Journal of Clinical Immunology, Vol. 12, No. 5, 1992

Cold-Dependent Activation of Complement: Recognition, Assessment, and Mechanism

KENNETH P. MATHEWS,[1,4] ROBERT A. MENTYKA,[3] SANDRA L. CHAMBERS,[1] TONY E. HUGLI,[2] JACK H. HERSCHBACH,[1] and BRUCE L. ZURAW[1]

Accepted: May 7, 1992

Cold-dependent activation of complement (CDAC) is a phenomenon characterized by low hemolytic complement activity in chilled serum. Complement component levels are normal when measured immunologically, and there is normal hemolytic activity in EDTA plasma or serum maintained at 37°C. Little attention has been paid to CDAC except in Japan, and current unfamiliarity with it, even by clinical immunologists, can lead to confusion and unnecessary laboratory tests. A 66-year-old patient with a complex medical history is described whose complement tests showed abnormalities characteristic of CDAC. Evidence for classical complement pathway activation in the cold was obtained by CH_{50} measurements, by hemolytic C4 determinations, by C4a, C3a, and C4d generation, and by quantitating $C1\bar{s}$–$C1\bar{r}$–(C1 inhibitor)$_2$ complexes. A good correlation was observed among these parameters. Cryoprecipitates were absent. CDAC activity has persisted for over 5 years and is greater at 13 than at 4°C. Activation is ablated by heating at 56°C and restored by the addition of C1 to the heated serum. Adsorption by streptococcal protein G–Sepharose and precipitation by 2.5% polyethylene glycol support the hypothesis that CDAC is caused by aggregated IgG. The CDAC factor(s) also induces complement activation in normal serum but has not interfered with Raji cell or C1q binding tests or with FACS analysis. More limited studies of a second individual experiencing CDAC yielded similar results.

KEY WORDS: Complement; cryoproteins; IgG; anaphylatoxins; C1 inhibitor.

[1]Department of Molecular and Experimental Medicine, SRB-6, The Scripps Research Institute, La Jolla, California 92037.
[2]Department of Immunology, The Scripps Research Institute, La Jolla, California 92037.
[3]Division of Allergy, Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109.
[4]To whom correspondence should be addressed.

INTRODUCTION

Discrepancies between hemolytic complement activity in serum versus plasma have been reported in abstracts published beginning in 1970 (1–4). Although the initial emphasis was on the possible role of blood coagulation factors (1–3, 5), it was subsequently discovered that the serum abnormality was associated with cooling the serum *in vitro* (4, 6–14). The usual features of cold-dependent activation of complement (CDAC) include low CH_{50}, low hemolytic C4, and normal hemolytic C5–C9 in chilled serum, whereas these values are nearly normal in EDTA plasma and in serum kept at 37°C. In addition, immunologic measurements of complement components are normal even in chilled serum. C1 inhibitor (C1 INH) levels also are normal, and precipitating cryoglobulins and cryofibrinogens are absent. Although there have been unreported cases, apparently only 11 patients with CDAC have been reported in the United States (1–4, 10). In contrast, more than 40 such individuals have been described by Japanese investigators (5–9, 11–13). Many of these patients had chronic liver disease, and 5 of 574 patients with primary renal disease had CDAC (9). In addition, Akasaki *et al.* (9) found evidence of CDAC in 5 of 8100 apparently healthy Japanese.

CASE REPORTS

Patient 1

Patient 1 was a 66-year-old woman who was initially seen by us in 1985 because of swelling and burning of the tongue. The complex medical history included hyperparathyroidism with resection of a parathyroid adenoma, thyroid nodule resection, splenectomy for splenic artery aneurysm, border-

0271-9142/92/0900-0362$06.50/0

Table I. Initial Complement Measurements on Sera of Patients 1 and 2[a]

	Patient 1	Normal	Patient 2	Normal[b]
CH_{50}	<22 u	104–188	0	64
C1q	24.3 mg/dl	14–20		
C3	153 mg/dl	80–231	86 mg/dl	80–231
C4	25 mg/dl	10–46	20 mg/dl	10–46
C1 INH	15.5 mg/dl	11–23		
Functional C1 INH	51,200	51,000	12,800	12,800
Hemolytic complement titers				
C1	32,000	256,000	32,000	64,000
C2	12,500	12,800	1,200	6,400
C3	64,000	96,000	4,800	6,400
C4	0	64,000	24	24,000
C5	96,000	192,000	96,000	64,000
C6	128,000	128,000	64,000	64,000
C7	128,000	128,000	64,000	64,000
C8	64,000	128,000	48,000	48,000
C9	16,000	16,000	24,000	16,000

[a]Tests performed elsewhere before being evaluated by us.
[b]Normal functional serum titers can vary on different days.

line polycythemia, a previous (but unsubstantiated) diagnosis of von Willebrand's disease, glaucoma, and hypertension. Aside from multiple surgical scars, the physical examination was unremarkable; the tongue appeared normal at that time.

Considering hereditary angioedema as a possible cause of the tongue swelling, a gastroenterologist had obtained C3 and C4 levels and a CH_{50}. The former were normal, but the CH_{50} was immeasurably low. These tests were repeated together with other measurements noted in Table I. The most striking result was the absence of measurable hemolytic C4 activity in the presence of a normal C4 level as measured immunologically. Subsequent tests showed similar results except that hemolytic C2 was only one-fourth of control levels.

The following additional abnormalities have been noted: WBC of 13,500, with 63% lymphocytes and increased platelets; Hgb, Hct, RBC, red cell mass, and total blood volume indicative of mild polycythemia; serum protein electrophoresis showing a polyclonal increase in γ-globulins; IgG elevated to 1750 mg/dl and IgA elevated to 524 mg/dl, with a normal IgM and IgE; IgG subclass measurements showing only IgG1 to be increased; C3b increased on immunofixation but normal alternative pathway activation following inulin stimulation; C1q binding of 14% (borderline, 13–16%); a Raji cell test of 254 μg AHG Eq/ml (normal, <50); and a rheumatoid factor titer of 1:20 in chilled serum and negative at 1:20 in 37°C serum.

The most relevant normal or negative tests were the urinalysis, erythrocyte sedimentation rate (3 mm/hr), C-reactive protein, antinuclear antibody, anti-DNA, hepatitis B antigens and antibodies, virus antibody screen, and cold agglutinins. In three tests there was an absence of cryoglobulin, cryofibrinogen, or heparin-precipitable cryoprotein after 7 days' observation and no decrease in C4 binding protein. Alpha-1 protease inhibitor and antithrombin III levels were normal. Tests for antimitochondrial, anti-smooth muscle, anti-parietal cell, anti-thyroglobulin, and anti-microsomal antibodies have been negative, as well as tests for von Willebrand's disease and coagulation abnormalities. Most liver function tests were normal. Subsequently a diagnosis of probable lymphocytic leukemia has been made based on persisting lymphocytosis and 5% of cells in the bone marrow appearing to be lymphoblasts. Flow cytometry in 1986 showed a T cell lymphocytosis with atypical surface phenotype. The patient has not experienced significant adverse symptoms on exposure to cold.

Patient 2

Patient 2, a 27-year-old married female, had fortuitously been found in 1984 to have an immeasurably low CH_{50} level on three occasions when she served as a control for complement tests. Her antinuclear antibody test had been positive at a dilution of 1:320 (homogeneous pattern), she had been experiencing Raynaud's phenomenon for about 10 years, and during the preceding year she had a marked facial reaction to sun exposure. The medical history included recurrent urinary tract

infections with pyelonephritis, recurrent staphylococcal infections including septic arthritis of the left knee, perirectal abscess, and deep venous thromboses. Physical examination was essentially within normal limits except for evidence of mitral valve prolapse. She was brought to our attention when we presented patient 1 at a local conference.

Complement measurements (Table I) showed strikingly decreased CH_{50} and hemolytic C4 values in the presence of normal C4 levels as measured immunologically. Subsequent immunologic measurements of C1q and C1 inhibitor were normal, and cryoglobulins could not be demonstrated in two tests lasting 7 days. Supporting a clinical diagnosis of probable systemic lupus erythematosus, the homogeneous ANA test titer had risen to 1:1280 by 1987, and DNA binding by the Farr test was elevated at 28%. Tests for anti-ENA antibodies have been positive, but anti-RNP, anti-Sm, anti-Ro, and anti-La tests have been negative. Other negative or normal results have included anti-cardiolipin antibodies, VDRL, WBC and differential, hepatitis B surface antigen, and nasal and rectal swabs for *Staphylococcus aureus*. The patient's parents and two siblings had been found to have normal CH_{50} levels and normal C4, C3, and C1 inhibitor values when measured immunologically.

More recently patient 2 has experienced recurrent arthralgias and episodes of pleuritic chest pain, and there have been two spontaneous abortions. Synovectomy of the left knee has been followed by the placement of a total knee prosthesis. Following sun exposure in 1990 she had a severe exacerbation of her disease including neurological symptoms. Exposure to cold has not produced difficulty other than Raynaud's phenomenon.

MATERIALS AND METHODS

Some blood samples were clotted for 1 hr at 4°C, centrifuged at that temperature, snap-frozen in aliquots in liquid N_2, and stored at −70°C; other blood samples were placed in tubes prewarmed to 37°C, transported to the laboratory in warmed sand at 37°C, centrifuged at 37°C, and immediately snap-frozen in aliquots; and additional blood samples were collected in tubes containing EDTA or heparin and the plasma snap-frozen in aliquots after centrifugation at 4°C. CH_{50}, hemolytic C4, and hemolytic C1 were assayed according to the methods of Kabat and Mayer (15), Cooper and Muller-Eberhard (16), and Borsos and Rapp (17), respectively. Similar procedures were used in another laboratory in the tests reported in Table I. Our hemolytic C4 values are based on reciprocal serum dilutions where $Z = 1$ (16). C4a and C3a were determined by radioimmunoassay using an Upjohn Co. kit, and C4d/C4 ratios were measured by rocket immunoelectrophoresis as described by Milgrom *et al.* (18). Experiments involving mixtures of serum containing CDAC factor(s), or precipitates therefrom, and normal serum employed the procedure of Atkinson *et al.* (10).

$C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$ Assays

Anti-C1s serum was obtained from a goat immunized with C1s purified by the method of Sim (19), the antiserum being purified by affinity chromatography. C1 INH was purified by the method of Salvesen *et al.* (20). $C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$ complexes were measured by the enzyme-linked immunosorbent assay (ELISA) procedure described by Harpel and Cooper (21) and subsequently modified according to the method of Nilsson and Back (22). Briefly, ELISA plates were coated overnight at 4°C with 1 μg/ml of the affinity-purified goat anti-C1s or $F(ab')_2$ fragments therefrom. Following blocking with gelatin solution, test serum diluted in phosphate-buffered saline (PBS)–Tween with 20 m*M* EDTA was introduced into the wells, and after overnight incubation at 4°C $C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$ complexes were quantitated following sequential reactions with 2 μg/ml mouse monoclonal anti-C1 INH, 1:5500 human adsorbed peroxidase-conjugated goat anti-mouse IgG serum (Caltag Labs, San Francisco, CA), and enzyme substrate. Control wells were coated with normal goat IgG. In agreement with Nilsson *et al.* (22), it was found that in order to compare results at different serum dilutions, it is necessary to precipitate out inhibitory C1qrs complexes by the addition of polyethylene glycol (PEG) 6000 to a final concentration of 6% (w/v) before diluting the serum with EDTA-containing buffer. Resulting OD readings were compared with a linear standard curve generated by serial dilutions of PEG-precipitated normal human serum which had been activated by 1 mg/ml of heat-aggregated γ-globulin and which had arbitrarily been assigned a value of 1000 units (23). The intraassay coefficient of variation averaged ±5.9%, and previous freezing and thawing of sera three times did not influence the results.

Protein G Adsorption Tests

Protein G–Sepharose was purchased from Pharmacia (Piscataway, NJ). Patient or control EDTA plasma was agitated for 30 min at room temperature (RT) with a double volume of washed protein G–Sepharose beads or anti-human albumin-conjugated Sepharose. $CaCl_2$ was added to the supernatants to a concentration of 14 m*M*. After removing the clots, the tubes were incubated for 2.5 hr at 12°C and divided into aliquots for C4d/C4 and $C1\bar{r}$–$C1\bar{s}$–$(C1\ INH)_2$ tests. Controls included unadsorbed plasma.

Inhibition by Heat and Restoration with C1

A redissolved euglobulin precipitate of normal serum was the source of the C1 used in this test as well as in the hemolytic assays. Aliquots of patient 1 serum obtained at 37°C were refrozen at once or were incubated for 2 hr at 4°C before refreezing. Other aliquots were heated at 56°C for 15 min before being either immediately refrozen, incubated for 2 hr at 4°C, incubated for 2 hr at 4°C following the addition of C1, or incubated for 2 hr at 4°C after the addition of C1 which had previously been heated at 56°C for 30 min. The C1 was dissolved in PBS containing 0.15 m*M* $CaCl_2$, and either this or buffer alone was added to 3 vol of serum in all aliquots. The final concentration of reconstituted C1 approximated that of normal serum. The various aliquots were subdivided for subsequent testing for C4d/C4 ratio, $C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$ content and hemolytic C4.

RESULTS

Recognition of CDAC

Attention initially was focused on the striking discrepancy between patient 1's total absence of C4 hemolytic activity and the presence of normal C4 levels as measured immunologically. Considering the possibility of an unusual C4 abnormality (e.g., abnormally glycosylated or incompletely processed pro-C4), sera from eight family members were tested for CH_{50}, hemolytic C4, and C4 antigen, but all were normal. Activation of the classical pathway would be the most obvious possibility, but lack of clinical evidence for immune complex disease, normal ESR, absence of precipitable cryoproteins, normal levels of C1q, C4, and C3 antigen, nearly normal levels of hemolytic C2, and exclusion of C1 inhibitor deficiency at first detracted from this consideration. However, measurement of C4a and C3a serum levels provided striking evidence of C4 and C3 cleavage, but unlike most cases of *in vivo* complement activation, the total activatable C4 and C3 levels also were somewhat above normal (Table IIA). Although there was a substantial amount of C3a detected, the percentage C3 cleavage was lower than C4 cleavage as is common in fluid-phase classical complement pathway activation. Of even greater interest was the concomitant normal C4a level measured in EDTA plasma, and subsequently it was shown that CH_{50} and hemolytic C2 levels also were normal in patient 1's EDTA or citrate plasma. These data suggested the diagnosis of CDAC. It resulted from the procedure at that time of sending blood for serum hemolytic complement levels to the pathology laboratory on ice, whereas in EDTA plasma C1 activation is inhibited by the chelation of Ca^{2+} by EDTA.

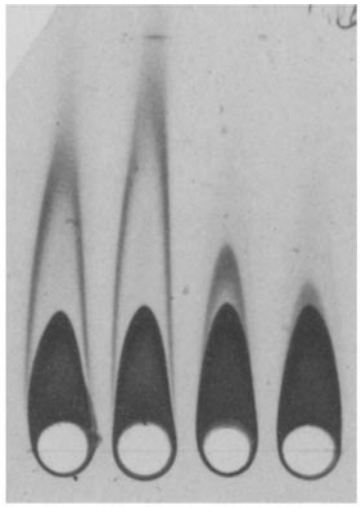
Table II. Anaphylatoxin Measurements by Radioimmunoassay

	C4a (ng/ml)	C3a (ng/ml)
A. Specimens collected at 4°C		
Patient 1 serum	16,980 (96)[a]	51,250 (31)
Normal serum	1,013 (8.7)	5,110 (4.3)
Patient 1 serum + zymosan + aggregated IgG	17,756	165,000
Normal serum + zymosan + aggregated IgG	11,620	119,000
Patient 1 EDTA plasma	181	202
Normal EDTA plasma	175	97
B. Patient 1 specimens collected at 37°C		
Hours incubated at 37°C		
0	289 (1.3)	4,600 (7.7)
1.5	465 (2.1)	5,016 (8.4)
3	959 (4.3)	3,760 (6.3)
Hours incubated at 4°C		
0	587 (2.6)	3,280 (5.5)
1.5	4,413 (19.6)	7,145 (11.9)
3	23,092 (100)	8,983 (15.0)

[a]Percentage of activatable C4 or C3.

Testing for CDAC

In general, documenting CDAC involves demonstrating functional classical pathway activation in chilled serum but not in (a) cold EDTA plasma or (b) serum obtained from blood maintained at 37°C. The latter requires meticulous specimen collection (see Materials and Methods) but permits one to study mechanisms of CDAC by subsequent con-



Incubation	4°C	13°C	24°C	none
C4d/C4 ratio	2.37	3.06	1.19	1.13
$C1\bar{s}$-$C1\bar{r}$-(C1 INH)$_2$	337[a]	1157	177	85
Hemolytic C4	20[b]	<5	90	100

[a] ELISA units
[b] % of normal

Fig. 1. Rocket immunoelectrophoresis for C4d/C4 ratio, $C1\bar{s}$–$C1\bar{r}$–(C1 INH)$_2$ ELISA measurements and hemolytic C4 titers on freshly thawed patient 1 37°C serum (12-8-86) before and after 4 hr at 4, 13, and 24°C.

trolled activation in the cold. As in some previously reported cases, our attention was directed to a complement abnormality by finding an unexpectedly low CH_{50} level, and subsequent CH_{50} measurements in warm and chilled serum and in EDTA plasma yielded a pattern typical for CDAC. The CH_{50} levels in chilled serum from patient 1 all were <22 u (N = 6), 37°C serum levels were 110 ± 13 u (N = 7), and EDTA plasma levels were 78 and 105 u. However, other methods of measuring complement activation provide more incisive evidence of classical pathway activation, greater sensitivity, or more convenience.

Figure 1 shows measurements of CDAC activity in freshly thawed serum from patient 1 obtained on 12-8-86 before and after incubation for 4 hr at 24, 13, or 4°C. Measurements were performed by rocket immunoelectrophoresis (IEP) for C4d generation, hemolytic C4 titration, and $C1\bar{s}$–$C1\bar{r}$–(C1 INH)$_2$ complex testing. Quantitation of IEP results was

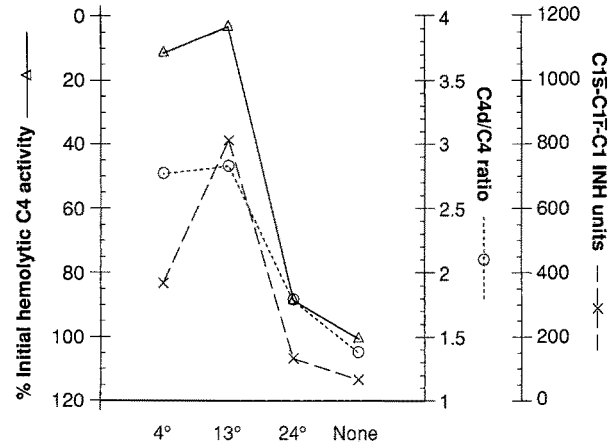


Fig. 2. Measurements of percentage initial hemolytic activity (△), C4d/C4 ratio (○), and $C1\bar{s}$–$C1\bar{r}$–(C1 INH)$_2$ ELISA units (X) before and after incubating patient 1 serum obtained at 37°C (4-12-88) for 4 hr at 24, 13, or 4°C.

achieved by measuring planimetrically the area encompassed by the upper C4d rocket and the area of the more sharply delineated C4 rocket; normally the C4d/C4 ratio is <1.2 (18). For comparison with the other data, the hemolytic C4 titers are expressed as a percentage of the unincubated specimen value. Figure 2 shows similar evidence for CDAC activity as assessed by these three tests in serum obtained on 4-12-88; additional tests yielded the same results on serum obtained on 4-4-90. The finding of greater CDAC activity at 13 than at 4°C was confirmed in five additional experiments including three using patient 2 serum. Earlier observations employing CH_{50} measurements of serum from patient 1 showed no evidence of CDAC at 32 or 29°C, but beginning complement activation was observed at 26°C. Serum obtained from patient 1 at 37°C required chilling for 3 hr at 4°C to achieve maximal complement activation. As shown in Table IIB, measurements of C4a and C3a generation in chilled patient 1 serum or recalcified EDTA plasma yielded results similar to those of the previously mentioned tests. Normal serum does not show this response to cold. Patient 2 serum also showed greater C4a generation at 4 than at 37°C, while C4a and C3a levels in EDTA plasma were significantly lower (data not shown). Another test for CDAC factor(s) is to demonstrate its capacity to activate complement in normal serum after mixing in the cold (10). The higher level of $C1\bar{s}$–$C1\bar{r}$–(C1 INH)$_2$ complexes and lower hemolytic C4 values seen in column 3 in Table III than in column 5 indicate that activation of normal serum complement has occurred. The fact

Table III. Activation of Normal Serum Complement by Incubation with CDAC Serum at 4°C for 24 hr

	(1) KMs[a] + VB^{++}[b]	(2) Pt. 1[c] + VB^{++}	(3) Pt. 1s + KMs	(4) BZs[a] + KMs	(5) (1 + 2)
C4d/C4 ratio	1.0	2.33	>2.62	1.0	
$C1\bar{s}$-$C1\bar{r}$-$(C1\ INH)_2$ (ELISA units)	33	470	774	25	503
Hemolytic C4 (% normal)	49	0	<2	79	49

[a]Normal serum.
[b]Veronal buffer with Ca and Mg.
[c]CDAC patient serum.

that the C4d/C4 ratio in column 3 exceeds the average of columns 1 and 2 also supports this conclusion. Similar results were obtained in six other experiments.

Assessing the Mechanisms of CDAC

Supporting previous data suggesting that the CDAC factor(s) is IgG (10), Table IV shows results representative of three experiments in which patient 1 plasma and control EDTA plasma were batched-adsorbed by protein G–Sepharose. In other experiments it was shown that more than 99% of the IgG was removed by this procedure. Results of both the C4/C4d and the $C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$ tests indicate an accompanying marked loss in CDAC activity which was much greater than that following adsorption with anti-human serum albumin Sepharose. It was necessary to do these adsorptions in EDTA plasma (followed by recalcification) rather than in serum, because the latter procedure resulted in significant complement activation.

Table IV. Adsorption of CDAC Factor(s) from EDTA Plasma by Protein G–Sepharose

	Unadsorbed		Adsorbed	
	25°C	12°C	Protein G, 12°C	Anti-albumin, 12°C
Patient 1[a]				
C4d/C4 ratio	1.05	2.17	1.10	1.41
$C1\bar{s}$-$C1\bar{r}$-$(C1\ INH)_2$	0.436[b]	1.440	0.322	0.900
Normal[a]				
C4d/C4 ratio	1.13	1.14	1.15	
$C1\bar{s}$-$C1\bar{r}$-$(C1\ INH)_2$	0.589	0.454	0.382	

[a]EDTA plasma recalcified after adsorption.
[b]OD value.

Table V shows the results of an experiment in which patient 1 and normal sera were precipitated by 2.5% PEG in PBS with 20 m*M* EDTA for 2 hr at 4°C. After washing with 2.5% PEG in PBS, the precipitates were (largely) dissolved in 37°C Veronal buffer, mixed with normal serum, incubated for 22 hr at 4°C, and aliquoted for C4d/C4, $C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$, and hemolytic C4 assays. As shown in Table V, results from all three tests showed substantial complement activation at 4°C after mixing the redissolved PEG precipitate from patient 1 with normal serum, whereas the PEG precipitate from normal serum (B.Z.) did not have this effect. This indicates that the CDAC factor(s) is included among the substances precipitated by 2.5% PEG.

Table V. Activation of Complement in Normal Serum by 2.5% Polyethylene Glycol (PEG) Precipitate of Patient 1 Serum

	Pt. 1 ppt.[a–c]	Pt. 1 ppt. + VB^{++}[d]	KMs[e] + Pt. 1 ppt[a]	KMs + VB^{++}	KMs + N1s ppt.[f]	N1s ppt.[f] + VB^{++}	KMs[b,c]
C4d/C4 ratio	ND[g]	ND	2.12	1.0	1.0		1.0
$C1\bar{s}$-$C1\bar{r}$-$(C1\ INH)_2$ (ELISA units)	0	0	428	33	38	0	31
Hemolytic C4 (% normal)		0	<1	49	46	0	86

[a]Redissolved patient 1 PEG precipitate.
[b]Not incubated at 4°C. All others incubated for 24 hr at 4°C.
[c]Undiluted.
[d]Equal volume of Veronal buffer with Ca^{2+} and Mg^{2+}.
[e]Normal serum.
[f]Redissolved PEG precipitate from another normal serum (BZ).
[g]Not detectable; rockets too small to quantitate.

Table VI. Inhibition of CDAC by Heat and Restoration by C1

	Unheated serum		Heated 15 min at 56°C			
	Not incubated	2 hr at 4°C	Not incubated	2 hr at 4°C	2 hr at 4°C + C1	2 hr at 4°C + heated C1
C4d/C4 ratio	1.04	1.83	1.77	1.81	2.67	1.88
$C1\bar{s}$-$C1\bar{r}$-$(C1\ INH)_2$	46[a]	121	~5	~5	105	9
Hemolytic C4 (% normal)	66	21	12	11	~0	12

[a]ELISA units.

In view of the significantly low levels of hemolytic C4 in our patients' sera and the variable and sometimes almost-normal levels of hemolytic C2 and C1, consideration was given to the possible presence of a cold-reactive autoantibody to C4 causing secondary activation of the classical pathway. This was explored by a series of more than 30 ELISA tests using wells coated with human C4 or with goat anti-human C4, the latter being employed to test for possible IgG:C4 complexes. Patient 1 serum usually gave higher OD readings than control sera, but it also gave higher OD measurements in wells coated with various heterologous materials: specific autoantibodies to C4 could not be demonstrated.

Considering the possibility that CDAC might have a direct effect on C4 other than through an autoantibody mechanism, the effect of heating patient 1 serum at 56°C for 15 min was examined. As expected, heating per se caused some C4 cleavage and loss of C4 hemolytic activity in addition to loss of CDAC (Table VI), but CDAC activity was restored by the addition of C1 but not by heat inactivated C1. This requirement for C1, which was reproduced in another experiment, is characteristic for classical complement pathway activation and opposes a direct effect of CDAC factor(s) on C4 or C4 binding protein.

Effect of a CDAC Factor(s) on Other Tests

Since CDAC factor(s) may be a form of aggregated IgG, patient 1 serum obtained at 37°C was incubated for 3 or 5 hr at either 4 or 37°C, and the effect on Raji cell tests was noted. The positive results, averaging 197 μg AHG Eq/ml (normal, <50 AHG Eq/ml), were essentially the same after incubation at either temperature. Likewise chilling the serum for 3 or 5 hr at 4°C did not change the borderline results of patient 1's C1q binding assay. Since FACS analyses involve incubating cells in the cold and about 26% of patient 1's lymphoid cells did not demonstrate the usual markers, the effect of incubating normal peripheral WBC with her heparinized plasma for 1.5 hr at 4°C was determined to see if her plasma might interfere with this test. However, there was no change in the percentage of cells expressing CD3, CD4, CD8, or CD19 markers as compared with unincubated cells or cells incubated with normal plasma.

DISCUSSION

Since CDAC is an *in vitro* phenomenon, afflicted individuals do not experience difficulty upon exposure to cold as in some other immune cryopathies such as cryoglobulinemia, cryofibrinogenemia, cold urticaria, or cold hemolysin syndrome. CDAC also clearly differs in mechanism from cases of cold-promoted activation (CPA) of blood coagulation factor VII (24) mediated through contact system activation and from cold-dependent consumption of C3 (25) mediated through the alternative complement pathway. Hishitani *et al.* (26) have reported that the occurrence of a CDAC-type phenomenon in patients with liver disease often is associated with precipitable mixed cryoglobulins. However, centrifugation of serum from our patients 1 and 2 after 7 days at 4°C repeatedly yielded no visible precipitate, and others have failed to find cryoprecipitates in cases not associated with liver disease. Another difference between CDAC and some cases of cryoglobulinemia is a decreased level of C1q and C4 antigen in the latter (27), whereas the lack of immune complex sequestration *in vitro* results in normal values in CDAC even though split products can be detected and hemolytic activity is compromised. Thus, in accord with most other investigators, we have used the term CDAC in a more restricted sense which excludes cryoglobulinemia

and the cold hemolysin syndrome. Perhaps a better term would be "idiopathic cold-dependent activation of complement."

CDAC was initially recognized by discrepant CH_{50} and hemolytic C4 values between serum obtained at 4°C and those measured in EDTA plasma. Here it has been shown that measurements of C4d/C4 ratios, C4a, C3a, and $C1\bar{s}$–$C1\bar{r}$–$(C1\ INH)_2$ complexes also can be used to document CDAC and that results obtained by these various methods parallel each other. No doubt other tests of classical complement pathway activation also could be utilized. The choice of which procedure to use therefore becomes largely a matter of convenience, rocket immunoelectrophoresis for C4d/C4 ratios and CH_{50} measurements being relatively simple. Hemolytic C4 measurements clearly provide the greatest sensitivity, but for some research purposes this can be disadvantageous; e.g., some lots of serum already had low hemolytic C4 values when blood supposedly obtained and processed at 37°C was not meticulously handled, and in some instances there was so much loss of hemolytic C4 activity at 4°C that it was difficult to appreciate the difference from results at 13°C that were more easily recognized by other methods (Fig. 2). This may be one reason why previous investigators have not noted that the optimal temperature for CDAC may be between 4°C and room temperature. Possibly more of the factor(s) responsible for CDAC aggregates at the lower temperature (i.e., 4°C), but consequent complement activation is enhanced at a higher temperature (e.g., 13°C). The data indicating complement activation when normal serum is mixed with CDAC serum at 4°C (Table III) confirm similar previous results of Kitamura *et al.* (8) and Atkinson *et al.* (10). The opportunity to test serum from patient 1 over a period of more than 5 years establishes that CDAC may be more than a transient phenomenon.

Our observation that CDAC activity is reduced by adsorption with streptococcal protein G–Sepharose (Table IV) supports the conclusion of Atkinson *et al.* (10) that it is IgG based on partial purification by ammonium sulfate precipitation at 4°C followed by DEAE column chromatography at room temperature. The additional observation that CDAC activity is precipitated by 2.5% PEG strongly implies that polymeric IgG is involved, since monomeric IgG would not be expected to precipitate significantly at this PEG concentration (28). The presence of CDAC activity in 2.5% PEG precipitates, as shown by mixing with normal serum (Table V), augments the finding of Takemura *et al.* (12) that such precipitates are anticomplementary. In interpreting these results it should be noted that, in addition to precipitating proteins, PEG has been reported to enhance the aggregation of IgG by IgM rheumatoid factor (29). Hishitani *et al.* (26) reported that the precipitable cryoglobulin in their (mainly) liver disease patients which produced features of CDAC was a mixed cryoglobulin consisting of IgM and polyclonal IgG. Patient 1 serum, however, had only minimal rheumatoid factor activity, and the partial purification procedure employed by Atkinson *et al.* (10) should largely exclude IgM. Many monoclonal IgG cryoimmunoglobulins are IgG3, but patient 1 did not have an elevated IgG3 serum level. Accordingly, one may speculate that the factor(s) responsible for CDAC in our patients, and in others without cryoprecipitates, contains polymeric IgG in a form too small or otherwise incapable of precipitating spontaneously in the cold. An alternative would be antigen-containing immune complexes of similar size and physical properties. In patient 1, however, the extensive testing noted above for antibodies to many exogenous and autologous antigens has yielded negative results. Regardless of the exact nature of the CDAC factor(s) in these patients, they appear to act through activation of the classical complement pathway. Unexpectedly high hemolytic C2 levels in relation to hemolytic C4 have also been noted in patients with hereditary angioedema (30) and might be due to reduced cleavage of C2 in the absence of C4. The cascade principle of complement activation of course would predict less evidence for C1 than C4 activation. Autoantibodies to C4 with secondary activation of the classical pathway could not be demonstrated.

ACKNOWLEDGMENTS

The assistance of R. J. Ziccardi, G. D. Till, E. S. Tucker, J. L. Wagner, P. M. Pan, K. Cox, B. Bradt, and C. Kane with various aspects of this work is gratefully acknowledged. The sustained cooperation of both patients also was greatly appreciated.

This work was supported in part by NIH Grants AI10386, AI32834, HL25658, and RR-00833 to the Scripps Clinic and Research Foundation and 5 MD1-RR00042-22 to the university of Michigan Clinical Research Center.

REFERENCES

1. Gewurz H, Baron J, Ratnoff DD, Yachnin S: Coagulation-associated consumption of complement: A case with dissociation between serum and plasma complement activities. J Clin Invest 49:39A, 1970
2. Glovsky MM, Alenty A: Coagulation related deficiencies of early components of C (C1, C4, C2) in human serum. J Immunol 111:305, 1973
3. Gewurz H: Nonimmune activation of C: Two new phenomena. J Immunol 111:305, 1973
4. Glovsky M, Inai S, Nagaki K, Tejima H: Permutations in early components of complement (C1, C4, C2) in man. Fed Proc 33:647, 1974
5. Inai S, Kitamura H, Fujita T, Kohima J, Nasaki K: Differences between plasma and serum complement in patients with chronic liver disease. Clin Exp Immunol 25:403–409, 1976
6. Kitamura H, Nagaki K, Inai S: Cold activation of the classical complement pathway found in patients with chronic liver disease. J Immunol 116:1738–1739, 1976
7. Kondo M, Hosokawa K, Masuda M: Cold activation of complement. I. Presence of coagulation-related activator. J Immunol 117:486–490, 1976
8. Kitamura H, Nagaki K, Inoshita K, Iida K, Inai S: The cold activation of the classical complement pathway: The cause of the differences between plasma and serum complement in liver cirrhosis. Clin Exp Immunol 27:34–37, 1977
9. Akagaki Y, Fujiwara Y, Nakanishi I, *et al.*: The difference between serum and plasma complement activity in primary renal disease. Clin Nephrol 14:75–80, 1980
10. Atkinson JP, Gorman JC, Curd J, *et al.*: Cold dependent activation of complement in systemic lupus erythematosus. A unique cause for a discrepancy between clinical and laboratory parameters. Arth Rheum 24:592–601, 1981
11. Matsuo T, Matsuoka Y, Umegaki K: Studies on inhibition of cold activation of complement. Jap J Clin Pathol 30:543–546, 1982
12. Takemura S, Hotta T, Matsumura N, Yoshikawa T, Kondo M: Cold activation of complement. Arth Rheum 25:1138–1140, 1982
13. Yukiyama Y, Yoshida K, Hirose S: Complement activation at low temperature. Arerugi 33:275–281, 1984
14. Newell S, Gorman JC, Bell E, Atkinson JP: Hemolytic and antigenic measurements of complement. A comparison of serum and plasma samples in normal individuals and patients. J Lab Clin Med 100:437–444, 1982
15. Kabat EA, Mayer MM: Experimental Immunochemistry, Springfield, IL, Thomas, 1967, p 133
16. Cooper NR, Muller-Eberhard HJ: A comparison of methods for the molecular quantitation of the fourth component of complement. Immunochemistry 5:155–169, 1968
17. Borsos T, Rapp HJ: Chromatographic separation of the first component of complement and its assay on a molecular basis. J Immunol 91:851–858, 1963
18. Milgrom H, Curd JG, Kaplan RA, Muller-Eberhard HJ, Vaughan JH: Activation of the fourth component of complement (C4): Assessment by rocket immunoelectrophoresis and correlation with the metabolism of C4. J Immunol 124:2780–2785, 1980
19. Sim RB: The human complement system serine proteases C1r and C1s and their proenzymes. Methods Enzymol 80:26–42, 1981
20. Salvesen GS, Catauese JM, Kress LF, Travis J: Primary structure of the reactive site of human C1-inhibitor. J Biol Chem 260:2432–2436, 1985
21. Harpel PC, Cooper NR: Circulating C1 inactivator-C1s-C1r complexes: Quantification by an enzyme-linked differential antibody immunosorbent assay. Clin Res 30:563A, 1982
22. Nilsson T, Back O: Determination of C1s-C1 inhibitor complexes in plasma by means of an enzyme linked immunosorbent assay. Clin Exp Immunol 60:178–182, 1985
23. Waldo FB, West CD: Quantitation of $(C1\ INH)_2$ C1r-C1s complexes in glomerulonephritis as an indicator of C1 activation. Clin Immunol Immunopathol 42:239–249, 1987
24. Gzendlik C, Lammle B, Duckert F: Cold promoted activation and factor VII, prekallikrein and C1 inhibitor. Thrombos Haemostas 53:242–244, 1985
25. Day NK, Geiger H, McLean R, Resnick J, Michael A, Good RA: The association of respiratory infection, recurrent hematuria, and focal glomerulonephritis with activation of the complement system in the cold. J Clin Invest 52:1698–1706, 1973
26. Hishitani Y, Inoi M, Kakehi K, Mozai T: Cold dependent activation of complement by mixed cryoglobulin. Bull Osaka Med School 28:83–92, 1982
27. Corvetta A, Spaeth PJ, Ghirelli PA, *et al.*: Complement activation and impaired capacity to solubilize immune complexes or to prevent their formation in essential mixed cryoglobulinemia. Diag Immunol 1:315–323, 1983
28. Digeon M, Laver M, Riza J, Bach JF: Detection of circulating immune complexes in human sera by simplified assays with polyethylene glycol. J Immunol Methods 16:165–183, 1977
29. Faaber P, van den Bersselaae LAM, van de Putte L, van den Berg WB: Circulating immune complexes and rheumatoid arthritis: the induction of complex formation between rheumatoid factor and IgG by polyethylene glycol. J Rheumat 16:1304–1309, 1989
30. Gronski P, Bodenbender L, Kanzy E-J, Seiler FR: C4-binding protein prevents spontaneous cleavage of C3 in sera of patients with hereditary angioedema. Complement 5:1–12, 1988