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Anaphylatoxins inhibit C2 production

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SUMMARY

Anaphylatoxins C5a and C3a and their des Arg derivatives inhibited C2 production by mononuclear phagocytes. C5a and $C5a_{des\,Arg}$ which were approximately equipotent ($IC50 = 10^{-10}$ mol/l) were more effective than C3a ($IC50 = 5 \times 10^{-8}$ mol/l) which was approximately 10–20-fold more potent than $C3a_{des\,Arg}$ $IC50 = 5 \times 10^{-6}$ mol/l). Inhibition of C2 production was only reversed slightly by the addition of either indomethacin or ETYA to the cultures. Intracellular levels of cAMP, were increased by anaphylatoxins. The level of cAMP showed a good inverse correlation with C2 levels in the culture supernatants. The data suggest that the reduction in C2 production produced by anaphylatoxins may be mediated by an increase in intracellular cAMP.

Keywords anaphylatoxins C2 synthesis mononuclear phagocytes

INTRODUCTION

The anaphylatoxins C3a and C5a are generated during complement activation, following proteolysis of the chains of C3 and C5 respectively (Hugli & Müller-Eberhard, 1979). These peptides play a major role in the inflammatory response because of their effects on a different population of cells. Activities ascribed to anaphylatoxins include smooth muscle contraction, release of vasoactive amines from mast cells and basophils, neutrophil aggregation, secretion of lysozomal enzymes and stimulation of the directional migration of polymorphonuclear leucocytes and monocytes (Hugli & Müller-Eberhard, 1979; Damerau, Grunefeld & Vogt, 1980a; Damerau *et al.*, 1980b). The molecules formed by the removal of the *C*-terminal arginine residues by digestion with plasma carboxypeptidase-*N* are termed $C3a_{des\,Arg}$ and $C5a_{des\,Arg}$, the activities of which are reduced markedly (Hugli & Müller-Eberhard, 1979; Damerau *et al.*, 1980b). We now describe another function of anaphylatoxins, the inhibition of the production of the second complement component (C2) by human monocytes.

MATERIALS AND METHODS

Anaphylatoxins. C3a and C5a were isolated from yeast activated porcine serum, as described elsewhere (Damerau *et al.*, 1980b). ε-amino caproic acid (EACA, 1 mol/l) was included during yeast yeast activation to prevent cleavage of the *C*-terminal arginine by plasma carboxypeptidase-*N*. $C3a_{des\,Arg}$ and $C5a_{des\,Arg}$ were prepared in the same manner, except that EACA was omitted

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(Damerau *et al.*, 1980b). These preparations were homogenous by acid disc electrophoresis and showed the appropriate reactions with rabbit anti-C3$a_{des\,Arg}$ and rabbit anti-C5$a_{des\,Arg}$ in immunodiffusion analysis.

Prior to their addition to cultures the anaphylatoxins were millipore filtered to ensure sterility.

Cultures of mononuclear phagocytes. (a) Monocyte monolayers were prepared from Ficoll-hypaque separated mononuclear leucocytes which had been isolated from the heparinized venous blood of normal healthy volunteers (Lappin & Whaley, 1980). Monolayers were prepared in Linbro tissue culture plates and cultured in RPMI 1640 containing 20% heat-inactivated (2 h, 56°C) fetal calf serum (FCS). Over 95% of the cells were monocytes as shown by staining with non-specific esterase, and their ability to phagocytose latex particles (Whaley, 1980). The cultures were maintained at 37°C in a humidified 5% CO_2/air atmosphere. Anaphylatoxins were added either on day 0 before the onset of C2 production or on day 5 when C2 production was maximal.

Samples of supernatants were harvested, on alternate days, beginning on day 1, following the addition of anaphylatoxin to the culture. Cultures to which anaphylatoxin had been added on day 0 were terminated on day 7, while those which had received anaphylatoxin on day 5 were terminated on day 10. Cultures were terminated by removal of the supernatants which, like the previous samples, were stored at −70°C. Following gentle washing the monolayers were lysed in 2% sodium dodecyl sulphate (SDS), for DNA determination (Einstein, Schneeberger & Colten, 1976).

(b) Synovial fluid macrophages were isolated from the synovial fluid of patients with rheumatoid arthritis as described previously (De Ceulaer, Papazoglou & Whaley, 1981). These cells were cultured under the same conditions as monocytes.

(c) Guinea-pig peritoneal macrophages were harvested 4 days following the intraperitoneal injection of 40 ml 0·1% casein solution. The cells were washed in RPMI 1640 and then plated out and cultured as described for monocytes.

Monocyte cultures for cyclic nucleotide levels. Monolayers were set up by the same procedure as that adopted for the cultures concerned only with the measurement of C2 production. However, the cell content of the monolayers was increased three-fold by increasing the seeding density of the mononuclear leucocyte suspension. This increase was required for accurate determination of cyclic nucleotides. Cultures were set up in triplicate—after 2 h the supernatant was removed from two of the monolayers; one monolayer was lysed in 10% trichloracetic acid for cAMP/cGMP determination, the other was lysed in 2% SDS for DNA determination. The supernatant of the remaining culture was sampled on day 3 and removed completely on day 7, and the monolayer lysed in 2% SDS for DNA determination. The supernatant was assayed for its C2 content.

Cyclic nucleotides. cAMP and cGMP were measured by radioimmunoassay using a modification of the method of Harper & Brooker (1975). The results were expressed as fmoles/μg DNA.

C2 assay. The C2 content of culture supernatants was measured by haemolytic assay (Rapp & Borsos, 1971).

The results were expressed as effective molecules/μg DNA.

RESULTS

Effect of anaphylatoxins on C2 production

C3a and C5a, and their des Arg derivatives were associated with a dose-dependent reduction of the concentration of C2 in the culture supernatants (Fig. 1). The 50% inhibitory concentration of C3a (5×10^{-8} mol/l) was higher than that for C5a (10^{-10} mol/l). C3$a_{des\,Arg}$ was less potent than C3a, the IC50 being 5×10^{-6} mol/l. However C5$a_{des\,Arg}$ appeared to be equipotent with C5a. As the DNA content of the anaphylatoxin treated monolayers did not differ from controls, and the cells appeared healthy by light microscopic examination, and excluded trypan blue, we concluded that the reduced C2 concentration was not due to cell death or loss of cells from the monolayer. Increased degradation of C2 was excluded in an experiment in which exogenous C2 was added to the control and anaphylatoxin treated cultures. The rates of degradation of C2 were the same in both sets of cultures. We therefore concluded that C2 production was decreased in anaphylatoxin treated cultures.

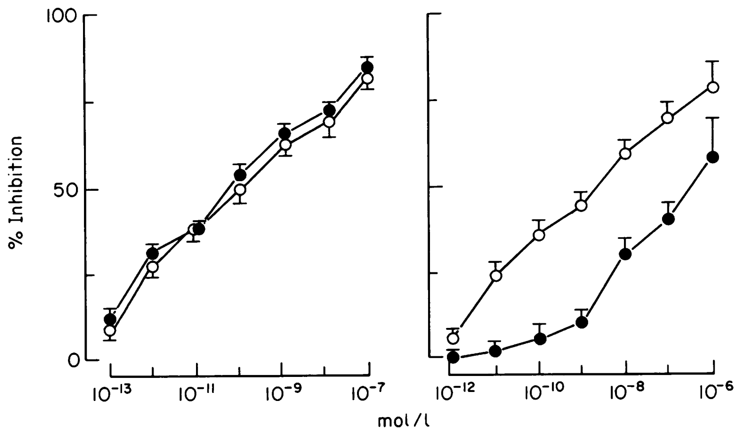

Fig. 1. A comparison of the effects of (a) C5a (●——●) with $C5a_{des\,Arg}$ (O——O, and (b) C3a (●——●) with $C3a_{des\,Arg}$ (O——O) on monocyte C2 production. Anaphylatoxin added on day 0 and C2 levels measured on day 7 of culture. Results expressed as % inhibition of C2 in control culture. Each point represents the mean ± s.e. of three cultures.

The degree of inhibition of C2 production was the same for monocytes treated with anaphylatoxins on day 0 or on day 5 (data not shown).

The anaphylatoxins exerted the same inhibitory effect on C2 production by synovial fluid macrophages and guinea-pig peritoneal macrophages. C5a and $C5a_{des\,Arg}$ were approximately equipotent (IC50 10^{-9} mol/l) whereas $C3a_{des\,Arg}$ (IC50 10^{-7} mol/l) showed reduced efficacy compared with C3a (IC50 5×10^{-8} mol/l).

Effect of prostaglandin synthetase inhibitors

ETYA and indomethacin (10^{-4} mol/l) enhanced C2 synthesis in the untreated and the anaphylatoxin treated cultures (Fig. 2). However, despite the increased synthesis of C2 in the anaphylatoxin treated cultures, the C2 concentrations were much lower than levels in the control cultures which were exposed to prostaglandin synthetase inhibitors.

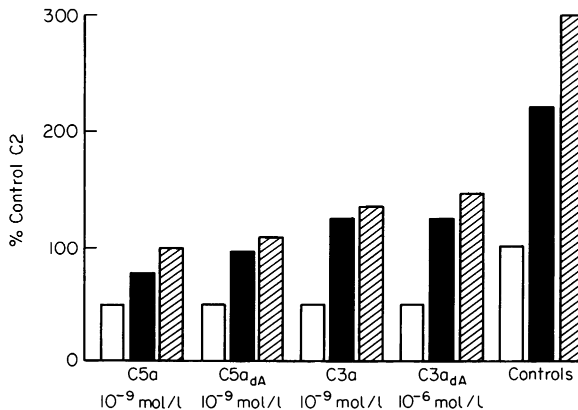

Fig. 2. Effect of prostaglandin (PG) synthetase inhibitors on inhibition of C2 production by anaphylatoxins. Anaphylatoxins and PG synthetase inhibitors added on day 0 and C2 levels measured on Day 7. Results expressed as % of level of C2 in control untreated cultures. No PG synthetase inhibitor (□), indomethacin 10^{-4} mol/l (■), ETYA 10^{-4} mol/l (▨). Results represent the mean of two experiments.

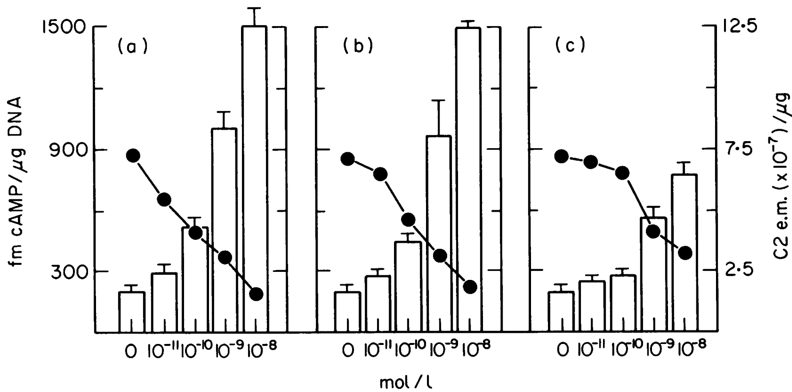

Fig. 3. Effect of anaphylatoxins on cAMP levels and C2 production. cAMP levels (mean ± s.e. of three cultures) measured after 2 h incubation. C2 levels (single point determination on replicate cultures) measured on day 3. (a) $C5a_{des\,Arg}$; (b) C5a; (c) C3a.

Anaphylatoxins and cyclic nucleotides

When added to human monocyte cultures C3a, C5a and $C5a_{des\,Arg}$ all produced dose-dependent increases in cAMP levels (Fig. 3).

C5a and $C5a_{des\,Arg}$, which were approximately equipotent, produced the greatest increases in cAMP levels. C3a, which was far less effective in reducing C2 production, caused a smaller increase in cAMP.

$C3a_{des\,Arg}$ was not tested in view of its almost insignificant effect on C2 production.

C2 concentrations were inversely correlated with intracellular cAMP (−0·9593; $P<0{\cdot}001$; Fig. 4).

Cyclic GMP levels (16·3 fmoles/μg DNA ± s.e. 0·7) were not significantly altered by the anaphylatoxins.

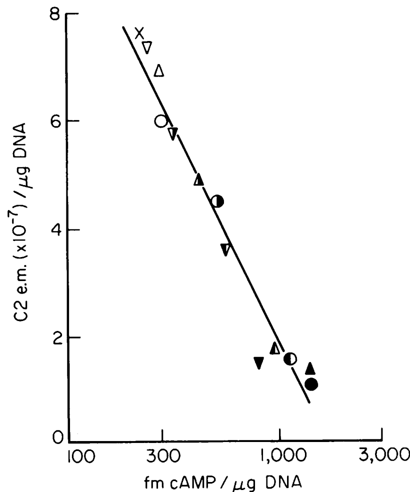

Fig. 4. Relationship between cAMP level and C2 production. Different concentrations of C3a (▼), C5a (▲) and $C5a_{des\,Arg}$ (●), were added to cultures. The shading pattern indicates the concentration of anaphylatoxin used (e.g. for C5a 10^{-8} mol/l ●; 10^{-7} mol/l ◐; 10^{-6} mol/l ○; 10^{-5} mol/l ◑). Control culture = X. $r=0{\cdot}9593$.

DISCUSSION

The phlogistic potential of anaphylatoxins is now well documented; important activities being the ability to increase vascular permeability and attract leucocytes into the inflammatory lesion (Hugli & Müller-Eberhard, 1979). The data presented in this paper suggest that anaphylatoxins may help control the inflammatory response by inhibiting the production of C2, the limiting component of the classical pathway. The conclusion that anaphylatoxins inhibited production of C2 was based upon the findings that the anaphylatoxins were not cytotoxic, and that C2 degradation was not increased in anaphylatoxin treated cultures. The criteria employed for assaying cytotoxicity were cell morphology, trypan blue exclusion and the normal DNA content of anaphylatoxin treated monolayers. Although we have not assessed release of lactate dehydrogenase, an enzyme which is a sensitive marker of cell injury, we feel that our data shows that the anaphylatoxins in the concentrations used did not cause cell death. This conclusion is supported by the findings of Goodman and his colleagues (Goodman, Weigle & Hugli, 1980) who showed that C3a was not cytotoxic for a number of cell types even at concentrations of 100 μg/ml. Furthermore, McCarthy & Henson (1979) were unable to demonstrate LDH release from rabbit alveolar macrophages after exposure to C5a and $C5a_{des\ Arg}$ at concentrations of 5 μg/ml. However, we have not excluded the possibility that anaphylatoxins cause a generalized inhibition of metabolism of mononuclear phagocytes, which produces a secondary inhibition of protein production. However, lysozyme synthesis which has been used as an indicator of normal macrophage function, is not affected by C5a or $C5a_{des\ Arg}$ (McCarthy & Henson, 1979).

In the C2 biosynthesis system the C5 derived anaphylatoxins were more potent than those from C3. It is of interest that C5a and $C5a_{des\ Arg}$ were equipotent whereas $C3a_{des\ Arg}$ showed the characteristic reduction of activity compared with C3a, which has been observed in other systems (Hugli & Müller-Eberhard, 1979). The possibility exists that C5a is rapidly converted to $C5a_{des\ Arg}$ by the enzyme carboxypeptidase, a secretory product of macrophages (Kreuzpainter, Damerau & Brade, 1982) and which could be present in trace quantities in FCS. If this is the case then the explanation of the obvious differences between the potencies of C3a and $C3a_{des\ Arg}$ may be a reflection of the relatively large concentrations of C3a required to produce 50% inhibition of C2 production. The studies of Damerau *et al.* (1980b) showed that $C5a_{des\ Arg}$ possessed 30–40% of the spasmogenic and leucocyte aggregating activities of C5a. It is possible, that by the construction of our dose–response curves (10-fold dilutions) that small reductions of inhibitory activity were overlooked. However, it is apparent that the ability to inhibit C2 synthesis is an integral function of the $C5a_{des\ Arg}$ molecule. The IC50 for C5a and $C5a_{des\ Arg}$ was of the order of 10^{-10} mol/l, thus this is one of the most powerful expressions of the activity of the C5 derived anaphylatoxins. This activity of anaphylatoxins is not species restricted as it can be observed in cultures of guinea-pig peritoneal macrophages.

The mechanism by which C2 production is inhibited by anaphylatoxins is not due to the generation of prostaglandins, as neither ETYA nor indomethacin reversed the effect. However the inhibition of C2 production was related to increased intracellular cAMP. We have shown previously that agents which increase cAMP decrease C2 production and those that decrease cAMP increase C2 synthesis (Lappin & Whaley, 1981, 1982). It appears therefore that cAMP may act as a second messenger between membrane associated events and C2 synthesis. Whether anaphylatoxins exert their effects directly or indirectly on adenyl cyclase or phosphodiesterase requires investigation.

The biological significance of this activity of anaphylatoxins is unknown. It can be postulated that it provides an important feedback control on complement activation. Synthesis of C2 by mononuclear phagocytes would increase the formation of $C\overline{42}$ and $C\overline{423b}$, the classical pathway C3 and C5 convertases (Whaley & Ferguson, 1982). The cleavage of C3 and C5 results in the formation of C3a and C5a which could then act on the mononuclear phagocytes to inhibit further C2 production.

In view of the rapid conversion of C3a and C5a to their des Arg derivatives by carboxypeptidase-*N* or -B, $C5a_{des\ Arg}$ is probably the most important anaphylatoxin in the control of C2 production by mononuclear phagocytes *in vivo*.

C2 is only one of a number of biologically active products synthesized by mononuclear phagocytes. The effect of anaphylatoxins on the production of these other molecules requires investigation.

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