Clin. exp. Immunol. (1979) 37, 310–322.

Studies on the nature of heat-labile anti-complementary activity in normal human serum

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(*Accepted for publication* 27 *November* 1978)

SUMMARY

Heat-labile anti-complementary activity (ACA) appears in normal human serum during storage or heating as endogenous haemolytic activity disappears. Following gel filtration of unheated serum, two peaks of heat-labile ACA are present. The ACA of both whole and fractionated serum has previously been attributed to the presence of heat-labile immunoglobulin aggregates or immune complexes. Our data demonstrate that the heavy peak of ACA obtained by gel filtration does not bind to ^{125}I-Clq or to Raji cells, and that its effect is abolished by C$\bar{1}$INH, suggesting that it represents C$\bar{1}$ rather than immunoglobulin aggregates or immune complexes. The lighter peak of ACA in fractionated serum has the functional characteristics of C$\bar{1}$s and free C$\bar{1}$s is demonstrable in fractions containing this activity. The ACA of whole serum likewise has functional characteristics of C$\bar{1}$. The anti-complementary effect of C$\bar{1}$ on guinea-pig complement would not be evident in the complement fixation assay until most endogenous haemolytic activity in human serum has been inactivated, either by heat or by storage. C$\bar{1}$INH only partially inhibits this ACA in serum or in solutions containing isolated C$\bar{1}$ in high concentrations. These observations indicate that heat-labile ACA in whole or fractionated sera is due to the presence of C$\bar{1}$ and C$\bar{1}$s and that this activity cannot be taken as evidence for the presence of immune complexes.

INTRODUCTION

Normal human serum develops heat-labile anti-complementary activity (ACA) during storage or heating as endogenous haemolytic activity disappears. This ACA, which has been recognized since the turn of the century, has frequently been a source of speculation in the medical literature (Noguchi, 1906). Erlich & Sachs (1902) first proposed that heat transforms complement into a 'complementoid' with a higher avidity for sensitized erythrocytes than that possessed by complement itself. In 1911, Zinsser & Johnson reported that heat-labile ACA also appears in unheated serum during storage as endogenous haemolytic activity disappears and that this activity is due to a globulin whose activity is abolished by heating at 56°C. Subsequent studies demonstrated that ACA develops in stored serum even when bacterial contamination is prevented (Kyutoku, 1919).

Unheated human serum, when fractionated by gel filtration chromatography, contains two peaks of ACA (Castanedo & Williams, 1967; Nielsen & Svehag, 1976). ACA in the higher molecular weight peak has been attributed both to heat-labile immunoglobulin aggregates in normal serum (Nielsen & Svehag, 1976) and to immune complexes in pathological serum (Hodgson, Potter & Jewell, 1977; Hilton, Moore & Howat, 1978). Castenedo & Williams (1967) suggested that the lighter ACA, which elutes between IgG and albumin, represents immunoglobulin which aggregates following gel filtration. The ACA which appears in normal serum during heating has also been attributed to immunoglobulin aggregates (Nielsen & Svehag, 1976).

Immunoglobulin aggregates, if actually present in normal serum, would be expected to alter the results

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obtained in a variety of immunological assays including those which measure immune complexes or which use cells possessing Fc receptors. If these proposed immunoglobulin aggregates develop *in vivo*, their presence might lead to the same pathological effects as those of immune complexes. Our investigations were conducted to define the nature of heat-labile ACA in whole and fractionated normal serum. The results demonstrate that this ACA is not due to immunoglobulin aggregates and suggest that it can be explained by the presence of activated $C1^{hu}$ ($C\bar{1}^{hu}$) and Cls ($C\bar{1}s$).

MATERIALS AND METHODS

Serum. Normal serum from healthy, fasting donors was stored in small aliquots at −70°C. Serum from a patient with, hereditary angioneurotic oedema (HANE) was kindly provided by Dr M. Blumenthal. Serum from a patient with common variable hypogammaglobulinaemia contained diminished IgM (13 mg%) and IgG (300 mg%) and no detectable IgA.

Complement components. Functionally pure $C\bar{1}^{hu}$, $C\bar{1}$INH and $C2^{hu}$ were purchased from Cordis Laboratories (Miami Florida), reconstituted and stored in aliquots at −70°C. Clq was isolated from fresh serum as described previously (Yonemasu & Stroud, 1971).

The euglobulin fraction of serum. This was prepared by dialysing normal human serum at 4°C overnight against 0·005 M phosphate buffer, pH 7·5 The precipitate was collected by centrifugation, washed twice in 0·005 M phosphate buffer, pH 7·5 and dissolved in 0·3 M NaCl 0·005 M phosphate buffer, pH 7·5.

Heat-aggregated human γ-globulin. Cohn fraction II (Cutter Laboratories, Berkeley, California), diluted to 82·5 mg/ml in normal saline, was heated in a water bath at 60°C for 20 min to produce immunoglobulin aggregates.

Buffers. Veronal buffered normal saline, pH 7·4 ($\mu = 0{\cdot}15$) containing $1{\cdot}5 \times 10^{-4}$M calcium and 5×10^{-4}M magnesium (VB^{++}) was used in all experiments unless otherwise specified.

Other reagents. Bovine trypsin, soybean trypsin inhibitor and plasmin were purchased from Sigma Chemical Company (St. Louis, Missouri). Aprotinin and heparin were purchased from Calbiochem (San Diego, California) and Upjohn (Kalamazoo, Michigan) respectively.

Fractionation of serum. Components of human serum were separated by upward flow on 2·6 × 100 cm columns of Sephadex G-200 (Pharmacia, Piscataway, New Jersey) using phosphate buffered normal saline, pH 7·4. Ultrogel AcA 22 (LKB) Stockholm, Sweden) was also used for column chromatography. The protein concentration of the eluted fractions was determined by measuring the absorbance at 280 nm. IgM, IgG, albumin and Cls were assayed by immunodiffusion in agar gel using commercial antisera (Behring Diagnostics, Sommerville, New Jersey).

Complement fixation assay. This assay was modified from the method of Johnson, Mowbray & Porter (1975) and has been described in detail elsewhere (Soltis *et al.*, 1979a). Results were expressed as a percentage of haemolysis using control tubes which contained VB^{++} in place of serum as 100%.

^{125}I-Clq binding test. This was performed as described previously (Zubler *et al.*, 1976).

The Raji cell radioimmunoassay. This was kindly performed by Drs Sandra Ristow and C. McKhann of the Department of Surgery, University of Minnesota.

Titration of haemolytically active $C1^{hu}$. This was performed as described by Rapp & Borsos (1970) using an $EAC4^{uu}$ intermediate and $C2^{hu}$.

Heat inactivation of serum This was conducted at either 53°C or 56°C. Heating at 53°C for 90 min. effectively inactivates endogenous haemolytic activity while producing less aggregation of immunoglobulin than does heating at 56°C (Soltis *et al.*, 1979a).

RESULTS

ACA of fractionated, unheated human serum

Two peaks of ACA were found when fresh normal serum or serum stored at −70°C was fractionated by gel filtration. The heavier peak eluted with the void volume from Sephadex G-200. When Ultrogel AcA 22 was used, this heavy ACA eluted between IgM and IgG as an intermediate sized substance (Fig. 1). The lighter peak eluted after the main portion of IgG and before albumin from Sephadex G-200 and with the latter part of the large protein peak containing albumin and IgG from Ultrogel AcA 22. Both peaks were heat-labile at 53°C and 56°C. In order to determine whether fractions eluting between these two peaks contained ACA which was masked by haemolytic activity, aliquots were heated at 53°C from 0 to 90 min and tested. No ACA developed.

Binding of serum fractions containing heavy ACA to ^{125}I-C1q and Raji cells

As has previously been suggested (Nielsen & Svehag, 1976) the size of the heavy peak of ACA is

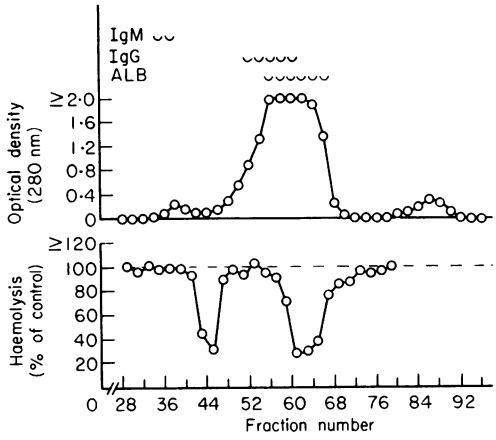

FIG. 1

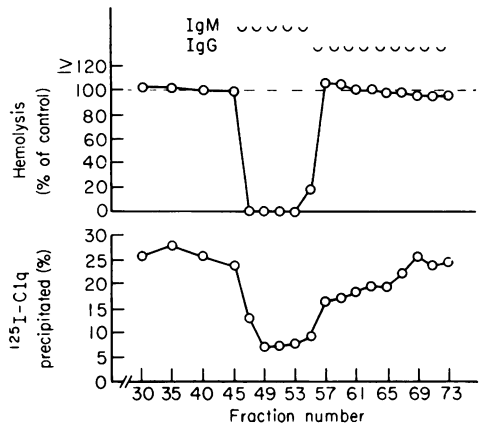

FIG. 2

FIG. 1. Anti-complementary activity (ACA) of unheated normal serum fractionated by gel filtration on Ultrogel AcA 22. 50 μl of the fractions were tested for ACA without heat inactivation. ACA was present in two peaks. Heavy ACA eluted between IgM and IgG and light ACA eluted in the major protein peak with albumin.

FIG. 2. ^{125}I-Clq binding activity of fractions of unheated normal serum obtained by gel filtration chromatography on Sephadex G-200 compared to the anti-complementary activity of the same fractions tested in the complement fixation assay. In contrast to what would be expected for immunoglobulin aggregates, anti-complementary fractions bound less ^{125}I-Clq than did other fractions.

compatible with immunoglobulin aggregates. Immunoglobulin aggregates, to be anti-complementary in the complement fixation assay, should also bind to ^{125}I-Clq. Fractions of unheated normal serum containing heavy ACA, however, actually bound less ^{125}I-Clq than did other fractions (Fig. 2).

The Raji cell radioimmunoassay detects IgG aggregates or IgG- containing immune complexes by their binding to receptors on lymphoblastoid cells (Theofilopoulos, Wilson & Dixon, 1976). No binding was found when fractions containing heavy ACA were tested. The failure of the heavy ACA to react with Raji cells or ^{125}I- Clq suggests that heavy ACA is not due to immunoglobulin aggregates.

Comparison of heavy ACA with $C\bar{1}^{hu}$

Sephadex is known to activate Cl^{hu} (Laurell & Siboo, 1966) and this macromolecule elutes between IgM and IgG on gel filtration chromatography. Since $C\bar{1}$INH, which regulates the activity of $C\bar{1}^{hu}$ in serum, would elute later, the activity of $C\bar{1}^{hu}$ in the eluate would be unopposed. Fluid phase inactivation of the guinea-pig complement (GPC) components might be expected in the complement fixation assay when fractions containing $C\bar{1}^{hu}$ are tested, resulting in ACA. To study the possibility that heavy ACA is due to $C\bar{1}^{hu}$, the following three experiments were performed.

Heat lability of heavy ACA and $C\bar{1}^{hu}$

Aliquots containing either heavy ACA, obtained by gel filtration, or two CH_{50} units of functionally pure $C\bar{1}^{hu}$ were heated at 53°C or 56°C for 0 to 90 min (Fig. 3). The heat-lability of both substances was similar at the two temperatures. The ACA of immunoglobulin aggregates, formed by prolonged storage of human γ-globulin under aseptic conditions at 4°C, was not altered by heating at either temperature (data not shown).

Effect of trypsin on heavy ACA, $C\bar{1}^{hu}$*, and aggregated IgG*

Trypsin at low concentrations has been reported to activate Cl^{hu} and, at higher concentrations, to inactivate already activated $C\bar{1}^{hu}$ (Ratnoff & Naff, 1967). The effect of trypsin on heavy ACA, $C\bar{1}^{hu}$ and aggregated IgG was studied by incubation at 37°C. After 30 min, the trypsin was neutralized by the addition of soybean trypsin inhibitor and the samples were tested in the complement fixation assay.

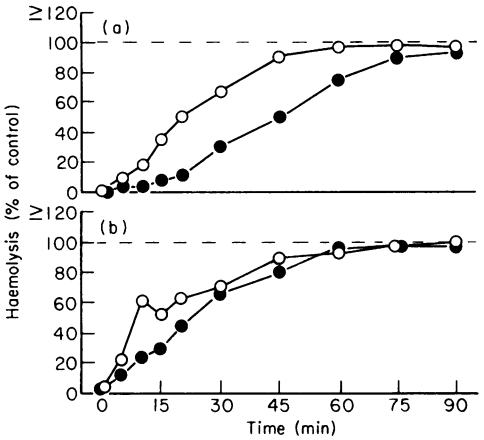

FIG. 3

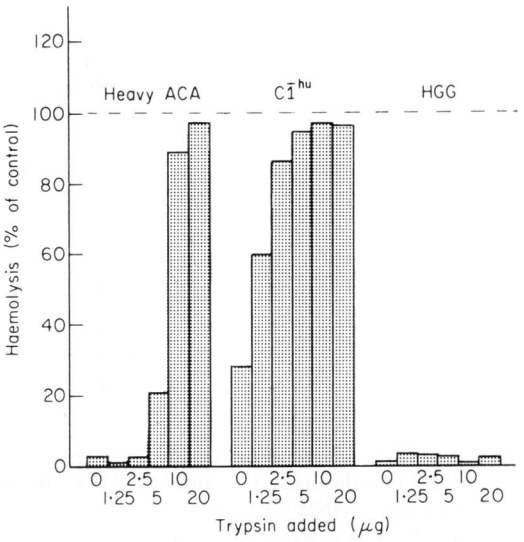

FIG. 4

FIG. 3. Comparison of heat-lability of anti-complementary activity (ACA) of $C\bar{1}^{hu}$ and heavy peak at 53°C and 56°C. 50 μl aliquots of functionally pure $C\bar{1}^{hu}$ containing 2 CH_{50} units (a) and of heavy peak ACA (b) were heated at 53°C (●—●) or at 56°C (○—○). The heat lability of both was similar at the two temperatures.

FIG. 4. The effect of trypsin on anti-complementary activity (ACA) of aliquots of the first peak from Sephadex G-200 (heavy ACA), $C\bar{1}^{hu}$ and aggregated human γ-globulin (HGG), each diluted to be almost totally anti-complementary. 50 μl of the substances tested were incubated with amounts of trypsin varying from 1·25 to 20 μg. After 30 min at 37°C, the trypsin was neutralized by adding 20 μg of soybean trypsin inhibitor and the samples were tested in the complement fixation assay. Control tubes containing trypsin and soybean trypsin inhibitor did not inhibit haemolysis. Pre-incubation with trypsin eliminated the ACA of the heavy peak and $C\bar{1}^{hu}$ but not aggregated γ-globulin.

Pre-incubation with trypsin abolished the ACA of the heavy peak and $C\bar{1}^{hu}$ but had no effect on aggregated IgG (Fig. 4).

Effect of CĪINH on ACA

Heavy ACA is similar to $C\bar{1}^{hu}$ in size, heat-lability, sensitivity to trypsin and its effect in the complement fixation assay. If the heavy ACA were due to the effect of $C\bar{1}^{hu}$ unopposed by CĪINH, the addition of CĪINH to these samples should diminish this anti-complementary effect. Heavy ACA was abolished by preincubation at 37°C for 30 min with 3·125, 6·25 and 12·5 units of CĪINH (Fig. 5). The ACA of 2 CH_{50} units of $C\bar{1}^{hu}$ was also abolished by CĪINH. CĪINH in buffer, at concentrations greater than 12 units, was anti-complementary, as was CĪINH in excess when added either to heavy ACA or $C\bar{1}^{hu}$, suggesting that free CINH exerted an anti-complementary effect on GPC (Gigli, Ruddy & Austen, 1968). These data indicate that heavy ACA is due to $C\bar{1}^{hu}$.

Characterization of light ACA

The lighter anti-complementary peak which eluted from Sephadex G-200 or Ultrogel AcA 22 between IgG and albumin was then characterized. Aliquots of light ACA, heated at 53°C, were similar to $C\bar{1}^{hu}$ in heat lability. The following three possibilities seemed likely to explain the light ACA, based on its size and heat lability. (1) Plasmin, generated from plasminogen during coagulation, has been reported to activate $C1^{hu}$ (Ratnoff & Naff, 1967) and cleave $C3^{hu}$ (Gigli, Ruddy & Austen 1968). Preliminary experiments testing plasmin in buffer at physiological concentrations demonstrated that it was anti-complementary when tested with GPC and that this effect was abolished at 53°C. (2) CĪINH, which is anti-complementary (Fig. 5), heat-labile and inhibits $C\bar{1}^{gp}$ more efficiently than $C\bar{1}^{hu}$ (Gigli *et al.*, 1968). (3) CĪs, which might become dissociated from the native CĪ complex in buffers lacking calcium. To test

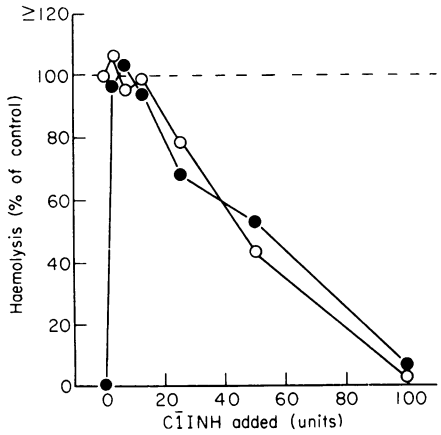

FIG. 5. Anti-complementary activity (ACA) of the first anti-complementary peak obtained by gel filtration (heavy ACA) following incubation with C1INH (●—●). 50 μl aliquots of heavy ACA were incubated with concentrations of C1INH varying from 3·125 to 100 units at 37°C. After 30 min, the samples were tested for ACA. The heavy peak was totally anti-complementary. Between 3·125 and 12·5 units of C1INH abolished the ACA of this material while higher concentrations increased ACA. C1INH in buffer (○—○), at concentrations above 12·5 units, was also anti-complementary.

TABLE 1. Effects of trypsin, C1INH, aprotinin and heparin on light ACA compared with their known actions on C1INH, plasmin and C1s

Substances tested	Expected effects on ACA of			Effect on light ACA
	C1INH	Plasmin	C1s	
Trypsin	↓		↓	↓
C1INH	↑	↓	↓	↓
Aprotinin		↓	?↑	↑
Heparin	↑			—

these three possibilities, aliquots of the light peak were pre-incubated with four different substances (trypsin, C1INH, aprotinin and heparin) which might alter their ACA in differing ways. The expected effects and those actually found are listed in Table 1.

Pre-incubation with trypsin might be expected to diminish the ACA of C1s and C1INH (Ratnoff & Naff, 1967; Harpel, 1970) while having no effect on the ACA of plasmin. Aliquots of the light peak were incubated with trypsin at 37°C; after 30 min the trypsin was neutralized with soybean trypsin inhibitor and residual ACA was tested. Fig. 6a demonstrates that this treatment diminished ACA.

Light ACA was incubated with aliquots of C1INH for 30 min at 37°C. If the light ACA were due to C1INH, additional C1INH would be expected to augment its effect. In contrast, C1INH would reduce the effect of C1s and plasmin (Nagaki & Inai, 1976). C1INH diminished light ACA (Fig. 6b).

Aprotinin (Trasylol), a potent inhibitor of plasmin, would be expected to diminish its ACA. It has also been reported to augment the activity of $C\bar{1}^{hu}$ (Delage, Simard & Lehner-Netsch, 1976). Aprotinin does not have a known effect on C1INH. Fifty μl aliquots of the light peak were pre-incubated at 37°C for 30 min with increasing concentrations of aprotinin (125–1000 units). Concentrations between 125 and 500 units had no effect on ACA, but 1000 units did produce a slight increase in ACA compared to control tubes containing aprotinin, VB^{++} and GPC (data not shown).

Finally, heparin has recently been shown to augment the activity of C1INH (Nagaki & Inai, 1976; Rent et al., 1976). To test its effect, 0·0031–0·05 units of heparin were pre-incubated for 30 min at 37°C with 50 μl aliquots of the light peak. There was no change in the ACA of the samples.

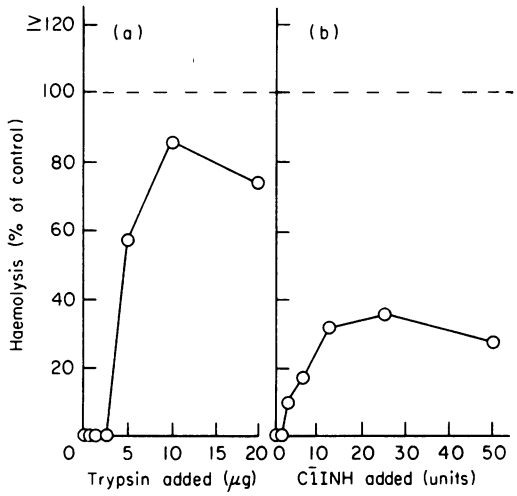

FIG. 6. (a) The effect of trypsin on the light peak of anti-complementary activity (ACA). 50 μl aliquots of the light peak were incubated with varying amounts of trypsin at 37°C. After 30 min the trypsin was neutralized by adding 20 μg soybean trypsin inhibitor. Pre-incubation with trypsin almost completely eliminated the ACA of the light peak. (b) The effect of C1INH on the ACA of the light peak. 50 μl aliquots of the light peak were incubated at 37°C with 0–50 units of C1INH and tested after 30 min. C1INH diminished, but did not eliminate, the ACA of the light peak.

Comparison of these results with those expected for C1INH, plasmin and C1s suggested that light ACA was most likely to be due to the presence of free C1s (Table 1). The presence of C1s in these fractions was demonstrated by immunodiffusion against anti-C1s in agar gel.

ACA in heated normal human serum

The preceding data indicate that ACA in unheated normal serum, when fractionated by gel filtration chromatography, can be explained by $C\bar{1}^{hu}$ and C1s rather than aggregated immunoglobulin. In a previous paper, we showed that ACA also becomes apparent in normal serum heated at temperatures ranging from 47°C to 62°C for up to 90 min, as endogenous haemolytic activity disappears. At 47°C and 50°C, ACA remains after 90 min, while at 53°C this ACA is almost completely inactivated by 90 min. At 56°C and above, ACA diminishes briefly and then progressively increases with continued heating. The late, heat-stable ACA was shown to represent immunoglobulin aggregation (Soltis *et al.*, 1979a).

Comparison of ACA with ^{125}I-C1q binding in normal and hypogammaglobulinaemic sera

Heat-labile ACA in heated serum might develop due to a mechanism different from that found in fractionated unheated serum. Because the heating used to inactivate endogenous haemolytic activity might also induce changes in immunoglobulins, the possibility that this heat-labile ACA might be due to immunoglobulin aggregates was studied. Aliquots of normal and hypogammaglobulinaemic sera were heated at 53°C for 20 min, a time when heat-labile ACA is maximal, and tested using the complement fixation and ^{125}I-Clq binding assays. The data, compared with those obtained from unheated serum and after heating at 56°C for 90 min, are shown in Fig. 7. A marked disparity was found between results obtained with the two assays. Heating at 56°C for 90 min, which produces immunoglobulin aggregates (Soltis *et al.*, 1979a), resulted in increased ^{125}I-Clq binding while producing only minimal inhibition of haemolysis. In contrast, heating at 53°C for 20 min produced no increase in ^{125}I-Clq binding compared to unheated serum, but led to a marked inhibition of haemolysis. These results suggest that heat-labile ACA is not due to the formation of immunoglobulin aggregates which would be expected to bind to ^{125}I-Clq. The observations that heat-labile ACA is present to the same extent in both normal and hypogammaglobulinaemic sera, and that greater ACA develops in normal serum during heating at 47°C and 50°C than at higher temperatures also argue against this explanation.

Evidence that $C\bar{1}^{hu}$ accounts for heat-labile ACA

Another possible explanation for heat-labile ACA is the presence of $C\bar{1}^{hu}$ in serum prior to heating. The anti-complementary effect of $C\bar{1}^{hu}$ on GPC would not be apparent in the complement fixation assay until heat inactivation of endogenous human haemolytic complement activity is nearly complete, since the ratio of human complement to GPC in this assay is approximately 16:1. As a result, even a substantial degree of ACA would not be apparent when testing whole, unheated human serum.

The most conclusive evidence to demonstrate that heat-labile ACA is due to $C\bar{1}^{hu}$ would be the lack of heat-labile ACA in $C\bar{1}^{hu}$ deficient serum and its appearance following the addition of $C\bar{1}^{hu}$. $C\bar{1}^{hu}$ deficiency is extremely rare and such a serum was not available. Other methods of depleting serum of $C\bar{1}^{hu}$ activity (euglobulin precipitation, diisopropyl phosphorofluoridate, pentosan-poly-sulfo-ester) all have associated technical problems that would make it difficult to interpret experiments designed to test the role of $C\bar{1}^{hu}$ in heat-labile ACA. Consequently, the following experiments were designed to provide indirect evidence that heat-labile ACA in heated serum is due to $C\bar{1}^{hu}$.

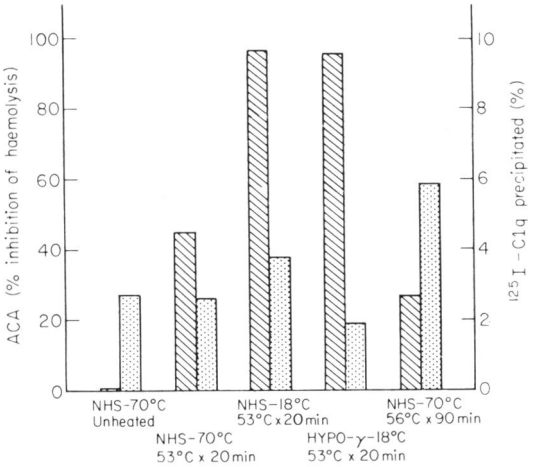

FIG. 7

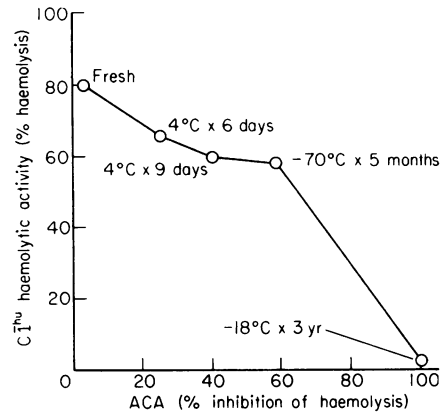

FIG. 8

FIG. 7. Comparison of anti-complementary activity (ACA) (▨) with ^{125}I-Clq binding (▩) of sera following heating. After heating at 53°C for 20 min, both the hypogammaglobulinaemic serum (HYPO-γ) and normal serum previously stored at −18°C for 18 months (NHS-18°C) had strong ACA (96–97% inhibition of haemolysis), but did not bind more ^{125}I-Clq than unheated normal serum. By contrast, serum heated at 56°C for 90 min, which produces immunoglobulin aggregates, was less anti-complementary but bound more ^{125}I-Clq than the same serum heated at 53°C for 20 min.

FIG. 8. Correlation of $C1^{hu}$ haemolytic activity with heat-labile anti-complementary activity (ACA). Sera which had been stored at varying temperatures and times were tested for $C1^{hu}$ haemolytic activity using an $EAC4^{gp}$ intermediate. These sera were then tested for ACA after heating at 53°C for 20 min. An inverse relationship existed between $C1^{hu}$ haemolytic activity and ACA.

Correlation of the loss of $C\bar{1}^{hu}$ haemolytic activity with the development of heat-labile ACA

Following activation, $C\bar{1}^{hu}$ loses haemolytic activity when tested in some assays, while it retains the ability to inactivate complement and to hydrolyze several synthetic amino acid esters (Lepow *et al.*, 1956; Lepow, Ratnoff & Levy, 1958). Different conditions used for the storage of serum might lead to varying degrees of activation of $C\bar{1}^{hu}$. If $C\bar{1}^{hu}$ accounts for heat-labile ACA, those sera having the most ACA after heating at 53°C for 20 min should have the least $C\bar{1}^{hu}$ haemolytic activity before heat inactivation. Fig. 8 demonstrates that an inverse relationship does exist between heat-labile ACA and $C\bar{1}^{hu}$ haemolytic activity.

ACA of hereditary angioneurotic oedema (HANE) serum

Patients with HANE lack functional $C\bar{1}$INH. If heat-labile ACA is due to $C\bar{1}^{hu}$, HANE serum should

demonstrate considerably greater ACA than normal serum due to the uncontrolled action of $C\bar{1}^{hu}$ on GPC. Unheated serum from an asymptomatic patient with HANE was totally anti-complementary up to a sixteen-fold dilution. To demonstrate that the ACA of this serum was due to $C\bar{1}^{hu}$, aliquots of unheated HANE serum, diluted sixteen times, were pre-incubated with $C\bar{1}$INH at 37°C for 30 min. $C\bar{1}$IHN completely inhibited ACA.

Enhancement of ACA by aprotinin

Aprotinin has been shown to augment the activity of $C1^{hu}$ and $C\bar{1}^{hu}$ (Delage *et al.*, 1976) and might be expected to enhance the ACA of heated serum if this effect were due to $C\bar{1}^{hu}$. Aprotinin, 0–1000 KIU, was added to aliquots of heated serum and the samples were tested for ACA after 30 min at 37°C. Control tubes, omitting heated serum, were not anti-complementary. Aprotinin increased ACA, further suggesting that ACA in serum was due to $C\bar{1}^{hu}$.

Effect of trypsin on ACA

At low concentrations, trypsin activates $C1^{hu}$, while at higher concentrations it inactivates $C\bar{1}^{hu}$ (Ratnoff & Naff, 1967). If $C\bar{1}^{hu}$ in heated serum were responsible for heat-habile ACA, incubation of heated serum with trypsin, prior to adding GPC, should also abolish ACA. To test this possibility, aliquots of heated serum, diluted 1:4 with VB^{++} to minimize the effects of anti-tryptic factors in the serum, were incubated with varying concentrations of trypsin. After 30 min at 37°C, the trypsin was neutralized by adding soybean trypsin inhibitor and the samples were tested. The ACA of normal serum, heated at 53°C for 20 min, was increased when incubated with low concentrations of trypsin and was eliminated by higher concentrations of trypsin (Fig. 9).

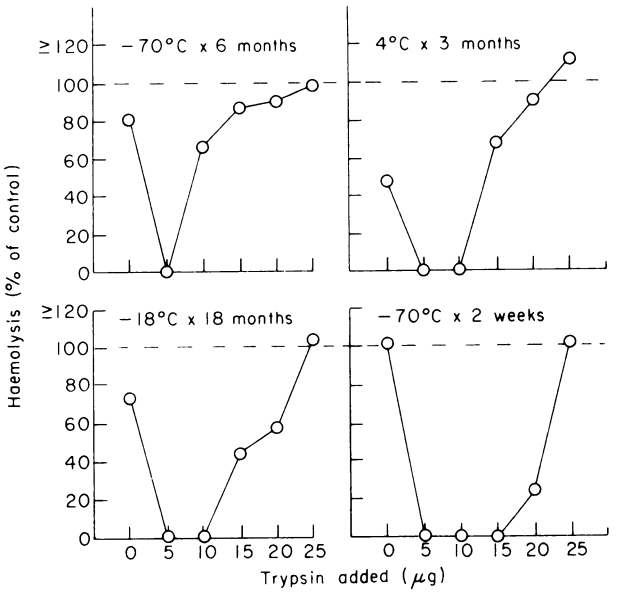

FIG. 9

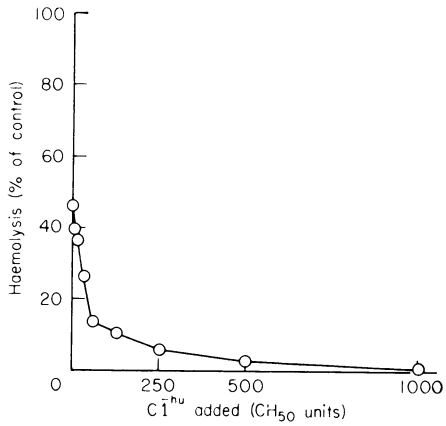

FIG. 10

FIG. 9. The effect of trypsin on anti-complementary activity (ACA) in normal serum. Aliquots of serum, stored at different temperatures for varying times, were diluted 1:4 and heated at 53°C for 20 min. Varying amounts of trypsin were added and after 30 min at 37°C the trypsin was neutralized by adding 25 μg of soybean trypsin inhibitor. The samples were then tested in the complement fixation assay, Control tubes containing trypsin and soybean trypsin inhibitor did not inhibit hemolysis. At low concentrations trypsin increased ACA, while at higher concentrations it was eliminated.

FIG. 10. Enhancement of anti-complementary activity (ACA) by exogenous $C\bar{1}^{hu}$. Functionally pure $C\bar{1}^{hu}$ was added to aliquots of serum, previously heated at 53°C for 20 min. After 30 min at 37°C the samples were tested for ACA. The addition of $C\bar{1}^{hu}$ progressively increased ACA in heated sesum.

The relationship of interactions between $C\bar{1}^{hu}$ and C1INH to heat-labile ACA

These data suggest that $C\bar{1}^{hu}$ can explain the occurrence of ACA in heated serum. Since C1INH completely blocks the ACA of diluted HANE serum, of $C\bar{1}^{hu}$ in small amounts and of the heavy peak from gel filtration chromatography, the appearance of heat-labile ACA in many sera presumably containing normal amounts of C1INH would appear to be a contradiction. The following experiments were conducted to examine the interplay between $C\bar{1}^{hu}$ and C1INH in serum, and their relative effects on heat-labile ACA.

Enhancement of ACA by endogenous $C\bar{1}^{hu}$

If endogenous C1INH in heated serum were still capable of blocking $C\bar{1}^{hu}$ activity, the addition of $C\bar{1}^{hu}$ should not increase ACA. Serum which had previously been heated at 53°C for 20 min was incubated for 30 min at 37°C with functionally pure $C\bar{1}^{hu}$ and then tested for ACA (Fig. 10). The incremental addition of $C\bar{1}^{hu}$ to heated serum progressively increased ACA. This increase was considerably less than the ACA of $C\bar{1}^{hu}$ in buffer, where two CH_{50} units were totally anti-complementary. These results suggest that endogenous serum factors, presumably C1INH, are capable of substantially, but not completely, limiting the effect of fluid phase $C\bar{1}^{hu}$ on GPC.

Effect of exogenous C1INH on heat-labile ACA and $C\bar{1}^{hu}$

The previous data might be interpreted as showing that insufficient amounts of C1INH were present in heated serum. If so, further addition of C1INH should diminish heat-labile ACA. An alternative interpretation would be that C1INH was already present in optimal amounts but was unable to completely block the effect of relatively large amounts of endogenous $C\bar{1}^{hu}$. The addition of C'INH would than have little effect on, or might even increase (see Fig. 5) ACA. Two experiments were performed to test

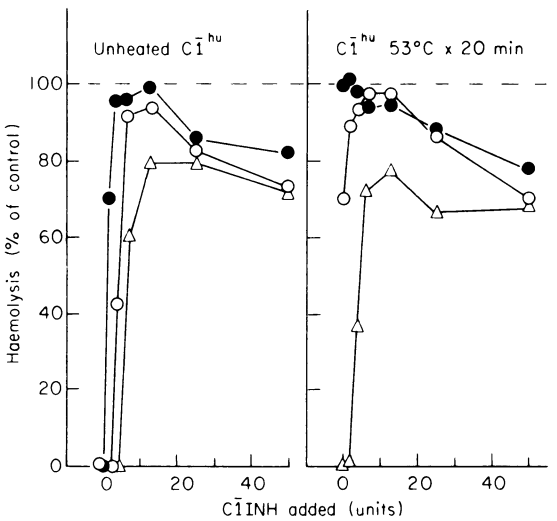

FIG. 11

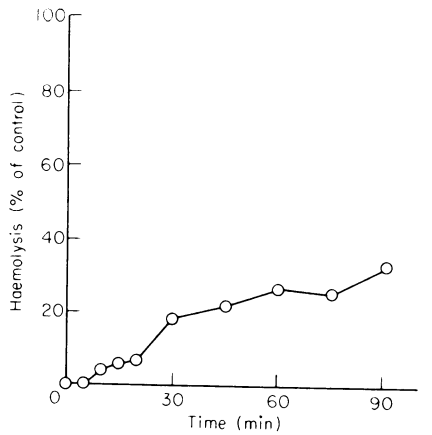

FIG. 12

FIG. 11. The effect of C1INH on the anti-complementary activity (ACA) of functionally pure $C\bar{1}^{hu}$. Aliquots of $C\bar{1}^{hu}$ containing 2 (●—●), 20 (○—○), or 50 (△—△) CH_{50} units were tested unheated (a) or after heating at 53°C for 20 minutes (b). C1INH (0–50 units) was added and after 30 min at 37°C the samples were tested for ACA. C1INH eliminated the ACA of small quantities of $C\bar{1}^{hu}$ but its effect on the ACA of larger quantities of $C\bar{1}^{hu}$ was incomplete.

FIG. 12. Inhibition of the anti-complementary activity (ACA) of $C\bar{1}^{hu}$ in serum by endogenous C1INH. Aliquots of the dissolved euglobulin fraction of serum. containing $C\bar{1}^{hu}$, were recombined in proportions equivalent to whole serum with aliquots of the pseudoglobulin fraction and then heated at 53°C. At various time intervals samples were removed and tested for ACA. Prior to heating, the recombined serum was totally anti-complementary and this ACA persisted up to 90 min.

these possibilities. First, C$\bar{1}$INH was added to aliquots of heated serum (53°C for 20 min) to final concentrations of 0–2000 units/ml and then incubated at 37°C for 30 min. Over a wide range of concentrations C$\bar{1}$INH did not diminish the ACA of heated serum, while concentrations greater than 200 units/ml increased ACA.

To further test the possibility that C$\bar{1}$INH would not completely inhibit the ACA of large quantities of heated or unheated $C\bar{1}^{hu}$, aliquots of $C\bar{1}^{hu}$ (2–50 CH_{50} units), either unheated or after heating at 53°C for 20 min, were incubated with C$\bar{1}$INH (Fig. 11). Although the ACA of 2 CH_{50} units of unheated $C\bar{1}^{hu}$ was completely eliminated by incubation with 12·5 units of C$\bar{1}$INH, the ACA of larger quantities of unheated $C\bar{1}^{hu}$ was not completely inhibited. Similar results were obtained when C$\bar{1}$INH was added to heated $C\bar{1}^{hu}$. An excess of C$\bar{1}$INH increased ACA at all concentrations of $C\bar{1}^{hu}$ tested. These results further support the concept that C$\bar{1}$INH cannot block heat-labile ACA due to the large amounts of $C\bar{1}^{hu}$ in serum.

Inhibition of $C\bar{1}^{hu}$ by endogenous C$\bar{1}$INH

The inability of endogenous C$\bar{1}$INH to completely eliminate the ACA of $C\bar{1}^{hu}$ in serum was further demonstrated as follows. $C1^{hu}$ was activated by precipitating $C1^{hu}$ with the euglobulin fraction of serum. The euglobulin fraction, adjusted to its original serum concentration and ionic strength, was totally anti-complementary up to a 64-fold dilution. Pre-incubation with trypsin totally eliminated the ACA of this fraction, as would be expected for $C\bar{1}^{hu}$. The unheated pseudoglobulin fraction, which contained C$\bar{1}$INH, was pro-complementary. The two fractions were recombined in proportions equivalent to whole serum and aliquots were heated at 53°C (Fig. 12). Prior to heating the reconstituted serum was totally anti-complementary and this ACA persisted with heating up to 90 min.

DISCUSSION

Two peaks of ACA are apparent in serum after gel filtration chromatography. The heavier anti-complementary peak appears to be due to the presence of activated Cl^{hu}, based on the following characteristics: (1) size, (2) heat-lability at 53°C and 56°C, (3) sensitivity to trypsin, and (4) inhibition by C$\bar{1}$INH. The lighter anti-complementary peak is also heat-labile and has functional characteristics of free C$\bar{1}$s when incubated with trypsin, plasmin, C$\bar{1}$INH or aprotinin. The presence of Cls was demonstrated in the lighter peak fractions by immunodiffusion.

Cl^{hu} might become activated *in vitro*, either during storage or by Sephadex during gel filtration chromatography (Laurell & Siboo, 1966). After $C\bar{1}^{hu}$ has been separated from C$\bar{1}$INH during gel filtration chromatography, $C\bar{1}^{hu}$ would be able to inactivate $C2^{hu}$ and $C4^{hu}$ during fluid phase incubation. It seems probable that $C2^{gp}$ and $C4^{gp}$ might also serve as substrates for $C\bar{1}^{hu}$. Thus $C\bar{1}^{hu}$ would be anti-complementary by depleting these components in the fluid phase segment of the complement fixation assay.

Cl^{hu} is a trimolecular complex whose components are loosely associated in the presence of calcium. During gel filtration, using buffers which do not contain calcium, it seems likely that C$\bar{1}$s partially dissociates from the parent molecule, explaining the lighter ACA. The recent demonstration by Laurell, Mårtensson & Sjöholm (1976) that free Clr-Cls complexes can be detected in serum in the presence of calcium, and by Bartholomew & Esser (1977) that free Cls may be present in serum, support this concept.

For reasons discussed earlier, the data suggesting that heat-labile ACA in heated serum is due to $C\bar{1}^{hu}$ are, of necessity, circumstantial. The following observations support this hypothesis: (1) ACA developed as Cl^{hu} haemolytic activity disappeared, suggesting activation of Cl^{hu}. (2) Trypsin at low concentrations enhanced and at higher concentrations eliminated ACA in heated normal sera, including those stored at −70°C, −18°C and 4°C. These results are consistent with known effects of trypsin on $C\bar{1}^{hu}$ and $C\bar{1}^{hu}$ (Ratnoff & Naff, 1967). (3) Addition of $C\bar{1}^{hu}$ to heated serum augmented ACA, suggesting that the effect of endogenous C1INH was already incomplete. (4) Aprotinin, known to enhance the activity of $C\bar{1}^{hu}$, also increased ACA in heated serum. (5) HANE serum, which lacks functional C$\bar{1}$INH, was totally anti-complementary up to a sixteen-fold dilution even prior to heating, and the addition of C$\bar{1}$INH to

diluted HANE serum eliminated this ACA, demonstrating that this activity was due to $C\bar{1}^{hu}$. Additional evidence to support the presence of $C\bar{1}^{hu}$ in normal serum has been provided by several recent studies (Laurell *et al.*, 1976; Loos, Borsos & Rapp, 1972; Ziccardi & Cooper, 1978).

The presence of ACA, which appeared to be due to $C\bar{1}^{hu}$ in normal serum containing $C\bar{1}$INH, led us to explore the role of $C\bar{1}$INH in regulating the ACA of fluid phase $C\bar{1}^{hu}$. $C\bar{1}$INH was able to inhibit the ACA of a small amount of heated or unheated $C\bar{1}^{hu}$ but never totally abolished ACA of larger amounts of $C\bar{1}^{hu}$. An excess of $C\bar{1}$INH was, in itself, anti-complementary. The addition of exogenous $C\bar{1}$INH to heated normal serum either failed to diminish ACA or, in higher concentrations, actually increased ACA. As more $C\bar{1}^{hu}$ was activated in serum, endogenous $C\bar{1}$INH was less able to block ACA. These data suggest that the ACA of $C\bar{1}^{hu}$ in serum was already maximally, though incompletely, inhibited by $C\bar{1}$INH.

On the basis of these and previous data, we propose the following hypothesis to explain the occurrence of heat-labile ACA in normal serum. Isolated $C\bar{1}^{hu}$ has two known fluid phase effects on complement Kondo, Gigli & Austen (1972) and Gigli & Austen(1969) have demonstrated that the first, i.e. the ability to inactivate $C2^{hu}$, is both exquisitely heat-labile and blocked by $C\bar{1}$INH. $C\bar{1}^{hu}$ also inactivates $C4^{hu}$. This function is less heat-labile and is not effectively blocked by $C\bar{1}$INH. Thus, if $C\bar{1}^{hu}$ became activated in serum, its effect on $C4^{hu}$ would not be completely inhibited. This effect on $C4^{hu}$, together with loss of $C1^{hu}$ haemolytic activity which may follow $C1^{hu}$ activation (Lepow *et al.*, 1956; 1958), no doubt contributes to the disappearance of endogenous haemolytic activity found during incubation of serum at 37°C or after prolonged storage at 4°C or −18°C. $C2^{gp}$ and $C4^{gp}$ probably also serve as substrates for $C\bar{1}^{hu}$. Because the amount of GPC used in the complement fixation assay is small, even a substantial degree of inactivation of GPC would not be detected until the endogenous haemolytic activity of human serum is eliminated. Thus ACA, which is presumably present in unheated serum, is not recognized until serum is heated, separated by gel filtration or stored in such a way that all endogenous haemolytic activity is lost. After heating at 53°C for 20 min, $C\bar{1}^{hu}$ would partially retain its ability to inactivate fluid phase C4, including $C4^{gp}$. This relatively heat-stable function is progressively lost with continued heating so that ACA would eventually disappear.

The mechanism by which $C1^{hu}$ becomes activated in serum is unknown. Many freshly drawn and separated sera have only minimal or no heat-labile ACA. During storage ACA progressively increases. Experiments designed to study the effect of inhibition of plasmin or to determine the effects of minute quantities of immunoglobulin aggregates in serum, both potential sources of $C1^{hu}$ activation, did not explain the activation of $C1^{hu}$ (unpublished observations).

Our data do not support previous suggestions that the heat-labile ACA in heated serum or in either peak obtained by gel filtration chromatography of normal unheated serum was due to immunoglobulin aggregates. Our data, in fact, argue against the presence of immunoglobulin aggregates: (1) heat-labile ACA developed to the same degree in normal and hypogammaglobulinaemic sera; (2) there was a marked disparity between ACA and ^{125}I-Clq binding in experiments conducted with serum heated at 53°C and 56°C; (3) fractions from the heavy ACA peak did not bind to Raji cells and actually showed diminished reactivity with ^{125}I-Clq; (4) the ACA of immunoglobulin aggregates was not altered by trypsin, in contrast to results found with heat-labile serum and heavy peak ACA; and (5) the ACA of IgG aggregates, formed during storage at 4°C, was not heat-labile. In contrast, we have shown previously that the late occurring, heat stable ACA which develops during heating at temperatures at or above 56°C is due to the formation of immunoglobulin aggregates (Soltis *et al.*, 1979a).

These findings have several practical implications. The presence of heat-labile ACA in serum potentially interferes with routine complement fixation tests, designed to detect a specific antigen or antibody, by diminishing sensitivity. Heat inactivation of serum at 53°C for 90 min prior to testing eliminates most ACA in normal sera stored at −70°C without producing the degree of immunoglobulin aggregation that occurs at 56°C. This modification should allow for increased sensitivity in routine complement fixation tests.

Our interest in the complement fixation test relates to its use in testing for antigen-antibody complexes. These findings indicate that the demonstration of ACA in fractions of pathological sera obtained by gel

filtration cannot be taken as evidence for the presence of immune complexes as has been previously suggested (Hodgson *et al.*, 1977), since normal sera contain ACA in these fractions. Heating normal serum at 53°C for 90 min might be expected to increase the specificity of complement fixation assays for immune complexes. Unfortunately, although complement-fixing immunoglobulin aggregates are not detectable in normal serum following heating at 53°C, samples containing increased IgG and/or decreased albumin concentrations do form detectable immunoglobulin aggregates at this temperature (Soltis *et al.*, 1979a), and even during storage at 4°C (Soltis *et al.*, 1979b). Most pathological sera in which immune complexes have been sought contain such protein abnormalities. Heat inactivation at 53°C or at any other temperature would be likely to produce a greater degree of immunoglobulin aggregation in pathological sera than in controls, thus falsely suggesting the presence of immune complexes. These observations demonstrate the difficulties encountered when interpreting results obtained using assays designed to detect immune complexes in heated serum.

This work was supported by a grant from the The National Foundation for Ileitis and Colitis, Inc.

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