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Interaction of Complex Polysaccharides with the Complement System: Effect of Calcium Depletion on Terminal Component Consumption

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Complex polysaccharides and lipopolysaccharides can activate the terminal components of complement by either the classical (antibody, C1, C4, and C2) or alternative complement pathways, but the relative importance of either pathway for terminal component consumption in normal serum is poorly understood. Since classical complement pathway function requires both calcium and magnesium ions, whereas the alternative pathway requires only magnesium ions, selective chelation of calcium ions in serum can be used to block the classical complement pathway while leaving the alternative pathway intact. In these studies, ethyleneglycol-bis-(β-aminoethyl ether)*N*, *N'*-tetraacetic acid, a potent chelator of calcium, was used to block the classical complement pathway in normal guinea pig serum. Consumption of the terminal complement components by endotoxin, inulin, and zymosan in such serum was strikingly depressed when compared to serum containing an intact classical complement pathway. These studies demonstrate that in normal serum, both the classical and alternative complement pathways participate in the consumption of the terminal complement components by complex polysaccharides and lipopolysaccharides.

The complement (C) system, through activation of its terminal components (C3 through C9), functions as an important immune effector and mediates numerous biological activities which are critical for host defense (16, 18, 19, 30). It is now clear that the terminal components can be activated in a number of ways including by the classical C pathway (antibody, C1, C4, and C2), by the alternative C pathway, and by the direct cleavage of C components by proteolytic enzymes (8, 17, 31, 32; P. A. Ward, M. C. Conroy, and I. H. Lepow, Fed. Proc. **30**:355). Immune complexes of antigen with immunoglobulin (Ig)G or IgM antibody have generally been considered to activate C3 through C9 predominantly via the classical C pathway since the interaction of these complexes with C in serum results in the consumption of C1, C4, and C2 as well as C3 through C9 (11). On the other hand, complex polysaccharides and lipopolysaccharides (i.e., inulin, zymosan, and endotoxin) are known to consume only minute quantities of C1, C4, and C2 in normal serum while depleting large quantities of C3 through C9 (8, 11, 23). These polymeric substances also consume C3 through C9 in serum from guinea pigs congenitally devoid of C4 and promote cleavage of alternative C pathway components in normal serum (6, 12, 13, 26). For these reasons, it has been assumed that endotoxins, inulin, and zymosan activate the terminal C components predominantly through the alternative C pathway (26). We have previously demonstrated, however, that depletion of C4 in normal guinea pig serum by a C4 inactivator derived from shark serum (14) resulted in a depressed consumption of C3 through C9 by endotoxin which could be restored by the readdition of small quantities of purified C4 (29). In addition, we showed that endotoxin could activate the terminal components of C in the absence of the alternative C pathway by interacting with "natural antibody," C1, C4, and C2 (22).

Complex polysaccharides and lipopolysaccharides can activate C3 through C9 via the alternative C pathway alone as seen in congenitally C4-deficient guinea pig serum (6) or by the classical C pathway alone using purified C components (22). The relative importance of either pathway for the consumption of the terminal C components in the more physiological environment of normal serum, however, remains unclear. This report attempts to define

the role of the classical C pathway in the activation of C3 through C9 in normal serum by determining the effects of calcium depletion on the ability of various C activators to consume the terminal C components.

MATERIALS AND METHODS

Sources of guinea pig serum. Pooled, normal guinea pig serum was purchased from Texas Biologicals, Inc. (Fort Worth, Tex.) or obtained from strains of normal, National Institutes of Health, multipurpose guinea pigs. Serum devoid of the fourth component of C was obtained from congenitally C4-deficient guinea pigs. Levels of Ca^{2+} and Mg^{2+} in guinea pig serum are normally 2×10^{-3} M and 10^{-3} M, respectively (2).

Endotoxic LPS. Endotoxic lipopolysaccharides (LPS) were isolated from *Proteus vulgaris* according to the method of Galanos et al. (7).

Inulin. Pyrogen-free inulin was purchased from Nutritional Biochemicals, Inc. (Cleveland, Ohio).

Zymosan. Type A zymosan (lot no. 7B651) was obtained from Fleischmann Laboratories (Standard Brands, Inc., New York).

AHGG. Aggregated human gamma globulin (AHGG) was prepared by heating human immune serum globulin (Armour Pharmaceutical Co., Phoenix, Ariz.) for 10 min at 61 C.

CVF. Cobra venom factor (CVF) was purified from the venom of *Naga naga* (Miami Serpentarium, Miami, Fla.) by the method of Nelson et al. (20).

Chelators. Ethyleneglycol-bis-(β-aminoethyl ether) *N, N'*-tetraacetic acid (EGTA) (Sigma Chemical Co., Cleveland, Ohio) and disodium ethylenediaminetetraacetate (EDTA) (Fisher Scientific, Inc., Fairlawn, N.J.) were used as chelating agents. EGTA and EDTA were prepared as 0.1 or 0.2 M solutions in distilled water, brought to pH 7.4 with NaOH, and mixed with equal volumes of gelatin-Veronal buffer (GVB) (15) to make stock solutions of 0.05 M or 0.1 M EGTA and EDTA. MgEGTA (0.05 M) was prepared by mixing equal volumes of 0.1 M $MgCl_2$ dissolved in GVB and 0.1 M EGTA. MgEGTA (0.1 M) was prepared in a similar manner. Where indicated, sera were incubated with a sufficient amount of EDTA, EGTA, or MgEGTA to yield a final concentration of 0.01 M chelator.

ShEA. Sheep erythrocytes were collected in equal volumes of Alsever solution and sensitized with anti-sheep hemolysin (ShEA) (Sylvana Chemical Co., Millburn, N.J.) according to the method of Rapp and Borsos (25).

Hemolytic complement titrations. Hemolytic C3 through C9 activity was assayed by a minor modification (9) of the method of Mayer (15). Briefly, ShEA carrying optimal amounts of C1, 4, and 2 (ShEAC $\overline{142}$) were added to serum which had been serially diluted in GVB containing 0.02 M EDTA. Percentage of hemolysis was determined spectrophotometrically. GVB containing supplemental Mg^{2+} and Ca^{2+} is designed as GVB^{2+} (15).

RESULTS

Effects of calcium depletion on the consumption of C3 through C9 in normal guinea pig serum by endotoxin, inulin, and zymosan. Cleavage of the terminal components of C by the classical C pathway depends on enzymatic processes which require both calcium and magnesium ions (15). The alternative C pathway, however, requires only magnesium ions to activate the terminal C components (4, 13). EGTA is a potent chelator of Ca^{2+} ($K_a = 10^{10.9}$) but a weak binder of Mg^{2+} ($K_a = 10^{5.2}$) (2) and has successfully been used to block the classical C pathway in normal serum while leaving the alternative C pathway intact (3, 4). EGTA was therefore added to normal guinea pig serum to determine the effect of Ca^{2+} depletion on the ability of LPS, inulin, and zymosan to consume C3 through C9 (Table 1). These data demonstrate that chelation of both Ca^{2+} and Mg^{2+} by EDTA effectively blocked C3 through C9 consumption by all the C activators used. Depletion of Ca^{2+} by EGTA or MgEGTA did not depress the ability of CVF to consume C3 through C9, thereby suggesting that the alternative C pathway was intact and, indeed, Ca^{2+} independent. The ability of AHGG or ShEA to consume C3 through C9 in such serum was almost completely blocked by EGTA. Ca^{2+} depletion by EGTA inhibited the ability of LPS, zymosan, and inulin to consume C3 through C9 by 43 to 64%.

Experiments were also performed to determine the degree of inhibition of C3 through C9 consumption by EGTA using a wide dose range of complex polysaccharides (Table 2). The data indicate that calcium depletion resulted in inhibition of terminal C component consumption by doses of complex polysaccharides which produced from 16 to 95% consumption of C3 through C9 in nonchelated serum.

Effects of calcium depletion on the consumption of C3 through C9 in C4 deficient guinea pig serum by endotoxin, inulin, and zymosan. Serum from guinea pigs genetically devoid of C4 support the consumption of C3 through C9 solely by the alternative C pathway except under conditions where large excesses of C1 are added to the serum (5). Serum deficient in C4 was therefore used in an effort to prove that the concentration of EGTA used in normal guinea pig serum did not inhibit the alternative C pathway or otherwise directly impair the consumption of C3 through C9 by the materials under study (Table 3). The addition of 0.01 M EGTA did not affect the ability of zymosan,

TABLE 1. *Effect of differential cation chelation on C3 through C9 consumption in normal guinea pig serum*

Complement activator[a]	Consumption (%) of C3 through C9 after addition of:[b]				Inhibition by EGTA (%)[d]
	None[c]	EDTA	EGTA	MgEGTA	
CVF (10 μliters)	96	<5	96	94	<5
CVF (0.02 μliters)	93	<5	94	ND[e]	<5
Zymosan (0.5 mg)	91	<5	51	44	43
LPS (*Proteus vulgaris*, 0.05 mg)	84	<5	46	ND	46
Inulin (0.25 mg)	86	<5	31	39	64
AHGG (0.25 mg)	92	<5	7	12	93
ShEA (2×10^8 cells)	62	<5	6	9	91

[a] The indicated C activator contained in 0.3 ml of GVB was added to 0.1 ml of guinea pig serum which had previously been incubated (37 C for 5 min) with 0.1 ml of the appropriate chelator (0.05 M) or with 0.1 ml of GVB. After incubation at 37 C for 60 min, the residual C3 through C9 titers were determined and compared to the C3 through C9 titer of guinea pig serum (0.1 ml) incubated (37 C for 60 min) with either 0.4 ml of GVB or 0.3 ml of GVB plus 0.1 ml of the appropriate chelator.

[b] Percentage of consumption was determined by comparing the residual C3 through C9 titer of serums incubated with the indicated C activator with the residual C3 through C9 titer of the appropriate control. C3 through C9 titers (CH50 units/ml) in sera not incubated with C activators were as follows: EDTA = 1,045; EGTA = 1,059; MgEGTA = 1,009; no chelator = 1,250.

[c] Each reaction mixture contains 0.3 ml of GVB^{2+}, 0.1 ml of guinea pig serum, and 0.1 ml of the indicated C activator contained in GVB.

[d] Percentage of inhibition was determined by comparing the percentage of consumption of C3 through C9 by the indicated C activator in serum containing EGTA with the percentage of consumption by the same C activator in serum containing GVB alone.

[e] ND = Not done.

inulin, or CVF to consume C3 through C9 in Ca^{2+}-depleted, C4-deficient serum, thus demonstrating the intactness of the alternative C pathway in the presence of 0.01 M EGTA. Consumption of C3 through C9 by LPS was slightly depressed by the addition of EGTA to C4-deficient serum. The addition of excess Ca^{2+} to EGTA-treated, C4-deficient serum did not restore this minimally depressed ability of LPS to consume C3 through C9 in such sera, thereby implying that EGTA has a slight "detoxifying" effect on LPS (4).

Effects of calcium depletion on the kinetics of C3 through C9 consumption in normal guinea pig serum by endotoxin, inulin, and zymosan. The foregoing experiments demonstrate that Ca^{2+} depletion in normal serum significantly diminishes the total consumption of C3 through C9 by LPS, inulin, and zymosan. To determine more precisely the role of Ca^{2+} in the consumption of C3 through C9 by these agents, MgEGTA was added to normal serum, and the kinetics of C3 through C9 consumption by inulin and zymosan were determined (Fig. 1). Since EGTA itself slightly depresses the ability of LPS to consume C3 through C9, LPS was not used in these experiments. MgEGTA (0.01 M) did not depress the kinetics of C3 through C9 consumption by CVF, again suggesting that the alternative C pathway was not inhibited. Serum treated with MgEGTA was, however, markedly defective in its ability to support C3 through C9 consumption by inulin and zymosan. At 10 min after interaction with zymosan, normal serum was depleted of almost 85% of available C3 through C9, whereas Ca^{2+}-deficient serum was depleted of only 20% of the available C3 through C9.

Effects of calcium depletion on the consumption of C3 through C9 in minimally diluted serum by endotoxin, inulin, and zymosan. Previous studies have demonstrated that alternative C pathway components are more susceptible to dilution in serum than are the classical C pathway components (27, 28). To be certain that the results of our experiments were not affected by the dilution of serum used (1:5 final dilution), experiments were performed in minimally diluted serums (4:5 final dilution). Depletion of Ca^{2+} in minimally diluted serum again depressed the ability of LPS, inulin, and zymosan to consume C3 through C9 (Table 4).

DISCUSSION

The availability of a number of pathways by which the terminal C components can be activated is well established (8, 17, 19, 26). The biochemistry of the classical C pathway consisting of antibody, C1, C4, and C2 has been well defined (25), whereas the characterization of

TABLE 2. *Effect of calcium depletion on C3 through C9 consumption in normal guinea pig serum by various amounts of complex polysaccharides*

Complement activator[a]	Consumption (%) of C3 through C9 after addition of:[b]		Inhibition by EGTA (%)[d]
	None[c]	EGTA	
Zymosan (mg)			
0.05	16	11	32
0.10	80	50	38
0.25	93	45	52
0.50	95	49	49
Inulin (mg)			
0.05	71	39	46
0.10	84	34	60
0.25	92	51	45
LPS (*Proteus vulgaris*) (mg)			
0.05	84	46	46
0.10	85	58	32
0.25	90	61	33

[a] The indicated C activator contained in 0.3 ml of GVB was added to 0.1 ml of guinea pig serum which had previously been incubated (37 C for 5 min) with 0.1 ml of the appropriate chelator (0.05 M) or with 0.1 ml of GVB. After incubation at 37 C for 60 min, the residual C3 through C9 titers were determined and compared to the C3 through C9 titer of guinea pig serum (0.1 ml) incubated (37 C for 60 min) with either 0.4 ml of GVB or 0.3 ml of GVB plus 0.1 ml of the appropriate chelator.

[b] Percentage of consumption was determined by comparing the residual C3 through C9 titer of serums incubated with the indicated C activator with the residual C3 through C9 titer of the appropriate control. C3 through C9 titers (CH50 units/ml) in sera not incubated with C activators were as follows: EGTA = 956; no chelator = 1,185.

[c] Each reaction mixture contains 0.3 ml of GVB^{2+}, 0.1 ml of guinea pig serum, and 0.1 ml of the indicated C activator contained in GVB.

[d] Percent inhibition was determined by comparing the percentage of consumption of C3 through C9 by the indicated C activator in serum containing EGTA with the percent consumption by the same C activator in serum containing GVB alone.

the components of the alternative C pathway is presently undergoing extensive investigation. The elucidation of the pathophysiological significance of the multiple pathways of C activation is important for many reasons, not the least of which are clinical, since certain human diseases seem to be mediated predominantly by activation of one or the other pathways (10, 24, 34, 35). Complex polysaccharides and LPS clearly activate the alternative C pathway and deplete normal serum of relatively small quantities of C1, C4, and C2 when compared to the depletion of these components by aggregated gamma globulins or immune complexes containing IgG or IgM antibody. In addition, consumption of C3 through C9 by endotoxin, inulin, or zymosan does not require immune antibody and occurs in the serum from guinea pigs congenitally devoid of C4, albeit at a somewhat diminished level as compared to normal guinea pig serum (6). It should be emphasized, however, that although polymeric substances such as endotoxins deplete serum of relatively small quantities of C1, C4, and C2, it is erroneous to assume that these components are not critical for consumption of C3 through C9. For example, membranous substances such as endotoxins and zymosan could very efficiently utilize small quantities of natural antibody, C1, C4, and C2 for consumption of C3 through C9. A role for natural antibody in the consumption of C by endotoxin has been demonstrated by the finding that serum absorbed with a given preparation of endotoxin had a diminished capacity to support C consumption by an identical, but not by an antigenically dissimilar, type of endotoxin (22). In addition, utilization of less than 5% of the available C1, C4, or C2 in serum by polymeric substances would not be detected by most hemolytic complement assays and could be critical for efficient consumption of the terminal C components. This indeed was found to be the case in previous studies where normal guinea pig serum containing approximately 20,000 CH50 units of C4 per ml was treated with a C4 inactivator to depress the C4 titer to 20

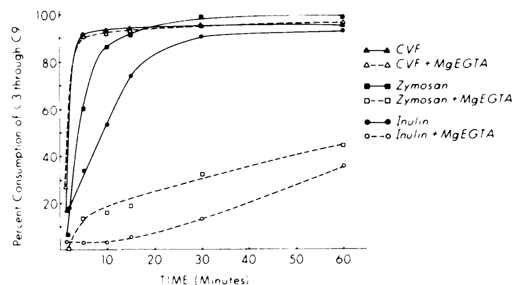

FIG. 1. *The indicated C activators contained in 0.9 ml of GVB were incubated at 37 C for various times with 0.3 ml of normal guinea pig serum containing 0.3 ml of MgEGTA (0.05 M) or GVB^{2+}. Portions (0.2 ml) were removed at the indicated times and placed in ice-cold 0.02 M EDTA-GVB, and the residual C3 through C9 titers were determined. The amount of C activators used were as follows: CVF = 30 μliters; zymosan = 1.5 mg; inulin = 0.75 mg. Percentage of consumption was determined by comparing the residual C3 through C9 titers of serums treated with C activators with residual C3 through C9 titer of the appropriate control.*

TABLE 3. *Effect of differential cation chelation on C3 through C9 consumption in C4-deficient guinea pig serum*

Complement activator[a]	Consumption (%) of C3 through C9 after addition of:[b]			Inhibition by EGTA (%)[d]
	None[c]	EDTA	EGTA	
CVF (0.02 μliters)	93	<5	94	<5
Zymosan (1 mg)	77	<5	76	<5
Inulin (0.5 mg)	79	<5	78	<5
LPS (*Proteus vulgaris*, 0.1 mg)	69	<5	52	25
ShEA (2×10^8 cells)	7	<5	8	<5

[a] The indicated C activator contained in 0.3 ml of GVB was added to 0.1 ml of C4-deficient guinea pig serum which had previously been incubated (37 C for 5 min) with 0.1 ml of the appropriate chelator (0.05 M) or with 0.1 ml of GVB. After incubation at 37 C for 60 min, the residual C3 through C9 titers were determined and compared to the C3 through C9 titer of C4-deficient guinea pig serum (0.1 ml) incubated (37 C for 60 min) with either 0.4 ml of GVB or 0.3 ml of GVB containing 0.1 ml of the appropriate chelator.

[b] Percentage of consumption was determined by comparing the residual C3 through C9 titers of serums incubated with the indicated C activator with the residual C3 through C9 titers of the appropriate control. C3 through C9 titers (CH50 units/ml) in sera not incubated with C activators were as follows: EDTA = 1,825; EGTA = 1,829; no chelator = 1,880.

[c] Each reaction mixture contains 0.3 ml of GVB^{2+}, 0.1 ml of guinea pig serum, and 0.1 ml of the indicated C activator contained in GVB.

[d] Percentage of inhibition was determined by comparing the percent consumption of C3 through C9 by the indicated C activator in serum containing EGTA with the percentage of consumption by the same C activator in serum containing GVB alone.

CH50 units per ml. The ability of endotoxin to consume C3 through C9 was markedly depressed in such serum but was fully restored by the readdition of approximately 200 CH50 units of purified C4 (29). Although it is evident that activation of C3 through C9 by complex polysaccharides and lipopolysaccharides can proceed solely via the alternative C pathway (5) or via the classical C pathway (22) under certain situations, information regarding the importance of either pathway in the more physiological milieu of normal serum is fragmentary. This present study exploits the fact that the addition of EGTA or MgEGTA to normal serum selectively blocks the classical C pathway while leaving the alternative pathway intact. In serum depleted of Ca^{2+}, consumption of C3 through C9 by CVF proceeds unhindered, whereas activation of C3 through C9 by aggregated gamma globulin or sensitized ShEA is profoundly depressed. These data strengthen the contention that in normal serum, immune complexes containing IgG or IgM antibody activate the terminal components of C predominantly via the classical C pathway. The ability of endotoxin, inulin, and zymosan to consume C3 through C9 in Ca^{2+} depleted normal serum and thus solely via the alternative C pathway is strikingly depressed when compared to serum containing both functional pathways. The degree of depression of C3 through C9 consumption, however, is not as profound as that seen with aggregated gamma globulin or ShEA.

These findings clearly demonstrate the par-

TABLE 4. *Effect of calcium depletion on C3 through C9 consumption in minimally diluted normal guinea pig serum*

Complement activator[a]	Consumption (%) of C3 through C9 after addition of:[b]		Inhibition by MgEGTA (%)[d]
	None[c]	MgEGTA	
CVF (40 μliters)	98	96	<5
Zymosan (2.0 mg)	90	32	64
Inulin (1.0 mg)	82	53	36
LPS (0.4 mg)	42	7	83
AHGG (1.0 mg)	91	0	100

[a] The indicated C activator contained in 0.05 ml of GVB was added to 0.4 ml of guinea pig serum which had previously been incubated (37 C for 5 min) with 0.05 ml of MgEGTA (0.1 M) or with 0.05 ml of GVB. After incubation at 37 C for 60 min, the residual C3 through C9 titers were determined and compared to the C3 through C9 titer of guinea pig serums (0.4 ml) incubated (37 C for 60 min) with either 0.1 ml of GVB or 0.05 ml of GVB plus 0.05 ml of MgEGTA (0.1 M).

[b] Percentage of consumption was determined by comparing the residual C3 through C9 titer of serums incubated with the indicated C activator to the residual C3 through C9 titer of the appropriate control. C3 through C9 titers (CH50 units/ml) in sera not incubated with C activators were as follows: MgEGTA = 920; no chelator = 1,186.

[c] Each reaction mixture contained 0.05 ml of GVB, 0.4 ml of guinea pig serum, and 0.05 ml of the indicated C activator contained in GVB.

[d] Percentage of inhibition was determined by comparing the percent consumption of C3 through C9 by the indicated C activator in serum containing MgEGTA with the percentage of consumption by the same C activator in serum containing GVB alone.

ticipation of both the classical and alternative C pathways for consumption of the terminal C components by complex polysaccharides as suggested previously (1, 21, 29). The data moreover indicate that maximally efficient consumption of C3 through C9 by endotoxin, inulin, and zymosan in normal serum requires an intact classical pathway.

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