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Stimulation of human rheumatoid synovial cells by non-lethal complement membrane attack

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SUMMARY

The effects of non-lethal complement attack on cultured human rheumatoid synovial cells have been investigated by measuring a variety of parameters. Within 3–4 min of initiating non-lethal complement membrane attack there was a rise in reactive oxygen metabolite release from cultured synovial cells, which slowly returned to basal levels over a period of 45 min. The response was dependent on the formation of the complete C5b–9 complex. Prostaglandin E_2 was also released during non-lethal attack in a biphasic manner, an early phase of release occuring within the first hour and a second, larger phase commencing at 4 hr and rising to levels of over 1000 ng/10^6 cells at 24 hr, compared to control levels at this time of less than 100 ng/10^6 cells. This response was dependent on the formation of the C5b–8 complex but did not require C9. Removal of extracellular calcium reduced release of prostaglandin E_2 to background levels, and inclusion of an inhibitor of protein synthesis abolished the second phase of release but not the first phase. Non-lethal attack caused release of small amounts of leukotriene B_4 but no detectable release of tumour necrosis factor.

INTRODUCTION

Formation of the membrane attack complex (MAC) of complement on a metabolically inert target such as an aged erythrocyte causes rapid, efficient colloid osmotic lysis of the cell (Mayer, 1972). Nucleated cells are, however, much more resistant to lysis, particularly by the homologous MAC. Resistance is predominantly the result of active recovery processes which rapidly remove the potentially cytolytic MAC from the cell surface (Ohanian, Schlager & Borsos, 1977; Morgan, Dankert & Esser, 1987). Cells which escape lysis in this way may nevertheless be transiently altered by non-lethal attack.

Stimulation of a variety of cellular activities, including the production of reactive oxygen metabolites (ROM) and metabolites of arachidonic acid (leukotrienes and prostaglandins), by non-lethal complement membrane attack has been demonstrated in several nucleated cell types, including neutrophils, monocytes, renal cells and oligodendrocytes (reviewed by Morgan, 1989). These non-lethal pro-inflammatory effects may be of importance in disease pathogenesis *in vivo*.

In rheumatoid arthritis, complement activation has been demonstrated and the MAC localized in the rheumatoid synovium (Sanders *et al.*, 1986; Morgan, Daniels & Williams 1988). Cell death is not a major feature of the disease, but release of biologically active molecules, including interleukin-1, prostaglandin E_2 and collagenase, from rheumatoid synovium has been demonstrated and implicated in the bone and cartilage destruction occurring in the joint (Dayer *et al.*, 1976; Wood, Ihrie & Hamerman, 1985; Hopkins, Humphreys & Jayson, 1988; Neale, Williams & Mathews, 1989). The mechanisms by which synovial cells are activated to produce these factors are as yet undefined.

We have previously demonstrated that non-lethal amounts of the MAC stimulate ROM release from human synoviocytes in suspension (Morgan *et al.*, 1988). The aim of the present study was to investigate the effects of non-lethal complement attack on adherent cultured human rheumatoid synoviocytes. Cells were stimulated to release ROM and metabolites of arachidonic acid, specifically prostaglandin E_2 and leukotriene B_4. ROM release was dependent on the formation of the complete MAC, whereas prostaglandin release required only C5b–8. Prostaglandin E_2 release was dependent on the presence of extracellular calcium and was inhibited by an agent that blocked protein synthesis. The mechanisms responsible for these observations and the possible relevance of these highly toxic molecules to the pathogenesis of rheumatoid synovitis will be discussed.

Abbreviations: LTB_4, leukotriene B_4; MAC, membrane attack complex; NHS, normal human serum. PGE_2, prostaglandin E_2; ROM, reactive oxygen metabolite; TNF, tumour necrosis factor.

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MATERIALS AND METHODS

Reagents

Tissue culture media and sera were obtained from Flow

Laboratories (Rickmansworth, Herts) and Biological Industries (Cumbernauld, Strathclyde). Unless stated all other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset) and BDH Chemicals (Poole, Dorset).

Sera

Normal human serum (NHS) was obtained from healthy volunteers. C9- and C8-depleted sera were obtained by passage of NHS over specific immunoaffinity columns, as described previously (Morgan *et al.*, 1983; Abraha, Morgan & Luzio, 1988).

Antibodies

Sheep anti-human 5′-nucleotidase was a kind gift of Dr J. P. Luzio (University of Cambridge, Cambridge). A mouse monoclonal antibody to human C9 (MC 47) was raised against native C9, as described previously (Morgan *et al.*, 1983).

Complement proteins

C5b6 was purified according to published methods (Podack, Kolb & Muller-Eberhard, 1978). C7 was purified by a modification of the method of DiScipio & Gagnon (1982). C8 and C9 were purified by immunoaffinity chromatography as described previously (Abraha *et al.*, 1988; Morgan *et al.* 1983).

Cells

Synovium was obtained fresh from rheumatoid patients undergoing synovectomy and the synovial membrane stripped from underlying tissue. Monolayers of synovial cells were then established essentially as described by Wernick *et al.* (1985). Briefly, the membrane was diced with scissors and placed in an enzyme mix (bacterial collagenase 1 mg/ml and bovine testicular hyaluronidase 10 μg/ml) for 3–4 hr at 37°, to digest connective tissue and allow release of cells into the medium. The digest was then filtered through sterile muslin and the filtrate spun at 350 *g* for 10 min at room temperature. The cell pellet was resuspended in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal calf serum (FCS) previously warmed to 37°. Cells were plated out in 24-well tissue culture plates and incubated at 37° in 95% O_2/5% CO_2. After 48 hr the medium was removed, the wells washed with DMEM to remove unattached cells and the remaining adherent cells cultured in DMEM/FCS medium as described above. At confluence (Day 5) cells were subcultured by trypsinization and replating at 1×10^4 cells/well in 24-well plates. To avoid between-study variation, all experiments were conducted at the second or third passage on confluent cells, each well containing about 5×10^4 cells, as assessed by trypsinization and counting cells from several wells in a haemocytometer.

Titration of non-lethal attack

Two strategies were employed to subject cells to complement membrane attack. (i) Adherent cells in serum-free DMEM were incubated for 30 min at 37° with 5 μg/ml sheep anti-5′-nucleotidase to antibody sensitize. These cells were then incubated with NHS at various dilutions for 30 min at 37°. (ii) Cells were subjected to reactive lysis in serum-free DMEM. Synoviocytes were incubated for 30 min at 37° with C5b6 (0·01 μg/ml–20 μg/ml) and C7 (10 μg/ml). They were then washed in serum-free DMEM and incubated for a further 30 min at 37° with C8 (10 μg/ml) and C9 (10 μg/ml).

Cell death in both cases was assessed by measuring lactate dehydrogenase release, in a microplate modification of published methods (Bergmeyer, 1965). The maximum dose of NHS or C5b6 at which no detectable cell lysis occurred was defined as the non-lethal dose for all subsequent experiments.

ROM measurement

Confluent, adherent antibody-sensitized cells were washed in serum-free DMEM and then 1 ml of serum-free DMEM containing 11 μM luminol added to each well. The tissue culture plate was placed on a microcomputer-controlled X, Y table of a home-built luminometer designed to measure luminescence in individual wells of a multi-well plate. One-hundred microlitres of a 1:2 dilution of NHS (final dilution 1:22) were injected into the well and the chemiluminescence was measure by a centronics photomultiplier tube connected to a RT11 computer, digital luminometer and recorder. Controls included sera depleted of C8 or C9 and a 100% lysis control utilizing the bee venom pore-forming toxin melittin (10 μg/ml; Sigma Chemical Co.).

Eicosanoid generation

The generation of PGE_2 and LTB_4 were estimated following non-lethal complement attack using NHS or reactive lysis with C5b6.

For complement attack utilizing whole serum, antibody-sensitized synoviocytes were incubated with a 1:20 dilution of NHS (or serum depleted of C8 or C9) for various times at 37°. Cells subjected to reactive lysis were incubated with C5b6 (0·7 μg/ml) and C7 (10 μg/ml) for 30 min at 37°, washed in serum-free DMEM, and incubated with C8 (10 μg/ml) and C9 (10 μg/ml) at 37° over the same time–course as described above for whole serum (controls included the omission of C9 or C8 plus C9).

Synoviocytes were also subjected to attack using reactive lysis in the absence of extracellular divalent cations. Cells were incubated with C5b6 and C7 and washed as described above, C8 and C9 added in the presence of EDTA at a final concentration of 10 mM, and the cells further incubated for various times.

The effects of non-lethal attack using reactive lysis were also examined in the presence of an inhibitor of protein synthesis. Actinomycin D, an inhibitor of mRNA synthesis, was added at a final concentration of 5 μg/ml at the same stage as C8 and C9. Cells were incubated for various times as described above.

At the end of each incubation supernatants were decanted, centrifuged to remove cell debris and stored at −70°. The cells were extracted overnight at −20° with 0·5 ml of 100% methanol. The extracted lipids were dried down in a Univac rotary vacuum evaporator (Uniscience Ltd, London), and reconstituted in Tris-isogel buffer (0·1 M Tris-HCl, 0·14 M NaCl, 0·1% gelatin and 0·01% NaN_3, pH 7·4) before assay.

Measurement of PGE_2 and LTB_4

PGE_2 and LTB_4 were measured by specific radioimmunoassay. [^{3}H]PGE_2 (184 Ci/mmol) and [^{3}H]LTB_4 (210 Ci/mmol) were purchased from Amersham International (Aylesbury, Bucks). PGE_2 standard was obtained from Sigma, and specific polyclonal antibody from Bioclinical Services (Cardiff). Synthetic LTB_4 standard was a kind gift from Dr B. Spur (Institute Henri Beaufor, Paris, France) and specific anti-LTB_4 antibody was kindly provided by Dr J. Rokach (Merk Frost, Montreal, Canada).

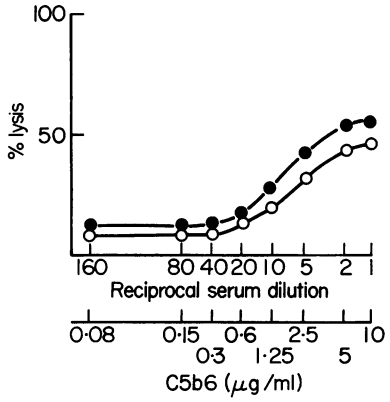

Figure 1. Complement killing of synoviocytes. Synoviocytes in culture on 24-well plastic plates were: (i) optimally antibody sensitized and incubated with various dilutions of NHS for 30 min at 37° (●); (ii) subjected to reactive lysis using C5b6 as the limiting factor (○). Cell death was assessed by measurement of lactate dehydrogenase release. Each point is the mean of four measurements obtained from four separate cell populations.

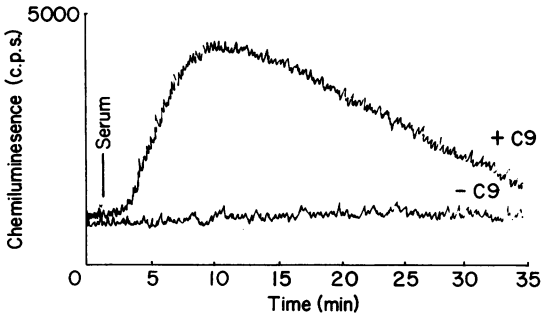

Figure 2. Release of reactive oxygen metabolites. Optimally antibody-sensitized synoviocytes *in situ* on plastic (5×10^4 cells per well) were incubated with non-lethal levels of NHS or NHS-C9 (1:22) in the presence of luminol at 37° on the X, Y table of a home-built luminometer. The top trace shows the luminol enhanced chemiluminescence produced by cells incubated with whole NHS. The bottom trace shows the chemiluminescence produced when cells are incubated with NHS depleted of C9. The traces shown are typical of those obtained in four different experiments.

The radioimmunoassays were performed in duplicate in total volumes of 300 μl of Tris-isogel buffer and contained 100-μl samples of supernatant, cell extract or standard, 100 μl of [^{3}H]eicosanoid and 100 μl of specific antibody in 3·5-ml polypropylene tubes (Sarstedt, Leicester, Leics). The reaction mixtures were incubated overnight at 4° for PGE_2 and at 37° for LTB_4. The non-protein-bound eicosanoids were precipitated by addition of 200 μl of 1% w/v charcoal (Norit SX-1; BDH) coated with dextran T70 (Pharmacia LKB, Milton Keynes, Bucks) in Tris-isogel buffer, followed by centrifugation at 2000 *g* for 15 min at 4°. Supernatants were decanted and mixed with 3·5 ml of Optiphase MP scintillant (LKB Instruments, Poole, Dorset), and the radioactivity measured in a Rack beta liquid scintillation counter (LKB). Synthetic LTB_4 was detected over a linear portion of the radiological binding curve at dilutions ranging from 0·1 to 4 ng/ml, while PGE_2 was measured in the concentration range 0·8–50 ng/ml.

Tumour necrosis factor (TNF) measurements

Cells were again subjected to reactive lysis as for PGE_2 and LTB_4 measurements and the supernatants were assayed for TNF in a cell cytotoxicity assay as described by Matthews & Neale (1987).

RESULTS

Titration of non-lethal attack

Serum as complement source. Synoviocytes were relatively resistant to lysis, end-point cell death being less than 60% even at a 1:2 dilution of NHS. At serum dilutions of below 1:20 there was no detectable lysis (Fig. 1). This dilution was therefore chosen as the maximum non-lethal dose in subsequent experiments.

Reactive lysis. C5b6 was used as the limiting factor with C7, C8 and C9 in excess. As shown in Fig. 1, no significant synovial cell lysis occurred at concentrations of C5b6 below 0·6 μg/ml. This concentration was therefore chosen as the non-lethal dose in all experiments using reactive lysis.

Total cell lysis was achieved in each case by incubating cells with 10 μg/ml mellitin.

ROM release

Following addition of serum (final dilution 1:22) to antibody-sensitized cells, ROM release, as measured by luminol chemiluminescence, was detectable after approximately 3 min. Release of ROM returned slowly back to basal levels over a period of 45 min (Fig. 2). The peak level of luminol-enhanced chemiluminescence from 5×10^4 cells was 5000 counts per second. The response was entirely dependent on the presence of C9, serum depleted of this component causing no ROM release (Fig. 2).

PGE_2 production

When reactive lysis was used, PGE_2 release into the supernatant, as measured by radioimmunoassay, was detectable within 1 hr of initiating non-lethal attack. A second, larger phase of release began after 4–5 hr and continued to rise throughout the 24-hr time-course, reaching levels of 1000 ng/10^6 cells (Fig. 3a), compared with control levels at this time of less than 100 ng/10^6 cells. Intracellular levels of PGE_2 began to rise above background levels (50–100 ng/10^6 cells) approximately 4 hr after initiation of attack and also rose in a time-dependent fashion over 24 hr, reaching levels of 600 ng/10^6 cells (Fig. 3a).

When serum was used as a complement source no release of immunoreactive PGE_2 into the supernatant was detected during non-lethal attack. However, within 30 min of initiating complement attack intracellular levels of PGE_2 were elevated and continued to rise to a peak at 4 hr of about 350 ng/10^6 cells compared with resting levels of less than 50 ng/10^6 cells (Fig. 3b). After 24 hr intracellular PGE_2 had returned to basal levels (Fig. 3b).

Using the reactive lysis system, the dependence on terminal components of PGE_2 release was investigated. Removal of C9 had no significant effect on PGE_2 release, but removal of C8 resulted in abolition of the response.

Removal of extracellular calcium using EDTA virtually abolished production and release of immunoreactive PGE_2. In the supernatant, PGE_2 was only detectable at 24 hr, and even then did not exceed control levels (Fig. 4a). In the cell extract no PGE_2 was detectable above control levels at any time (Fig. 4b).

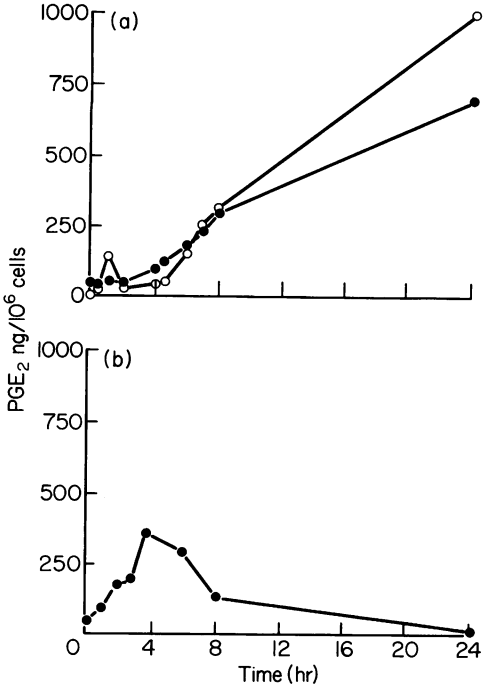

Figure 3. PGE_2 generation by human synoviocytes. (a) PGE_2 was measured at various time-points in supernatants (○) and cell extracts (●) of synoviocytes subjected to non-lethal reactive lysis at 37° over a 24-hr period. (b) PGE_2 was measured at intervals in cell extracts (●) of optimally antibody-sensitized synoviocytes incubated with a non-lethal dose of NHS at 37° over a 24-hr time-course. The results show the amount of PGE_2, expressed in terms of ng/10^6 cells detectable in supernatants and extracts at each time-point. Each point is the mean of four measurements from four separate cell populations.

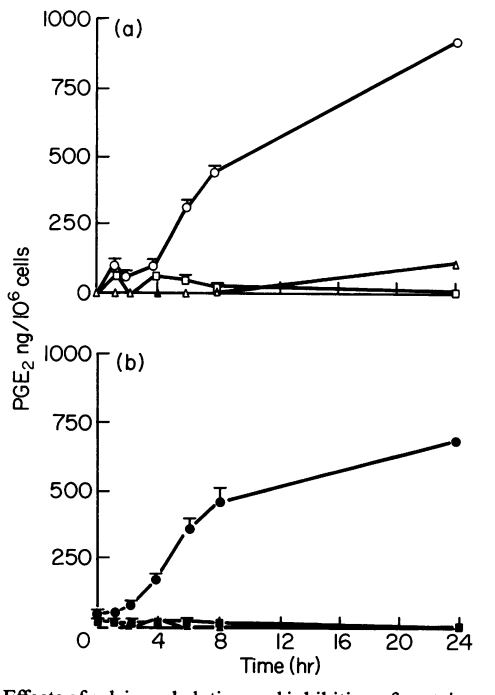

Figure 4. Effects of calcium chelation and inhibition of protein synthesis. Synoviocytes were subjected to non-lethal 'reactive lysis' at 37° and supernatants and cell extracts assayed for PGE_2 as described in Fig. 3. (a) PGE_2 release into the supernatant. (b) PGE_2 in the cell extracts. The results compare the effects of addition of EDTA (△, ▲) or actinomycin D (□, ■) at the initiation of non-lethal attack with cells attacked in the absence of these substances (○, ●). Each point is the mean of four different measurements from four separate cell populations.

Inhibition of protein synthesis using actinomycin D (5 μg/ml) also profoundly affected PGE_2 production and release. The smaller, early phase of release was little affected by the presence of actinomycin D, but the larger, late phase of release was completely abolished (Fig. 4a). In the cell extract PGE_2 was not detectable above background levels at any time in the presence of actinomycin D (Fig. 4b).

LTB_4 production

LTB_4 was detectable 30 min after initiating non-lethal attack using reactive lysis and was present only in the fluid phase. The concentration reached a maximum of 1·8 ng/10^6 cells at 30 min (background levels less than 0·1 ng/ml) and then rapidly returned to the basal level. No release of LTB_4 was found at later points in the time-course and there was no detectable LTB_4 in the cell extract at any time.

TNF production

Over the 24-hr time-course there was no detectable release of TNF from synoviocytes attacked non-lethally using either serum or purified components as a source of complement. Synoviocytes incubated with *E. coli* endotoxin (1 μg/ml) released about 0·5 ng/ml TNF over the time-course of the experiment. The detection limit of the assay was 0·2 ng/ml.

DISCUSSION

The experiments presented here demonstrate that cultured human rheumatoid synovial cells are relatively resistant to lysis by homologous complement, and that non-lethal complement attack *in vitro* causes the release of active metabolites which may be of importance in the pathogenesis of rheumatoid synovitis.

The release of reactive oxygen metabolites (ROM) from cultured synoviocytes *in situ* was initiated within 3 min of addition of the complement source and was a relatively long-lasting response. In our previous studies of ROM release from synoviocytes in suspension, the response to non-lethal complement attack was more rapidly initiated and of shorter duration, perhaps reflecting the more abnormal state of the cells after trypsinization and removal from surface attachments (Morgan *et al.*, 1988). In both cases the responses were shown to be dependent on the formation of the complete C5b–9 complex.

In attempts to demonstrate complement-induced PGE_2 production by synoviocytes *in vitro*, we initially used the same strategy adopted for the ROM studies, that is, attacking antibody-sensitized cells with non-lethal serum dilutions. Although increased PGE_2 production in the cell extracts was observed, we could detect no release into the cell supernatant. However, using purified complement components in a non-lethal 'reactive lysis' system the release of large amounts of PGE_2 into the fluid phase was evident. Serum at the concen-

trations used in these experiments did not affect the assay, eliminating the possibility that the inability to detect release in the initial experiments was due to the presence in serum of factors which interfered with measurement of immunoreactive PGE_2. The kinetics of intracellular PGE_2 production in response to serum also differed markedly from the response to reactive lysis, the response being much more rapid and transient in the former case. The reasons for these differences are as yet unclear, but it is likely that other serum factors, including other active complement products such as C3a and C5a, by directly influencing prostaglandin metabolism, modulate or mask the effects of the MAC. By using the reactive lysis system for subsequent experiments these complicating factors were eliminated. Release of PGE_2 using non-lethal 'reactive lysis' occurred in a biphasic manner.The first phase of release was detectable within 30 min, peaked at 1 hr and then fell back to the basal level. The fall in PGE_2 levels after this early peak strongly implies that released PGE_2 is metabolized in the fluid phase. Attempts to identify the products of metabolism are at present underway. The second, larger phase of release began about 4 hr after initiation of attack and continued over the 24-hr time-course of the experiments, reaching levels of 1000 ng/10^6 cells. PGE_2 release from unattacked cells at this time-point was about 100 ng/10^6 cells, a level which is in agreement with the 'constitutive' secretion of PGE_2 from synoviocytes described by others (Dayer *et al.*, 1976). The probability that breakdown of PGE_2 occurs throughout the time-course makes calculations of the rate of release impossible, but the net effect of such metabolism would be to reduce the amounts detectable at each time-point, making the levels attained all the more remarkable. The biphasic kinetics of PGE_2 release from synoviocytes are difficult to explain mechanistically. The first phase is likely to be due to direct stimulation (via increased intracellular calcium concentration) of pre-existing cyclo-oxygenase enzymes by the MAC. The second phase of PGE_2 generation, which occurs hours after initiating attack, at a time when no MAC will remain on the cell, may result from *de novo* synthesis of cyclo-oxygenase pathway enzymes. To test this hypothesis we attacked synoviocytes in the presence of the mRNA synthesis inhibitor actinomycin D. The early phase of release persisted but the second, larger phase was abolished. These results are in accordance with the hypothesis. Non-lethal amounts of the MAC therefore not only stimulate existing enzymes but also directly or indirectly promote enzyme synthesis. this is to the best of our knowledge the first demonstration of a direct effect of the MAC on protein synthesis.

Synoviocytes also released LTB_4 in response to non-lethal complement attack. LTB_4 release in response to non-lethal complement attack has previously been reported in renal mesangial cells (Lovett *et al.*, 1987) and in oligodendrocytes (Shirazi, Imagawa & Shin, 1987). Release of LTB_4 from synoviocytes was monophasic, resembling the first phase of PGE_2 release. The response was again transient, implying metabolism in the fluid phase. The enzymes responsible for leukotriene synthesis, unlike those responsible for prostaglandin synthesis, are present in relatively few cell types, principally neutrophils, eosinophils, monocytes, macrophages and mast cells (Salmon & Higgs, 1987). The demonstration of LTB_4 production by rheumatoid synoviocytes is further evidence in favour of the suggestion that a proportion of these cells are macrophage-derived (Burmester *et al.*, 1983).

Complement-mediated release of PGE_2 from synoviocytes was little affected by removal of C9, whereas ROM release was completely abolished. In the absence of C8, PGE_2 release was also abolished. Dependence on formation of the C5b–8 complex rather than the complete MAC has previously been reported for complement-mediated release of eicosanoids from human neutrophils (Seeger *et al.*, 1986), though some cell types appear to require C9 (Imagawa *et al.*, 1987). The dissociation of ROM production from eicosanoid production on the basis of requirement for C9 implies that the intracellular signals responsible for initiating these responses are different. Both PGE_2 and ROM production require calcium and are probably mediated by increased intracellular free calcium concentration (Morgan, 1989). It is therefore likely that the intracellular signals differ quantitatively rather than qualitatively, the limited degree of membrane perturbation and ion flux caused by C5b–8 insertion causing a rise in intracellular calcium which, though sufficient to initiate PGE_2 production, is insufficient to cause production of ROM (Ramm, Whitlow & Mayer 1982; Morgan, 1984).

Several cytokines, including IL-1 (Gowen *et al.*, 1984; Wood *et al.*, 1985; Duff *et al.*, 1985; Hopkins *et al.*, 1988), IL-6 (Waage *et al.*, 1989) and TNF (Neale *et al.*, 1989; Gitter *et al.*, 1989; Ziff, Cavender & Haskard, 1988), have been demonstrated in rheumatoid synovial fluid and implicated in joint destruction. The factors initiating cytokine production are not known. The MAC has been shown to stimulate release of cytokines from renal cells (Lovett *et al.*, 1987) and is therefore a candidate. Using a bioassay capable of detecting TNF at a concentration of 0·2 ng/ml (4 ng/10^6 cells in our assay system) no TNF release was detectable from synovial cells during non-lethal complement attack. Whether release occurs at levels below this remains to be investigated. Synoviocytes were stimulated to release TNF by endotoxin, demonstrating the capacity of this cell type to synthesize this mediator. Activation of release of IL-1 and IL-6 is currently under investigation.

The evidence presented in this study demonstrates that synoviocytes in culture are induced to produce several highly active substances when subjected to non-lethal complement attack. These substances may play an important role in the damage caused at inflammatory sites and may be involved in the pathogenesis of rheumatoid synovitis.

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