Function of the Classical and Alternate Pathways of Human Complement in Serum Treated with Ethylene Glycol Tetraacetic Acid and MgCl$_2$-Ethylene Glycol Tetraacetic Acid

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An immunochemical and functional analysis of the classical and alternate complement pathways in human serum was performed in the presence of 10 mM ethylene glycol tetraacetic acid (EGTA) and MgCl$_2$-EGTA (MgEGTA), chelating agents which have been recently utilized as a means of distinguishing between these two complement pathways. Total hemolytic activity, integrity of the C1 complex, hemolytic activity of C2, conversion of factor B (C3 proactivator), and complement-dependent bactericidal activity were studied. The effect of these chelators on activation of complement pathways by Escherichia coli, by sensitized erythrocytes as a prototype of activators of the classical pathway, and by zymosan as a prototype of alternate (properdin) pathway activators was studied. Human serum containing 10 mM EGTA, which provides almost no ionized calcium and considerably less ionized magnesium than unchelated serum, allowed consumption of complement via the alternate (properdin) pathway, but blocked the classical pathway as judged by disintegration of the C1 complex and lack of utilization of C2. However, activity of the alternate complement pathway in EGTA serum, as judged by conversion of factor B and bactericidal activity against gram-negative bacteria, was distinctly suboptimal. Addition of magnesium ion in a concentration equimolar to EGTA (MgEGTA serum), while still providing conditions in which the C1 complex dissociated, significantly enhanced alternate complement pathway-mediated bactericidal activity. However, in MgEGTA serum considerable fluid-phase activation of the alternate pathway, as indicated by decrease in 50% hemolytic complement (CH$_50$) titers and conversion of factor B to its active form in the absence of any activating challenge, was observed. Moreover, some fluid-phase consumption of C2 was observed in MgEGTA serum, even though, as mentioned, the C1 complex was shown to be dissociated under these conditions. MgEGTA-related activation of C2 and of the alternate (properdin) pathway of complement was significantly enhanced by the presence of zymosan and E. coli. These results indicate that use of the chelating agents EGTA and MgEGTA to differentiate between classical and alternate pathway activation of human complement is more complex than has hitherto been suggested. In EGTA serum, spontaneous activation of either pathway does not occur but bactericidal activity, as a measure of biologic function of complement, is suboptimal. In MgEGTA serum, bactericidal activity is fully expressed, but there is considerable instability, in terms of fluid-phase activation, in Mg$^{2+}$-dependent components of both pathways. Thus, caution is indicated in the use and interpretation of the effects of these chelating agents on biologic functions mediated by either pathway of human complement.

The complement system in man participates in host immune reactions as a result of activation of the classical (C142) and the alternate (properdin) complement pathways. Acquired defects in or depletion of the complement system are thought to play a role in abnormal host defense mechanisms in several disease states including immune complex disease (10, 25), sickle cell anemia (14), and liver disease (4). A better understanding of these abnormalities requires methods whereby the function of one or another of the two complement pathways can be easily evaluated.

We have previously applied the concept of...
differential chelation of divalent cations using ethylene glycol tetraacetic acid (EGTA) as a means of distinguishing between the classical (C142 mediated) and alternate (properdin) pathways of complement activation (8). This approach has been frequently employed in several studies to ascertain the function of complement in patients with sickle cell anemia (14), in paroxysmal nocturnal hemoglobinuria (2), in activation of B lymphocytes (22), in endotoxin-induced complement changes (7), and in phagocytosis of cryptococci (5) and of gram-positive and gram-negative bacteria (9). Despite these numerous examples of applying EGTA and/or MgCl2-EGTA (MgEGTA) as chelating agents for differentiation of both complement pathways, a detailed analysis of the effect of these chelators on such divalent cation-dependent complement components as the C1 complex, C2, and the properdin pathway is still lacking.

The present study was undertaken to evaluate the functional and immunochemical integrity of the human complement system in chelated serum by measuring total hemolytic activity, the immunochemical pattern of the C1 complex, hemolytic activity of C2, the immunochemical pattern of factor B (C3 proactivator), and complement-dependent bactericidal activity. Different chelating conditions providing varied concentration of magnesium and calcium ions were compared. Three known activators of complement pathways, sensitized erythrocytes, zymosan, and Escherichia coli, were used. The data suggest that the use and interpretation of the effect of chelating agents on the complement system of man requires caution since it is more complex than had previously been thought.

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MATERIALS AND METHODS

Human blood. Blood was obtained by venipuncture from healthy volunteers and was allowed to clot for 1 h at room temperature, and the serum was used fresh or, in most experiments, after storage at −70°C for 1 week or less.

Chelators. The preparation of 100 mM stock saline solutions of ethylenediaminetetraacetate (EDTA), EGTA, EGTA supplemented with an equimolar concentration of MgCl2 (MgEGTA), and the source of these reagents have been described previously (8). Stock solutions were added to serum or buffer to provide a final concentration of 10 mM.

Complement activation (fixation) experiments. Serum with or without chelators was incubated at 37°C for 30 min in the presence or absence of three activating substances. These included E. coli “E,” a clinical isolate from the bacteriology laboratory of Vanderbilt Hospital, zymosan (Z), and sheep erythrocytes sensitized with anti-sheep hemolysin (EA). E. coli “E” was maintained on Trypticase soy agar slants, grown overnight at 37°C in Trypticase soy broth, centrifuged, washed three times with saline, and standardized after resuspension in saline by optical density determination at 500 nm based on previously determined curves relating optical density to viable colony counts by standard bacterial enumeration techniques. Z (Nutritional Biochemicals Corp., Cleveland, Ohio) was boiled for 1 h, washed three times in saline, and resuspended in serum at a final concentration of 2 mg/ml (26). EA were prepared according to the method of Rapp and Borsos (28) from sheep erythrocytes (Baltimore Biological Laboratory, Cockeysville, Md.) and anti-sheep hemolysin (Difco Laboratories, Detroit, Mich.), and concentrated as described previously (8). Normal saline was substituted for either the activating substances or the chelator in control experiments. After incubation, the activating substances were separated by centrifugation (2,500 × g for 10 min at 4°C) and the supernatant serum was analyzed for complement activity after addition of 0.1 ml of 100 mM CaCl2/ml of serum to those samples initially containing either EGTA or MgEGTA.

Determination of hemolytic complement activity (HA, titers). Serum samples were analyzed by the method of Rapp and Borsos (28).

Determination of C2 hemolytic activity. Serum samples were analyzed according to the method of Rapp and Borsos (28), using purified guinea pig C1, (Cordis Laboratories, Miami, Fla.). Purified human C2 from the same source was used as a standard.

Immunoelectrophoresis. This was performed in 1% agarose on microscope slides in veronal buffer of ionic strength 0.05 and pH 8.0 according to the method of Scheidegger (32). In studies utilizing chelated serum, it was necessary to include the chelator in a final concentration of 10 mM in both the agarose and buffer by the method of Nagaki and Stroud (24).

Analysis of C1 complex. Serum samples with and without chelators were analyzed by immunoelectrophoresis using monospecific rabbit anti-human C1s antiserum kindly provided by Robert Stroud, University of Alabama Medical School, Birmingham (24).

Assay of conversion factor B (C3 proactivator, C3PA) to factor B (C3 activator, C3A). Nonchelated and chelated serum samples incubated with and without the challenge particles for 30 min at 37°C were analyzed by immunoelectrophoresis using monospecific anti-C3A antiserum (Behring Diagnostics, Summerville, N.J.) by the methods of Götte and Müller-Eberhard (12). The degree of conversion was estimated visually and graded from 0 to ++ +.

Bactericidal assays. The following seven bacterial strains were employed in these assays. E. coli “E” and Salmonella typhi were clinical isolates obtained from the bacteriology laboratory, Vanderbilt University Hospital. E. coli “C” and Proteus rettgeri were kindly provided by Charles E. McCall, Bowman Gray School of Medicine. All four strains were selected for their exquisite sensitivity to the complement-mediated

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serum bactericidal system. Bacteria were maintained on Trypticase soy agar slants and were grown over- night at 37°C prior to each experiment. To obtain log-phase organisms for bactericidal assays, bacteria were transferred with a standardized inoculum loop from an overnight culture to fresh Trypticase soy broth and allowed to grow at 37°C from 150 to 250 min, depending on the bacterial strain.

At the start of each experiment, 0.2-ml aliquots of a 10^{-3} dilution of the log-phase culture were added to plastic tubes (75 by 12 mm) containing 1.8 ml of serum and 0.2 ml of either stock chelator solution or normal saline. All tubes were warmed to 37°C immediately prior to inoculation. The final concentration of bacteria in each tube was approximately 3 × 10^{9} per ml. The tubes were incubated at 37°C with gentle end-over-end tumbling on a tube rotator. Trypticase soy agar pour plates were made from 0.1-ml aliquots removed from each tube at timed intervals; plates were incubated for at least 24 h before colony enumeration. “Complete killing” was defined as the absence of viable bacteria in aliquots removed from the bacteria-serum mixture after 1 h of incubation. “Significant killing” was defined as a reduction in the number of viable bacteria to less than 10% of the original inoculum by the end of 1 h (6).

RESULTS

Immunochromatographic analysis of the classical and alternate complement pathways in human serum chelated with EGTA. Three steps in complement activation require divalent cations: Ca^{2+}-dependent assembly of C1 complex (15), Mg^{2+}-dependent C2 activation (16), and Mg^{2+}-dependent activation of the properdin pathway (12, 26, 31). Although normal human serum contains approximately 10^{-3} M ionized calcium and magnesium, serum chelated with 1 × 10^{-3} M EGTA contains 10^{-11} M Ca^{2+} and 1.6 × 10^{-4} M Mg^{2+} (2, 13). Under conditions of almost complete depletion of Ca ion and borderline depletion of Mg ion by EGTA, some steps of complement activation will be inhibited. The following experiments were performed to document in detail the pattern of the inhibitory effect of EGTA.

(i) Immunochromatographic demonstration of the C1 complex disintegration in the presence of EGTA. Nagaki and Stroud, using antiserum monospecific for purified C1s, demonstrated that the immunoelectrophoretic mobility of C1 was altered in the presence of EDTA and became similar to the mobility of purified C1s either in the presence or absence of the chelator (24). This was taken as evidence that dissociation of C1 into its subcomponents (C1q, C1r, and C1s) could be demonstrated by immunoelectrophoresis. As illustrated in Fig. 1, dissociation of the C1 complex took place also in EGTA serum in a manner quite similar to that observed in EDTA serum used as a positive control. C1s migrated toward the anode, indicating it dissociation from the C1 complex. In contrast, in nonchelated serum (negative control), material immunoreactive with anti-C1s remained at the origin in the macromolecular C1 complex. Thus, the trimeric C1 component of complement dissociated in EGTA serum.

(ii) Consumption of total hemolytic complement activity and C2 activity in EGTA serum. The effect of EGTA on the functional integrity of the complement system and of the C2 component was assessed. For this purpose the consumption of total hemolytic activity and of C2 hemolytic activity by the activating substances was measured. Three activators were used: E. coli and Z. known to activate the alternate pathway, and EA as a prototype of classical pathway activating agents. In the control, unchelated human serum, E. coli caused almost complete consumption of CH_{50} and C2 (Fig. 2). Z and EA also caused complete consumption of total hemolytic complement activity but the pattern of C2 consumption differed: EA challenge produced 100% and Z challenge 45% consumption of C2, respectively. These patterns of consumption in unchelated serum suggest involvement of both the classical (C142) and alternate (properdin) pathways in complement activation by E. coli and Z.

Human serum chelated with EGTA showed a different pattern of decomplementation. E. coli and Z still caused consumption of total complement (CH_{50}) 70% and 98%, respectively. However, consumption of C2 was blocked. Both whole complement fixation (CH_{50}) and C2 fixation by EA were almost completely blocked in EGTA serum. These results indicate that chelation of serum by EGTA prevented activation of the classical pathway by EA and also by E. coli and Z. Hence, consumption of total complement activity by E. coli and Z observed in EGTA serum represented activation of the alternate (properdin) pathway.

(iii) Activation of the alternate (properdin) pathway as measured by conversion of factor B (C3 proactivator) in EGTA serum. Consumption of whole complement (CH_{50}) but not C2 in EGTA serum incubated with E. coli and Z provided indirect evidence that the alternate complement pathway was activated under these conditions. Direct evidence for alternate pathway activation was obtained by immunoelectrophoretic analysis of factor B (C3 proactivator) conversion to factor B (C3 activator). Using antiserum monospecific to C3 ac-
Fig. 1. Dissociation of C1 complex in chelated serum. Immunoelectrophoresis of nonchelated serum or serum containing the designated chelators was carried out as described. Experiments using chelated serum were carried out with the same concentration of chelator in the agarose gel. Anti-C1s antibody is in the center trough. The movement of material reactive with anti-C1s antibody toward the anode took place in the presence of all three chelators. In nonchelated serum this material remained at the cathodal side of the well. Anode is at left.

Fig. 2. Consumption of total hemolytic complement activity and C2. Unchelated serum, EGTA serum, and MgEGTA serum were incubated with E. coli (10¹⁰ organisms/ml of serum), Z (2 mg/ml of serum), and EA (2 x 10⁹/ml of serum). Incubation was carried out for 30 min at 37 C before sedimentation of challenges and determination of residual CH₅₀ and C₂ titers (see text). Figures represent percentage of control (normal human serum) expressed as mean value plus or minus the standard error of the mean.

I Activator, the change in electrophoretic mobility of material reacting with this antisera was observed in nonchelated human serum incubated with E. coli or Z but was not seen in serum incubated with EA (Fig. 3). Such a change in electrophoretic mobility indicates conversion of factor B (C3 proactivator) to factor B (C3 activator) (12) due to activation of the alternate (properdin) pathway. In EGTA serum, conversion of C3 proactivator to C3 activator was observed in the presence of E. coli and Z but not with EA (Fig. 3). However, the extent of C3 proactivator conversion was consistently smaller in EGTA serum than in unchelated serum. This immunochemical analysis of factor B indicates that activation of the alternate (properdin) pathway of complement by E. coli and Z occurs in EGTA serum but to a lesser degree than in unchelated serum.

(iv) Complement-mediated bactericidal activity of human serum in the presence of EGTA. Gram-negative bacteria are target cells for the complement system in human blood, and evaluation of bactericidal activity of human serum represents one of the most sensitive indexes of the biologic function of complement. Therefore, complement-mediated bactericidal activity of human serum was evaluated under the chelating conditions studied. In unchelated human serum, bactericidal activity against complement-sensitive strains of E. coli, S. typhi, and P. rettgeri was complete within 10
Fig. 3. Conversion of factor B (C3 proactivator) to factor B (C3 activator) in unchelated and chelated human serum incubated with E. coli, EA, and Z. The concentrations of the challenge material, time of incubation of serum, and inclusion of chelators in the immunoelectrophoresis system are the same as described in Fig. 1 and 2. Anti-factor B (C3A) antibody is in the center troughs. Nonconverted factor B occupied a position anodal from the well; conversion to C3A was associated with movement of the immunoreactive material in a cathodal direction. Anode is at right.

to 20 min (curve A in four panels of Fig. 4). In serum containing 10 mM EGTA, bactericidal activity was inconsistent (curve C in four panels of Fig. 4). Thus, these results indicate further that EGTA serum provides suboptimal conditions for activation of the complement system as manifested by bactericidal activity. It must be noted that heat-labile bactericidal activity in EGTA serum must represent alternate pathway activity since, as demonstrated, the classical pathway activation is completely blocked.

Immunohemical and functional characterization of the classical and alternate complement pathways in human serum chelated with MgEGTA. The results of the experiments described above indicated that chelation of human serum with EGTA causes disintegration of the C1 complex and blocks consumption of C2 preventing activation of complement by classic pathway activators such as EA. Further, serum chelated with EGTA permits activation of the alternate (properdin) pathway but at an apparently suboptimal rate. Reasoning that this suboptimal rate might be due to a suboptimal concentration of Mg$^{2+}$ ($1.6 \times 10^{-6}$ M), EGTA serum was supplemented with equimolar concentration of Mg$^{2+}$ ($1 \times 10^{-4}$ M) to produce a final concentration of $3 \times 10^{-3}$ M Mg$^{2+}$ (2). Under these conditions, the concentration of ionized calcium is $10^{-10}$ M. Thus, chelation with

Fig. 4. Bactericidal activity of human serum against four complement-sensitive bacterial strains in unchelated fresh serum (A), MgEGTA serum (B), and EGTA serum (C). Controls consisted of heat-inactivated serum containing MgEGTA (D) or EGTA (E). All chelators were in 10 mM concentration.
MgEGTA provides near normal concentrations of ionized magnesium but almost total chelation of ionized calcium. MgEGTA serum was studied in a parallel series of experiments assessing the immunochemical and functional properties of both complement pathways.

(i) **Immunoochemical demonstration of the C1 complex disintegration in the presence of MgEGTA.** Addition of MgEGTA to human serum caused the material immunoreactive with anti-C1s antibody to migrate toward the anode in a manner similar to EDTA and EGTA (Fig. 1). This was taken as evidence that the C1 complex was dissociated in MgEGTA serum.

(ii) **Consumption of total hemolytic complement activity and C2 activity in MgEGTA serum.** The pattern of complement depletion in human MgEGTA serum was different from that observed in either nonchelated or EGTA serum (Fig. 2). Significant decrease in whole complement (69%) and C2 (38%) titers was observed after incubation of MgEGTA serum for 30 min at 37°C in the absence of any challenge material (fluid-phase activation). Both E. coli and Z produced complete consumption of whole complement and a high degree of consumption of C2. The fluid-phase activation manifested by the spontaneous and significant decrease in whole complement titers (in the absence of discomplementing challenge materials) and to a lesser degree in C2 titers was unanticipated and indicated that C2 and perhaps other components of complement pathways were modified or unstable in MgEGTA serum.

(iii) **Activation of the alternate (properdin) pathway as measured by conversion of factor B (C3 proactivator) in MgEGTA serum.** Activation of factor B as measured by a change in electrophoretic mobility was observed in MgEGTA serum in the absence of any challenge material whatsoever (control), or with EA which, as described above, did not activate the alternate pathway in nonchelated or EGTA serum (Fig. 3). Furthermore, conversion of C3 proactivator (factor B) to C3 activator was more complete than in EGTA serum after incubation with E. coli or Z. These results provide evidence that conditions for activation of the alternate (properdin) pathway by various activating substances (E. coli, Z) are more favorable in MgEGTA serum than in EGTA serum. However, spontaneous and partial fluid-phase alternate pathway activation in MgEGTA serum in the absence of activating substances considerably complicates interpretation of the data with activating substances.

(iv) **Complement-mediated bactericidal activity of human serum in the presence of MgEGTA.** When human serum chelated with MgEGTA was tested for complement-dependent bactericidal activity, complete killing of all four tested gram-negative bacterial strains was regularly observed (curve B in four panels of Fig. 4). There was a characteristic delay in the onset of bactericidal activity in MgEGTA serum compared to unchelated serum. Nevertheless, complete killing of the organisms tested occurred during first 40 min of incubation in MgEGTA serum.

**DISCUSSION**

The results of the present study will be discussed in relation to: (i) the role of divalent cations in the function of the classical and alternate complement pathways; (ii) the nature of complement activation in the presence of EGTA and MgEGTA; and (iii) the complexity of the action of the chelators due to the biologic effects of different absolute and relative concentrations of ionized calcium and magnesium.

The importance of divalent cations in the complement system was initially reported in 1906 by Cernovodeanu and Henri (3). Mayer et al. found in their classic studies that Mg²⁺ markedly enhanced the hemolytic activity of complement (21) and Levine et al. indicated that Ca²⁺ was required for the function of C1 whereas Mg²⁺ was necessary for the action of C2 (16, 17). Subsequent studies of Pillemer et al. postulated that magnesium ion was an essential component of the properdin system (26). Sandberg and Osler confirmed that Mg²⁺ was required for alternate (properdin) pathway activity (31), and Müller-Eberhard and Götze have suggested that magnesium ion is required for the function of factor D (C3PA convertase) (23). Thus, calcium ion is required during the early C1-dependent step of the classical pathway activation and magnesium ion is necessary for the C2-dependent step in the classical pathway and for the function of the properdin system. These different divalent ion requirements in the two pathways prompted the idea to utilize chelators not only to block calcium-dependent and magnesium-dependent complement steps (15–17) but also to differentiate one pathway from other (8). This approach has been employed in several investigations of the function of complement in several different systems (2, 5, 7, 9, 10, 22).

Our data support these findings but with some qualifications. First, immunochemical analysis of the C1 complex using anti-C1s antibody indicated disintegration of C1 in EGTA serum in a manner analogous to the effect of EDTA as previously shown by Nagaki...
and Stroud (24). Second, EGTA blocked fixation of complement via the classical pathway by EA. This blocking effect was assessed by measurement of total hemolytic activity and C2 hemolytic activity. Third, as predicted, EGTA serum allowed consumption of complement by E. coli and Z, both known to activate the alternate pathway (12, 18, 26). Activation of the alternate (properdin) pathway was documented immunochemically by conversion of factor B (C3 proactivator) to factor B (C3 activator). However, this conversion was not complete in EGTA serum challenged with E. coli or Z (Fig. 3). Likewise, bactericidal activity, examined as a sensitive biologic end point of the complement system, was not fully expressed in EGTA serum. Thus, although inhibition of the classical (C142) pathway of complement was documented by several means in EGTA serum, the alternate (properdin) pathway did not function at an optimal rate. The suboptimal function of the alternate pathway in EGTA serum is best explained by suboptimal concentration of ionized magnesium.

An additional limitation of the use of EGTA serum or plasma is that the low magnesium ion concentration in the presence of EGTA may be detrimental to a variety of biologic systems, including the structural integrity of target cells. For example, magnesium ion is considered to be the critical divalent cation for maintaining the structural integrity of the E. coli cell wall (1, 30). Experiments from this laboratory reported elsewhere (1) have demonstrated that the relatively complement-resistant strain E. coli ATCC 25911 was killed more readily in EGTA serum than in MgEGTA serum. This is exactly opposite to the effects of these two chelators on relatively complement-sensitive strains, as illustrated in Fig. 4. This effect of EGTA on the complement-resistant strain was attributed to cell wall damage as a consequence of magnesium ion deprivation, rendering it much more sensitive to the serum bactericidal system.

As discussed above, the concentration of magnesium ion in EGTA serum was distinctly suboptimal for activation of the alternate (properdin) pathway as measured by incomplete conversion of factor B and by inconsistent complement-mediated bactericidal activity. This suboptimal activity in EGTA serum has led to the addition of equimolar concentration of magnesium ion to EGTA. MgEGTA serum prepared in this fashion and used in this and other laboratories (R. B. Johnston, Jr., personal communication) is deficient in ionized calcium (10−10 M) but the concentration of Mg2+ is increased to 3.0 × 10−3 M. Under these chelating conditions, the C1 complex was dissociated as demonstrated by electrophoretic mobility of C1s, but Mg-dependent steps such as activation of C2 and the properdin pathway became spontaneously triggered or unstable in the fluid phase during incubation of serum at 37°C. Despite this fluid-phase activation, complement-dependent bactericidal activity was fully and consistently expressed in MgEGTA serum.

It appears that MgEGTA, added just prior to a test reaction, can be a useful reagent for indicating the presence or absence of alternate pathway-mediated biologic activity of complement but that caution should be exercised in the quantitation of the phenomena observed under these conditions.

The spontaneous fluid-phase activation or instability of Mg-dependent reactions (C2 step and properdin pathway) in MgEGTA serum merits emphasis. This phenomenon was temperature dependent since it occurred in MgEGTA serum incubated alone at 37°C; incubation of MgEGTA serum at 4°C prevented spontaneous activation of C2 or conversion of C3 proactivator. The presence of a particulate matrix such as EA enhanced these MgEGTA-related processes of C2 consumption or of alternate pathway activation causing appreciable loss of whole complement activity.

The consistent fall in C2 titer in MgEGTA serum both in the presence and absence of challenge particles is unexplained. Since the C1 complex was observed to dissociate into its component parts in MgEGTA serum as well as in EGTA serum, it seems unlikely that activation of the classical pathway based on an intact C1 complex could account for this observation. Since C2 titers did not decrease in EGTA serum after the same manipulations, the presence of the chelator per se could not account for this phenomenon. Since magnesium is required for the reactivity of C2 in immune hemolysis (16), it is reasonable to postulate that some aspect of the C2 activating mechanism may be spontaneously triggered under conditions of relative magnesium ion excess in a manner analogous to spontaneous activation of factor B. Since the C1 complex is dissociated in MgEGTA serum, the observed decrease in C2 titers in MgEGTA serum cannot be ascribed to the newly described C1 bypass activator pathway (20). However, there is a possibility that under conditions of a relative excess of Mg ion, C1s is able to act on C2. Polley and Müller-Eberhard observed inactivation of C2 during its purification in 2 × 10−3 M EDTA, pH 6.0, and they attributed this phenomenon (and C4 inactivation) to C1s (27). Gigli and Austen demonstrated that fluid-phase
activation of C2 by C1 involves unmasking the C2 receptor site on C1s which is otherwise blocked by C1r (11). Such unmasking may be accelerated by a relative excess of Mg ion in MgEGTA serum.

The spontaneous activation of the alternate (properdin) pathway in MgEGTA serum as manifested by conversion of C3 proactivator is probably due to the appreciable excess of magnesium ion in relation to calcium ion. The alternate pathway of complement activated in the presence of target cells by the relative excess of Mg$^{2+}$ in MgEGTA serum will trigger the membrane lesion responsible for complete killing of gram-negative bacteria (Fig. 4) or for lysis of paroxysmal nocturnal hemoglobinuria erythrocytes as previously demonstrated by Bryant and Jenkins (2). The critical condition for this spontaneous activation is not the absolute concentration of magnesium but rather the ratio of ionized magnesium to ionized calcium as recently suggested by May et al. (20) and Lambert et al. (P. H. Lambert, L. Perrin, and J. C. Cerottini, J. Immunol. 111:307).

The concept emerging from these and other studies is summarized in Table 1. In vitro elimination of calcium and magnesium ion by chelation with EDTA inhibits the classical and alternate pathways. Chelation of human serum with EGTA inhibits the classical pathway due to elimination of Ca$^{2+}$ but leaves enough Mg$^{2+}$ to allow the function of the alternate pathway at a suboptimal rate resulting in incomplete bactericidal activity. Human serum containing MgEGTA has an increased total concentration of Mg$^{2+}$ compared to nonchelated serum but still vanishingly small concentration of Ca$^{2+}$. These divalent ion concentrations result in the fluid phase activation of the Mg$^{2+}$-dependent C2 step and of the properdin pathway. However, complement-dependent bactericidal activity is fully expressed. The ratio of ionized magnesium to ionized calcium in MgEGTA serum is approximately 30 million; normal serum has approximately equal concentrations of both ions. Thus, although both EGTA and MgEGTA can be used as reagents permitting alternate pathway activation, their effects are considerably more complex than has hitherto been suggested and caution is needed in interpretation of biologic effects studied in the presence of these chelators.

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**LITERATURE CITED**


**TABLE 1. Summary of data on the function of the classic and alternate complement pathways in human serum under conditions of divalent cation chelation**

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Mg$^{2+}$</th>
<th>Ca$^{2+}$</th>
<th>Approximate ratio of Mg$^{2+}$ to Ca$^{2+}$</th>
<th>Complement pathways</th>
</tr>
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<tbody>
<tr>
<td>Unchelated</td>
<td>$10^{-3}$ M</td>
<td>$10^{-3}$ M</td>
<td>1.0</td>
<td>Functional</td>
</tr>
<tr>
<td>10 mM EDTA serum</td>
<td>$10^{-4}$ M</td>
<td>$10^{-11}$ M</td>
<td>$10^2$</td>
<td>Functional</td>
</tr>
<tr>
<td>10 mM EGTA serum</td>
<td>$1.6 \times 10^{-4}$ M</td>
<td>$5.0 \times 10^{-11}$ M</td>
<td>$3.1 \times 10^4$</td>
<td>Suboptimally functional</td>
</tr>
<tr>
<td>10 mM MgEGTA serum</td>
<td>$3.0 \times 10^{-3}$ M</td>
<td>$10^{-10}$ M</td>
<td>$3.0 \times 10^7$</td>
<td>Partially inhibited$^a$</td>
</tr>
</tbody>
</table>

$^a$ C1 complex dissociated; C2 activated in a fluid phase.

$^b$ Factor B (C3 proactivator) activated in a fluid phase.