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THE DEMONSTRATION OF ALTERNATE COMPLEMENT PATHWAY ACTIVITY BY '--ETC(U)
JAN 77 C E HAWLEY, W A FALKLER DABB05-72-A-0666
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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE *(and Subtitle)* Demonstration of Alternate Complement Pathway Activity by Fusobacterium Polymorphum in the Presence of EGTA	5. TYPE OF REPORT & PERIOD COVERED Manuscript for publication Sep 75 - Jul 76
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Charles E. Hawley, William A. Falkler, Jr.	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, D.C. 20012	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS Ph.D. Thesis, Univ. of Maryland
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command, ATTN: (SGRD-RP), Washington, D.C. 20314	12. REPORT DATE 12 January 1977
	13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS *(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)* UNCLASSIFIED
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

ADA034404

16. DISTRIBUTION STATEMENT *(of this Report)*

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17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

DDC RECEIVED JAN 14 1977 D

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Alternate Pathway; EDTA/EGTA; Fusobacterium Polymorphum.

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A complement consumption assay was used to investigate alternate pathway activation by Fusobacterium polymorphum in guinea pig sera treated with the divalent cation chelators, EDTA and EGTA. In the presence of 0.001 M EGTA, a cell wall preparation of F. polymorphum and inulin reduced the hemolytic complement activity to the same degree as that observed in the non-chelated serum controls. However, there was no hemolytic complement activity in the EGTA chelated sera against EA. A 0.001 M concentration of EDTA inhibited the anticomplementary activity of F. polymorphum, inulin, and the hemolysis of EA. (continued on reverse)

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It was also shown that a concentration of 0.001 M Mg^{++} reversed the inhibitory effect of EDTA on the classical pathway EA system and the anticomplementary activity displayed by F. polymorohum cell walls and inulin. The addition of the same concentration of Mg^{++} to the EDTA tests did not augment the consumption of complement via the alternate pathway, nor did it reverse the inhibition of the classical pathway. It is concluded that the anticomplementary activity demonstrated by the cell wall preparation and inulin in normal GPC' did not depend upon a competent classical pathway but occurred via an alternate complement pathway. It was also shown that there was a competitive interaction between Ca^{++} and Mg^{++} for common EDTA binding sites.

The Demonstration of Alternate Complement Pathway Activity by *Fusobacterium polymorphum* in the Presence of Ethylene Glycol Tetraacetic Acid

(9) Doctoral Thesis

by

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(11) 12 Jan 77

(12) 26p.

(15) DABB05-72-A-0666
PHS-DE-04161

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Short title: *F. polymorphum* Alternate Pathway Activity

This investigation was supported by Public Health Service Grant DE 04161-01 from the National Institute of Dental Research and Public Health Service Training Grant PHS T01 DE 00088-13. Participation of the senior author in this research was made possible through the U.S. Army Training Contract, DABB05-72-A0666. The work was performed during graduate studies at the Dental School, University of Maryland as partial fulfillment of requirements for the Doctor of Philosophy degree in microbiology.

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Abbreviations used in this paper:

EDTA	ethylene diamine tetra acetic acid
EGTA	ethylene glycol tetra acetic acid
C'	complement
GPC'	guinea pig complement
EA	hemolysin sensitized ovine erythrocytes
LPS	lipopolysaccharide
F	*Fusobacterium*
PBS	0.01 M phosphate buffered saline (0.15 M NaCl) pH 7
SRBC	sheep red blood cells
VBD	veronal buffered diluent
LBCF	Laboratory Branch Complement Fixation

Abstract

A complement consumption assay was used to investigate alternate pathway activation by *Fusobacterium polymorphum* in guinea pig sera treated with the divalent cation chelators, EDTA and EGTA. In the presence of 0.001 M EGTA, a cell wall preparation of *F. polymorphum* and inulin reduced the hemolytic complement activity to the same degree as that observed in the non-chelated serum controls. However, there was no hemolytic complement activity in the EGTA chelated sera against EA. A 0.001 M concentration of EDTA inhibited the anticomplementary activity of *F. polymorphum*, inulin, and the hemolysis of EA. It was also shown that a concentration of 0.001 M Mg^{++} reversed the inhibitory effect of EDTA on the classical pathway EA system and the anticomplementary activity displayed by *F. polymorphum* cell walls and inulin. The addition of the same concentration of Mg^{++} to the EDTA tests did not augment the consumption of complement *via* the alternate pathway, nor did it reverse the ihhibition of the classical pathway. It is concluded that the anticomplementary activity demonstrated by the cell wall preparation and inulin in normal GPC' did not depend upon a competent classical pathway but occurred *via* an alternate complement pathway. It was also shown that there was a competitive interaction between Ca^{++} and Mg^{++} for common EDTA binding sites.

Chelators with the ability to remove Ca^{++} and Mg^{++} from solution have gained popularity in establishing an effective separation of the classical and alternate complement pathways (1,2,3,4,5). Early studies by Levine *et al.* (1) revealed that EDTA at a concentration of 0.0007 M could exhibit the classical consumption of hemolytic activity in guinea pig sera. Similarly, 0.0057 M EDTA could inhibit the same activity in guinea pig sera, but the inhibition could be reversed by the addition of 0.007 M Ca^{++} and 0.007 M Mg^{++} to the reaction mixture. It was assumed that these divalent cations were essential in the fixation of complement by immune complexes.

Fine *et al.* (2) have used EDTA to inhibit the consumption of total human complement by EA, zymosan, and *E. coli* at 37 C. It was found that EDTA (0.001 M) could inhibit the anticomplementary activity of all three agents. However, the same concentration of EGTA selectively inhibited the decomplementation by EA while the anticomplementary activity of zymosan and *E. coli* cell suspensions proceeded according to patterns seen in unchelated sera. It was proposed that a comparison of anticomplementary activity in EDTA and EGTA chelated sera could serve as a means of distinguishing the Ca^{++} dependent classical pathway from the Mg^{++} dependent alternate complement pathway. EDTA and EGTA have near identical association constants for Ca^{++} (1×10^{11}). While EDTA has a Mg^{++} binding constant of 1×10^{9}, EGTA is less effective in binding Mg^{++} at 1×10^{5} (2,3,4,5). Fine (3) studied the effects of the same chelators on GPC'. It was observed that LPS was progressively "detoxified" in chelated sera, and that its anticomplementary activity could only be demonstrated in normal GPC'. However, when the LPS was bound to ovine erythrocytes, E-LPS, it showed no evidence of this chelator associated reduction in anticomplementary potency. The anticomplementary activities of EA, E-LPS, and zymosan were inhibited in EDTA treated sera while only zymosan was able to decomplement EGTA treated sera. Differences in the reactivity of LPS and E-LPS were demonstrated after GPC' had been absorbed with E-LPS and

then challenged with E-LPS, LPS, and zymosan. LPS and zymosan each displayed the same anticomplementary activity in the E-LPS absorbed and non-absorbed GPC'. However, the activity of E-LPS was depressed in the E-LPS absorbed sera. These results indicated that LPS activated both the alternate and classical pathways, but that LPS activated only the classical pathway when bound to erythrocyte membranes.

Snyderman and Pike (4) showed that C3-C9 consumption by cobra venom factor, inulin, zymosan, EA, and LPS could be inhibited in GPC' chelated with EDTA at a concentration of 0.001 M. It was further noted that the anticomplementary activities of EA, LPS, inulin, and zymosan against C3-C9 in normal GPC' were depressed in GPC' treated with EGTA at a final concentration of 0.001 M. The activity of cobra venom factor was unaffected by the same concentration of EGTA. The activity could not be enhanced by the addition of Mg^{++} to the EDTA treated sera. LPS, inulin, cobra venom factor, and zymosan displayed the same consumption of C3-C9 in either EGTA treated or untreated C4-deficient guinea pig sera. Since complement consumption by LPS, inulin, and zymosan was greater in normal GPC' than in EGTA chelated or C4-deficient sera, it was assumed that these factors consumed the terminal complement components in normal GPC' through both the alternate and classical pathways. The enhanced activity in normal sera probably represented the contribution of the classical pathway which on the basis of EA reactivity did not operate in the EGTA treated sera. Cobra venom factor apparently did not consume complement to any degree through the classical pathway.

DesPrez *et al.* (5) compared the function of the classical and alternate pathways in human sera using either 0.001 M concentrations of EGTA and EDTA. Some assays were supplemented with 0.001 M Mg^{++}. Sera chelated with EGTA showed alternate pathway consumption by zymosan and *E. coli* cells. The alternate pathway activity was further established electrophoretically by the observed disso-

ciation of C'1 and the conversion of C3 proactivator (C3PA) to C3 activator (C3A). Also there was no consumption of C'2 in the presence of EGTA. However, there was a marked reduction of total complement by both zymosan and *E. coli*. There was no hemolytic activity in the EGTA treated assays against EA. In non-chelated sera, zymosan and *E. coli* cells showed 100% consumption of total complement and C'2. The addition of Mg^{++} to the EGTA treated sera had no effect on reversing the instability of C'1, but there was increased consumption of C'2 by zymosan, *E. coli*, and EA. These same investigators (5) also noted the dissociation of C'1, the decomplementation of C'2, and the conversion of C3PA in C3A ~~in~~ EGTA and Mg^{++} treated sera which did not contain zymosan or *E. coli*.

This paper examines the anticomplementary activity of a cell wall preparation from the gram negative anaerobe, *Fusobacterium polymorphum*, in guinea pig sera with EDTA or EGTA. In tests supplemented with Mg^{++}, we attempt to clarify the dynamic interaction of divalent cations with either EGTA or EGTA.

Materials and Methods

Microorganisms: *Fusobacterium polymorphum*, ATCC #10953, was grown anaerobically in brewer jars (Gas-Pak System, Baltimore Biological Laboratories, Cockeysville, Md.). A liquid modified tryptone medium (6) was used.

Cell Wall Preparation: After 48 h incubation period, the cells were harvested by centrifugation at 10,000 x g at 4 C. The cells were washed three times in 0.01 M phosphate buffered saline (0.15M NaCl) pH 7.2 (PBS). The cells were resuspended in isotonic saline and sonicated (Branson Instruments, Inc., Stamford, Conn.) at 6 amps in a dry ice/ethanol bath (-40 C) using eight 30 sec bursts. The sonicate was then centrifuged at 2,000 x g for 10 min at 4 C. The sediment was washed 5 times in deionized distilled water at 20,000 x g for 15 min or

until the wash supernatants showed no absorbance at 260 nm (Gilford Laboratory Instruments, Inc., Oberlin, Ohio). This was used as an index for complete removal of cytoplasmic material from the sonicated debris (7). The resulting cell wall preparations were lyophilized (Virtis Co., Inc., Gardiner, N.Y.) and designated FP cell walls. Prior to use in the complement consumption assay, the preparation was reconstituted in deionized (Bantam Demineralizer, Barnstead Still and Sterilizer Co., Boston, Mass.) distilled water at 5,000 µg/ml.

Complement Titration: Complement titrations were performed according to a modified LBCF (8) protocol.

Modifications included the preparation of a cation starved VBD, designated VBD^{--}, which was prepared by mixing 1 part of a stock salt solution (5x concentrated) with 4 parts of a gelatin water solution. The buffer was made fresh daily and kept on ice at all times. The 5x stock was prepared by adding the following to a 1 liter volumetric flask:

NaCl	42.5 g
Na5, 5-diethyl barbiturate	5.1 g
Deionized-distilled water	250.0 ml
HCl 1N	17.3 ml
Dextrose	50.0 g

SRBC in Alsever's solution were washed three times in VBD^{--} and the packed volume resulting from centrifugation at 600 x G for 10 min was resuspended in 32.33 volumes of buffer. This produced a 3.0% suspension of SRBC containing approximately 6.7×10^5 cells/mm^3 (8).

Complement Consumption Assay: A complement consumption assay was employed to determine the consumption of total complement activity in GPC' and GPC' that had been treated with the divalent cation chelators,(Sigma Chemical Co., St. Louis, Mo.) ethylene diamine tetraacetic acid (EDTA) and ethylene glycol-bis-N, N'-tetra acetic acid (EGTA). The methods of Bladen *et al.* (9), Gewurz *et al.* (10), Gewurz *et al.* (11), and Phillips *et al.* (12) were used as the basis for

the complement consumption assay. In order to test the effect of the chelators, modifications were made in the basic assay according to the principles reported by Mayer (14), Fine *et al.* (2), and Fine (3). Tests were set up in 15 ml glass centrifuge tubes in the following manner: 0.1 ml reconstituted GPC' and 0.1 ml of a 0.01 M solution of the chelator in saline were incubated at 4 C for 15 min. To this was added 0.1 ml of the anticomplementary agent (500 µg FP cell walls or 500 µg inulin), and VBD^{--} (0.7 ml) to make the final volume of the test 1.0 ml. In some cases, 0.1 ml of a 0.01 M solution of anhydrous $MgSO_4$ in saline was added at the same time as the anticomplementary reagents, and this was followed with 0.6 ml VBD^{--}. The tests were then incubated at 37 C for 1 h. At the end of the incubation period, the tubes were centrifuged at 6,000 x g in the cold and the supernatants were carefully removed with Pasteur pipettes from the pellet of anticomplementary material. The supernatants were then divided into aliquots of 0.4 ml each. One of the aliquots was recalcified with 0.05 ml of a 0.01 M solution of anhydrous $CaCl_2$ in saline, while the other, which was to become a non-recalcified control, was treated with 0.05 ml of saline. Recalcification was permitted to occur in the cold for 1 h. In addition, saline control tests were run in parallel with the assays containing EDTA, EGTA, or combinations of these chelators with $MgSO_4$. At the end of the incubation period, a complement titration was performed on the VBD^{--} controls. The correct dilution and the volume of thtt dilution which contained 1 $C'H_{50}$ unit were used to calculate the number of $C'H_{50}$ units in the original 0.1 ml GPC' placed in the assay. This value was established as the control level of unconsumed complement.

The residual GPC' in experimentals, in positive controls, and in negative controls was measured by single point analysis of partial lysis using the conversion factors calculated from the Von Krogh equation (13). A 0.1 ml sample of each test was diluted to the same degree as the negative control in the complement titration. Two other more concentrated dilutions were made to detect

reduced $C'H_{50}$ unit activity. Each dilution (0.3 ml) was added to a serologic tube containing 0.5 ml VBD^{--}. To this mixture, 0.2 ml EA was added. The tubes were shaken and incubated in a 37 C water bath for 30 min. After incubation the degree of hemolysis was measured by comparison with the color standards, and the number of residual $C'H_{50}$ units in the test calculated. The difference between the amount of GPC' in the VBD^{--} controls determined by complement titration and the amount of residual complement established by the single point analysis method was recorded in $C'H_{50}$ units consumed and in percent $C'H_{50}$ units consumed.

Results

The hemolytic activity of normal GPC' against EA was compared with the hemolytic activity of GPC' in the presence of either EDTA or EGTA at final concentrations of 0.001 M. After 1 h in a water bath at 37 C, normal GPC' was diluted 1:400 before 1 $C'H_{50}$ unit could be titrated. After the same incubation period, EDTA or EGTA chelated GPC' did not display hemolytic activity with EA in dilutions that ranged from 1:50 to 1:400. The failure to find residual $C'H_{50}$ unit activity at these dilutions of chelator treated GPC' represented a greater than 87.5% inhibition of classical pathway activity.

The effect of the chelator, EGTA, on the anticomplementary activity of FP cell walls was tested in the complement assay. These tests used 0.1 ml GPC' and 0.1 ml 0.01 M EGTA which were mixed and incubated for 15 min at 4 C before the addition of 0.1 ml of 500 µg FP cell walls or inulin (500 µg), and 0.7 ml VBD^{--}. The test systems were incubated for 1 h at 37 C, centrifuged in the cold (4 C) at 6,000 x G for 10 min. After the recalcification period, the tests were titrated for residual complement activity with EA. The results are reported in Figure 1. The percent anticomplementary activity of FP cell walls

at 500 µg/ml of test was 44.5% (solid bar A) in EGTA treated GPC' and 47.9% (solid bar B) in non-chelated saline controls. The anticomplementary activity of inulin at 500 µg/ml test was 58.8% (solid bar C) in EGTA chelated GPC' and 53.9% (solid bar D) in untreated saline controls. As shown by the EGTA treated VBD^{--} controls, the activity of EA was inhibited by 76.4% (open bar E).

In other tests with EGTA treated sera, GPC', 0.1 ml, was incubated with 0.1 ml 0.01 M EGTA for 15 min at 4 C prior to adding 0.1 ml 0.01 M $MgSO_4$, 0.1 ml FP cell walls (500 µg) or inulin (500 µg), and 0.6 ml VBD^{--} buffer. The results of the test are presented in Figure 2. FP cell walls were capable of decomplementing EGTA/Mg^{++} treated GPC' of 50.7% (solid bar A) of the $C'H_{50}$ units present in the test. A similar finding was observed when inulin was used as the anticomplementary agent, and complement was consumed by 58.8% (solid bar C). Without recalcification, all EGTA/Mg^{++} tests demonstrated that 79.0% of the original $C'H_{50}$ unit activity could not be measured by complement titration with Ca^{++} (open bars A and C). FP cell walls were shown to consume complement by 50.7% in non-chelated saline controls (open bar B and solid bar B). Inulin reduced the GPC' activity in the same EGTA free tests by 59.8% (open bar D and solid bar D). In the non-recalcified EGTA/Mg^{++} treated VBD^{--} negative controls (open bar E), the hemolytic activity with EA was reduced by 71.7%.

The tests to determine the effect of EDTA on the anticomplementary activity of FP cell walls and inulin were performed in the same manner as the EGTA chelated tests. The results are shown in Figure 3. Based on the activity of VBD^{--} controls, EDTA was effective in inhibiting the consumption of GPC' by FP cell walls and inulin. After recalcification, both tests showed activity (solid bars A and C) which was equal to the activity missing in the recalcified EDTA treated controls (solid bar E) which were incubated without either of the anticomplementary agents. If the EDTA treated tests were not recalcified, there was a greater than 79.9%

(open bar A) inhibition of complement activity in the FP cell wall treated tests, and a 74.4% (open bar C) inhibition with inulin treatment. In the absence of the EDTA, both FP cell walls and inulin were able to consume complement activity by 42.4% (open and solid bars B) and 47.0% (open and solid bars D) respectively. Compared to the missing $C'H_{50}$ unit activity in the recalcified EDTA VBD^{--} negative control, 8.1% (solid bar E), hemolysis of the EA was inhibited by 79.3% in EDTA treated VBD^{--} negative controls (open bar E) that were not recalcified before titration.

The EDTA tests were modified by the addition of Mg^{++}. After the incubation of 0.1 ml GPC' with 0.1 ml 0.01 M EDTA for 15 min at 4 C, 0.1 ml 0.1 $MgSO_4$ was added to the test at the same time as 0.1 ml FP cell walls (500 µg) and 0.6 ml VBD^{--} buffer. The 1.0 ml test was incubated, sedimented, and recalcified in the manner presented for EDTA chelated tests. The results of this test are reported in Figure 4. As seen in the EDTA test, the non-titratable $C'H_{50}$ unit activity in the recalcified FP cell wall treated EDTA chelated tests (solid bar A) was the same, 11.6%, as that demonstrated by the recalcified EDTA chelated VBD^{--} negative control (solid bar D). The missing $C'H_{50}$ unit activity was 49.9% in the non-recalcified EDTA chelated and Mg^{++} supplemented test (open bar B) and 52.2% in the recalcified EDTA chelated and Mg^{++} supplemented test (solid bar B). A similar anticomplementary effect with FP cell walls was demonstrated by the 47.8% reduction in complement activity in both the recalcified (solid bar C) and the non-recalcified (open bar C) saline controls that did not receive EDTA. In the VBD^{--} negative controls that were chelated with EDTA and supplemented with Mg^{++}, both the recalcified (solid bar E) and the non-recalcified (open bar E) aliquots showed the same level of complement activity, 11.6%, which was identical to that observed in the non-chelated, but Mg^{++} supplemented VBD^{--} controls (open and solid bars F). However, EDTA alone restricted the ability of the non-recalcified VBD^{--} negative control to react with EA by 66.4% (open bar D),

Discussion

It has been reported (2,3,4,5) that the divalent cation chelators, EGTA and EDTA, each have characteristic and individual binding affinities for Mg^{++}, while both chelators display the near identical association constants for Ca^{++} (1×10^{11}). The low binding of Mg^{++} by EGTA ($1 \times 10^{5.2}$) has been used by the above investigators as a method of confirming exclusive activity of the Mg^{++} dependent alternate complement pathway while the Ca^{++} dependent classical pathway was inhibited at the same time.

Preliminary studies were performed with EGTA and EDTA in order to establish their effect on the classical EA system. A comparison of the $C'H_{50}$ unit activity in normal GPC' and chelator treated GPC' after 1 h incubation at 37 C suggests that there was a greater than 87% inhibition of the EA system in the presence of either EDTA or EGTA. Once this inhibition was established, EA were used in subsequent chelator studies as an index of potential classical pathway activity during the 1 h incubation period of the complement consumption assay. As revealed by the missing hemolytic activity in the non-recalcified chelator treated VBD^{--} controls (74.9%, 71.1%, 79.3%, and 66.4%), this inhibitory effect of EDTA and EDTA remained essentially unchanged throughout the additional centrifugation and recalcification phases of the complement consumption assay. This would indicate that any C'1 dissociation produced by the chelation of Ca^{++} in EDTA and EGTA treated GPC' was maintained throughout the time frame of the assay unless the tests were recalcified with $CaCl_2$.

With at least a 76.4% inhibition of the classical EA system by EGTA, FP cell walls and inulin were shown to consume GPC' to the same degree as shown by these agents in non-chelated normal GPC'. A comparison of the EGTA and control saline tests with FP cell walls and inulin indicated that there was no enhanced total complement consumption in the presence of an uninhibited classical

pathway. It also implies that there were no reactive antibodies in GPC' against the FP cell walls. This is in contrast to the report by Snyderman and Pike (4) who stated that EGTA inhibited the anticomplementary activity of inulin, LPS, and zymosan on normal C3 through C9 by 64%, 46%, and 43% respectively. They also showed that this same inhibition by EGTA in normal GPC' could not be observed in C4-deficient guinea pig sera. Their findings suggested that both the alternate and a natural antibody mediated classical pathway were involved in the C3-C9 consumption in normal GPC' by these agents.

Phillips *et al.* (12) presented evidence that LPS, when attached to SRBC as E-LPS, was found to consume GPC' by both the classical and alternate pathways. Fine (3) also implicated classical pathway involvement when the consumption of GPC' by E-LPS could be inhibited by EGTA, but that the anticomplementary activity of LPS alone was probably not affected by the same chelator. Fine (3) further indicated that LPS alone underwent "detoxification" in the presence of EGTA, but that this process was inhibited when LPS was bound as E-LPS. It would seem then that LPS is more stable in its biologic activity when it is membrane bound. On the basis of results presented here, it would appear that the anticomplementary factor(s) in cell walls are more resistant to "detoxification" by EGTA than laboratory LPS preparations.

It had also been expressed (5) that the alternate complement pathway was suboptimal in its activity in the presence of EGTA. This was thought to be due to magnesium starvation, because when the EGTA was supplemented with Mg^{++}, normal alternate complement pathway activity was restored (5). In the results reported in Figure 2, it was shown that the addition of Mg^{++} to GPC' and EGTA did not enhance the consumption of $C'H_{50}$ units by either FP cell walls or inulin. As judged by the non-recalcified VBD^{--} controls, the addition of Mg^{++} to the reaction mixture during the incubation phase had no effect upon inhibition of the EA system by EGTA.

The tests which employed the chelator EDTA (Figure 3) demonstrated an 80% inhibition of the classical complement pathway in the EA system as shown by the missing complement activity in the non-recalcified EDTA controls. In addition, the recalcified experimental tests of FP cell walls and inulin showed that the consumption of $C'H_{50}$ units by these anticomplementary agents had been inhibited by EDTA in the test incubation. Similar inhibition of an alternate complement pathway by EDTA has been reported by Fine (3) with zymosan, by Fine *et al.* (2) with zymosan and *E. coli* whole cells, and by Snyderman and Pike (4) with zymosan, LPS, and inulin.

It was thought that the Mg^{++} dependent alternate complement pathway might be partially or completely restored with the addition of $MgSO_4$ to the complement consumption assay containing EGTA. The results showed that the addition of Mg^{++} restored the alternate pathway to full activity as shown by the 52.2% consumption of GPC' by FP cell walls. This was identical to the percent consumption by the same agent in saline controls. It was also surprising to find that the classical complement pathway, as shown by the EA system, had been restored to full activity. This was revealed by the equal levels of C'H unit activity in non-recalcified EDTA/Mg^{++} controls and the non-chelated controls that were supplemented with Mg^{++}. Data of this nature has not been reported in the complement literature. In explanation, one can speculate on the presence of a common cation binding site on EDTA where there may be a constant dynamic interaction of both Ca^{++} and Mg^{++} which have the same affinity for that site. The addition of excess Mg^{++} to the test system established a competitive interaction between the two cations and the EDTA binding sites which increased the availability of previously bound Ca^{++} in quantities sufficient to satisfy the cation requirements of the classical complement pathway and allow for reassociation of C'1.

On the basis of the results presented here, it is proposed that *F. polymorphum* has the potential for alternate pathway activation in the guinea pig complement system. This previously undemonstrated activity would indicate that these organisms may display similar activity in the human complement system. Since it has been shown (14,15,16) that human sera contains antibodies reactive with antigenic preparations of *Fusobacterium* species, it appears that any contribution to the anticomplementary activity by these immunoglobulins could be controlled in assays utilizing the chelator system presented here. Such studies are under way in our laboratories.

Acknowledgments

This work was partially supported by a NIDR research grant #DE-04161- . The authors wish to thank James R. Mongiello for his technical assistance and to express our appreciation to Gertrude A. Kraft for the preparation of this manuscript.

The Materials and Methods presented in this manuscript are basic to current mission oriented research activities at USAIDR, Washington, D.C.

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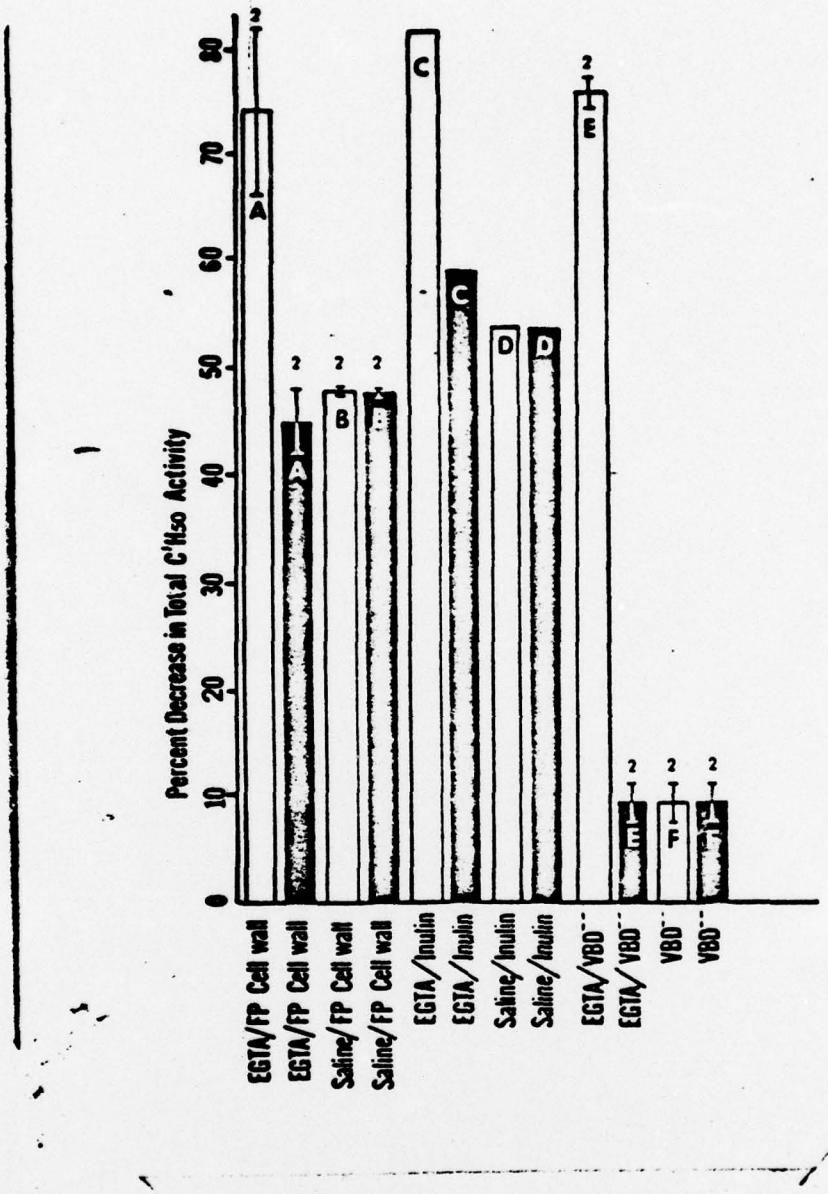
Percent Decrease in Total C'H50 Activity
0
10
20
30
40
50
60
70
80
A
B
C
D
D
E
F
EGTA/FP Cell wall
EGTA/FP Cell wall
Saline/FP Cell wall
Saline/FP Cell wall
EGTA/Inulin
EGTA/Inulin
Saline/Inulin
Saline/Inulin
EGTA/VBD⁻⁻
EGTA/VBD⁻⁻
VBD⁻⁻
VBD⁻⁻

Figure 1.

Figure 1. This figure shows the results of EGTA chelation tests using VBD^{--} buffer. GPC' was treated with 0.01 M EGTA prior to exposing it to the anticomplementary effects of FP cell walls and inulin at 500 µg/ml of test. Open bars indicate the activity of tests prior to recalcification. Solid bars show the activity of tests after recalcification. The vertical lines represent the range of data in the tests above and below the mean. The number of tests is indicated.

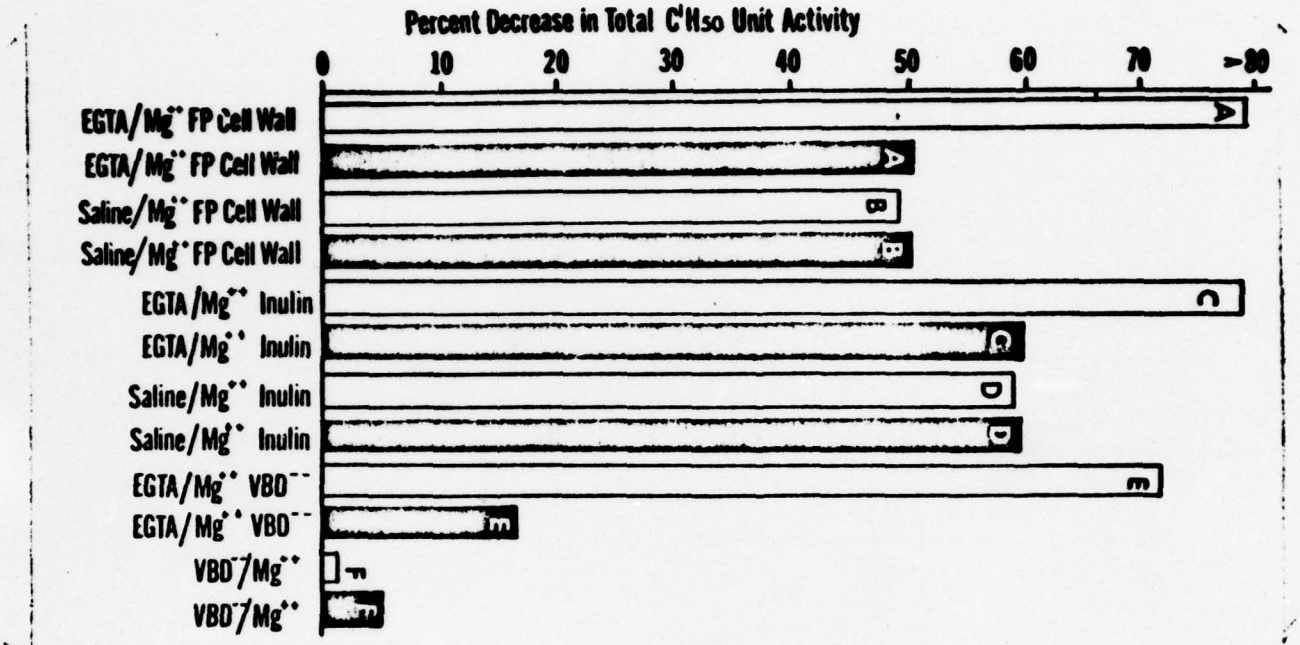
Percent Decrease in Total C'H50 Unit Activity
0
10
20
30
40
50
60
70
>80
EGTA/Mg++ FP Cell Wall
EGTA/Mg++ FP Cell Wall
Saline/Mg++ FP Cell Wall
Saline/Mg++ FP Cell Wall
EGTA/Mg++ Inulin
EGTA/Mg++ Inulin
Saline/Mg++ Inulin
Saline/Mg++ Inulin
EGTA/Mg++ VBD--
EGTA/Mg++ VBD--
VBD-/Mg++
VBD-/Mg++
A
A
B
B
C
C
D
D
E
E
F
F

Figure 2.

Figure 2. This figure shows the complement consumption by 500 µg of either FP cell walls or inulin in GPC' that was chelated with 0.01 M EGTA and supplemented with 0.01 M Mg^{++}. The buffer in this test was VBD^{--}. The open bars refer to the activity of the tests prior to recalcification. Solid bars show the activity of tests after recalcification.

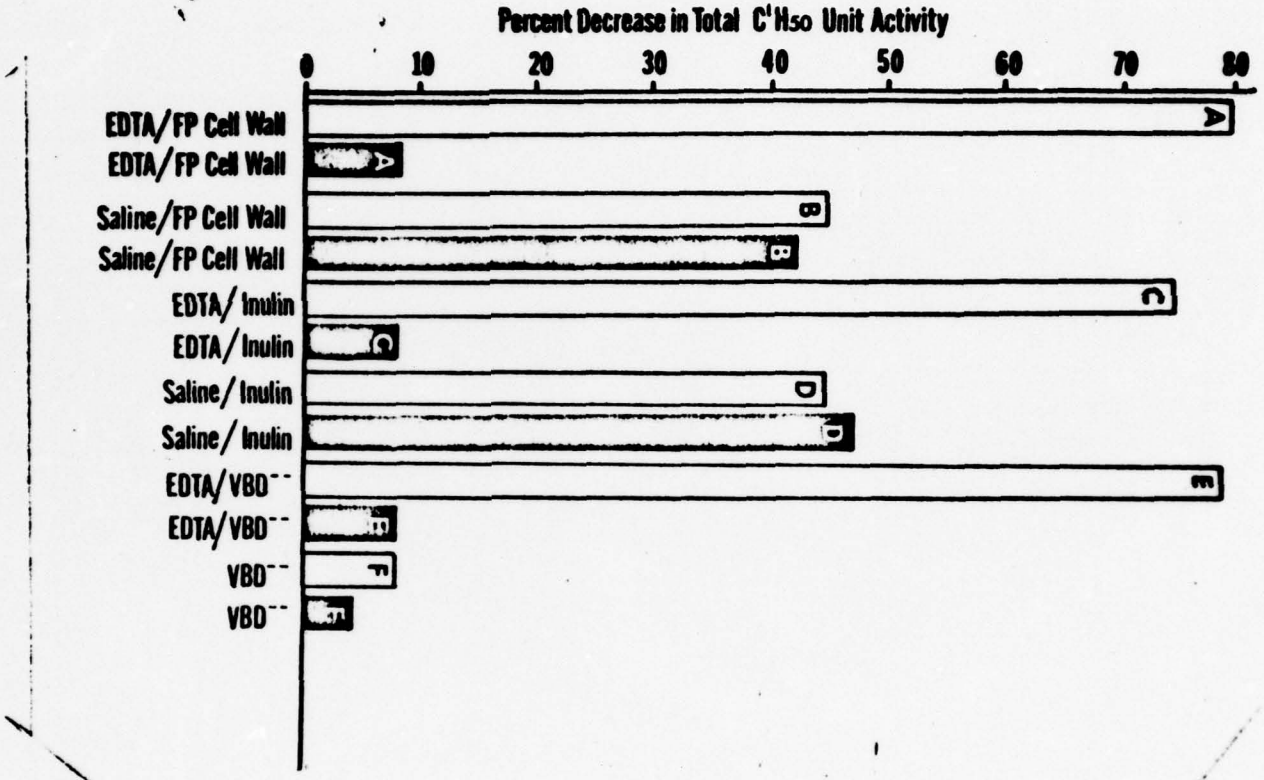
Percent Decrease in Total C'Hso Unit Activity
0
10
20
30
40
50
60
70
80
EDTA/FP Cell Wall
A
EDTA/FP Cell Wall
A
Saline/FP Cell Wall
B
Saline/FP Cell Wall
B
EDTA/ Inulin
C
EDTA/ Inulin
C
Saline/ Inulin
D
Saline/ Inulin
D
EDTA/VBD--
E
EDTA/VBD--
E
VBD--
F
VBD--
F

Figure 3.

Figure 3. This figure shows the complement consumption by 500 µg of FP cell walls and inulin in GPC' chelated with 0.01 M EDTA. The open bars refer to the activity of tests prior to recalcification. Solid bars show the activity of tests after recalcification.

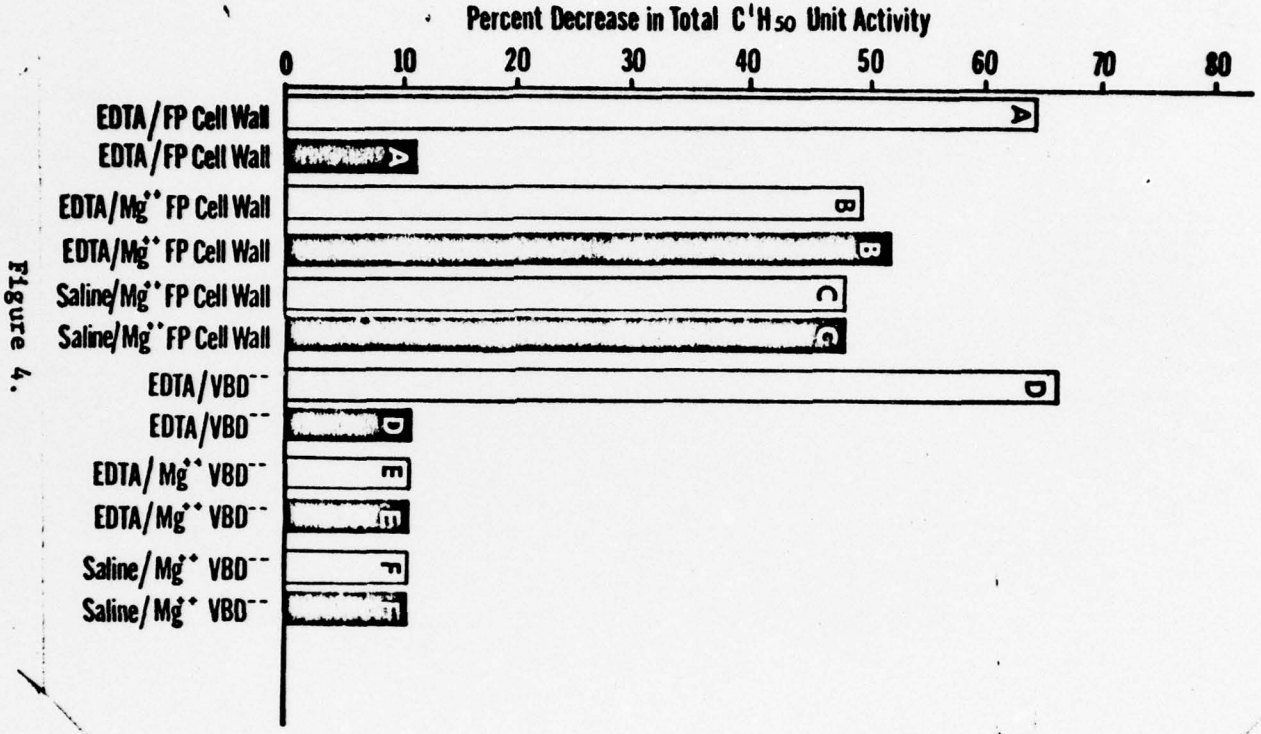
Percent Decrease in Total C'H50 Unit Activity
0
10
20
30
40
50
60
70
80
EDTA/FP Cell Wall
EDTA/FP Cell Wall
EDTA/Mg++ FP Cell Wall
EDTA/Mg++ FP Cell Wall
Saline/Mg++ FP Cell Wall
Saline/Mg++ FP Cell Wall
EDTA/VBD--
EDTA/VBD--
EDTA/Mg++ VBD--
EDTA/Mg++ VBD--
Saline/Mg++ VBD--
Saline/Mg++ VBD--
A
A
B
B
C
C
D
D
E
E
F
F

Figure 4.

Figure 4. This figure shows the complement consumption of FP cell walls at 500 g/ml of the complement consumption assay in GPC' that was either chelated with 0.01 M EDTA alone or chelated with 0.01 M EDTA and supplemented with the addition of an equal concentration of Mg^{++}. Open bars indicate the activity of tests before recalcification. Solid bars indicate the activity after recalcification.