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IN VITRO INHIBITION OF THE CLASSICAL PATHWAY OF HUMAN COMPLEMENT BY POLYMYXIN B

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Abstract—Polymyxin B was found to be an inhibitor of the classical pathway of the complement system. The main sites of inhibition were the interaction of $EAC\overline{14}$ with C2 and $EAC\overline{142}$ with C3. It also inhibited $EAC\overline{1\text{-}9}$ formation from $EAC\overline{1\text{-}3}$ and C5-9 though slightly less efficiently. It did not inhibit C3/C5 convertase of the alternative pathway or its formation. The inhibition of the classical pathway was reversible since hemolytic activity was almost completely restored after dialysis.

In recent years, several synthetic inhibitors of complement (C)§ components have been developed and used for a number of distinct purposes. They have been used to study the possibility of immunosuppression at C level [1], to gain insight into the nature of active sites of C enzymes such as $C1\bar{s}$, $C1\bar{r}$ and C3 [2–6], and to prepare cellular intermediates [7–9] without the use of purified C components. Use of C inhibitors has also been made to protect C components during purification from the contaminating enzyme(s), e.g. the inhibitors of $C1\bar{s}$ protected C2 during purification [4, 10]. These are a few examples of the many uses of C inhibitors.

Because of their diverse uses, we are interested in finding out new inhibitors of different components of C. This paper describes that polymyxin B inhibits classical pathway at different steps. It does not inhibit the activity or formation of C3/C5 converse of the alternative pathway. The ability of polymyxin B to inhibit $EAC\overline{142}$ formation from $EAC\overline{14}$ and C2 could be utilized to prepare $EAC\overline{14}$ cells without the use of purified components.

MATERIALS AND METHODS

Chemicals. Polymyxin B (Fig. 1) was obtained from Pfizer, Brussels, Belgium. All other chemicals used were of high quality.

Buffers. The following buffers were used. GVB: isotonic veronal buffered saline containing 0.1% gelatin, pH 7.4, ionic strength = 0.147, prepared as described by Mayer [11]. GVB^{2+}: GVB containing 0.15 mM $CaCl_2$ and 1.0 mM $MgCl_2$, pH 7.4, prepared using complement fixation diluent tablets (Oxoid Ltd, U.K.). G1-GVB^{2+}: isotonic buffered saline containing 2.5% glucose, 0.07 M NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.41 M sodium diethylbarbiturate and 0.1% gelatin, pH 7.4. Sucrose-GVB^{+}: GVB containing 4.5% sucrose and 0.15 mM $CaCl_2$, pH 7.4. Mg^{2+}-EGTA-GVB^{+}: GVB containing 2 mM Mg^{2+} and 8 mM EGTA, pH 7.4. EDTA-GVB: 40 mM EDTA in GVB, pH 7.4.

Erythrocytes, antisera and complement. Sheep and rabbit erythrocytes, rabbit antiserum against boiled sheep erythrocytes stromata and guinea pig complement were obtained from the National Institute of Public Health, Bilthoven, The Netherlands.

A pool of sera obtained from fourteen healthy volunteers served as a source of complement. Serum of a patient suffering from localized frontoparietal scleroderma *en coup de sabre* [12] having undetectable total classical pathway activity due to genetic deficiency of C2, was used as a source of C2-deficient serum. EDTA-C was prepared by diluting guinea-pig serum 1/12.5 with 0.04 M EDTA-GVB. R3 was prepared by incubating normal human serum pool with an equal volume of saturated KBr as described by Tack and Prahl [13]. Serum of a patient suffering from chronic discoid lupus erythomatosus and alopecia associated with the genetic deficiency of C5 was used as a source of C5-deficient serum. R3 and C5 deficient sera did not show any hemolytic activity of the classical pathway but they fully recovered hemolytic activity by the addition of purified C3 and C5, respectively. All the complement components used in this study were obtained from Cordis Laboratories, Miami, FL, and were functionally pure. Unless mentioned otherwise, human complement components were used and activities are expressed as defined by Cordis Laboratories.

Assessment of inhibition of the classical pathway. Classical pathway activity was assessed according to the method of Mayer [11] with slight modification. The incubation mixture, in a total volume of 1.0 ml, contained EA (2×10^7) and normal human serum

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§ Abbreviations used: C, complement; C1, C2 etc., complement component first, second etc.; E, sheep erythrocytes; EA, optimally sensitized sheep erythrocytes; $EAC\overline{1}$-n, sensitized erythrocytes carrying C components up to Cn where n indicates complement component; EDTA, ethylenediamine tetraacetate; EGTA, ethyleneglycol-bis (aminoethyl) tetraacetate; PBS, phosphate buffered saline, pH 7.4; R3, normal human serum pool rendered deficient in C3; CoVF, cobra venom factor; T_{max}, time required for maximum $EAC\overline{142}$ cell formation. Other abbreviations used have been defined in the Materials and Methods section.

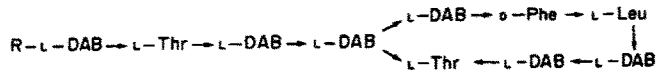

Fig. 1. Structure of polymyxin B. R = Polymyxin B_1: (+)-6-methyloctanoyl; Polymyxin B_2: 6-methylephtanoyl. DAB = α, γ-diaminobutyric acid. Polymyxin B is a mixture of polymyxins B_1 and B_2.

pool (1/437.5) in GVB^{2+} with (varying concentrations) or without polymyxin B. The mixture was incubated at 37° for 1 hr with shaking. Tubes were then cooled and 2.0 ml cold saline was added. After centrifugation the extinction of the supernatant was read at 412 mμ. Spontaneous and 100% lysis controls were always included. The percentage of lysis was calculated and the average number of hemolytic sites per cell (Z) was determined using the formula $Z = -1n(1-y)$; y being the degree of lysis. The results were presented in terms of Z/Z_0 values, where Z represents the average number of hemolytic sites per cell in the presence of inhibitor and Z_0 in its absence.

Assessment of inhibition of the alternative pathway. Alternative pathway assay was carried out by the method described by Kaneko *et al.* [14]. The incubation mixture, in a volume of 0.8 ml containing normal human serum pool (1/16.6) in Mg^{2+}-EGTA-GVB^+ with or without polymyxin B, was preincubated at 30° for 10 min. Rabbit erythrocytes (2×10^7) in 0.2 ml of Mg^{2+}-EGTA-GVB^+ were then added and the total mixture was further incubated at 37° for 60 min with shaking. Appropriate controls were always included. The percentage lysis was measured and Z/Z_0 values calculated as described above for the classical pathway.

Preparation of cellular intermediates. $EAC\overline{14}$ cells were prepared by incubating 20×10^8 EA (in 20 ml sucrose-GVB^+) with 40 ml normal serum pool (diluted 1/175 in sucrose-GVB^+) and 40 ml sucrose-GVB^+ at 32° for 30 min followed by two washings with sucrose-GVB^+ and three with G1-GVB^+. $EAC\overline{1\text{-}3}$ cells were prepared by incubating 15×10^8 EA (in 15 ml GVB^{2+}) with 60 ml GVB^{2+} and 0.24 ml C5-deficient serum for 30 min at 32°. After incubation the cells were washed five times with GVB^{2+} and the cell concentration was adjusted to 1×10^8/ml using GVB^{2+}. Other cellular intermediates were prepared in incubation mixtures as described below.

Measurement of the effect of polymyxin B on the various steps of complement cascade. For testing the effect of polymyxin B on $EAC\overline{14}$ cell formation, 1.0 ml of incubation mixture containing 2×10^7 EA cells and C2 deficient serum in a final dilution of 1/1250 in G1-GVB^{2+} with and without polymyxin B was incubated at 32° for 30 min. The cells were then washed with 9.0 ml cold G1-GVB^{2+}. C2 (0.2 ml) containing 20 units was added and the cell suspension was incubated at 32° for 2 min. EDTA-GVB and C-EDTA were then added and incubated as described above. The reaction of $EAC\overline{14}$ with C2 was measured by incubating $EAC\overline{14}$ cells (2×10^7) with C2 (20 units) in G1-GVB^{2+} in a total volume of 0.5 ml at 32° for 2 min with and without polymyxin B. EDTA-GVB and C-EDTA were then added and incubated as described above. The reaction of $EAC\overline{142}$ with C3 was measured by incubating $EAC\overline{14}$ cells (2×10^7) with C2 (20 units) in a total volume of 0.5 ml in G1-GVB^{2+} at 32° for 2 min followed by the addition of 20 units of C3 with or without polymyxin B in 0.5 ml G1-GVB^{2+}. The mixture was incubated further at 32° for 15 min. The cells were washed with 9.0 ml of cold G1-GVB and then lysed by incubating with 1 ml of R3 (1/250) at 37° for 1 hr with shaking.

The reaction of $EAC\overline{1\text{-}3}$ with C5-C9 was studied by incubating 0.2 ml of $EAC\overline{1\text{-}3}$ cells (2×10^7) with 0.5 ml R3 (1/50) and 0.3 ml Gl-GVB^{2+} with and without polymyxin B at 37° for 1 hr with shaking.

The effect of polymyxin B on the early steps of the alternative pathway was investigated by monitoring its effect on CoVF mediated destruction of C3 in normal human serum. One-tenth of a millilitre of normal serum (1/45) was incubated with CoVF (30 units) with and without polymyxin B (3×10^{-4} M) in a total volume of 0.5 ml of GVB^{2+} at 37° for 1 hr. The remaining C3 was estimated by the addition of 0.3 ml R3 (1/70) and 0.2 ml EA (1×10^8 ml) followed by incubation at 37° for 1 hr with shaking.

Formation of $EAC\overline{14}$ cells from EA, NS and polymyxin B in presence of Ca^{2+} and Mg^{2+}. To 40 ml of NS (1/150 in GVB^{2+}) were added 10 ml polymyxin B (1×10^{-2} M in GVB^{2+}), 30 ml GVB^{2+} and 20 ml EA (20×10^8 in GVB^{2+}). The mixture was incubated at 37° for 1 hr and then cells were washed twice with 1×10^{-3} M polymyxin B in GVB^{2+} and thrice with Gl-GVB^{2+}. The cell concentration was then adjusted to 1×10^8/ml.

The $EAC\overline{14}$ cells thus formed were characterized by testing their ability to form $EAC\overline{142}$ in presence of C2 as follows. Cells (2×10^7) were incubated with (20 units in 0.2 ml) or without C2 in a total volume of 0.5 ml of G1-GVB^{2+} at 37° for different time intervals from 0.25 to 20 min. EDTA-GVB and C-EDTA were added and lysis observed following 1 hr incubation at 37° with shaking.

RESULTS

Polymyxin B was able to inhibit the total classical as well as the alternative pathway activities (Fig. 2). In order to decrease the Z/Z_0 value by 50%, a concentration of about 2×10^{-4} M was needed in the classical pathway and about 4×10^{-4} M in the alternative pathway assay, suggesting that polymyxin B is a fairly strong inhibitor of the complement system.

In order to pinpoint the site of inhibition, the effect of polymyxin B on the various steps of the complement cascade was investigated. The results are presented in Fig. 3 and Table 1. Polymyxin B did not inhibit the formation of $EAC\overline{14}$ cells when EA and C2-deficient serum were used as reactants. The

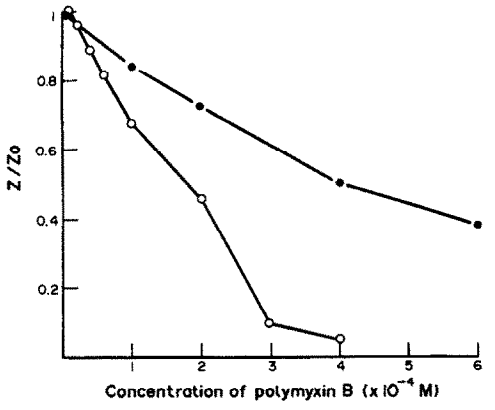

Fig. 2. Inhibition of complement by polymyxin B: O—O, classical pathway; ●—●, alternative pathway. The results are average of duplicate determinations.

interactions of EAC$\overline{14}$ cells with C2, EAC$\overline{142}$ with C3 and EAC$\overline{1\text{-}3}$ with C5–C9 were inhibited. Since inhibition of interaction of EAC$\overline{1\text{-}3}$ with C5–C9 represented collective inhibition of various steps from C5 to C9 which was comparatively weak, it was not considered necessary to study the effect of polymyxin B on different steps of complement activation from C5–C9. CoVF mediated consumption of C3 was not inhibited by polymyxin B. This observation strongly suggested that the observed inhibitory effect on the total alternative pathway could perhaps be due to the ability of polymyxin B to inhibit reaction steps involving later components of complement common to both pathways.

Incubation of normal serum with 5×10^{-4} M polymyxin B almost completely inhibited classical pathway activity but the removal of polymyxin B by dialysis restored 87% of classical pathway activity, indicating that the inhibition was reversible.

EAC$\overline{142}$ formation and decay profiles in presence and absence of polymyxin B (Fig. 4) showed that polymyxin B did not accelerate the rate of decay of EAC$\overline{142}$ cells suggesting that its observed inhibitory effect on EAC$\overline{142}$ formation was probably not due to knocking out of C2 from EAC$\overline{142}$ complex.

The ability of polymyxin B to inhibit EAC$\overline{142}$ formation from EAC$\overline{14}$ and C2 could be exploited to prepare EAC$\overline{14}$ cells. Incubation of EA with normal serum in GVB^{2+} in presence of polymyxin B (see Materials and Methods) seemed to cause sensitization of EA up to EAC$\overline{14}$ stage. Polymyxin B appeared to block further sensitization. The EAC$\overline{14}$ cell thus formed showed EAC$\overline{142}$ formation and decay as a function of time in presence of C2 but not in its absence (Fig. 2).

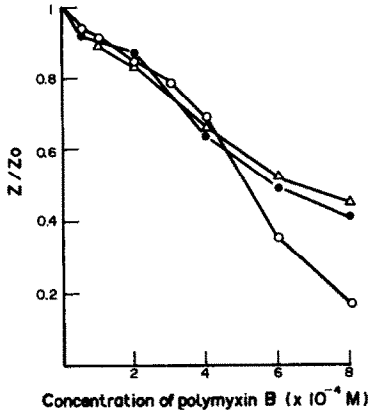

Fig. 3. Effect of polymyxin B on various reaction steps of the classical pathway cascade. Effect on formation of: O—O, EAC$\overline{142}$ from EAC$\overline{14}$ and C2; ●—●, EAC$\overline{1\text{-}3}$ from EAC$\overline{142}$ and C3; and △—△, EAC1-9 from EAC$\overline{1\text{-}3}$ and C5-C9. There was no inhibition of EAC$\overline{14}$ formation from EA and C2 deficient serum. The results are the average of quadruplicate determinations.

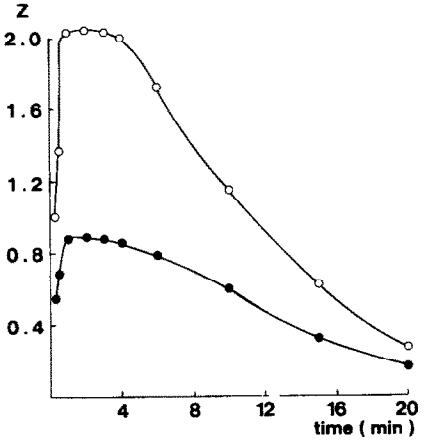

Fig. 4. Effect of polymyxin B on EAC$\overline{142}$ formation and decay: O—O, without inhibitor; ●—●, 6×10^{-4} M polymyxin B. The results are an average of duplicate determinations.

Table 1. Inhibition of different steps of classical pathway activation by polymyxin B

System	Cellular intermediate formation	Concentration required for 50% decrease in Z/Z_0 value (M)
1. EA + C2-deficient serum	EAC$\overline{14}$	*
2. EAC$\overline{14}$ + C2	EAC$\overline{142}$	5.1×10^{-4} M
3. EAC$\overline{142}$ + C3	EAC$\overline{1\text{-}3}$	6×10^{-4} M
4. EAC$\overline{1\text{-}3}$ + C5-C9	EAC$\overline{1\text{-}9}$	6.6×10^{-4} M

Experimental conditions are described in the text.
* No inhibition.

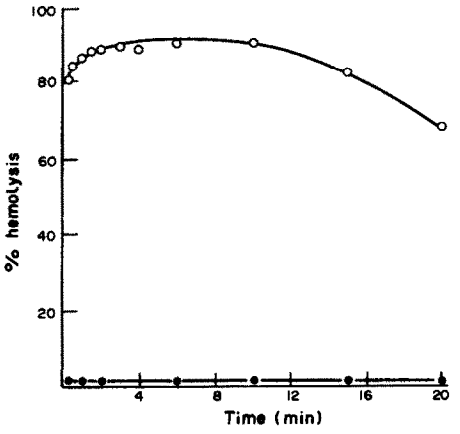

Fig. 5. $EAC\overline{142}$ cell formation and decay profile of $EAC\overline{14}$ cells prepared by incubation of EA with normal serum in presence of polymyxin B: ○—○, $EAC\overline{142}$ formation in presence of C2 and C-EDTA; ●—●, in presence of C-EDTA.

DISCUSSION

It has been demonstrated that animals depleted of complement by means of CoVF fail to develop Arthus reaction [15], experimental allergic encephalomyelitis (EAE) [16] and experimental myasthenia gravis [17]. Several low molecular weight inhibitors have also been shown to suppress complement mediated diseases in humans and in experimental animals [1]. More recently, strong suppression of EAE [18] and experimental [19] and human [20] vasculitis by complement inhibitors has been demonstrated. These observations indicate that complement inhibiting drugs may cause suppression of complement mediated disease processes. Several laboratories are therefore developing new inhibitors of the early steps of the classical or alternative pathway.

The present work describes the inhibition of the classical pathway by an antibacterial agent, polymyxin B. An antibacterial agent with complement inhibiting activity is likely not only to eliminate the bacteria in infectious diseases, but also to suppress simultaneously the complement mediated tissue destructive phases such as glomerulonephritis, vasculitis and ulceration of eye in certain diseases.

Polymyxin B was seen to inhibit the classical pathway and this was most effective at the steps of interaction of $EAC\overline{14}$ with C2 and $EAC\overline{142}$ with C3 although later steps of complement activation were also inhibited. Polymyxin B did not accelerate the decay of C2 from $C\overline{142}$ complex suggesting the nature of its effect on $C\overline{142}$ to be inhibitory rather than via disassembly of the complex. Polymyxin B does not appear to effect C1 as formation of $EAC\overline{14}$ from EA and C2-deficient serum was not inhibited by polymyxin B. It does not appear to inhibit total alternative pathway as alternative pathway C3/C5 convertase and its formation was not effected.

The ability of polymyxin B to inhibit $EAC\overline{142}$ formation from $EAC\overline{14}$ and C2 was exploited to prepare $EAC\overline{14}$ cells. When normal serum was incubated with EA in presence of Ca^{2+}, Mg^{2+} and polymyxin B under appropriate conditions, cells were formed which could be lysed by C-EDTA and C2 but not by C-EDTA alone. $EAC\overline{142}$ formation and decay by these cells in presence but not in absence of C2 suggested that cells obtained were $EAC\overline{14}$ cells. Kitamura *et al.* [21] have reported similar results with methylprednisolone, an inhibitor of $EAC\overline{142}$ formation.

The inhibition of the classical pathway by polymyxin B makes this compound a good candidate for studying its effect on experimental models of classical pathway mediated diseases. It can perhaps also be used in *in vitro* studies in which alternative pathway is to be studied while keeping classical pathway dormant.

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REFERENCES

1. S. S. Asghar, *Pharmac. Rev.* **36**, 223 (1984).
2. L. Levine, *Biochim. biophys. Acta* **18**, 283 (1955).
3. D. H. Bing, *Biochemistry* **8**, 4503 (1969).
4. S. S. Asghar, K. W. Pondman and R. H. Cormane, *Biochim. biophys. Acta* **317**, 539 (1973).
5. N. R. Cooper and E. L. Bocker, *J. Immunol.* **98**, 119 (1967).
6. N. R. Cooper and H. J. Müller-Eberhard, *Fedn Proc. Fedn Am. Socs. exp. Biol.* **26**, 361 (1967).
7. K. Hong, T. Kinoshita, W. Miyazaki, T. Izawa and K. Inoue, *J. Immunol.* **122**, 2418 (1979).
8. K. Hong, T. Kinoshita, H. Kitajima and K. Inoue, *J. Immunol.* **127**, 104 (1981).
9. K. Hong, T. Kinoshita, W. Miyazaki, T. Izawa and K. Inoue, *J. Immunol.* **127**, 109 (1981).
10. M. A. Kerr, *Biochem. J.* **183**, 615 (1979).
11. M. M. Mayer, in *Experimental Immunochemistry* (Eds. E. A. Kabat and M. M. Mayer), 2nd Edn, p. 180. Charles Thomas, Springfield, IL (1967).
12. R. F. H. J. Hulsmans, S. S. Asghar, A. H. Siddiqui and R. H. Cormane, *Archs Dermatol.* **122**, 76 (1986).
13. B. F. Tack and J. W. Prahl, *Biochemistry* **15**, 4513 (1976).
14. I. Kaneko, D. T. Fearon and K. F. Austen, *J. Immunol.* **124**, 1194 (1980).
15. G. C. Cochrane, in *Progress in Allergy* II, p. 1. Karger, Basel (1967).
16. M. A. Morariu and A. P. Dalmasso, *Ann. Neurol.* **4**, 427 (1978).
17. V. A. Lennon, M. E. Seybold, J. Lindstrom, C. G. Cochrane and R. Yulevitch, *J. exp. Med.* **147**, 973 (1978).
18. R. C. van der Veen, S. S. Asghar, B. Uitdenhaag, H. J. van der Helm and O. R. Hommes, *Neuropharmacol.* **24**, 1139 (1985).
19. S. S. Asghar, K. P. Dingemans, A. Kammeijer, W. R. Faber and M. Y. M. Abdal Mawla, *Complement* **3**, 40 (1986).
20. S. S. Asghar, W. Westerhof, P. K. Das, F. Ch. Jansen and R. H. Cormane, *Archs Dermatol. Res.* **277**, 504 (1985).
21. F. Kitamura, K. Shimada, T. Suzuki and K. Nishioka, *J. Immunol. Meth.* **85**, 363 (1985).