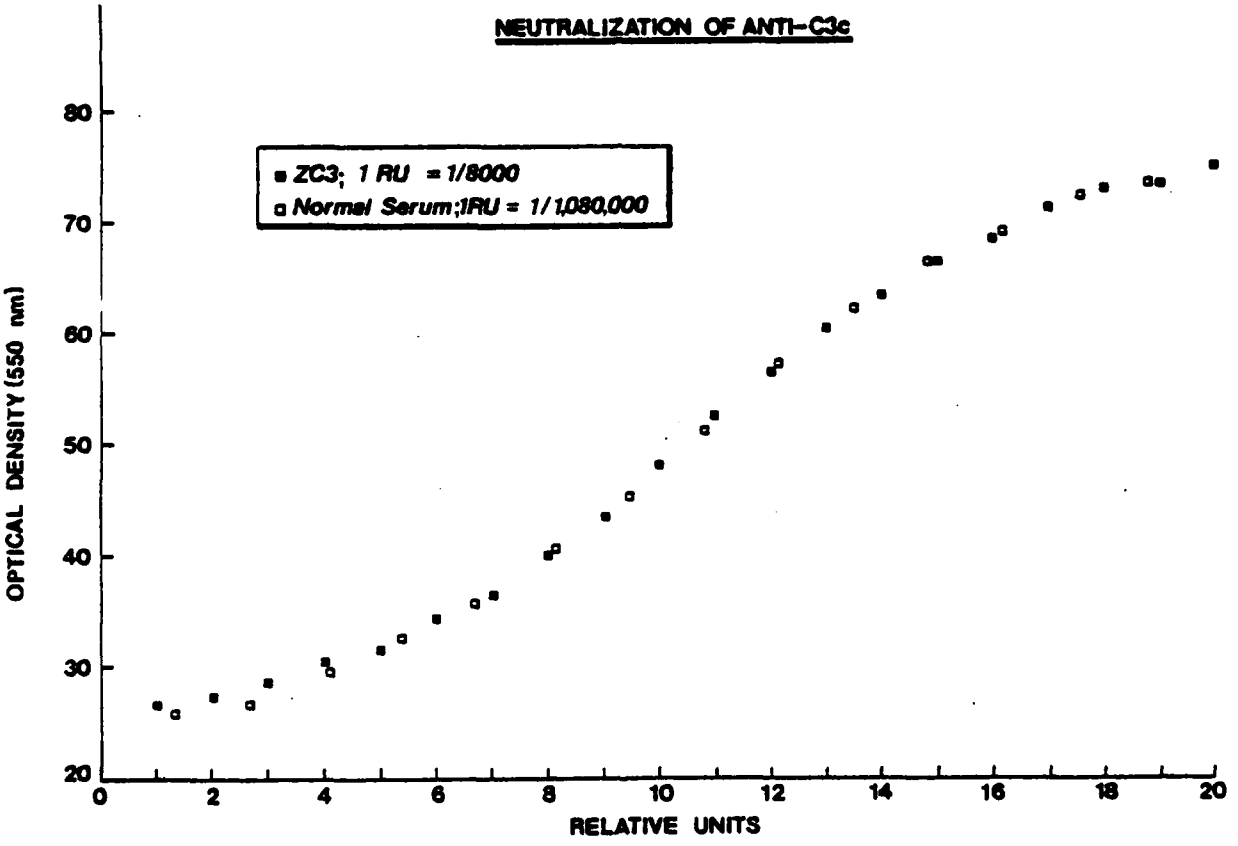


Figure 5

Neutralization of anti-C3c (target 1/200,000) with various dilutions of ZC3 and of RBC stored at 4°C for 17 days (E stored). The ability of anti-C3c to agglutinate EC43 after the neutralization procedure is shown as a function of the relative amount of ZC3 (1 RU = 5.2×10^9 molecules) or E stored (1 U = 1.34×10^8 cells).

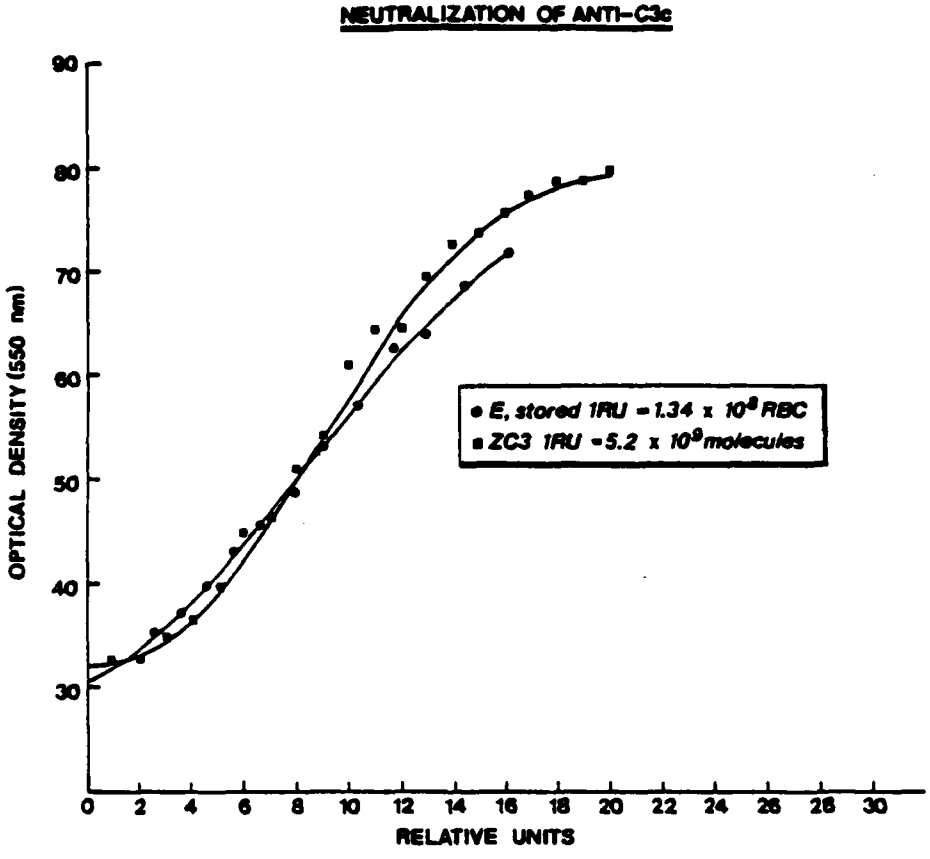


Figure 6

Neutralization of anti-C3c (target = 1/200,000) with various dilutions of ZC3 and EC43. The ability of anti-C3c to agglutinate EC43 after the neutralization procedure is depicted as a function of the relative amount of ZC3 (1 RU = 5.2×10^9 molecules) or EC43 (1 U = 1.26×10^6 cells).

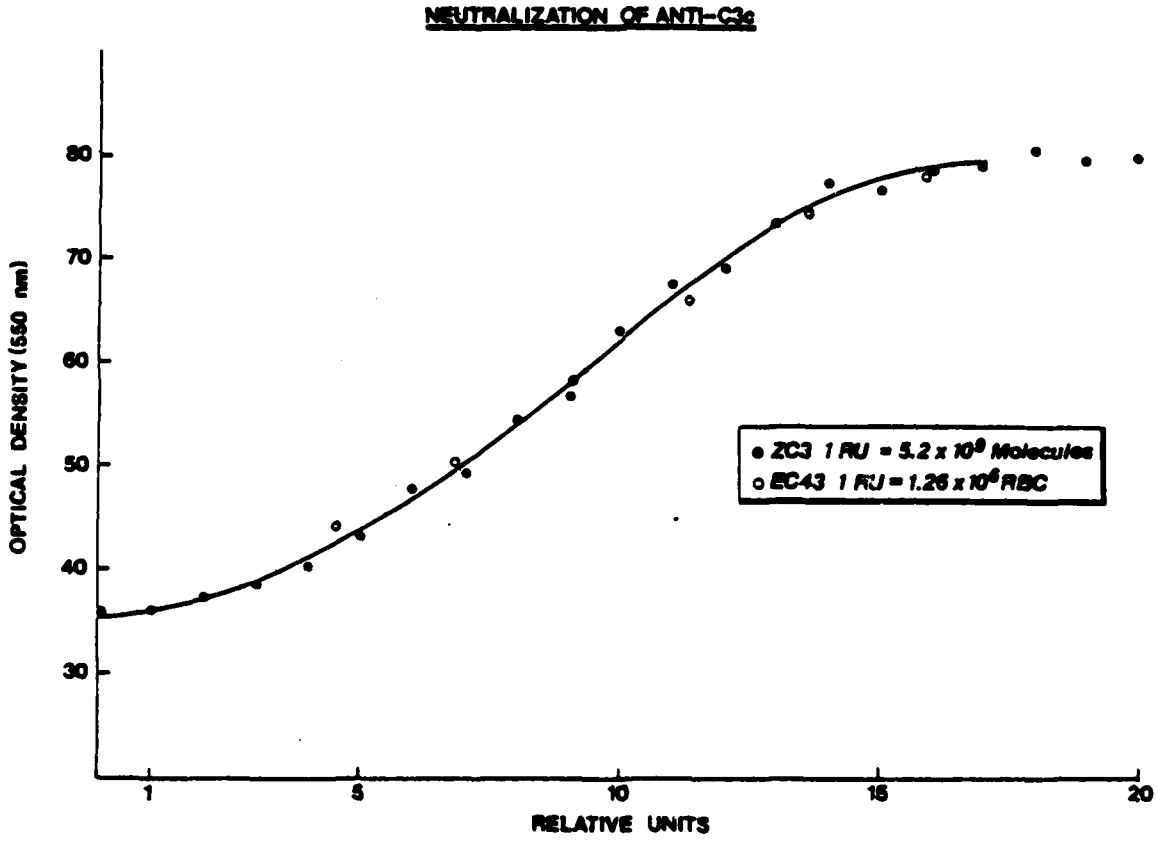
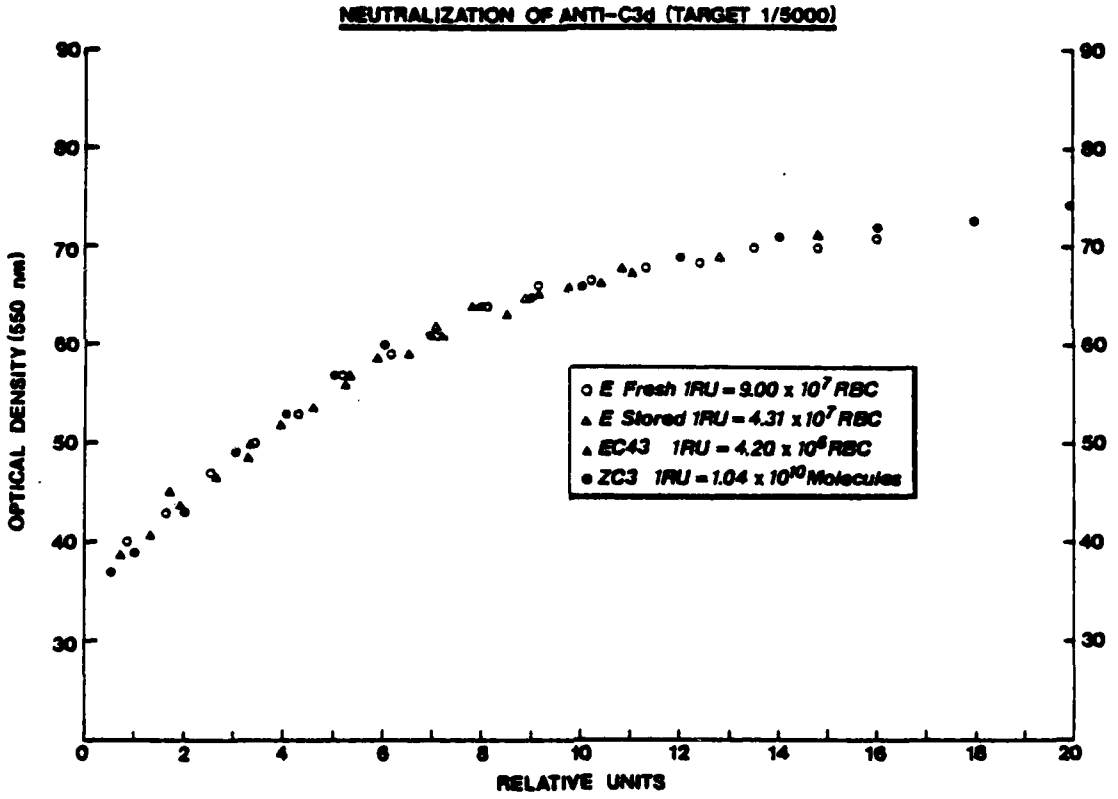


Figure 7

Neutralization of anti-C3d (target = 1/5000) with various dilutions of ZC3, E fresh, E stored, and EC43. The ability of anti-C3d to agglutinate EC43 in the AutoAnalyzer after the neutralization procedure is depicted as a function of the relative amount of various types of particle-bound C3.



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PUBLICATIONS SUPPORTED BY CONTRACT

Szymanski IO, Harper J, Odgren PR, Valeri CR: Freezing quality control red blood cells for antiglobulin test. In press. Transfusion.

Szymanski IO, Keegan M, Odgren PR: Uptake of Ig and complement by erythrocytes in low ionic strength solutions. Submitted for publication.

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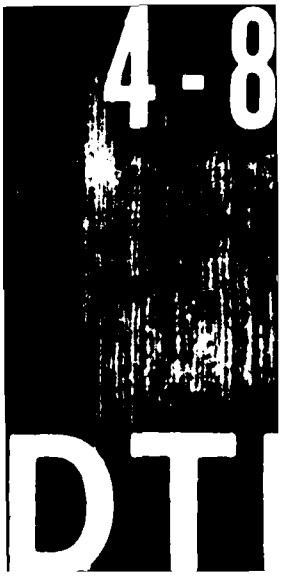
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