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THE COMPLEMENT SYSTEM IN CRYOGLOBULINAEMIA

INTERACTION WITH IMMUNOGLOBULINS AND LIPOPROTEINS

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SUMMARY

Serum from a patient with an IgM–lipoprotein cryoglobulin, both before and after removal of the cryoprecipitate at 0°C, had extremely low levels of whole complement (C), C1, C4 and C2, while amounts of the remaining components were normal or only slightly reduced. The cryoprecipitate, when added to fresh normal human serum, reproduced this pattern of C fixation.

Separation of the patient's serum at 37°C into its lipoprotein, IgG and IgM fractions revealed that the IgM alone would precipitate at 0°C. This precipitation was unaffected by the patient's IgG, but was markedly enhanced by extremely small amounts of the patient's $d < 1{\cdot}075$ lipoprotein fraction or of homologous very low density lipoprotein (VLDL). Aggregation occurred even at 37°C in the presence of VLDL. Fixation of semi-purified human C1 paralleled these results closely: it occurred with the patient's IgM alone at 0° but not at 37°C, while IgM in the presence of the patient's lipoprotein, or of VLDL from normal serum, fixed C1 strongly at 37° as well as at 0°C. Fab dimers and monomers prepared from the patient's IgM did not aggregate in the cold, even in the presence of lipoprotein, and did not inhibit the aggregation of intact IgM in the presence of VLDL, at any temperature.

All three highly purified IgM cryoglobulins, and three of four IgG cryoglobulins, fixed C1 strongly. The IgG preparation which failed to fix C1 was the only one which had lost its cryoprecipitability during purification.

Measurement of C3 or whole C levels may be an insensitive method for detecting C fixation in cryoglobulinaemia. It is suggested that analysis for C1, C4 or C2 should be employed instead.

INTRODUCTION

We present here a study of the factors affecting complement (C) fixation by an unusual

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IgM–lipoprotein cryoglobulin complex from a patient (Alex.) who had less than 1% of normal C1, C4 and C2 in her serum. This 75-year-old woman complained of generalized weakness and of pain in the lower extremities upon exposure to cold. A precipitate containing low density (LDL) and very low density (VLDL) lipoprotein with IgM, formed in her serum *in vitro* at temperatures below 33·5°C. Serum obtained at 37°C contained 4·1 g/dl of a monoclonal IgM, and lipoproteins with mobilities characteristic of the three major electrophoretic classes ($\alpha 1$, $\alpha 2$ and β) of normal human plasma lipoproteins. After precipitation at 20°C the monoclonal IgM was absent and only lipoproteins of $\alpha 1$ mobility remained. Purified normal LDL and VLDL added to the patient's 37°C serum precipitated with her cryoglobulin at 20°C.

MATERIALS AND METHODS

Reagents

Veronal buffers were prepared by mixing appropriate proportions of sodium chloride and sucrose–veronal buffers of $\mu = 0{\cdot}15$ and $\mu = 0{\cdot}009$ respectively (Rapp & Borsos, 1963). C components were purchased from Cordis Corporation (Miami, Florida), and cell intermediates containing C1 (EAC1), C4 (EAC4) and other C components were prepared from guinea-pig serum or guinea-pig C components (Borsos & Rapp, 1967; Linscott, 1969a).

Complement assays

'Whole C' assays were performed by adding serum dilutions to $2{\cdot}5 \times 10^7$ EA. After 60 min at 37°C, the mixture was centrifuged, and the optical density read at 413 nm after warming the tubes to 37°C to resolubilize any cryoprecipitate. C1 was measured with EAC4 at $\mu = 0{\cdot}065$. C4 was assayed with EAC1 at $\mu = 0{\cdot}065$ for 60 min at 37°C and then for 60 min at 0°C. C2 assays were carried out with EAC14 for 10 min at 30°C, $\mu = 0{\cdot}065$. C3 was titrated using EAC142 and purified C5–C9.

Cryoglobulin-containing serum from patient (Alex.)

Serum was separated at 37°C, and either used directly or kept at −70°C. Cryoprecipitates were prepared from this serum by incubating for 15–30 min at 0°C, followed by centrifugation in the cold and washing with cold buffer. The supernatant from such a precipitation is referred to as 'cryoglobulin-free serum'.

Lipoproteins (LDL and VLDL), which are capable of coprecipitating with the patient's IgM at 37°C, were separated by a modification of the ultracentrifugal method of Havel, Eder & Bragdon (1955). The non-protein solvent density of 37°C serum was adjusted to 1·075 g/ml, and layered under a salt solution of like density and centrifuged for $1{\cdot}5 \times 10^8$ g/min at 37°C. The supernatant fraction containing LDL, VLDL and a small fraction of the HDL was removed in the top millilitre and the protein pellet was redissolved in the lowest 3 ml of the tube's contents. Immunoglobulins were undetectable by immunodiffusion in the $d < 1{\cdot}075$ fraction. Similarly, LDL and VLDL could not be detected in the infranatant fraction. Purified HDL and LDL were prepared from the serum of normal subjects, and VLDL from the serum of a patient with primary hyperprebetalipoproteinaemia by repetitive preparative ultracentrifugation (Havel, Eder & Bragdon, 1955).

TABLE 1. Fixation of human complement components by cryoglobulin at different ionic strengths and temperatures

Mixture	Contents	Final temperature (°C)	Final ionic strength	Titre (corrected to 1 ml of undiluted serum)					Percentage of control titre				
				C	C1	C4	C2	C3	C	C1	C4	C2	C3
A1	NHS*	37	0·15	260	1,400,000	490,000	650	8500					
A2	NHS	0	0·15	265	1,400,000	500,000	650	8500					
A3	NHS	37	0·056	275	400,000	80,000	30	3500					
A4	NHS	0	0·056	330	600,000	190,000	300	4000					
B1	NHS+cryoppt.	37	0·15	<5	40,000	3300	<5	2500	<2	3	0·7	<0·8	29
B2	NHS+cryoppt.	0	0·15	33	100,000	3300	<5	2500	12	7	0·7	<0·8	29
B3	NHS+cryoppt.	37	0·056	<5	6000	300	<5	2500	<2	1·5	0·4		71
B4	NHS+cryoppt.	0	0·056	<5	7000	600	<5	3500	<2	1·2	0·3	<0·8	88
D	37°C pt. serum			1·6	900	3000	0	450	0·6	0·06	0·6	<0·1	5
E	Cryo-free pt. serum			15	1500	4000	0	1000	6	0·1	0·8	<0·1	12

Four 0·5-ml portions of normal human serum were mixed with washed cryoprecipitate prepared from 0·5 ml portions of fresh 37°C Alex. serum. After 30 min at 37°C, these mixtures (along with normal serum controls) were treated as follows: (B1) addition of 2 volumes (μ = 0·15) buffer followed by incubation at 37°C for 45 min; (B2) addition of 2 volumes (μ = 0·15) buffer followed by incubation for 15 min at 37°C and 30 min at 0°C; (B3) addition of 2 volumes (μ = 0·009) buffer (final μ = 0·056) followed by incubation for 45 min at 37°C; (B4) addition of 2 volumes (μ = 0·009) buffer followed by incubation for 15 min at 37°C and 30 min at 0°C. All tubes were then centrifuged at the same temperature as the final incubation stage, and the supernates diluted and tested for C, C1, C4, C2 and C3. Similar tests were made on a sample of Alex. serum held continuously at 37°C, and on a cryo-free sample of the same serum.

* NHS = normal human serum.

RESULTS

Levels of all nine C components, determined by microtitre assay (Cordis Corporation, Miami, Florida) of the patient's serum were nearly identical before and after removal of the cryoglobulin fraction: undetectable 'whole C' activity; less than 1% of normal C1, C4 and C2; 33–50% of normal C3; and 50–100% of normal C5, C6, C7, C8 and C9. No C1q was detectable in either the intact serum or the cryoprecipitate by immunodiffusion assay. The low C1, C4 and C2 levels in the intact serum as well as in the cryoglobulin-free serum suggested that C fixation had occurred *in vivo*. (This was confirmed by similar measurements on fresh serum which had never been allowed to cool below 37°C (Table 1).)

C fixation from normal serum by cryoprecipitate

Since the patient's cryoglobulin was predominantly IgM, one might expect it to fix C less completely in the cold but more completely at reduced ionic strength (Linscott, 1970). Mixtures of Alex. cryoprecipitate and normal serum were incubated under four different conditions of temperature and ionic strength and then assayed for C, C1, C4, C2 and C3. Similar tests were made on the patient's serum held strictly at 37°C, and after removal of the cryoprecipitate at 0°C (Table 1). C1 fixation was greater at 37°C than in the cold, at least at $\mu = 0{\cdot}15$, and greater at low ionic strength. C2 was almost unmeasurable in all four mixtures. Fixation of C3 occurred under all four sets of conditions, but was far less extensive than fixation of C1, C4 and C2. C2 appeared to be markedly unstable at 37°C, $\mu = 0{\cdot}056$ (control A3).

Anti-complement activity of different amounts of patient's serum and serum fractions

In order to compare the relative C-fixing activity of the patient's serum with and without its cryoglobulin content, portions of normal human serum were incubated with dilutions of: 37°C patient's serum; cryoglobulin-free patient's serum; or washed cryoprecipitate restored to original serum volume. The residual whole C activity in each mixture was then measured. The cryoglobulin-free patient's serum had essentially no anti-complementary activity. The washed cryoglobulin was even more anti-complementary than whole 37°C serum, probably because it was essentially devoid of active C components, while the 37°C serum contained small amounts of C1, C4, C2 and C3, and nearly normal amounts of C5–C9. These components may have partially compensated for the marked depletion of C1, C4, and C2 by the cryoglobulin.

C1 fixation by pure IgM and IgG cryoglobulins

Since aggregated IgM or IgG can fix C by itself (Augener *et al.*, 1971), purified cryoproteins of the IgG and IgM classes should be able to fix C1 alone, to the extent that they can aggregate under the conditions of the assay. This was tested by incubating 5·5 units of human C1 with 0·1 mg of each of several different purified IgM and IgG cryoglobulins under a variety of conditions. Each cryoglobulin was also analysed for C1 contamination by direct incubation for 2 hr with EAC4. The percentage of C1 fixed (Table 2) is complicated by the fact that more than 90% of the C1 added was inactivated at 37°C, $\mu = 0{\cdot}15$. C1 stability at $\mu = 0{\cdot}037$ was far better. It is clear that three out of four IgG and all three IgM cryoglobulins, and Alex. IgM fixed human C1 strongly under at least one set of conditions. As expected, fixation was best at 0°C, $\mu = 0{\cdot}037$ and much poorer at $\mu = 0{\cdot}15$.

TABLE 2. Fixation of human C1 by purified human immunoglobulins

	Conditions during C1 fixation reaction					
	30′ at 37°C $\mu = 0{\cdot}15$ no cfg.	30′ at 0°C $\mu = 0{\cdot}15$ cfg. at 0°C	15′ at 37°C 15′ at 0°C $\mu = 0{\cdot}15$ cfg. at 0°C	30′ at 0°C $\mu = 0{\cdot}037$ cfg. at 0°C	15′ at 37°C 15′ at 0°C $\mu = 0{\cdot}037$ cfg. at 0°C	Units of C1 in cryoppt. alone
C1 only (control) (units of C1 remaining)	0·34	1·32	0·62	4·72	5·44	
C1 + immunoglobulin (percentage of C1 fixed):						
Patient (Alex.)	0	58	53	77	94	0·37*
Bolt. (IgM cryo.)†	29	83	66	89	92	0·19
Polk. (IgM cryo.)	0	0	0	87	95	0·13
Nevi. (IgM cryo.)	65	80	83	89	97	0·03
Lear. (IgG1 cryo.)	86	90	91	>99	>99	0·03
Fran. (IgG3 cryo.)	>99	37	83	>99	>99	0·02
Ocon. (IgG2 cryo.)	52	86	81	87	91	0·05
Whit. (IgG1 cryo.)†	0	0	0	11	28	0·06
Ford. (IgA myeloma)	0	17	8	3	32	0·38

0·1 mg amounts of various human cryoglobulins were incubated with 5·5 units of human C1, under the conditions shown above. Residual C1 activity was measured in the 37°C sample without cooling and in the 0°C samples after cooling to 0°C and removal of cryoprecipitate by centrifugation.

* Patient's cryoglobulin was precipitated and washed in EDTA to minimize C1 contamination.

† No longer cryoprecipitable after purification.

TABLE 3. Fixation of human C1 by IgM, IgG and lipoprotein fractions from Alex. serum

Alex. serum fraction tested	Amount (mg)	Percentage of C1 fixed
None	—	—
IgM	0·01	0
IgG	0·01	28
Lipoprotein	0·01 protein	0
IgM + IgG		0
IgG + lipoprotein		23
IgM + lipoprotein		86
IgM + IgG + lipoprotein		87

0·01 mg amounts of various Alex. serum fractions were incubated at $\mu = 0{\cdot}096$ with human C1. Residual C1 activity was then measured.

Nevertheless, significant fixation did occur at physiological temperature and ionic strength with several preparations. An IgA myeloma protein and one IgG cryoglobulin which had lost its cryoprecipitability during purification, fixed C1 very weakly.

Fractionation of Alex. serum IgM and IgG components: their interaction with lipoprotein to fix human C1

Alex. d<1·075 lipoproteins were separated as described above. IgM and IgG were separated from the d<1·075 infranate using Sephadex G-200 at 37°C, and IgG was further purified by chromatography on DEAE–cellulose. Neither purified IgG nor d<1·075 lipoproteins precipitated at low temperature, but IgM did. This was markedly enhanced by addition of the lipoprotein fraction but not by IgG. These fractions were then tested, singly and in various combinations, for their ability to fix 1 unit of human C1 in 15 min at 37°C, followed by 15 min at 0°C. Only a combination of IgM and the lipoprotein fraction induced significant C1 fixation (Table 3). The small amount of activity in the IgG fraction is presumably due to spontaneous aggregation.

Substitution of homologous lipoprotein fractions for Alex. lipoprotein

HDL, LDL and VLDL were substituted for Alex. lipoprotein in experiments similar to the one described above. VLDL was found to be highly active in fixing human C1 when combined with Alex. IgM; LDL was about 1/200 as active (based on protein content) as VLDL, and HDL was inactive. A wide range of VLDL and IgM concentrations were then compared for ability to fix C1 at 0° and 37°C. It was found that as much as 0·1 mg of IgM, in the absence of VLDL, did not fix C1 at 37°C, where aggregation should be minimal; but at 0°C, where visible turbidity was evident, strong C1 fixation occurred with 0·1 and 0·01 mg of IgM. The addition of as little as 4 ng of VLDL protein was sufficient to permit C1 fixation by IgM at 37°C, and an optimal amount of VLDL resulted in significant C1 fixation by as little as 1 ng of IgM. A definite prozone effect was seen with high concentrations of IgM, but not of VLDL.

The specificity of the interaction between VLDL and Alex. IgM was determined by comparing three IgM cryoglobulins (separated from mixed IgG–IgM cryoproteins), one IgM from Waldenstrom's macroglobulinaemia and a normal human IgM, for their ability to fix C1 at 37°C with or without added VLDL. At a concentration of 10 μg each fixed part of the C1 added, but none showed any increase in C1 fixation upon the addition of 4 μg of VLDL. Thus, C1 fixation at 37°C by Alex. IgM required the specific interaction of her monoclonal IgM, with normal VLDL.

Light attenuation measurements of reversible IgM-VLDL interaction

Mixing Alex. IgM with normal serum VLDL produced a nearly instantaneous turbidity which was visible at 37°C but became much heavier in the cold. Turbidity was estimated by measuring the optical density (OD) at 300 nm, using a spectrophotometer (Beckman, model DU). Varying amounts of buffer, Alex. IgM or IgG, and VLDL from a normal serum were combined at 20°C and the OD measured as quickly as possible ('zero time'). The mixture was then incubated at different temperatures, with periodic measurements of OD (Fig. 1). Pure solutions of VLDL or Alex. IgG, and mixtures of VLDL and Alex. IgG showed essentially no change in OD at different temperatures. Alex. IgM aggregated rapidly when cooled to 0°C, but resolubilized upon warming. IgM aggregation was greatly enhanced

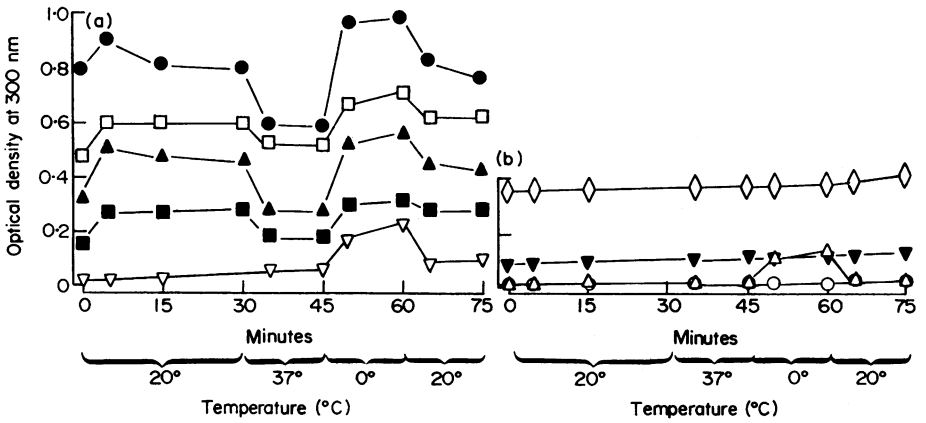

FIG. 1. Optical density of VLDL, Alex. IgM and IgG incubated singly or in combination at different temperatures in 0·15 M veronal buffer (pH 7·4). (●) 0·2 mg IgM+0·08 mg VLDL. (□) 0·05 mg IgM+0·08 mg VLDL. (▲) 0·2 mg IgM+0·02 mg VLDL. (■) 0·2 mg IgM+0·008 mg VLDL. (▽) 0·2 mg IgM+0·1 mg IgG. (◇) 0·08 mg VLDL. (▼) 0·02 mg VLDL. (△) 0·2 mg IgM. (○) 0·1 mg IgG.

by VLDL, but not by Alex. IgG. When VLDL was added to Alex. IgM, the turbidity increased very rapidly, with maximum effect usually within 5 min at 20°C, a temperature at which Alex. IgM alone showed no increase in OD. Raising the temperature from 20° to 37°C caused an almost immediate drop in the OD, while cooling to 0°C brought about a rapid rise. Reversibility occurred with warming. Detectable IgM aggregation occurred with as little as 8 μg of VLDL. That this occurred even at 37°C strongly suggests that important effects may also have taken place *in vivo*.

In order to determine whether the IgM–VLDL interaction resembled that of an antibody with its antigen, Fab monomers and dimers were prepared (Wang, Gergely & Fudenberg, 1973) from Alex. IgM. Neither showed any aggregation alone at 0°C at a concentration of 0·1–0·2 mg/ml, and neither caused any detectable aggregation of VLDL at any temperature tested (using 0·05 mg of Fab). Neither preparation had any inhibitory effect upon the aggregation of VLDL by a small amount of intact Alex. IgM, either in the cold or at higher temperatures.

DISCUSSION

This report concerns a cryoglobulin containing IgM and lipoprotein, with a critical temperature of 33·5°C. The IgM component was able to precipitate by itself at low temperature, and under these conditions to fix C1, but at 37°C aggregation and C1 fixation both required the presence of lipoprotein. Thus the condition essential for C activation was IgM aggregation with the lipoprotein probably playing a critical role *in vivo*.

The low C1, C4 and C2 levels in the patient's serum thus were probably due to C fixation *in vivo*. Supporting evidence includes: (1) the finding of a similar distribution pattern for all nine C components both in cryoglobulin-containing serum at 37°C, and in serum freed of its cryoglobulin by incubation and centrifugation in the cold; (2) very low C and C1 levels in serum which had never been allowed to cool below 37°C; and (3) depletion of C1, C4

and C2 from normal human serum by washed Alex. cryoglobulin with fixation of only a moderate amount of C3, a close duplication of the pattern seen in the patient's serum.

C1 fixation by cryoglobulins may involve mutually contradictory optima: C1 fixation is better with increasing aggregation (Augener *et al.*, 1971), but this requires a reduction in temperature which, in the case of IgM but not with IgG, also reduces the avidity with which C1 binds to the immunoglobulin (Linscott, 1969b). This effect probably accounts for the increased C1 and whole C activity in the patient's serum after removal of the cryoglobulin at 0°C (Table 1).

Pure IgG or IgM cryoglobulins which can precipitate by themselves are not uncommon. Some IgM types are unable to precipitate alone, but coprecipitate readily in the presence of normal IgG (Grey & Kohler, 1973; Meltzer *et al.*, 1966; Wang *et al.*, 1974). Similar behaviour has been reported for an IgM–β-lipoprotein cryoglobulin (Allen, 1966). Alex. IgM thus seems exceptional, in that it is capable of precipitating by itself at 0°C, but aggregates strongly with normal VLDL even at 37°C. Alex. IgM precipitation at 0°C is not due to contamination with lipoprotein, because as much as 0·1 mg of IgM alone did not fix C1 at 37°C, yet 0·1 μg of IgM fixed C1 when as little as 4 ng of VLDL was added.

Fab dimers and monomers from Alex. IgM did not interact detectably with VLDL, suggesting that the IgM–lipoprotein complex may involve some portion of the IgM molecule other than the combining sites for antigen.

Since there is no molecular species of lipid which is categorically restricted to LDL and VLDL, the IgM must be interacting with apoprotein, most, if not all, of which is believed to be located at the surface of these lipoprotein complexes. The markedly greater interaction of VLDL in comparison with LDL indicates that the protein species involved is not apolipoprotein B, the principal apoprotein of both lipoproteins, but one of a number of polypeptides of smaller molecular weight which are most abundant in VLDL (Shore & Shore, 1969), many of which are detectable in small amounts in LDL.

Since the C1-fixing activities of human IgG and IgM are directly proportional to their extent of aggregation (Augener *et al.*, 1971), it is not surprising that C1 fixation by other IgM and IgG cryoglobulins was poorer at 37°C than at 0°C in nearly all cases (Table 2). One each of the IgM (Bolt.) and IgG (Whit.) preparations tested had lost cryoprecipitability during purification. The Bolt. preparation fixed C1 well but Whit. did not, which is consistent with the observation that IgM monomers fix C1 much better than IgG monomers (Augener *et al.*, 1971).

Most investigators have measured C component levels by immunodiffusion, which fails to differentiate between active and inactive components. This may help to explain the small C decreases often reported in cryoglobulinaemia. Also, C3 may be a less sensitive indicator of C fixation in these cases than C1, C4 and C2 (Martinez & Kohler, 1971; Riethmuller *et al.*, 1966; and the present results). (A serum C profile virtually identical to that of Alex. has recently been observed by us in a patient with a mixed IgM–IgG cryoglobulin.) Whole C titres, too, are relatively insensitive in reflecting changes in the levels of individual components, unless these changes are quite large. Thus, many previous investigators may have failed to detect significant C changes in cryoglobulinaemia through an inappropriate choice of methods.

C1 fixation should be rapidly followed by interaction with C4, C2, C3 and later components, with consequent generation of anaphylatoxic and chemotactic fragments of C3 and perhaps of C5. These products facilitate histamine release and cellular infiltration.

The peripheral vasculitis commonly seen in patients with cryoglobulinaemia may well result from the generation of C-fixing cryoprotein aggregates at the lower temperatures in peripheral vascular beds, with subsequent lodging of these complexes within vessel walls due to changes in permeability brought about by local anaphylatoxin generation (Meltzer *et al.*, 1966; Costanzi, Coltman & Donaldson, 1969; Cochrane, 1971). Chemotactic C fragments could then promote cellular infiltration, and damage via mechanisms analogous to the Arthus reaction (Cochrane, 1971). While the patient described herein did not have urticaria, the pain in her extremities upon exposure to cold may have been related to the release of inflammatory mediators secondary to the fixation of C, or alternatively to physical obstruction of peripheral capillary beds by the lipoprotein–IgM complexes.

The ability of Alex. IgM and lipoprotein to aggregate *in vitro* at 37°C suggests that complex formation and C fixation can take place *in vivo* even at core temperature. Deposits of lipid-containing amyloid-like material seen in the lamina propria of Alex.'s small intestine may be due to coprecipitation of IgM with nascent chylomicrons, since those lipoproteins appear to contain essentially all of the apoprotein species found in VLDL.

In a patient such as Alex., with extremely low levels of C1, C4 and C2, and the likely presence of complexes which could readily react with a fresh supply of these components, the potential hazards of blood transfusion must be considered. The rapid generation of large amounts of anaphylatoxins and other mediators upon transfusion of any fluid containing significant amounts of the early C components seems a real risk. Transfusion with washed red blood cells would appear indicated in such a patient if blood replacement is required.

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