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IN VITRO INHIBITION OF THE CLASSICAL PATHWAY OF HUMAN COMPLEMENT BY A NATURAL MICROBIAL PRODUCT, COLISTIN SULPHATE

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Abstract—Colistin sulphate 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$\overline{14}$ formation from EA and C2-deficient serum, EAC$\overline{1-7}$ formation from EAC$\overline{1-3}$, C5, C6 and C7 and the interaction of EAC$\overline{1-7}$ with C8 and C9, though less efficiently. It did not inhibit formation of C3/C5 convertase of the alternative pathway. The inhibition of the classical pathway was reversible since hemolytic activity was completely restored after dialysis.

The complement system participates in the host defence against foreign antigens and micro-organisms and is also employed in the pathogenesis of the immune complex and autoimmune diseases [1, 2]. Evidence is beginning to emerge that the inhibition of the complement system by pharmacological means can arrest complement mediated disease processes. The subject of complement inhibiting drugs and their use in suppressing complement mediated diseases in experimental models and humans has been reviewed by Asghar [3] and Patrick and Johnson [4].

The development of specific inhibitors of complement components is important not only for clinical purposes but also for their use as tools in complement research. For example, benzamidine has been used during the preparation of C2* to protect it from the activity of contaminating C1$\bar{s}$ [5] and K-76COOH, an inhibitor of C5 [6] and C3b-inactivator [7], has been used for the preparation of EAC$\overline{1-3}$ and EAC$\overline{43}$ cells [8] without the use of purified complement components.

Our laboratory is also involved in the development of complement inhibitors. In previous communications interactions of diphenyldiamidines [9, 10], phenothiazine sulphonate [11] and substituted phenylindandiones [12, 13] have been described. This article describes the inhibition of complement by a natural microbial product, colistin sulphate (Fig. 1) and demonstrates this antibacterial agent's sites of inhibition on the complement cascade.

MATERIALS AND METHODS

Chemicals. Colistin sulphate (batch no 162132: 20600 units/mg, 17.1% sulphate) was obtained from Bergelnederland BV, Heerhugowaard, The Netherlands. All the 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 [14]; 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, England); Gl–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; 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" [15] 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 [16]. R5 was prepared by passing normal serum pool through a column of anti-C5 coupled sepharose in the presence of 0.01 M EDTA. R3 and R5 did not show any hemolytic activity of the

* 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; PEG, polyethylene glycol; PBS, phosphate buffered saline, pH 7.4; R3 and R5, normal human serum pool deficient in C3 and C5, respectively; CoVF, cobra venom factor; T_{max}, time required for maximum EAC$\overline{142}$ cell formation; FPLC, fast protein liquid chromatography; other abbreviations used have been defined in the Materials and Methods section.

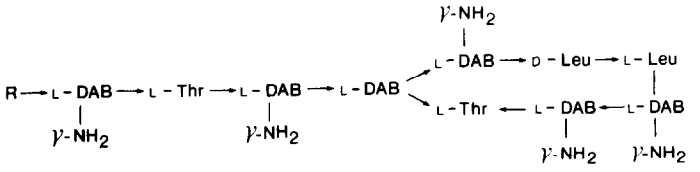

Fig. 1. Skeleton of colistin sulphate. Note the polyamine structure of the molecule. R = fatty acid moiety; DAB = α-γ-diaminobutyric acid.

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 their activities expressed in terms of units as defined by Cordis Laboratories.

Purification of $\overline{Cls}$. Human $\overline{Cls}$ was purified by treatment of preincubated (37°, 60 min) PBS soluble proteins of Cohn fraction I with equal volume of 10% PEG (6000) in PBS. The precipitate was dissolved in and dialysed against 0.067 M phosphate buffer, pH 7.4 containing 0.01 M EDTA. Some proteins in dialysed solution tended to precipitate and were removed by centrifugation at 16,000 rpm. The $\overline{Cls}$ preparation thus obtained was diluted 15 times and stored at −100° for use as crude enzyme. Part of this preparation was passed on mono Q column for FPLC chromatography (Pharmacia FPLC system). $\overline{Cls}$ was eluted in the region of 2.5–4.0 M NaCl concentration. The pooled $\overline{Cls}$ fractions from several runs had 34 units/ml enzyme with a specific activity of 154 units/mg protein.

Assay of $\overline{Cls}$. The esterolytic activity of $\overline{Cls}$ was measured using acetyl tyrosine ethyl ester as substrate. The hydrolysis of the substrate was monitored continuously with the help of pH-stat (Radiometer, Copenhagen, Denmark). In the titration vessel, a total volume of 2.0 ml contained 0.02 ml enzyme (34 units/ml), 0.125 ml substrate dissolved in cellusolve and required concentration of inhibitor in phosphate buffered saline, pH 7.4. The amount of 0.005 M NaOH required to maintain the pH of the mixture at 7.4 was recorded automatically. The reaction was carried out at 37°. One unit of $\overline{Cls}$ was defined as the amount of enzyme required to produce 0.5 μ equivalent H^+ in 15 min from the substrate.

Assessment of inhibition of the classical pathway. Classical pathway activity was assessed according to the method of Mayer [14] with slight modification. Incubation mixture, in a total volume of 1.0 ml, contained EA (2×10^7) and normal human serum pool (1/437.5) in GVB^{2+} with (in varying concentrations) or without colistin sulphate. 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. Appropriate controls were always included. The percentage of lysis was determined and the average number of hemolytic sites per cell (Z) was calculated using the formula $Z = -\ln(1 - Y)$ where Y represents 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.* [17]. 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 colistin sulphate, 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. 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 25×10^8 EA with 0.1 ml of C2 deficient serum in a total volume of 131 ml of GVB^{2+} at 32° for 30 min, followed by three washings with Gl–GVB^{2+}. The cell concentration was adjusted to 1×10^8 cells/ml in Gl–GVB^{2+}. $EAC\overline{4}$ cells were prepared by washing $EAC\overline{14}$ cells twice with EDTA–GVB followed by thorough washing with Gl–GVB^{2+}. Other cellular intermediates were prepared in incubation mixtures as described below.

Measurement of the effect of colistin sulphate on the various steps of complement cascade. The interaction of $EAC\overline{4}$ with Cl was studied by incubating 2×10^7 $EAC\overline{4}$ cells with 200 units of Cl in a total volume of 1.0 ml Gl–GVB^{2+} with and without colistin sulphate at 32° for 15 min. Cells were then washed with 9.0 ml of cold Gl–GVB^{2+} and incubated with 0.2 ml of a mixture of C2 and C3 (20 units each) in Gl–GVB^{2+} at 32° for 15 min. The reaction was stopped by adding 0.6 ml EDTA–GVB and cell lysis was effected by further addition of 0.2 ml C–EDTA followed by incubation at 37° for 60 min with shaking. Lysis was measured as described above. For testing the effect of colistin sulphate 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 Gl–GVB^{2+} with and without colistin sulphate was incubated at 32° for 30 min. The cells were then washed with 9.0 ml cold Gl–GVB^{2+}. 0.2 ml of C2 (20 units) was added and the cell suspension was incubated at 32° for 4 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 Gl–GVB^{2+} in a total volume of 0.5 ml at 32° for 4 min with and without colistin sulphate. 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 Gl–GVB^{2+} at 32° for 4 min followed by the addition of 20 units of C3 with or without colistin sulphate. The mixture was incubated further at 32° for 15 min. The cells were washed and then lysed by incubating with 1 ml C5–C9 reagent (20 units each in Gl–GVB^{2+}) at 37° for 1 hr with shaking.

The reaction of EAC$\overline{1423}$ with C5, C6 and C7 was studied by incubating EAC$\overline{14}$ cells (2×10^7) with C2 and C3 (20 units each) in Gl–GVB^{2+} in a total volume of 1.0 ml at 32° for 15 min. The mixture was then centrifuged and the supernatant removed. The cells were then suspended in 1 ml of C5–C7 reagent (20 units each in Gl–GVB^{2+}) and incubated at 32° for 20 min followed by centrifugation and removal of the supernatant. The cells were then put into 1.0 ml guinea-pig C8, C9 reagent (20 units each) and incubated at 37° for 1 hr with shaking.

The reaction of EAC$\overline{1-7}$ with human C8 and C9 was measured by incubating EAC$\overline{14}$ cells with C2, C3, C5–C7 reagent (20 units each) in Gl–GVB^{2+} in a total volume of 1.0 at 32° for 20 min. Following centrifugation and the removal of the supernatant the cells were suspended in 1.0 ml Gl–GVB^{2+} containing human C8 and C9 (20 units each) with and without colistin sulphate and incubated at 37° for 1 hr with shaking.

The effect of colistin sulphate on the early steps of the alternative pathway leading to C5-convertase formation was studied as follows: the incubation mixture having a total volume of 2.0 ml, containing 2×10^7 rabbit erythrocytes and R5 in a final dilution of 1/14.28 in Mg^{2+}–EGTA–GVB$^+$ with and without colistin sulphate. The mixture was incubated at 37° for 30 min and centrifuged to remove the supernatant. Cell lysis was produced by suspending the cells in 1.0 ml of 1/37.5 diluted R3 in Mg^{2+}–EGTA–GVB$^+$, followed by 60 min incubation at 37° with shaking.

The effect of colistin sulphate on the early steps of the alternative pathway was also investigated by monitoring its effect on CoVF mediated destruction of C3 in normal human serum. One-tenth ml of normal serum (1/45) was incubated with CoVF (30 units) with and without colistin sulphate (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.

The effect of colistin sulphate on the B-determinant of C3 was studied by a method described earlier [10, 11] with slight modification. Five ml of 1% agarose, with or without the incorporation of 4×10^{-3} M colistin sulphate, was poured into petri dishes (2 cm diameter). Wells (4 mm diameter) were cut into solidified agarose. The centre to centre distance between antigen and antibody wells was 1.0 cm. The antigen well contained normal serum pool and the antibody well contained anti-B-determinant antiserum. The slides were incubated at 37° overnight and read for visible lines of precipitation.

RESULTS

Colistin sulphate 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 5×10^{-5} M was needed in the classical pathway and about 5×10^{-4} M in the alternative pathway assay, suggesting that colistin sulphate is a fairly strong inhibitor of the complement system.

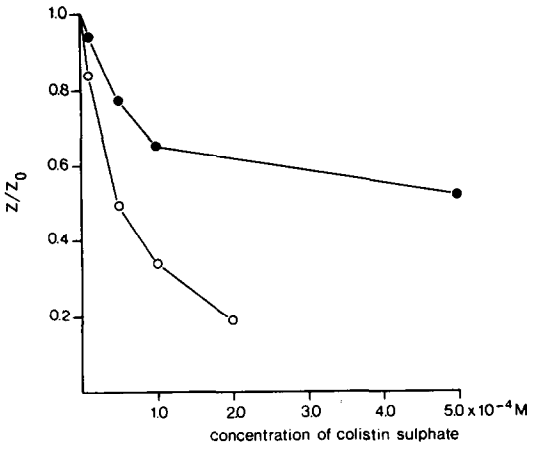

Fig. 2. Inhibition of complement by colistin sulphate: ○—○ classical pathway; ●—● alternative pathway. The results are the average of duplicate determinations.

In order to pinpoint the site of inhibition, the effect of colistin sulphate on the various steps of the complement cascade was investigated. It did not inhibit esterolytic activity of C1$\bar{s}$ at a concentration as high as 5×10^{-3} M. Interaction of EAC$\overline{4}$ with C1 was slightly enhanced by colistin sulphate at 3×10^{-4} M. Colistin sulphate inhibited the formation of EAC$\overline{14}$ cells when EA and C2-deficient serum were used as reactants (Fig. 3; Table 1). The interactions of EAC$\overline{14}$ cells with C2 and of EAC$\overline{142}$ with C3 were most strongly inhibited. EAC$\overline{142}$ formation

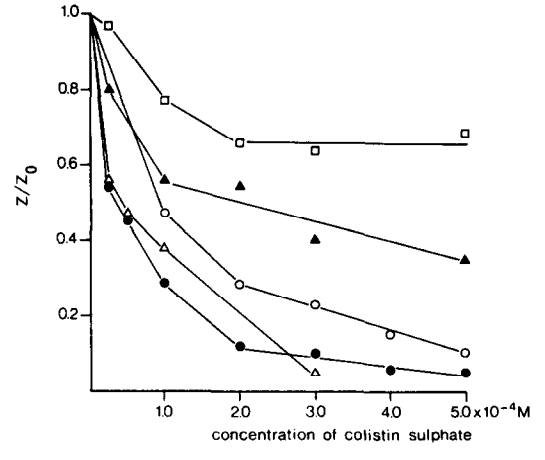

Fig. 3. Effect of colistin sulphate on various reaction steps of the classical pathway cascade. Effect on formation of ○—○ EAC$\overline{14}$ from EA and C2-deficient serum; ●—● EAC$\overline{142}$ from EAC$\overline{14}$ and C2; △—△ EAC$\overline{1-3}$ from EAC$\overline{142}$ and C3; ▲—▲ EAC$\overline{1-7}$ from EAC$\overline{1-3}$, C5, C6 and C7; and □—□ EAC$\overline{1-9}$ from EAC$\overline{1-7}$, C8 and C9. The results are the average of duplicate determinations.

Table 1. Inhibition of different steps of classical pathway activation by colistin sulphate

System	Cellular intermediate formation	Concentration required for 50% decrease in Z/Z_0 value (M)
1. EA + C2 deficient serum	$EAC\overline{14}$	9.25×10^{-5}
2. $EAC\overline{14}$ + C2	$EAC\overline{142}$	3.25×10^{-5}
3. $EAC\overline{142}$ + C3	$EAC\overline{1-3}$	4.25×10^{-5}
4. $EAC\overline{1-3}$ + C5–C7	$EAC\overline{1-7}$	2×10^{-4}
5. $EAC\overline{1-7}$ + C8–C9	$EAC\overline{1-9}$	*

Experimental conditions are described in the text.
* See Fig. 3.

and decay profiles in presence and absence of colistin sulphate (Fig. 4) showed that colistin sulphate 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 interactions of $EAC\overline{1-3}$ cells with C5, C6 and C7 and of EAC $\overline{1-7}$ with C8 and C9 were inhibited but comparatively less strongly. This indicated that the observed inhibitory effect on the total alternative pathway could perhaps be due to the ability of colistin sulphate to inhibit reaction steps involving later components of complement common to both pathways. Experiments designed to investigate inhibition at the early steps of the alternative pathway, i.e. at the stage of C5-convertase formation, showed that there was no inhibition of early components of the alternative pathway. CoFV mediated consumption of C3 was not inhibited by colistin sulphate. Alternative pathway C5-convertase formation in assay system, in which rabbit erythrocytes and R5 in Mg^{+}–EGTA were used to generate C5-convertase and R3 was used to bring about lysis, was also unaffected by colistin sulphate.

Colistin sulphate at 4×10^{-3} M did not appear to interact with the B-determinant of C3 to render it unable to react with anti-B-determinant antiserum.

Incubation of normal serum with 4×10^{-4} M colistin sulphate almost completely inhibited classical pathway activity but the removal of colistin sulphate by dialysis totally restored this activity, indicating that the inhibition was reversible.

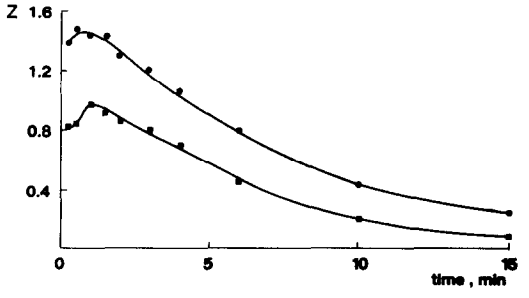

Fig. 4. Effect of colistin sulphate on $EAC\overline{142}$ cell formation and decay: ●—● without inhibitor; ■—■ 2×10^{-5} M colistin sulphate. Experimental conditions are same as described for reaction of $EAC\overline{14}$ with C2 except that reaction was carried out for different time intervals as indicated and 4 units of C2 (21.6 ng) were used instead of 20 units. The results are the average of duplicate determinations.

DISCUSSION

It has been demonstrated that animals depleted of complement by means of CoVF fail to develop Arthus reaction [18], experimental allergic encephalomyelitis (EAE) [19] and experimental myasthenia gravis [20]. Several low molecular weight inhibitors have also been shown to suppress complement mediated diseases in humans and in experimental animals [3]. More recently, strong suppression of EAE [21] and experimental [22] and human [23] 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, colistin sulphate. An antibacterial agent with complement inhibiting activity is likely not only to eliminate the bacteria in infectious diseases, but also to suppress simultaneously complement mediated tissue destructive phases such as glomerulonephritis, vasculitis and ulceration of eye in certain diseases.

Colistin sulphate 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 $EAC\overline{14}$ formation from EA and C2 deficient serum was also effected. By virtue of its polyaminestructure (Fig. 1) it may be expected that colistin sulphate might be dissociating C1 in a manner reminiscent of dissociation of C1 into C1q and $C1r_2$–$C1s_2$ by diamino or polyamino compounds as recently shown by Villiers *et al.* [24]. This does not appear to be the case, at least not at the concentrations used in this study, since the formation of EAC $\overline{14}$ from EAC4 and C1 was not inhibited by colistin sulphate and the $EAC\overline{14}$ thus formed were lysed in the presence of C2 and C-EDTA. The possibility of the inhibition of $C1\bar{s}$ was also ruled out because of lack of inhibition of esterolytic activity. The results of this study indicate that colistin sulphate probably does not knock out C2 but may inhibit C2 and C4. C3 did not appear to be appreciably inhibited as colistin sulphate was a weak inhibitor of the inter-

action of $EAC\overline{1-3}$ with C5, C6 and C7, did not inhibit the early steps of the alternative pathway involving C3 and did not react with the B-determinant. The B-determinant of C3 appears to be closely related to the hemolytic activity since those C3 breakdown products which do not have an intact B-determinant configuration also do not possess hemolytic activity. Certain complement inhibitors have been shown to interact with the B-determinant of C3 to render it incapable of interacting with anti-B-determinant [10, 11].

The total alternative pathway activity was inhibited to some extent by colistin sulphate but the early steps leading to the alternative pathway C3/C5-convertase formation were not inhibited. Thus the observed effect on total alternative pathway activity appeared to be due to the inhibition of later components common to both pathways.

Inhibition of the early steps of the classical pathway by colistin sulphate makes this compound a good candidate for studying its effect on experimental models of classical pathway mediated diseases. The strong inhibition of the interactions of $EAC\overline{14}$ with C2 and $EAC\overline{142}$ with C3 by this compound, and the comparatively weaker inhibition of $EAC\overline{14}$ formation, also suggests that it could perhaps be used for the preparation of $EAC\overline{14}$ cells from EA and normal serum under appropriate conditions.

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