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Cyclic nucleotides and their relationship to complement-component-C2 synthesis by human monocytes

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The time courses of changes in cyclic nucleotide levels in monocytes have been studied. Histamine and prostaglandin E_2 (PGE_2) produced a rapid rise in cyclic AMP (peak 15min) levels, which returned to normal within 4h, whereas cholera toxin, NaF and phosphodiesterase inhibitors produced slow sustained rises lasting over 24h. With the exception of isobutylmethylxanthine (10 μmol·l^{-1}), none of these reagents altered cyclic GMP levels. α_1-Adrenergic and nicotinic cholinergic receptor–ligand interactions and imidazole produced rapid and relatively short-lived falls in cyclic AMP, and rises in cyclic GMP. In contrast, prostaglandin synthetase inhibitors produced delayed but more sustained falls in cyclic AMP but no rises in cyclic GMP. Agents that increased cyclic AMP decreased complement-component-C2 production, and those that decreased cyclic AMP increased C2 production. Agents that increased cyclic GMP alone (ascorbate, nitroprusside and prostaglandin $F_{2\alpha}$) did not affect C2 production. Antigen–antibody complexes that stimulate C2 synthesis produced falls in cyclic AMP and rises in cyclic GMP similar to those produced by adrenergic and cholinergic ligands. Serum-treated complexes and anaphylatoxins, which inhibited C2 production, were associated with changes in cyclic AMP similar to those produced by histamine and PGE_2. These data suggest that there are two transmembrane signals involved in the regulation of C2 production by monocytes. The inhibitory signal is adenylyl cyclase activation. The stimulatory signal is not so obvious, but may be Ca^{2+} influx, since the time courses of changes in cyclic nucleotides produced by agents that stimulate C2 synthesis are identical, and α_1-adrenergic agonists cause the formation of Ca^{2+} channels.

The complement system consists of a series of plasma proteins that plays a major role in host defence. Although hepatocytes are thought to synthesize over 90% of the C3, C6 and factor B that are present in the plasma (Alper *et al.*, 1968; Alper & Rosen, 1976; Hobart *et al.*, 1977), local production of complement components occurs at sites of inflammation (Ruddy & Colten, 1974). Metabolic studies have shown that approximately half of the C3 present in the synovial fluid from inflamed joints is synthesized locally (Ruddy & Colten, 1974). The mononuclear phagocyte, which has been shown to synthesize a number of functionally active complement components, including C2, B, P, D̄ and C3 (Einstein *et al.*, 1976; Whaley, 1980; Strunk *et al.*, 1983), is the cell most likely to be responsible for this local synthesis. The observations that macrophages isolated from chronically inflamed synovial joints synthesize greater quantities of complement components than do cells

Abbreviations used: the nomenclature of complement components is that recommended by the World Health Organisation (1968); PGE_2, $PGF_{2\alpha}$, prostaglandins E_2 and $F_{2\alpha}$; IC, antigen–antibody complexes; SIC, serum-treated IC; CR_1, C3b receptor; ETYA, eicosatetra-5,8,11,14-ynoic acid; DTSP, dithiobis-succinimidyl propionate; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G; HBSS, Hanks balanced salt solution; FCS, foetal-calf serum; LDH, lactate dehydrogenase; IBMX, isobutylmethylxanthine.

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from non-inflamed joints, or monocyte-derived macrophages (De Ceulaer *et al.*, 1980), supports this notion.

The factors that control the synthesis of complement components by mononuclear phagocytes are only beginning to be defined. We have been investigating the effect of inflammatory mediators and defined pharmacological agents on complement synthesis, and have shown that agents such as histamine (Lappin & Whaley, 1980) and PGE_2 (Lappin & Whaley, 1982), which activate adenylyl cyclase, inhibit the synthesis of complement components, whereas imidazole, which decreases cyclic AMP, increases their synthesis (Lappin & Whaley, 1983). These observations suggested that changes in the intracellular concentrations of cyclic AMP may control the synthesis of complement components by mononuclear phagocytes. In other experiments in which we have investigated the effects of antigen–antibody complexes on complement synthesis, we have shown that antigen–antibody complexes (IC) that bind to Fc receptors have a stimulatory effect (McPhaden *et al.*, 1981), whereas serum-treated IC (SIC), which act on the C3b receptor (CR_1), are inhibitory (Whaley *et al.*, 1983*a*). The anaphylatoxins C3a, C5a and their desarginyl derivatives, also inhibit the synthesis of the second component of complement (C2) (Lappin *et al.*, 1983).

Here we present the results of experiments showing the kinetics of the changes of cyclic AMP and cyclic GMP levels in cultured human monocytes after exposure to defined pharmacological agents, IC, SIC and anaphylatoxins. We also show that the changes in cyclic AMP levels correlate inversely with changes in the concentrations of C2 in the monocyte culture medium.

Materials and methods

Reagents

Histamine dihydrochloride, PGE_2, $PGF_{2\alpha}$, indomethacin, phenylephrine, carbamylcholine, atropine, D-tubocurarine, IBMX, theophylline, sodium ascorbate (Sigma Chemical Co., Poole, Dorset, U.K.), chlorpheniramine maleate (Allen and Hanburys, Greenford, Middx., U.K.), cimetidine (Smith, Kline and French, Welwyn Garden City, Herts., U.K.), sodium nitroprusside and NaF (BDH, Poole, Dorset, U.K.), cholera toxin (Schwartz–Mann; from Becton–Dickinson, Cowley, Oxford, U.K.), were obtained from commercial sources. Yohimbine and prazosin (Dr. F. Boyle, Department of Pharmacology, University of Glasgow, Glasgow, Scotland, U.K.), ETYA (Mr. T. Collins, Roche Products Ltd., Welwyn Garden City, Herts., U.K.), were gifts. Aqueous solutions of histamine, chlorpheniramine maleate, cimetidine, phenylephrine, yohimbine, prazosin, carbamylcholine, atropine, D-tubocurarine, PGE_2 and $PGF_{2\alpha}$ were prepared immediately before their addition to cultures. Indomethacin and ETYA were dissolved in ethanol to give a final concentration of $1\,mol\cdot l^{-1}$. All solutions were diluted in medium immediately before addition to monocyte cultures. When substances that had been dissolved in alcohol were added to cultures, control cultures containing an equivalent concentration of alcohol without the compound were included. The alcohol concentrations used in these experiments ($\leqslant 0.01\%$) did not alter C2 synthesis or cyclic nucleotide levels.

Complement components, antisera and antigen–antibody complexes

C3b was prepared from purified C3 (Tack & Prahl, 1976) by using a solid-phase cobra-venom-factor-dependent C3 convertase (Gitlin *et al.*, 1975). C3b was polymerized by using DTSP (Pierce and Warriner, Chester, Cheshire, U.K.) as described previously (Hamilton *et al.*, 1983). Analysis of DTSP-polymerized C3b by SDS/polyacrylamide-gel electrophoresis run under non-reducing conditions revealed that 50% of the C3b was polymerized, and oligomers, ranging from dimer to hexamer and larger, were formed. Human CR_1 (Fearon, 1980) and the porcine anaphylatoxins C3a and C5a and their desarginyl derivatives (Damerau *et al.*, 1980) were prepared by published techniques. Antisera to C3 and CR_1 were prepared by the immunization of rabbits with the antigens emulsified in Freund's complete adjuvant followed 1 month later by three booster injections spaced at 2-week intervals with the antigens emulsified in Freund's incomplete adjuvant. The rabbits were bled 10 days after the final injection. Fab fragments of the IgG fractions of the antisera were prepared by papain digestion (Porter, 1959). IC were prepared at equivalence by using sterile bovine serum albumin and sterile ^{125}I-labelled immunoadsorbent-purified rabbit anti-(bovine serum albumin). IC were added to cultures within 5 min of their formation. SIC were prepared by solubilizing ^{125}I-labelled immune precipitates formed at equivalence with the normal human serum. SIC were separated from other complement components by gel-filtration chromatography using Sepharose 6B, and then sterilized by Millipore filtration (Whaley *et al.*, 1983*a*). Human IgG and IgG subclass myeloma proteins (a gift from Dr. D. R. Stanworth, University of Birmingham, Birmingham, U.K.) were cross-linked as described previously (Whaley *et al.*, 1983*b*).

Monocyte cultures

Mononuclear leucocytes were prepared from buffy-coat preparations of blood-bank donations. The buffy-coat suspensions were centrifuged (600*g* for 10min at 4°C) and the supernatant plasma removed and stored, and the buffy-coat leucocytes carefully removed from the surface of the red-cell pellet and resuspended in ice-cold HBSS. The mononuclear leucocytes, which were isolated by Ficoll–Hypaque gradient centrifugation (Boyum, 1968), were washed thrice in HBSS, resuspended in 5ml of RPMI 1640 (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.), containing L-glutamine (2mmol·l^{-1}), $NaHCO_3$ (9mmol·l^{-1}) and antibiotics (Antibiotic–Antimycotic solution; Gibco Europe, Paisley, Scotland, U.K.), and the cell count determined. The volume of the suspension was adjusted, by the addition of RPMI 1640 containing 20% (v/v) inactivated (2h at 56°C) FCS (Gibco), to give a final cell count of 1×10^7/ml).

A portion (500μl) of the cell suspension was added to each well of 24-well Linbro tissue-culture plates, and the cells incubated for 2h at 37°C in a humidified atmosphere of air/CO_2 (19:1). The non-adherent cells were removed by vigorous washing in RPMI 1640 (37°C), and the cells cultured in RPMI/FCS (1ml), under the conditions described above. For experiments in which IC or SIC were added to cultures, RPMI 1640 containing 10% heat-inactivated autologous serum was used as the culture medium in preference to RPMI/FCS. Autologous serum was obtained by incubation of the recalcified plasma at 37°C for 1h. After removal of the clot, the serum was heat-inactivated (2h at 56°C).

Monolayers prepared under these conditions consisted of over 95% monocytes as judged by morphology, phagocytosis of latex particles and staining for non-specific esterase.

Experiments were performed on monocytes that had been cultured for 5 days. On the fifth day of culture, the medium on each monolayer was removed, and replaced by fresh medium. In order to study the kinetics of changes in cyclic nucleotide levels, a fixed dose of the reagent under study was added to a series of cultures. At timed intervals the supernatant was removed and monocytes disrupted by the addition of 200μl of ice-cold ethanol to the wells. The culture plate was then placed at −20°C for 10min, after which time the ethanol and cell debris were transferred to Microcap tubes (Eppendorff). The wells were washed twice in ice-cold ethanol (200μl per wash) and the ethanol transferred to the Microcap tube. After centrifugation (Beckman Microfuge for 3min) the supernatant was retained, and the pellet washed once in 200μl of ethanol. After re-centrifugation the second supernatant was removed and added to the first supernatant. The pellet was discarded and the supernatants freeze-dried. By using ^{125}I-labelled tracers, the recoveries of cyclic AMP and cyclic GMP were 96 and 91% respectively. Results of assays were corrected for 100% recovery.

Initially the time points for these kinetic studies were 0, 5, 15 and 45s, 2, 5, 15 and 30min. However, it became obvious that, for all the reagents under investigation, peaks or troughs in cyclic nucleotide levels did not occur at intervals of less than 1min. We therefore decided to sample at 0, 2, 5, 15, 30 and 60min, 2, 4, 8 and 24h. At each point a control culture to which no reagent had been added was harvested in the same way. Replicate cultures were exposed to the reagent under study, or no reagent, and 24h later the supernatant was removed for measurement of C2 content by a stoichiometric haemolytic assay (Rapp & Borsos, 1971). The monolayer was washed and lysed in 2% (w/v) SDS and the DNA content measured by spectrofluorimetry (Einstein *et al.*, 1976). The DNA content of the monolayers used in the present study was between 2 and 4μg (i.e. between 3×10^5 and 6×10^5 monocytes/culture).

Dose–response curves were constructed once the time of the maximal change in cyclic nucleotide levels had been defined on the basis of kinetic assays. In these experiments, different concentrations of reagent were added to the cultures, and incubation continued until maximal change in cyclic nucleotide levels had occurred. Replicate cultures were incubated for 24h in order to measure the C2 content of the supernatants and the DNA content of the monolayers.

Cyclic nucleotide assays

The freeze-dried material from the ethanol extracts of the monolayers were dissolved in 500μl of acetate buffer (50mmol·l^{-1}), pH4.75, acetylated with acetic anhydride (130mmol·l^{-1}) and then assayed for their cyclic AMP and cyclic GMP content by using a modification of the method of Harper & Brooker (1975). For cyclic AMP, a 50μl sample of the acetylated sample, 100μl of sodium acetate buffer (50mmol/l), pH4.75, 25μl of ^{125}I-cyclic AMP (2nCi; The Radiochemical Centre, Amersham, Bucks., U.K.) and 100μl of antiserum to cyclic AMP (1:4 dilution of stock; Becton–Dickinson, Oxford, U.K.) were mixed in a test tube and incubated at 4°C overnight. Free and antibody-bound cyclic AMP were separated with charcoal. Standard curves were constructed by using cyclic AMP (Sigma) that had been acetylated as described above.

The cyclic GMP assay was similar to that described for cyclic AMP, except that 100μl of extract was used. The reaction volume was adjusted

by decreasing the volume of buffer to 50 μl, and then 25 μl (2.1 nCi) of ^{125}I-cyclic GMP (The Radiochemical Centre) and 100 μl of antiserum [1:300 dilution of stock antiserum used in cyclic GMP radioimmunoassay kits (The Radiochemical Centre)] were added.

In addition to background and 100%-binding controls, in some assays specificity controls were included. These consisted of unlabelled cyclic AMP, cyclic GMP or extracts that had been pretreated with cyclic nucleotide phosphodiesterase (Sigma). After digestion, the phosphodiesterase was inactivated by heating for 2min at 90°C. Digestion with phosphodiesterase always decreased cyclic nucleotide concentrations to zero.

All assays were performed in duplicate; when results of replicate assays differed by more than 10%, the assay was repeated. By using these assays we were able to measure confidently 5fmol of cyclic AMP and 2.5fmol of cyclic GMP.

In order to exclude the possibility that agents which inhibited C2 production were cytotoxic or were inhibiting monocyte protein synthesis nonspecifically, the following experiments were performed. Monocytes were cultured for 5 days as described above. After replacing the culture supernatant with fresh medium on day 5, the agent under study was added. In one series of experiments the ability of monocytes to exclude Trypan Blue was measured after 24h (Lappin & Whaley, 1980). In a second series, the supernatant was removed after 24h, and the washed monolayer was lysed in 1.5ml of Triton X-100 (0.05%). The supernatants and lysates were assayed for LDH content by a standard technique (Strunk *et al.*, 1980). LDH release was expressed as a percentage of the total (intracellular + extracellular). The lysozyme content of the supernatants was assayed by measuring the lysis of *Micrococcus lysodeikticus* (Sigma) by the method of Strunk *et al.* (1980). The results were expressed as units/μg of DNA. One unit of activity is that which will produce a ΔA_{450} of 0.001/min at pH6.4 and 25°C with a suspension of *Micrococcus lysodeikticus* (0.2mg/ml) as substrate in a 2.6ml reaction volume and a light-path of 1cm.

Results

Cyclic nucleotide levels in cultured monocytes

The mean levels of cyclic AMP and cyclic GMP in control monocytes (31 cultures) that had been cultured for 5 days were 277.0±19.3 (S.E.M.) fmol/μg of DNA and 21.4fmol/μg of DNA (±1.0) respectively. Levels in control untreated cultures remained constant throughout the 24h of study. Unless stated otherwise, all further results given represent the mean (±S.E.M.) for three separate cultures.

Studies of the time courses of elevations of cyclic AMP induced by pharmacological agents

In order to define the kinetics of changes in cyclic AMP levels in monocytes, we studied the actions of agents that (*a*) activate adenylyl cyclase by specific cell membrane receptors (histamine, PGE_2), (*b*) act on the regulatory unit of the holoenzyme (cholera toxin and NaF) and (*c*) inhibit phosphodiesterase activity (theophylline and IBMX).

Histamine (1 μmol·l^{-1}) and PGE_2 (10 μmol·l^{-1}) produced a rapid rise in cyclic AMP that reached a peak at 15min, and returned to control values by 8h (Fig. 1). NaF (100nmol·l^{-1}) and cholera toxin (10ng/ml) produced a slower increase in cyclic AMP that reached a peak at 2h and remained elevated at 24h (Fig. 1). Theophylline (100 μmol·l^{-1}) and IBMX (10 μmol·l^{-1}) also produced slower but sustained elevations in cyclic AMP and, as with agents acting on the regulatory unit of adenylyl cyclase, the peak level was achieved by 2h, and at 24h, cyclic AMP levels were still above control values (Fig. 1). In this series of experiments the mean control level of cyclic AMP was 300 (±13) fmol/μg of DNA, and the peak levels obtained with the agents studied were as follows: histamine, 669 (±22); NaF, 1469 (±25); cholera toxin, 1261 (±34); theophylline, 958 (±13); and IBMX, 951 (±8). The C2 levels in monocyte cultures 24h after exposure to these reagents were lower than those in control cultures (Fig. 1).

Cell viability was not influenced by these reagents, as assessed by morphology, Trypan Blue exclusion and DNA content of monolayers, LDH release and lysozyme synthesis (Table 1). Increased

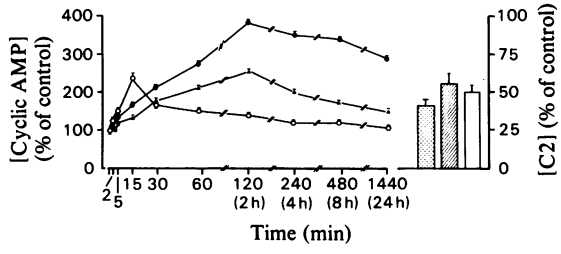

Fig. 1. *Effects of histamine (1 μmol·l^{-1}) (○, cyclic AMP; □, C2), NaF (100nmol·l^{-1}) (●, cyclic AMP; ■, C2) and IBMX (10 μmol·l^{-1}) (▲, cyclic AMP; ▨, C2) on intracellular concentrations of cyclic AMP (shown as a percentage of control levels) and C2 production (expressed as a percentage of control) by monocytes in culture, measured at 24h*

Each point represents the mean±S.E.M. from three experiments.

Table 1. *Lysozyme synthesis and LDH release in monocyte cultures exposed to agents that inhibit C2 production*

Agents were added to cultures on day 5. C2 production, lysozyme synthesis and LDH release were measured after 24h. The results represent the means ± S.E.M. for three cultures. Because of the number of agents under investigation, they could only be studied in small groups (Expts. 1–4).

	Expt. 1					Expt. 2	
Agent ...	Histamine	PGE_2	Cholera toxin	NaF	Control	IBMX	Theophylline	Control
Concn. ...	1 μmol·l^{-1}	10 μmol·l^{-1}	10ng/ml	100nmol·l^{-1}		10 μmol·l^{-1}	100 μmol·l^{-1}	
Lysozyme (units/μg of DNA)	17.2±0.8	20.8±1.2	20.7±0.6	18.1±0.2	19.2±0.3	18.7±0.6	18.1±0.4	19.1±0.8
LDH release (%)	18.8±1.9	20.5±1.1	23.3±2.7	21.2±2.4	21.2±1.0	15.9±0.5	15.2±0.2	17.7±0.4
Inhibition of C2 production (%)	62.4±0.8	64.8±0.8	72.8±0.5	48.8±2.4	-	35.1±5.5	48.9±8.7	-

	Expt. 3				Expt. 4		
Agent ...	SIC	Poly-C3b	C5a	Control	des-Arg-C5a	C3a	Control
Concn. ...	300ng/ml	10 μg/ml	1nmol·l^{-1}		1nmol·l^{-1}	1nmol·l^{-1}	
Lysozyme (units/μg of DNA)	16.4±0.2	17.9±0.1	16.3±0.3	16.7±0.3	22.2±0.4	22.3±0.8	21.5±0.3
LDH release (%)	16.4±0.2	15.2±1.5	16.3±0.1	16.9±0.1	22.2±0.6	25.9±0.6	24.8±0.6
Inhibition of C2 production (%)	63.1±1.2	58.7±3.3	65.6±5.3	-	58.1±1.0	50.0±5.8	-

degradation of newly synthesized C2 was excluded by showing that functionally purified C2 (Ruddy & Austen, 1967) was not degraded more rapidly in cultures exposed to the reagents under study. Thus decreased concentrations of C2 in the culture supernatants are due to reduced production. $PGF_{2\alpha}$ (10 μmol·l^{-1}) did not affect C2 production or cyclic AMP levels.

Studies of the time courses of decreases in cyclic AMP induced by pharmacological agents

The α-adrenergic agonist phenylephrine (1 μmol·l^{-1}) produced an immediate fall in intracellular cyclic AMP that reached the lowest level 30min after the addition of the agent to the culture and returned to normal after 8h (Fig. 2). The β-adrenergic agonist isoprenaline (100 μmol·l^{-1}) had no effect on cyclic AMP levels. The cholinergic agonist carbamylcholine (1 μmol·l^{-1}) and imidazole (10 μmol·l^{-1}) also produced marked lowering of cyclic AMP levels, the lowest level occurring after 30min and returning to control values by 8h. Indomethacin (1 μmol·l^{-1}) and ETYA (1 μmol·l^{-1}) also decreased cyclic AMP levels (Fig. 2). However, unlike the α-adrenergic and cholinergic ligands, the fall, which did not begin until after 15min of exposure, was gradual, with the lowest levels occurring after 60min and persisting throughout 24h. The mean control level of cyclic AMP for the sets of cultures treated with phenylephrine, indomethacin and ETYA and imidazole was 328 (±3) fmol/μg of DNA. The minimum levels for the reagents used were as follows: phenylephrine, 236 (±3); imidazole, 195 (±4); indomethacin, 227 (±2.5); and ETYA, 186 (±2.5). The mean control level for cultures treated with carbamylcholine was 367fmol/μg of DNA (±13.5) and the minimum level produced by this

Fig. 2. *Elevation of C2 production and decrease in cyclic AMP levels in human monocytes exposed to indomethacin (1 μmol·l^{-1}) (○, cyclic AMP; □, C2) and phenylephrine (1 μmol·l^{-1}) (▲, cyclic AMP; ▨, C2), and the effect of isoprenaline (100 μmol·l^{-1}) ▼, cyclic AMP; ▦, C2) on intracellular cyclic AMP and C2 production*

All results are expressed as percentages of the control value and are the means ± S.E.M. for three experiments.

ligand was 293 (±2.3). C2 levels were significantly elevated in cultures exposed to phenylephrine, carbamylcholine, imidazole, indomethacin and ETYA (Fig. 2).

Effect of agents on cyclic GMP levels

Histamine, PGE_2, cholera toxin, NaF, theophylline, indomethacin and ETYA did not affect cyclic GMP levels. At high concentrations (10 μmol·l^{-1}) IBMX produced a small elevation in cyclic GMP, which did not achieve statistical significance. Sodium nitroprusside (100 μmol·l^{-1}) and sodium ascorbate (10 μmol·l^{-1}) produced marked elevations in cyclic GMP, the peak level being reached after 60 min and beginning to fall by 8 h (Fig. 3). The rise in cyclic GMP after the addition of $PGF_{2\alpha}$ to the cultures was less pronounced, reaching a peak by 30 min and lasting less than 4 h.

The mean control cyclic GMP level in cultures treated with nitroprusside and $PGF_{2\alpha}$ was 14.3 fmol/μg of DNA (±0.3). The peak levels obtained with sodium nitroprusside and $PGF_{2\alpha}$ were 31.9 (±0.5) and 19.7 (±0.8) fmol/μg respectively. The mean control level of cyclic GMP in cultures treated with sodium ascorbate were 19.6 (±1.4) fmol/μg of DNA. The peak value produced by this agent was 50.7 (±6.2) fmol/μg. C2 levels in cultures exposed to sodium nitroprusside, sodium ascorbate and $PGF_{2\alpha}$ did not differ from those in control cultures.

Phenylephrine (1 μmol·l^{-1}), carbamylcholine (1 μmol·l^{-1}) and imidazole (10 μmol·l^{-1}) caused increases in cyclic GMP; the time courses and the size of the increases were similar to those produced by $PGF_{2\alpha}$. The level of cyclic GMP in the control cultures for phenylephrine and imidazole were 23.5 fmol/μg of DNA (±1.1) and the peak levels for these agents were 33.7 (±1.1) and 33.0 (±0.2) fmol/μg respectively. The mean control level in carbamylcholine-treated cultures was 22.5 (±1.7) fmol/μg of DNA and the peak level achieved with this agent was 45.2 (±2.1) fmol/μg.

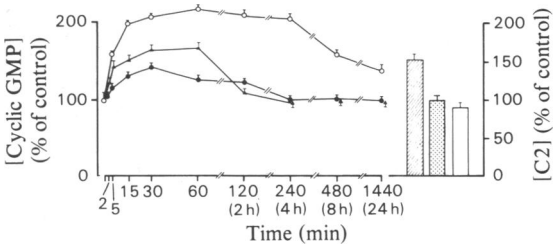

Fig. 3. *Effects of sodium nitroprusside (100 μmol·l^{-1}) (○, cyclic GMP; □, C2), $PGF_{2\alpha}$ (10 μmol·l^{-1}) (●, cyclic GMP; ■, C2) and phenylephrine (1 μmol·l^{-1}) (▲, cyclic GMP; ▨, C2) on monocyte cyclic GMP levels and C2 production, measured at 24 h (expressed as percentages of the control values)*

Each point represents the mean ± S.E.M. for three experiments.

Reversibility of effects of histamine and cholinergic and adrenergic ligands

Histamine (1 μmol·l^{-1}) was added to a series of monocyte cultures in the presence and absence of cimetidine (0.01–10 μmol·l^{-1}), chlorpheniramine (0.01–10 μmol·l^{-1}) or indomethacin (10 μmol·l^{-1}). Cyclic AMP levels were measured after 15 min and C2 levels after 24 h. Cimetidine (1 μmol·l^{-1}) completely inhibited the elevation in cyclic AMP and the fall in C2 production produced by histamine, whereas chlorpheniramine and indomethacin had no effect on these actions. Neither cimetidine nor chlorpheniramine alone had any effect on cyclic nucleotide levels or C2 synthesis (results not shown).

Carbamylcholine (1 μmol·l^{-1}) was added to a series of cultures in the presence or absence of D-tubocurarine (10000–1 nmol·l^{-1}) or atropine (10000–1 nmol·l^{-1}). Cyclic AMP and cyclic GMP levels were measured after 30 min and C2 levels in the supernatants of replicate cultures after 24 h. D-Tubocurarine (10 nmol·l^{-1}) abrogated completely the effects of carbamylcholine, whereas atropine was ineffective (results not shown). The changes in cyclic nucleotide levels and C2 synthesis produced by phenylephrine (1 μmol·l^{-1}) were inhibited by prazosin (10 nmol·l^{-1}) and phentolamine (1 μmol·l^{-1}), whereas yohimbine and propranolol (both at 10–0.1 μmol·l^{-1}) had no effect. None of these antagonists affected cyclic AMP levels or C2 production when added to cultures in the absence of any agonists (results not shown).

Effects of antigen–antibody complexes on cyclic AMP, cyclic GMP and C2 production

IC and DTSP-polymerized IgG, IgG_1 and IgG_3 produced falls in cyclic AMP levels and increased synthesis of C2 (Fig. 4). The fall in cyclic AMP began within the first few minutes of the addition of these reagents to the culture, and minimum levels were achieved within 30 min. After 4 h cyclic AMP levels returned to control values. This pattern of change is similar to that found for phenylephrine, carbamylcholine and imidazole (Fig. 2). The mean control level of cyclic AMP in cultures treated with IC and DTSP-polymerized IgG was 407 (±2) fmol/μg of DNA, and the minimum levels produced by the reagents under investigation were as follows: IC, 245 (±4.0); polymerized IgG, 260 (±12) fmol/μg. The control value for cultures treated with polymerized IgG subclasses was 122.4 fmol/μg of DNA (±2.6) and the minimum levels achieved with polymerized IgG_1 and IgG_3 were 97.7 (±1.4), and 91.7 (±1.1) fmol/μg respec-

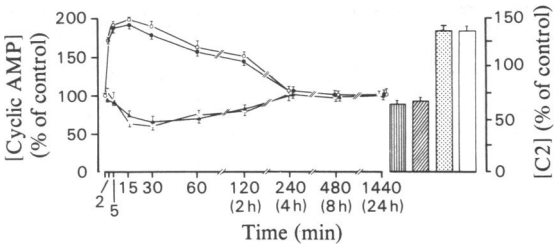

Fig. 4. *Effects of IC (300ng of IgG/ml) (△, cyclic AMP; □, C2), DTSP-polymerized IgG (10μg/ml) (▲, cyclic AMP; ▦, C2), SIC (300μg of IgG/ml) (○, cyclic AMP; ▨, C2) and DTSP-polymerized C3 (10μg/ml) (○, cyclic AMP; ▥, C2) on monocyte levels of cyclic AMP and C2 produced over 24h*

Each point represents the mean ± S.E.M. for three experiments.

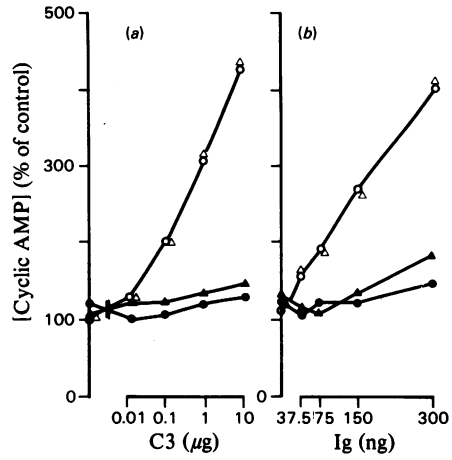

Fig. 5. *Role of C3b and CR_1 in SIC-mediated increase in cyclic AMP*

(*a*) Reversal of the elevation of cyclic AMP levels after addition of polymerized C3b (○) (0.01–10μg/ml) by the concomitant addition of Fab fragments of rabbit anti-(human C3) (●) and Fab fragments of rabbit anti-(human CR_1) (▲), and the failure of the Fab fragments of normal rabbit IgG (△) to influence the action of polymerized C3b. (*b*) An identical experiment performed with SIC alone (○) and in the presence of Fab anti-C3 (●), Fab anti-CR_1 (▲) and normal rabbit Fab fragments (△). Each point represents the mean for duplicate determinations.

tively. Polymerized IgG_2 and IgG_4 did not produce a fall in cyclic AMP and did not increase C2 production.

IC and DTSP-polymerized IgG, IgG_1 and IgG_3 caused elevations in cyclic GMP levels (results not shown) similar to those seen with phenylephrine, carbamylcholine, imidazole and $PGF_{2\alpha}$. The mean levels of cyclic GMP in the control cultures for those treated with IC and IgG were 16.4 (±2.5) fmol/μg of DNA, and peak levels achieved at 30min were 29.0 (±7.0) and 28.0 (±5.8) fmol/μg respectively. The mean control level for cultures treated with polymerized IgG subclasses was 29.1 (±0.7) fmol/μg of DNA. Peak levels achieved with IgG_1 and IgG_3 were 40.1 (±1.7) and 41.7 (±2.6) fmol/μg respectively. IgG_2 and IgG_4 had no effect on cyclic GMP levels.

SIC (300ng of IgG/ml) and polymerized C3b (10μg/ml) produced a rapid rise in cyclic AMP that reached a peak at 15min and returned to normal by 4h (Fig. 4). This pattern is similar to that produced by histamine and PGE_2 (Fig. 1). Levels of C2 were reduced in cultures treated with SIC and polymerized C3b. By using the criteria described above, the decreased C2 content in these cultures was due to reduced production. The mean control cyclic AMP level for this set of cultures was 404 (±17) fmol/μg of DNA. The mean peak level of cyclic AMP in cultures exposed to SIC was 802 (±0.5) fmol/μg, whereas after exposure to polymerized C3b it was 817 (±26) fmol/μg.

Reversal of effects of SIC and polymerized C3b on C2 production

The elevations in cyclic AMP produced by SIC (37.5–300ng/ml) and polymerized C3b (0.01–10μg/ml) were abrogated by the concomitant administration of Fab fragments (10μg/ml) of anti-CR_1 or anti-C3, but not by normal rabbit Fab fragments (Fig. 5). Fab fragments of anti-C3 and anti-CR_1 also prevented the reduction in C2 production in cultures exposed to SIC or polymerized C3b. Indomethacin ($10\,\mu mol \cdot l^{-1}$) did not affect the changes in cyclic AMP or C2 production produced by SIC or polymerized C3b.

Effect of anaphylatoxins on cyclic AMP and C2 production

C3a, C5a and des-Arg-C5a (all at $10\,\mu mol \cdot l^{-1}$) produced changes in cyclic AMP levels. After an initial rapid rise that reached a peak after 15min, the levels returned to control values by 4h (results not shown). The control cyclic AMP level in this series of cultures was 174 fmol/μg of DNA (mean for two cultures), and the peak levels for C3a, C5a and des-Arg-C5a were 518, 643 and 702 fmol/μg respectively. C2 levels were reduced in anaphylatoxin-treated cultures, the decrease being due to reduced production. Anaphylatoxins did not change levels of cyclic GMP.

Relationship between changes in cyclic AMP and C2 production

Does–response studies were performed on histamine, PGE_2, NaF, theophylline, IBMX, ETYA,

indomethacin and imidazole. Cyclic AMP levels were measured after 15min for histamine and PGE_2, 1h for NaF, theophylline and IBMX, 30min for imidazole and 60min for ETYA and indomethacin, which were the times required to achieve maximal changes. C2 levels were measured after 24h incubation in the presence of the reagent. When the cyclic AMP levels were plotted against the C2 concentrations there was an inverse correlation between C2 levels and log(peak cyclic AMP level). The correlation coefficient for those agents that increased cyclic AMP was -0.98, and was -0.97 for those that decreased cyclic AMP (results not shown).

Lysozyme synthesis and LDH release

Agents that inhibited C2 production did not reduce lysozyme secretion or increase LDH release (Table 1).

Discussion

Cyclic AMP provides a link between membrane signals and the expression of many cellular functions, and is therefore regarded as a second signal. Many mononuclear-phagocytic functions have been shown to be modulated by changes in intracellular cyclic AMP levels, including migration (Pick, 1972), response to MIF (migration-inhibitory factor) (Koopman *et al.*, 1973) interferon-induced tumoricidal activity (Schultz *et al.*, 1979) plasminogen activator (Vassalli *et al.*, 1976) and lysozomal enzyme secretion (Weissmann *et al.*, 1971), lipopolysaccharide-induced collagenase synthesis (McCarthy *et al.*, 1980) and Fc-receptor-mediated phagocytosis (Muschel *et al.*, 1977). Since pharmacological agents that increase cyclic AMP (histamine, prostaglandins and phosphodiesterase inhibitors) decrease the synthesis of C2 and other complement components by human monocytes (Lappin & Whaley, 1981), it appears that cyclic AMP is important in the regulation of this cellular function.

In order to confirm this conclusion, and to demonstrate whether the changes in the levels of cyclic AMP were closely related to changes in C2 synthesis, we undertook to measure levels of cyclic nucleotides in monocytes by radioimmunoassay. The levels of cyclic AMP and cyclic GMP measured in control cultures on day 5 are similar to those reported previously by other workers (Gallin *et al.*, 1978; Herlin & Kragballe, 1981). We waited until day 5 to study cyclic nucleotide levels because on day 0, immediately after plating out, there is a pronounced rise in monocyte cyclic AMP levels, which do not return to baseline values until 24h. This rise masks changes seen with agents that increase cyclic AMP.

The time courses of the increases in cyclic AMP were dependent on the site of action of the pharmacological agent under investigation. Adenylyl cyclase activators, acting via hormone receptors, produced an early rise in cyclic AMP that reached a peak after 15min and returned to baseline levels by 4h (Fig. 1). Agents that acted on the regulatory unit of adenylyl cyclase, or inhibited phosphodiesterase activity, produced much slower, but more sustained, elevations. The abrogation of the histamine effect by cimetidine rather than chlorpheniramine supports the idea that the elevation of cyclic AMP produced by histamine is mediated by adenylyl cyclase activation, as H2 receptors are known to activate adenylyl cyclase (Bourne *et al.*, 1971).

Two time-course patterns of decrease in cyclic AMP levels were produced. The first pattern, produced by the α-adrenergic agonist phenylephrine and the cholinergic ligand carbamylcholine, showed an early fall without any delay (Fig. 2). The second pattern of reduction, that produced by inhibitors of PG synthesis, occurred later and was more sustained. This difference in time course, together with the failure of prostaglandin synthetase inhibitors to increase levels of cyclic GMP, indicates that the mode of action is distinct from the former agents. The decrease in cyclic AMP, elevation of cyclic GMP and increased C2 production by phenylephrine was reversed by prazosin, but not by yohimbine, showing that these effects were mediated by α_1-adrenergic receptors. The observation that the actions of carbamylcholine on cyclic AMP, cyclic GMP and C2 production were reversed by D-tubocurarine rather than atropine, confirms that this ligand is acting on nicotinic receptors. As stimulation of α_1-adrenergic receptors leads to the formation of calcium channels (Takai *et al.*, 1981), the first pattern of cyclic AMP decrease could be due to activation of phosphodiesterase (Cheung, 1971) or to the modulation of adenylyl cyclase activity secondary to the intracellular influx of calcium. The fall in cyclic AMP produced by inhibitors of prostaglandin synthesis, could be explained on the assumption that endogenous production of prostaglandin regulates monocyte cyclic AMP levels. If so, then the time taken for the fall to occur could represent the combined time taken for the inhibitor to decrease prostaglandin production, and for the effect of the decrease to affect adenylyl cyclase activity.

The patterns of changes in cyclic AMP levels induced by polymerized C3b and serum-treated complexes were the same as those produced by histamine and PGE_2 (Fig. 4). The observation that the effects of these agents on cyclic AMP and C2 production were abrogated by Fab fragments of anti-C3 and anti-CR_1 show that the effects are

mediated by a C3 cleavage fragment bound to the complexes binding with CR_1. Furthermore, they suggest that CR_1-receptor–ligand interactions activate adenylyl cyclase. Recently, it has been shown that C3b stimulates prostaglandin synthesis by monocytes (Rutherford & Shenkin, 1983). The failure of indomethacin to abrogate the effects of SIC and polymerized C3b shows that prostaglandin formation is not the cause of the adenylyl cyclase activation or the reduction in C2 production after the addition of these agents to monocyte cultures. Anaphylatoxins, which inhibit C2 production, also increased cyclic AMP, the pattern of change suggesting that C3a and C5a receptors are linked to adenylyl cyclase-like histamine and PGE_2. Stimulation of prostaglandin production by anaphylatoxins does not account for the changes in cyclic AMP or C2 production, as shown by the failure of indomethacin to inhibit these effects.

The decreases in cyclic AMP produced by IC and polymerized IgG_1 and IgG_3 (Fig. 4) followed the same time courses as those produced by adrenergic and cholinergic ligands. It is therefore possible that IC and polymerized IgG_1 and IgG_3 act as calcium-channel formers. As stimulation of C2 production by IC is mediated by the Fc receptor (McPhaden *et al.*, 1981), and polymerized IgG_1 and IgG_3, but not IgG_2 and IgG_4, stimulate C2 production and decrease cyclic AMP levels, this would mean that the cross-linking of monocyte Fc receptors produces calcium-channel formation, as has been shown to occur in mast cells after cross-linking of IgE receptors (Curtain *et al.*, 1981).

The changes in monocyte cyclic AMP levels were always associated with changes in C2 production (Figs. 1, 2 and 4). The dose–response studies, which showed that cyclic AMP and C2 levels were inversely correlated, supports the idea that changes in cyclic AMP levels play an important role in the modulation of C2 production.

The results of Trypan Blue-exclusion tests and LDH-release assays show that agents which inhibit C2 production are not cytotoxic. Furthermore, as they do not inhibit lysozyme secretion, their inhibitory action is selective. Lysozyme secretion is unaffected by the state of macrophage activation, and is therefore considered to be constitutive (Gordon, 1980). In contrast, secretion of neutral proteinases such as plasminogen activator is induced by stimuli that cause macrophage activation. As synthesis of C2 is increased during macrophage activation (Littman & Ruddy, 1977; McPhaden *et al.*, 1982), it can be considered to be an induced secretory product. Like C2 synthesis, that of plasminogen activator is inhibited by increasing cyclic AMP, whereas lysozyme secretion continues unabated (Rosen *et al.*, 1978).

The results of these studies suggest that the transmembrane signal responsible for the decrease in C2 production is adenylyl cyclase activation. It is possible that the signal for stimulation of C2 synthesis is calcium-channel formation. If so, it remains to be determined whether the calcium influx stimulates C2 production by activating a calcium-dependent protein kinase, or whether the reduced levels of cyclic AMP play a role by inhibiting cyclic AMP-dependent protein kinase.

Finally, our data suggest that cyclic GMP does not play a significant role in the modulation of C2 production by monocytes.

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