Complement Activation During Storage of Blood Under Normal Blood Bank Conditions. Effects of Proteinase Inhibitors and Leukocyte Depletion

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During storage of CPD-A1 preserved whole blood factors of the complement cascade become activated, as evidenced by a rapid increase in the concentrations of C3a-desArg and C4a-desArg. After 10 to 14 days of whole blood storage, the elevations of C3a and C4a levels were highly significant. This increase was paralleled by an increase in the concentration of the lysosomal proteinase elastase from polymorphonuclear (PMN) granulocytes. By contrast, the concentration of the C3 activator complex C4b2b remained unchanged even after 3 weeks of storage. The supplementation of the anticoagulant CPD-A1 with the polyvalent-proteinase–inhibitor aprotinin and the specific elastase-inhibitor eglin C failed to inhibit complement activation, whereas leukocyte depletion could partially abolish the increase of the concentration of C4a, but had no effect on C3a concentrations. These observations support the notion that cleavage of C4 during storage of whole blood is partially leukocyte dependent, whereas the activation of C3 is possibly caused by the activation of the alternate pathway of the complement system by contact of plasma with plastic surfaces.

LONG-TERM STORAGE at 4°C of whole blood and of red blood cell components is a widely used routine procedure that has proven essential to meet the needs of modern medicine. The metabolism and the functional integrity of the cellular components of blood have been extensively studied during and after long-term storage.[1,2] Much less is known about variations of soluble plasma factors during storage of blood, but there is some evidence that complement activation occurs during blood storage at 4°C.[3,4]

The degradation of leukocytes and platelets during storage[5] may be accompanied by the release of components with potentially hazardous properties. During storage of blood, an increase in the concentrations of various proteinases of leukocyte origin, eg, elastase from polymorphonuclear (PMN) granulocytes, has been observed.[6,7] Because of their proteolytic activities, these proteinases possess the ability to activate the complement system[8,9] via the classical or the alternate pathway and may thereby be involved in the formation of anaphylatoxins.

It has been reported that the contact of blood with plastic surfaces may also activate the complement system by the alternate pathway.[10,11] Because blood is stored in plastic bags this might be another possibility for the formation of anaphylatoxins during storage of blood.

To investigate possible variations of anaphylatoxin concentrations in blood units during storage, we used sensitive radioimmunoassay techniques for quantification of the complement-derived antigens C3a, C4a, and C5a in stored blood. To analyze the cause and mechanism of complement activation during storage of blood we examined the concentrations of the C3 activator complex C4b2b and of PMN leukocyte elastase. Furthermore, we determined whether complement activation during storage of blood could be avoided either by supplementation of the CPD-A1 anticoagulant with the polyvalent proteinase inhibitor-aprotinin,[12] the specific elastase inhibitor eglin C,[13,14] or by removal of leukocytes by a filtration procedure[15] before storage of the blood. As indicators of complement activation we monitored the above-mentioned parameters.

MATERIALS AND METHODS

Normal blood units. Units of whole blood (approximately 500 mL) were routinely collected from healthy donors in CPD-A1 anticoagulant at the Bavarian Red Cross blood donation center in Munich and 10 mL of each unit were separated as pilot tubes into plastic vials and stored together with the unit under standard blood bank conditions at 4°C. Aliquots were taken from the pilot tubes under sterile conditions after variable storage periods and the plasma was frozen at −20°C (for the maximum of 4 months) until assaying. In addition, 5 U of routinely collected blood was stored under normal blood bank conditions and aliquots were drawn under sterile conditions into edetic acid/futhan coated tubes after various lengths of time and assayed immediately for C3a and C4a.

Proteinase inhibitor treated blood units. A total of 90 mL of blood was drawn from 10 healthy volunteer donors. Aliquots of 30 mL were added to 4.65 mL of CPD-A1 anticoagulant, which was supplemented with either 0.35 mL isotonic saline, 40 mg/mL recombinant eglin C (a generous gift from Professor H. Fritz), or 10,000 KIU/mL aprotinin (Bayer Co, Leverkusen, Germany). The final concentration of eglin C was 400 μg/mL, and of aprotinin was 100 KIU/mL. These aliquots of whole blood were then stored in 150 mL blood bags (Baxter Co, Unterschleißheim, Germany) under normal blood bank conditions. After different storage periods aliquots were taken under sterile conditions, centrifuged, and the plasma was frozen at −20°C until assaying.

Leukocyte-poor blood units. Six-hundred milliliters of whole blood, obtained from 10 healthy volunteers, was drawn into the first bag of a sterile two-bag system, containing CPD-A1 as an anticoagulant. A leukocyte adhesion filter (Pall Biomed Products Corp, Glen Cove, NY) was placed between the primary and secondary collecting bag and then half of each unit was filtered through the leukocyte adhesion filter. The system was divided and, after sealing, each bag was stored under normal blood bank conditions. Sample collecting and further processing was performed as described above.

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Human complement assays. The concentrations of human C3a-desArg, C4a-desArg, and C5a-desArg were determined by radio immunoassay according to the manufacturer's instructions (Amersham-Buchler Co, Braunschweig, Germany). The assays are specific for C3a, C4a, and C5a, but cross-react with the inactivated peptides C3a-desArg, C4a-desArg, and C5a-desArg respectively.[16,17] The concentrations of the C3 activator complex C4b2b was assayed by radial immunodiffusion (LC-Partigen C3 Aktivator, Behring Co, Marburg, Germany).

Elastase assay. The concentration of human elastase was determined as elastase-α1 proteinase inhibitor complex by enzyme immunoassay according to the manufacturer's instructions (E. Merck, Darmstadt, Germany).

Statistical analysis. The C3a data derived from the pilot tubes of routinely collected units of whole blood were analyzed statistically by means of the Student's *t*-test. For statistical analysis of the data derived from proteinase-inhibitor–treated and leukocyte-depleted units of whole blood we applied a paired two-tailed *t*-test as well as multiple comparisons according to Wilcoxon-Wilcox.

RESULTS

C3a-desArg and C5a-desArg levels were measured in 109 units of whole blood after variable storage periods. There was no significant increase in C5a-desArg concentrations within 2 weeks of storage under normal blood bank conditions (data not shown). By contrast, C3a-desArg levels increased from initial values of 124 ± 41 ng/mL continously during the 2-week storage period to values of 476 ± 281 ng/mL, as shown in Fig 1. After 10 days of storage, the elevation of the C3a-desArg concentrations reached a highly significant level ($P < .0025$) as compared with initial values.

To exclude inadvertent complement activation during sample processing and to avoid any misinterpretation caused by slightly different storage conditions, C3a and C4a were measured immediately without freezing in an additional 5 U of whole blood after appropriate storage periods. As depicted in Fig 2 the concentrations of C3a-desArg and C4a-desArg in any of the blood units continously increased during the 2-week storage period from means of 578 ± 186 ng/mL (C3a-desArg) and 400 ± 321 ng/mL (C4a-desArg) on day 2 to means of 1,222 ± 221 ng/mL and 1,782 ± 1,592

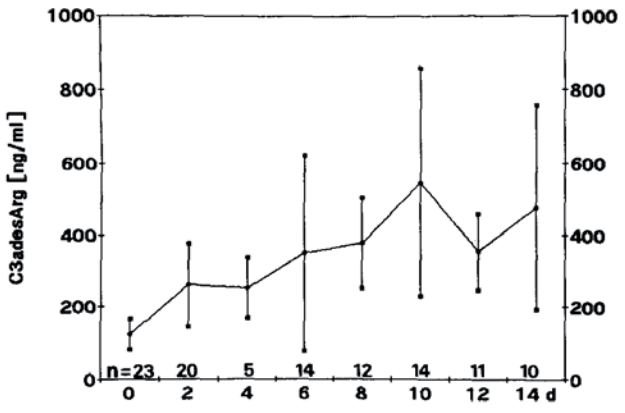

Fig 1. C3a concentrations (means) determined radioimmunologically in the pilot tubes of units of whole blood after variable periods of storage under normal blood bank conditions. SD is indicated by horizontal bars.

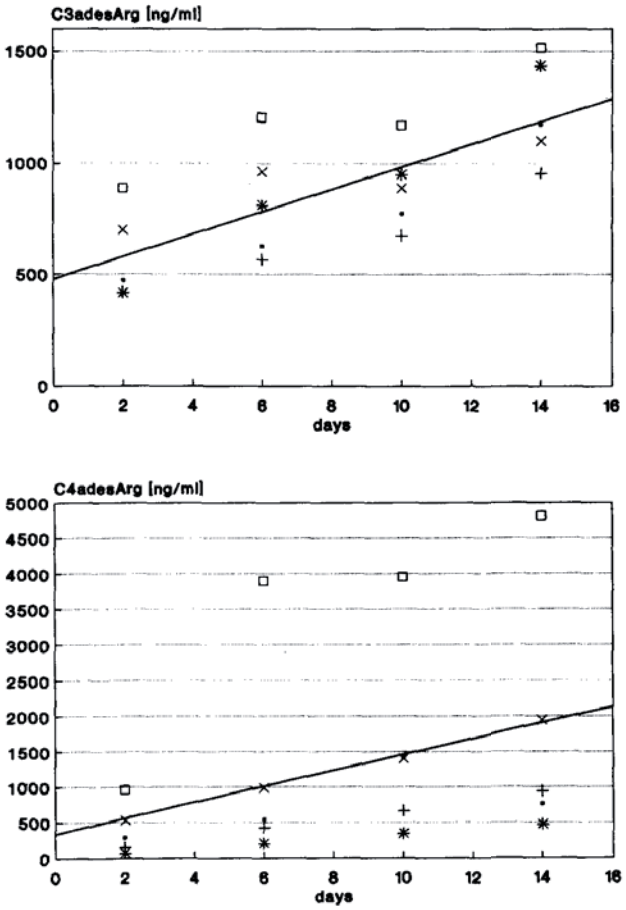

Fig 2. C3a (upper panel) and C4a (lower panel) concentrations in 5 U of whole blood, stored under normal blood bank conditions that were measured immediately after drawing the samples directly from blood bags after the indicated storage periods. Each symbol represents the values in an individual unit of whole blood. The lines represent the calculated trend of the mean values.

ng/mL on day 14. Despite the low number of observations, the differences in the concentrations of C3a-desArg and C4a-desArg reached a highly significant level ($P \leq .01$; multiple comparisons according to Wilcoxon-Wilcox) on day 14 as compared with day 2.

Supplementation of the blood aliquots with the proteinase inhibitor aprotinin to a final concentration of 100 KIU/mL or with the elastase specific proteinase inhibitor eglin C to a final concentration of 400 μg/mL corresponding to 5×10^{-5}M, failed to show any effect on the concentration of the different complement factors. The concentration of C3a-desArg peaked after 2 weeks of storage (Fig 3A). The initial values of 276 ± 35 ng/mL increased to 1,922 ± 218 ng/mL after 2 weeks of storage and remained relatively constant after that time. The supplementation of the blood units with aprotinin or eglin C had no effect on C3a-desArg levels. The concentration of C4a-desArg increased also with storage time from initial levels of 172 ± 16 ng/mL to 1,483 ± 233 ng/mL after 2 weeks, but, in contrast to C3a-desArg, it further increased with time (Fig 3B). Again the proteinase inhibitors aprotinin and eglin C had no effect on the production of this

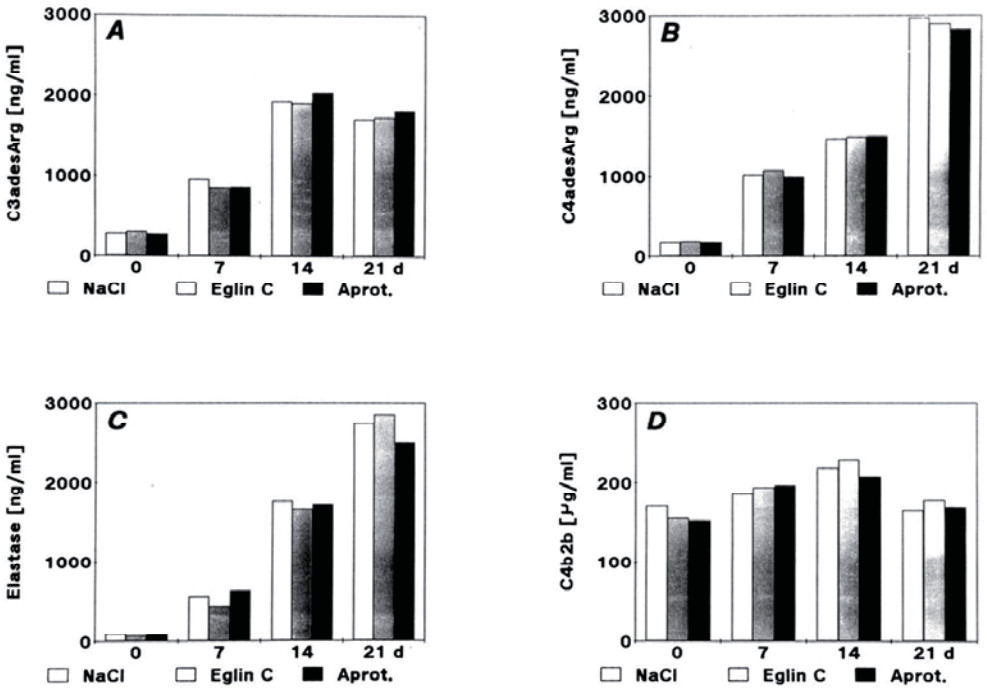

Fig 3. Mean values of the concentrations of (A) C3a, (B) C4a, (C) Elastase, and (D) C3 activator complex C4b2b during storage of whole blood under normal blood bank conditions. Before storage the anticoagulant was supplemented with either isotonic saline, eglin C (400 μg/mL), or aprotinin (100 U/mL) as indicated.

complement factor. No significant increase of C5a-desArg concentrations has been observed (data not shown).

As expected, elastase levels, which have been assayed as the complex of elastase with its natural inhibitor, also increased during storage of whole blood under normal blood bank conditions. The addition of eglin C significantly decreased the elastase concentrations as compared with the controls during the first 5 days of storage (Table 1) by competition with the natural plasma elastase inhibitor, α1-proteinase inhibitor (α1-PI), whereas aprotinin had no significant effect. However, in the later observation period the exogenous inhibitor eglin C seemed unable to compete further with α1-PI, because elastase levels measured in complex with α1-PI increased with time even in the presence of eglin C (Fig 3C).

To further investigate which pathway of the complement system becomes activated during storage of blood products, we measured the C3-activator complex C4b2b. As shown in Fig 3D there have been no major differences in the concentration of C4b2b during the storage period of three weeks. The addition of either of the two proteinase inhibitors had also no effect.

Table 1. Elastase Concentrations (ng/mL + SD) in Stored Whole Blood During the First Week of Storage Under Normal Blood Bank Conditions

Day	Control	Eglin C	Aprotinin
0	98 ± 50	75 ± 18 ($P > .05$)	92 ± 23 ($P > .05$)
1	132 ± 49	122 ± 43 ($P \leq .01$)	171 ± 67 ($P > .05$)
3	191 ± 59	143 ± 42 ($P \leq .01$)	180 ± 71 ($P > .05$)
5	358 ± 177	220 ± 119 ($P \leq .01$)	302 ± 196 ($P > .05$)
7	563 ± 263	438 ± 291 ($P > .05$)	635 ± 313 ($P > .05$)

Before storage, the CPD-A1 anticoagulant was supplemented with either isotonic saline (control), eglin C (400 μg/mL), or aprotinin (100 U/mL). Results of the statistical analysis for multiple comparisons according to the Wilcoxon-Wilcox test for the comparison of the effects on elastase concentrations of eglin C and aprotinin versus the controls are given as *P* values in brackets.

In another attempt to clarify the role of degrading leukocytes in complement activation during storage of blood products, we measured complement factors in leukocyte-depleted blood. Although this procedure had absolutely no effect on the increasing concentrations of C3a-desArg (Fig 4A), it clearly reduced ($P < .001$ at day 14; two-tailed *t*-test) the elevated levels of C4a-desArg (Fig 4B). Again, there have been no major differences in the concentration of the C3-activator complex in the leukocyte-rich samples during the storage period of 3 weeks, nor could leukocyte depletion alter these concentrations (data not shown).

DISCUSSION

The data presented in this report suggest that during storage of whole blood some components of the complement system become activated. Although individual values of C3a-desArg and C4a-desArg showed considerable variation in different units of whole blood, statistical analysis showed a highly significant increase during storage in the concentrations of these anaphylatoxins (Figs 1 and 2). Because the concentrations, which were measured immediately without prior frozen storage of plasma aliquots in 5 units of routinely collected whole blood, showed even higher C3a concentrations with mean values of more than 1,000 ng/mL on day 14 (Fig 2), inadvertent cleavage of C3 during sample processing, especially during storage of frozen plasma samples, seems to be unlikely. This divergence is possibly caused by differences in the chemical composition of the pilot tubes as compared with normal blood bags, or by differences in the volume-to-surface ratio. Interestingly, no increase in the concentration of C5a-

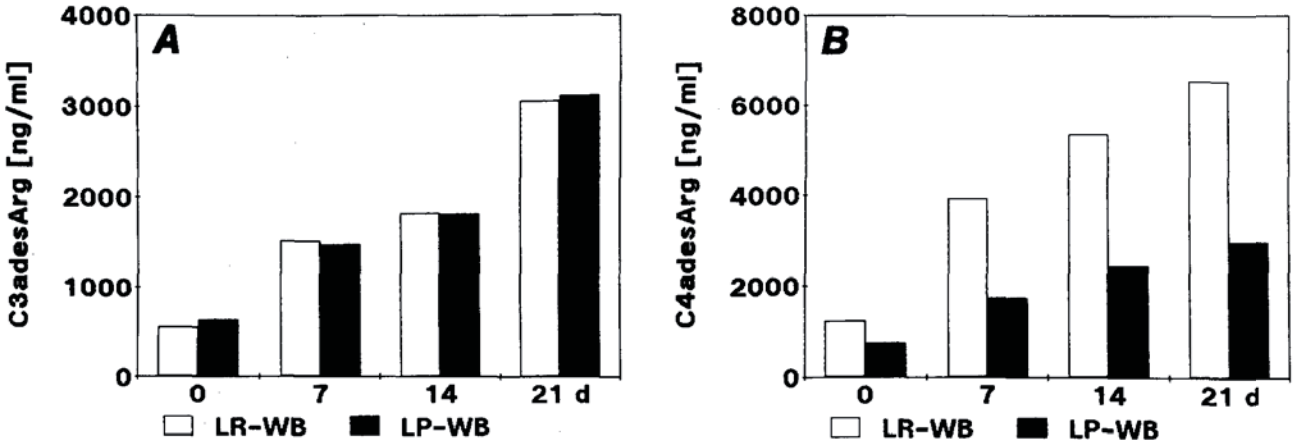

Fig 4. Mean values of the concentrations of (A) C3a, and (B) C4a of leukocyte-rich whole blood (LR-WB) and leukocyte-poor whole blood (LP-WB) during storage under normal blood bank conditions.

desArg could be observed. This might be attributable either to the very short half-life of approximately 1 minute for C5a-desArg,[18] which may be adsorbed on the leukocyte surface, or to a rapid inactivation of C3b, thus preventing the formation of the C5 convertase. On the other hand, the assay system used might not be sensitive enough to detect variations in C5a-desArg concentrations.

The complement activation may be caused by different mechanisms and may occur via the classical or the alternate pathway of the complement cascade. It has been reported that contact of blood with plastic surfaces can cause the activation of the alternate pathway of the complement system.[10,11,19,20] Elevated levels of C3a-desArg were also found after passing blood through dialysis membranes[21] or during cardiopulmonary bypass,[22] whereas these procedures had no effect on C4a-desArg.

In addition, isolated leukocyte proteinases, such as elastase from PMN granulocytes, are capable of activating the alternate pathway of the complement system as well as cleaving components of the classical pathway[6,8] in vitro. However, in vivo, elastase is rapidly inactivated by α1-PI.[23] Because the concentration of elastase was measured by an immunoassay that detects elastase in complex with α1-PI,[24] the given data provide no information about the enzymatic activity of elastase. However, because of the tremendous molar surplus of α1-PI over released elastase (more than 1,000-fold) and the rapid inactivation time (milliseconds), proteolytic activity of elastase in storage blood seems very unlikely. Moreover, because the addition of eglin C, a highly specific inhibitor of elastase,[13,14] also failed to reduce the increase of C3a-desArg and C4a-desArg, it is quite clear that elastase plays no major role in complement activation during storage of blood.

This notion is further supported by our data indicating identical C3a-desArg levels in leukocyte-rich whole blood (LR-WB) and in leukocyte-poor whole blood (LP-WB) (Fig 4A), because previous studies from this laboratory have shown that the increase in elastase levels during storage of blood is abrogated in leukocyte depleted units of whole blood.[7] Filtration procedures remove up to 98% to 99% of leukocytes from whole blood, as determined by hemocytometer counts.[7] The high efficacy of the filter system used in this study has also been shown by more sensitive techniques using a ploymerase chain reaction.[25] Therefore, we conclude that the elevation of C3a-desArg occurs independently of the presence of viable or degrading leukocytes.

By contrast, the concentration of C4a-desArg increased to a lesser extend in LP-WB as compared with LR-WB (Fig 4B), indicating that cleavage of C4 is at least partially attributable to the presence of leukocytes. But neither the addition of eglin C nor of aprotinin, which preferably inhibits plasmin and tissue kallikrein,[12] had any effect on C4a-desArg concentrations and it remains unclear which leukocyte factors contribute to the elevation of C4a-desArg during storage of blood products. But even in LP-WB, some increase in C4a-desArg concentrations occured with storage time, which might be caused by autoactivation of C4 at 4°C.[17]

As elevated concentrations of C4a-desArg might be interpreted as an indicator of the activation of the classical pathway of the complement cascade, we determined the concentrations of the C3 activator complex C4b2b as a second parameter of this pathway. No major differences in the concentrations of the C3 activator complex could be observed even with prolonged storage time (Fig 3C) and the addition of proteinase inhibitors or leukocyte depletion did not alter these concentrations. Therefore, and because all manipulations with the blood units were done under sterile conditions, it seems unlikely that complement activation in stored blood occurs via the classical pathway. These findings are in good agreement with an earlier report on an increase of the C3 breakdown product C3d during storage of blood. Similar to our results, no activator of the classical pathway could be found, as evidenced by C1q binding studies.[26] Taken together, the most probable mechanism that underlies the C3a-desArg elevation seems to be an activation of the alternate pathway of the complement system by contact of plasma with plastic surfaces.

Although the desArg peptides are no longer anaphylatoxins, the antibodies used in the radioimmunoassays for C3a-desArg and C4a-desArg cross-react with the anaphylatoxins C3a and C4a. Therefore, some anaphylactic poten-

tial of the investigated peptides cannot be ruled out by our data. Moreover, even the anaphylactically inactive C3a-desArg has some further biologically adverse properties. For example, concentrations of C3a-desArg in the range of 1 ng/mL and below have been reported to enhance platelet aggregation and the release of serotonin from platelets[27] that might lead to augmentation of hypercoagulability and thrombosis. Although controversial, reports have also been published dealing with an inhibitory potential of C3a-desArg in concentrations of 1,000 ng/mL on the cytotoxic activity of natural killer cells.[28,29] But because we measured C3a-desArg radioimmunologically, these data do not provide any information about functional activities of C3a-desArg derived from units of stored whole blood. Therefore, we are currently investigating to see if the plasma of stored whole blood has any platelet activating potential.

Nevertheless these data give rise to some theoretical concerns regarding the potential for adverse effects of the complement cleavage products we have measured in units of whole blood, although relevant clinical data about adverse reactions to transfusions because of activated complement factors have not yet been published.

REFERENCES

1. Valeri CR, Valeri DA, Gray A, Melaragno A, Dennis RC, Emerson CP: Viability and function of red blood cell concentrates stored at 4°C for 35 days in CPDA-1, CPDA-2, or CPDA-3. Transfusion 22:210, 1982
2. McCullough J, Carter SJ, Quie PG: Effects of anticoagulants and storage on granulocyte function in bank blood. Blood 43:207, 1974
3. Szymanski IO, Odgren PR: Studies on the preservation of red blood cells. Attachment of the third component of human complement to erythrocytes during storage at 4°C. Vox Sang 36:213, 1979
4. Szymanski IO, Swanton RE, Odgren PR: Quantitation of the third component of complement on stored red cells. Transfusion 24:194, 1984
5. McCullough JJ, Weiblen BJ: The relationship of granulocyte ATP to chemotactic response during storage. Transfusion 19:764, 1979
6. Lane TA, Lamkin GE: The effect of storage on degranulation by human neutrophils. Transfusion 25:155, 1985
7. Riedner C, Heim MU, Mempel W, Wilmanns W: Possibility to improve preservation of whole blood by leukocyte-depletion before storage. Vox Sang 59:78, 1990
8. Johnson U, Ohlsson K, Ohlsson I: Effects of granulocyte neutral proteases on complement components. Scand J Immunol 5:421, 1976
9. Venge P, Olsson I: Cationic proteins of human granulocytes. VI. Effects on the complement system and mediation of chemotactic activity. J Immunol 115:1505, 1975
10. Hed J, Johansson M, Lindroth M: Complement activation according to the alternate pathway by glass and plastic surfaces and its role in neutrophil adhesion. Immunol Lett 8:295, 1984
11. Sevastianov VI, Tseytlina EA: The activation of the complement system by polymer materials and their blood compatibility. J Biomed Mater Res 18:969, 1985
12. Fritz H, Wunderer G: Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. Drug Res 33:479, 1983
13. Seemüller U, Meier M, Ohlsson K, Müller H-P, Fritz H: Isolation and characterization of a low molecular weight inhibitor (of chymotrypsin and human granulocytic elastase and Cathepsin G) from leeches. Hoppe Seylers Z Phys Chem 358:1105, 1977
14. Baici A, Seemüller U: Kinetics of the inhibition of human leucocyte elastase by eglin from the leech Hirudo medicinalis. Biochem J 218:829, 1984
15. Domen RE, Williams L, Gilbert DM: Preparation of leukocyte-depleted red cells by filtration. Transfusion 27:504, 1987
16. Chenoweth DE, Hugli TE: Techniques and significance of C3a and C5a measurement, in Nakamura RM (ed): Future Perspectives in Clinical Laboratory Immunoassays. New York, NY, Liss, 1980
17. Gorski JP: Quantitation of human complement fragment $C4a_1$ in physiological fluids by competetive inhibition radioimmune assay. J Immunol Methods 47:61, 1981
18. Webster RO, Larsen GL, Henson PM: In vivo clearance and tissue distribution of C5a and C5a des arginine complement fragments in rabbits. J Clin Invest 70:1177, 1982
19. Fehr J, Craddock PR, Jacob HS: Complement (C') mediated granulocyte (PMN) and pulmonary dysfunction during nylon fiber leukapheresis. Blood 46:1054, 1975 (abstr)
20. Craddock PR, Fehr J, Brigham KL, Kronenberg RS, Jacob HS: Complement and leukocyte-mediated pulmonary dysfunction in hemodialysis. N Engl J Med 296:769, 1977
21. Chenoweth DE, Cheung AK, Henderson LW: Anaphylatoxin formation during hemodialysis: Effects of different dialyzer membranes. Kidney Int 24:764, 1983
22. Chenoweth DE, Cooper SW, Hugli TE, Stewart RW, Blackstone EH, Kirklin JW: Complement activation during cardiopulmonary bypass: Evidence for generation of C3a and C5a anaphylatoxins. N Engl J Med 304:497, 1981
23. Ohlsson K, Olsson I: Neutral proteases of human granulocytes. III. Interaction between human granulocyte elastase and plasma protease inhibitors. Scand J Clin Lab Invest 34:349, 1974
24. Neumann S, Gunzer G, Hennrich N, Lang H: "PMN-elastase assay": Enzyme immunoassay for human polymorphnuclear elastase complexed with α1-proteinase inhibitor. J Clin Chem Clin Biochem 22:693, 1984
25. Rawal BD, Schwadron R, Busch MP, Endow R, Vyas GN: Evaluation of leukocyte removal filters modelled by use of HIV-infected cells and DNA amplification. Blood 76:2159, 1990
26. Schifferli JA, Boralessa H, Howarth HL, Whitwam JG, Rees AJ: Complement in CPD-stored blood. Vox Sang 42:198, 1982
27. Polley MJ, Nachman RL: Human platelet activation by C3a and C3a desarg. J Exp Med 158:603, 1983
28. Charriaut C, Senik A, Kolb J-P, Barel M, Frade R: Inhibition of in vitro natural killer activity by the third component of complement. Role for the C3a fragment. Proc Natl Acad Sci USA 79:6003, 1982
29. Morgan EL, Weigle WO, Hugli TE: Anaphylatoxin-mediated regulation of human and murine immune responses. Fed Proc 43:2543, 1984