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Kinetics of the generation and action of chemical mediators in zymosan-induced inflammation of the rabbit peritoneal cavity

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1 Acute inflammation was induced by intraperitoneal injection of zymosan (yeast cell walls) in the rabbit.

2 Peritoneal inflammation was monitored by the local accumulation of intravenously-injected Evans blue dye (which binds to plasma albumin) and of polymorphonuclear leukocytes (PMNLs).

3 The zymosan-induced exudate fluid contained a microvascular permeability-increasing factor or factors which, unlike histamine and bradykinin, had a long duration of action when tested in rabbit skin and was dependent on circulating PMNLs.

4 Using radioimmunossay, high levels of rabbit C5a, or C5a des Arg, were detected in the exudate fluid and accounted for much of the permeability-increasing activity, as judged by skin bioassay after separation on Sephadex G-100.

5 The vasodilator prostaglandin, prostaglandin I_2 (PGI_2), was generated in the inflammatory reaction, as judged by the presence of high levels of 6-oxo-$PGF_{1\alpha}$ detected in the exudate by radioimmunossay. However, in contrast to observations in rabbit skin, inhibition of prostaglandin generation had a relatively small effect on peritoneal oedema formation.

6 C5a and C5a des Arg increase microvascular permeability by a PMNL-dependent mechanism in the rabbit. However, in response to zymosan, protein leakage was detected considerably earlier than PMNL accumulation. A hypothesis to account for this difference is proposed.

Introduction

Recent studies have established a link between three major features of acute inflammation: vasodilatation, increased microvascular permeability and leukocyte accumulation (Williams & Jose, 1981; Wedmore & Williams, 1981a). In these studies it was concluded that oedema formation in response to intradermal injection of zymosan (yeast cell walls) in the rabbit results from the concomitant extravascular generation of two chemical mediators. These two mediators, the complement-derived peptide C5a and a vasodilator prostaglandin, act synergistically to induce oedema. C5a and its des Arg metabolite increase the permeability of venules to plasma proteins and the prostaglandin increases the intravenular hydrostatic pressure by dilating arterioles (Williams & Peck, 1977; Williams & Jose, 1981). Further, increased venular permeability induced by C5a (unlike that induced by histamine and bradykinin) was found to be entirely dependent on circulating polymorphonuclear leukocytes (PMNLs): the suggestion being that extravascular C5a causes a rapid interaction between PMNLs and venular endothelial cells which, by an unknown mechanism, induces protein leakage (Wedmore & Williams, 1981a; Jose *et al.*, 1981a).

These conclusions are derived from experiments in the skin, a site which permits precise measurements of oedema formation using the local accumulation of intravenous radiolabelled albumin in response to multiple intradermal injections (e.g. up to 90 per animal). However, collection of substantial quantities of exudate fluid from skin is difficult. We have therefore utilized the peritoneal cavity in an attempt to obtain direct evidence of mediator generation and action. The peritoneal cavity offers the advantage that

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serial exudate samples can be collected in quantities suitable for analysis of mediators, protein extravasation and leukocyte accumulation. This paper describes the identification of C5a in zymosan-induced peritoneal exudate and the kinetics of C5a generation (measured by skin bioassay and radioimmunoassay), prostaglandin generation (by radioimmunoassay), PMNL accumulation and plasma protein leakage. Because of our interest in the role of endogenous vasodilator prostaglandins in oedema formation in a region of high basal blood flow, as compared to the low basal blood flow in dorsal skin, we also investigated the effects of indomethacin on the peritoneal inflammation.

A brief account of some early experiments on zymosan-induced peritoneal inflammation has been presented to the British Pharmacological Society (Forrest *et al.*, 1981).

Methods

Male New Zealand White rabbits (Buxted Farms, Sussex) weighing between 3.5 and 4.0 kg were used in all experiments.

Generation of inflammatory exudates in the peritoneal cavity

Rabbits were anaesthetized by an intravenous injection of Saffan: 0.5 ml kg^{-1} initially followed by maintenance doses as required. Evans blue dye (2.5% w/v) was injected intravenously (0.5 ml kg^{-1}) to monitor plasma protein extravasation into the peritoneal cavity. A sterile 14 gauge concentric polythene cannula and steel needle (Arterioveine, 11720: Vygon, 95440 Ecouen, France) were inserted into the peritoneum. The needle was withdrawn leaving the cannula in position, through which either 50 ml of a zymosan (obtained from boiled yeast cells) suspension (10 mg ml^{-1}) in sterile saline or saline alone were injected. An even distribution of zymosan in the peritoneum was achieved by using a large injection volume (50 ml) and by briefly gently massaging the peritoneum following the injection. A 3 ml sample of exudate was withdrawn immediately into sodium EDTA pH 7.2 and indomethacin (10 mM and 5.6 μM final concentrations, respectively) on ice. A 100 μl aliquot was removed for cell analysis and the remainder was immediately centrifuged (7800 *g* for 1 min) to ensure the rapid removal of cells and particulate matter. The supernatant was stored at −20°C. The cannula was left in position for further exudate collections. When 12 h time courses were being studied the cannula was removed after 6 h and re-inserted at 12 h. When time courses were not required, the cannula was removed immediately after the intraperitoneal injection and the total available exudate was recovered after 2 h.

Measurement of the inflammatory reaction in the peritoneal cavity

Plasma protein extravasation into the peritoneal cavity was monitored as the accumulation of intravenously-injected Evans blue dye. This was determined spectrophotometrically at 620 nm (A_{620}). In some experiments, the total extravasated albumin (mg) at 2 h was estimated from: (exudate dye concentration/plasma dye concentration) × total volume of exudate recovered (ml) × 40 mg ml^{-1} plasma albumin concentration.

The numbers of PMNLs were determined by diluting the exudate in gentian violet (1 ml 1% crystal violet; 1.5 ml glacial acetic acid; 97.5 ml distilled water) and counting the PMNLs in an improved Neubauer chamber microscopically.

Measurement of inflammatory mediators in peritoneal exudate fluid by use of rabbit skin bioassays

Peritoneal exudate fluid was injected (100 μl per site) into the shaved dorsal skin of rabbits and oedema formation was measured as the 30 min local accumulation of intravenously-injected ^{125}I-albumin as described previously (Williams, 1979). In some experiments, the duration of action of oedema-forming activity was measured by injecting samples at different intervals before the intravenous injection of ^{125}I-albumin. Prostaglandin E_2 (PGE_2, 3×10^{-10} mol per 100 μl) was then injected locally into the same skin sites to facilitate the measurements of oedema formation during the next 30 min (see text and Williams & Jose, 1981). On other occasions, rabbits were depleted of circulating PMNLs (<1% of normal) by an intravenous injection of nitrogen mustard (1.75 mg kg^{-1}) 4 days before the intradermal injections (Wedmore & Williams, 1981a).

Blood flow changes induced by injections of peritoneal exudate fluid into rabbit skin were measured as changes in the clearance of locally-injected ^{133}Xe as described previously (Williams, 1979).

Biochemical and immunological characterization of exudate and plasma samples

For gel permeation, exudate samples were concentrated 5 fold by lyophilisation and reconstitution in distilled water. Aliquots (5 ml) were applied to a column (2.5 × 40 cm) of Sephadex G-100 in 0.9% w/v NaCl solution (saline) at 2°C and eluted at a constant flow rate of 0.5 ml min^{-1}. Fractions of 3.5 ml were collected and analysed in the rabbit skin bioassay.

For ion exchange chromatography, exudate and plasma samples were adjusted to pH 6.0 using 0.1 M acetic acid, cooled to 2°C and dialysed against a buffer consisting of 5 volumes saline plus 1 volume 0.1 M sodium acetate pH 6.0. Samples were applied to columns (1.5 × 3.0 cm) of carboxymethyl (CM)-Sephadex C-25 equilibrated in dialysis buffer. The columns were then washed with 5 column volumes of dialysis buffer. The retained proteins were eluted with 5 column volumes of 1.0 M ammonium formate (pH 7), dialysed against 20 mM acetic acid, lyophilised and redissolved in 10 mM phosphate-buffered saline pH 7.2 (PBS). These samples were analysed in the rabbit skin bioassay (after removal of excess salts by passage through columns of Sephadex G-25M in PBS) and in immunodiffusion plates.

Ouchterlony double radial immunodiffusion tests were performed in 1% agarose in 20 mM PBS, pH 7.2, containing 10 mM EDTA and 2% polyethylene glycol 6000. Wells of 5 mm diameter were cut 5 mm apart, filled with 20 µl volumes of antiserum or test samples and incubated in a humid atmosphere at 2°C for 24 h. Rabbit C5a and the guinea-pig anti-rabbit C5a antiserum were prepared as described previously (Jose *et al.*, 1983).

Radioimmunoassay for C5a

Immunoreactive C5a (irC5a) was measured as described previously (Jose *et al.*, 1983). Briefly, samples were mixed with an equal volume of 22% polyethylene glycol 6000 and centrifuged to precipitate high molecular weight proteins including C5 and IgG. The C5a remaining in the supernatant (recovery: 85% from plasma and >95% from peritoneal exudates) was mixed with ^{125}I-rabbit C5a and guinea-pig anti-rabbit C5a in a competitive binding assay. Antibody-bound ligand was precipitated by use of protein A bacterial adsorbent and counted in a γ-counter.

Radioimmunoassay for prostaglandins

Prostaglandins were extracted from 1 ml aliquots of exudate fluid as described by Higgs & Salmon (1979) and measured by radioimmunoassay as described previously (Jose *et al.*, 1976). The cross-reaction data for the anti-6-oxo-$PGF_{1\alpha}$ and anti-PGE_2 antisera have been described by Hensby *et al.* (1981) and Jose *et al.* (1981b) respectively.

Drugs and chemicals

The following were used: Saffan (alphaxolone, 9 mg ml^{-1}; alphadolone, 3 mg ml^{-1}) from Glaxovet; Evans blue dye from Searle; mepyramine maleate from May and Baker; baker's yeast cells type II, PGE_1, PGE_2 and protein molecular weight standards from Sigma; histamine acid phosphate from British Drug Houses; nitrogen mustard (mustine hydrochloride) from Boots; Trasylol (aprotonin) from Bayer; ^{125}I-human serum albumin, ^{133}Xe in saline, [^{3}H]-PGE_2 and [^{3}H]-$PGF_{2\alpha}$ from Amersham International; [^{3}H]-6-oxo-$PGF_{1\alpha}$ from New England Nuclear; Sephadex products from Pharmacia; and protein A bacterial adsorbent from Miles. The following were generous gifts: indomethacin from Merck, Sharp and Dohme; mammalian tissue kallikrein from Bayer; $PGF_{2\alpha}$ and 6-oxo-$PGF_{1\alpha}$ from Dr J. Pike, Upjohn; and sheep antiserum to 6-oxo-$PGF_{1\alpha}$ from Dr L. Myatt.

Statistics

Data are given as the mean ± s.e.mean of n observations. Significance of the differences between populations was determined by use of Student's t test.

Results

Plasma protein exudation induced by an intraperitoneal injection of zymosan

Figure 1 shows the accumulation of albumin-bound Evans blue dye in the peritoneal cavity following an intravenous injection of the dye and an intraperitoneal injection of zymosan. The rate of protein leakage was

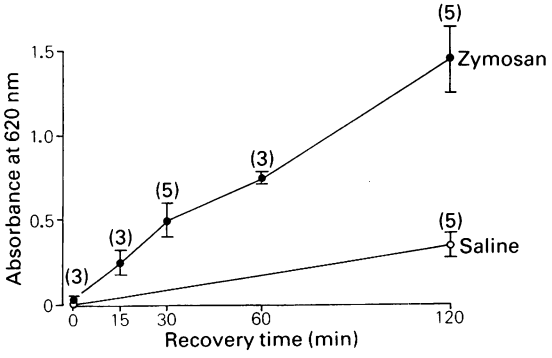

Figure 1 Intraperitoneal zymosan increases plasma protein extravasation into the peritoneal cavity. Rabbits were injected intravenously with Evans blue dye followed by an intraperitoneal injection (50 ml) of either a zymosan suspension in saline (10 mg ml^{-1}) (●) or saline (○). At timed intervals, exudate was withdrawn from the peritoneal cavity via an indwelling cannula into tubes containing EDTA. Particulate matter was removed by centrifugation and the Evans blue dye content of the exudate fluid determined spectrophotometrically by absorbance at 620 nm. Each point represents the mean result of fluids obtained from 3–5 rabbits and vertical lines show s.e.mean.

clearly substantially greater than in control rabbits injected with intraperitoneal saline. In a separate series of experiments (shown in Figure 5), the total volume of fluid recoverable after 2 h was 39.3 ± 2.4 ml after injection of zymosan ($n = 10$) and 20.8 ± 1.6 ml after injection of saline ($n = 3$) ($P < 0.01$). The total extravasated albumin in these samples was 266 ± 33 mg in the zymosan-induced exudates ($n = 10$) and 25 ± 5 mg in the saline-injected controls ($n = 3$) ($P < 0.01$). These results show that intraperitoneal injection of zymosan induces significant oedema formation.

Characterization of permeability-increasing activity in zymosan-induced peritoneal exudate fluid using the rabbit skin test system

In order to investigate the endogenous mediators responsible for protein extravasation in the cavity, peritoneal exudate fluids (centrifuged to remove zymosan and cells) obtained 2 h after administration of zymosan or saline were injected into rabbit dorsal skin and oedema formation measured using the accumulation of intravenously-injected ^{125}I-albumin over a 30 min period. Figure 2 shows the results of these experiments. Exudate fluids alone produced little oedema formation in skin. However, addition of PGE_2 to zymosan-induced exudate fluids resulted in large oedema responses in the skin, whereas saline-induced exudates + PGE_2 and PGE_2 alone caused only small responses. Thus, zymosan-induced exudate fluid contains a permeability-increasing substance(s) which induces oedema formation in rabbit skin when tested in the presence of a vasodilator prostaglandin (see Williams & Peck, 1977; Williams & Jose, 1981).

Histamine induced small oedema responses in skin and again these were greatly enhanced by the addition of PGE_2 (Figure 2). However, the permeability-increasing activity in zymosan-induced exudate fluid was clearly distinguishable from histamine by three

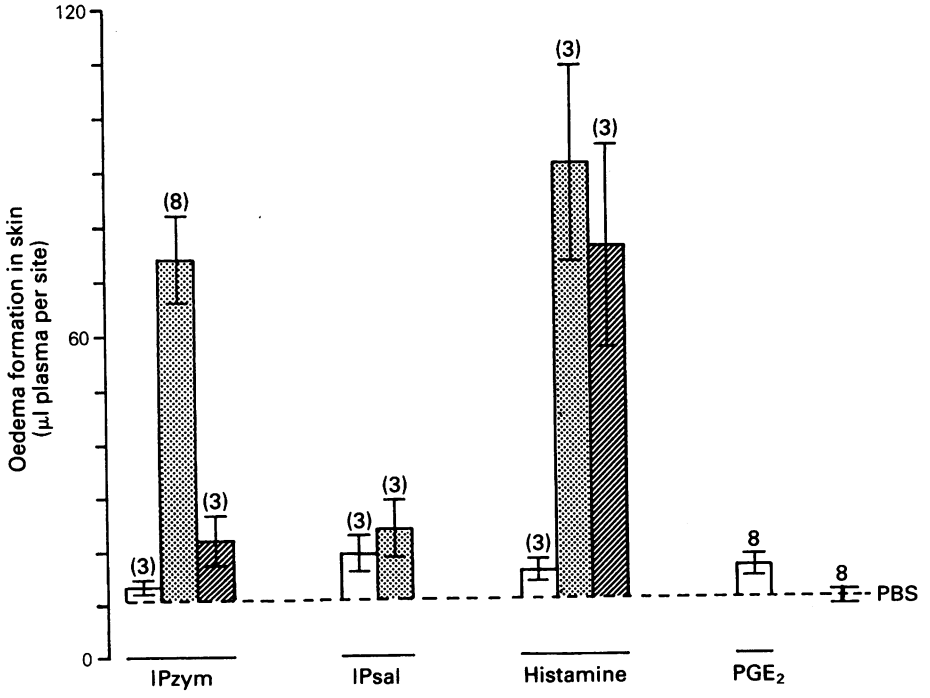

Figure 2 Comparison of the permeability-increasing characteristics of zymosan-induced peritoneal exudate fluid and histamine in normal and PMNL-depleted rabbits. Peritoneal exudate fluids from donor rabbits were collected 2 h after the intraperitoneal injection (50 ml) of either a zymosan suspension in saline (10 mg ml^{-1}) (IPzym, $n = 8$) or saline (IPsal, $n = 3$). Skin responses to peritoneal exudate fluids were tested in recipient rabbits (see Methods) and compared to those induced by intradermal histamine (2×10^{-8} mol per 100 μl). The oedema formation induced by samples injected alone (open columns) into normal rabbits; mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} mol per 100 μl) into normal rabbits (stippled columns); or mixed with PGE_2 into rabbits depleted of circulating PMNLs (hatched columns) is shown. The dashed horizontal line represents the plasma volume of skin sites injected with phosphate-buffered saline pH 7.2 (PBS). Each test mixture was injected into six replicate sites to give an average response per rabbit and the results are expressed as the mean ± s.e.mean of values obtained in the skin of 3–8 rabbits.

different criteria. (a) Responses to zymosan-induced exudate + PGE_2 were virtually abolished by depletion of circulating PMNLs; whereas, responses to histamine + PGE_2 were unaffected (Figure 2). (b) Addition of a histamine H_1-receptor antagonist (mepyramine, 10^{-8} mol per 100 μl) to zymosan-induced exudate + PGE_2 resulted in only a 15.0 ± 4.2% ($n = 4$ rabbits) reduction in oedema formation; whereas, the same dose of mepyramine produced a 93.5 ± 1.1% ($n = 4$) reduction in the oedema response to histamine + PGE_2 (doses as in legend to Figure 2). (c) The permeability-increasing activity of zymosan-induced exudate has a long duration of action when injected intradermally (see Methods), declining with a $t_{\frac{1}{2}} = 66.3 \pm 10.3$ min ($n = 6$ rabbits) which is not significantly different from that of rabbit purified C5a des Arg (5×10^{-11} mol per 100 μl; $t_{\frac{1}{2}} = 95.4 \pm 19.0$ min, $n = 6$). In contrast, responses to histamine (2×10^{-8} mol per 100 μl) declined with a $t_{\frac{1}{2}} = 8.3 \pm 1.5$ min ($n = 6$).

Kallikrein does not contribute significantly to the permeability-increasing activity in zymosan-induced exudate as shown by use of an inhibitor of this kinin-forming enzyme. Trasylol (1 μg = 7 kallikrein inhibitory units per 100 μl) reduced oedema formation induced by kallikrein (150 ng = 1.5 μ per 100 μl) + PGE_2 (3×10^{-10} mol per 100 μl) by 86.4 ± 3.6% ($n = 4$) but had no significant effect on responses to zymosan-induced exudate fluids + PGE_2, i.e. responses were increased by 10.7 ± 7.6% ($n = 4$). The permeability-increasing activity is not due to pre-formed kinins as indicated by molecular weight determinations (see below) and dependence on circulating PMNLs.

All these results demonstrate that the permeability-increasing activity in zymosan-induced peritoneal exudate fluids cannot be explained by the presence of histamine, histamine-releasers, kinins or kinin-forming enyzmes. The activity of the mediator or mediators is dependent on the presence of circulating neutrophils and has a long duration following intradermal injection.

Biochemical characterization of the permeability-increasing activity in zymosan-induced peritoneal exudate fluid

The molecular weight of the permeability-increasing activity in zymosan-induced exudate was determined using gel permeation chromatography. Exudate fluids collected 2 h after intraperitoneal injection were concentrated 5 fold by lyophilisation and reconstitution in distilled water. Samples were then applied to columns of Sephadex G-100. Effluent fractions were mixed with vasodilator prostaglandin (PGE_1 in this series of experiments) and assayed for oedema forming activity in rabbit skin. The activity in zymosan-induced exudate appeared as a single peak in the region corresponding to a molecular weight of approximately 18,000 (Figure 3). In three chromatographic separations, a mean value of 18,800 ± 800 daltons was obtained. No such activity

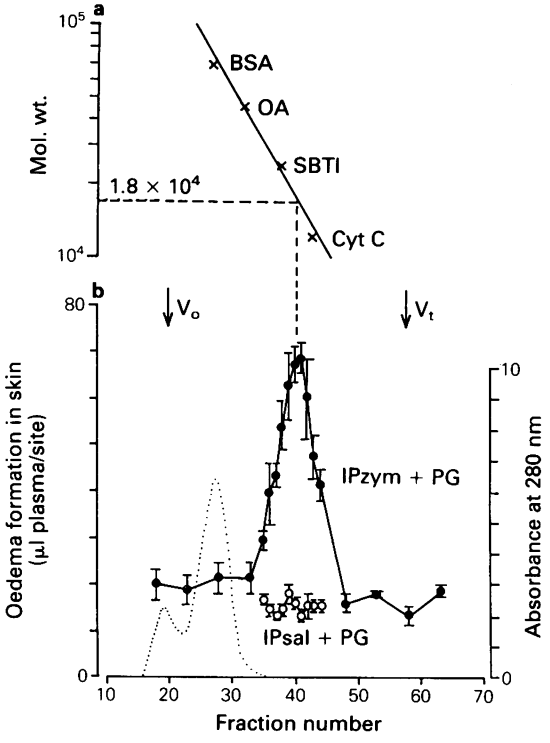

Figure 3 (a) The molecular weight of the permeability-increasing activity in zymosan-induced peritoneal exudate fluid collected 2 h following the intraperitoneal injection (50 ml) of either a suspension of zymosan in saline (10 mg ml^{-1}) (IPzym, ●) or saline (IPsal, ○) as determined by gel filtration in sephadex G-100 (see Methods). (b) The protein content of eluate fractions obtained from zymosan-induced exudate was determined by absorbance at 280 nm (stippled line). The permeability-increasing activity of fractions was determined by their ability to promote oedema formation in rabbit skin when mixed with prostaglandin E_1 (PG, 3×10^{-10} mol per 100 μl) as described previously. The results shown for permeability-increasing activity are compiled from three experiments.

(a) Shows a plot of the molecular weights of protein standards (bovine serum albumin, BSA, mol. wt. = 67,000; ovalbumin, OA, mol. wt. = 45,000; soya bean trypsin inhibitor, SBTI, mol. wt. = 21,500; cytochrome C, Cyt C, mol. wt. = 12,400) against their respective elution fractions. The void volume (V_o) was determined using thyroglobulin (mol. wt. = 670,000) and the total volume (V_t) using ammonium formate (mol. wt. = 63).

was observed in fractions obtained from saline-induced exudates. No activity was detected in the region of Vt where the low molecular weight permeability-increasing substances, such as histamine, bradykinin and leukotriene B_4, would appear. The permeability-increasing activity in zymosan-induced exudate was retained by the cation exchanger CM-Sephadex at pH 6.0, with the exception of a very small amount of unidentified activity (results not shown).

On the basis of these results, the major permeability-increasing activity in zymosan-induced exudate fluid is indistinguishable from rabbit C5a and C5a des Arg (Williams & Jose, 1981).

Identification of C5a in zymosan-induced peritoneal exudate using an anti-rabbit C5a antiserum

Figure 4 shows the result of an Ouchterlony double radial immunodiffusion test with guinea-pig anti-rabbit C5a antiserum in the centre well and test samples, concentrated using CM-Sephadex, in the surrounding wells. Exudate fluid obtained 2 h after intraperitoneal injection of zymosan gave a single precipitin band which showed co-identity with those produced by zymosan-activated plasma and purified C5a. Control plasma samples (incubated with saline) produced a weak band, possibly the result of low level complement activation by the heparin anticoagulant (Rent *et al.*, 1975). No precipitin band was seen with fluid obtained 2 h after intraperitoneal injection of saline.

The effect of inhibition of prostaglandin synthesis on zymosan-induced plasma protein exudation in the peritoneal cavity

The vasodilator prostaglandin, PGI_2, was generated in response to intraperitoneal zymosan as shown by the presence of high levels of its inactive breakdown product, 6-oxo-$PGF_{1\alpha}$ (Figure 5). Zymosan-induced exudate fluids contained only low levels (2–3%) immunoreactive PGE_2 (2.7 ± 0.7 ng ml^{-1}, $n = 4$) and $PGF_{2\alpha}$ (4.6 ± 1.6 ng ml^{-1}, $n = 5$) and these were not significantly different from those found after injection of saline (1.6 ± 0.8 ng PGE_2 ml^{-1}, $n = 4$; 2.2 ± 1.4 ng $PGF_{2\alpha}$ ml^{-1}, $n = 5$).

These observations might explain why zymosan-

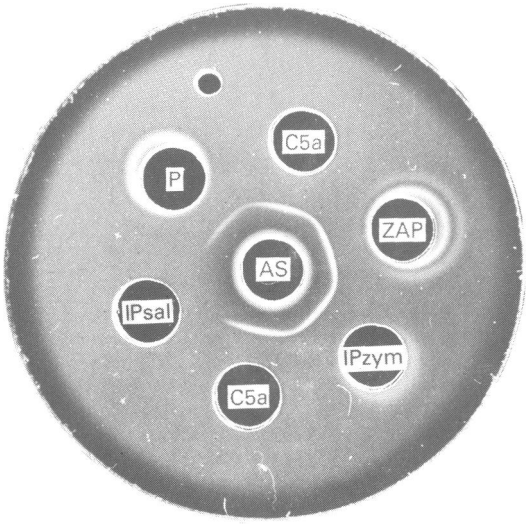

Figure 4 Immunological identity of C5a in zymosan-induced peritoneal exudate fluid using Ouchterlony double radial immunodiffusion. Guinea-pig anti-rabbit C5a antiserum (AS: 20 μl) was placed in the central well and 20 μl of each of the test solutions were placed in the outer wells. These solutions were: rabbit C5a des Arg (C5a, 0.8 μg); heparinized rabbit plasma incubated for 30 min at 37°C with saline (P) or zymosan (10 mg ml^{-1}) (ZAP); peritoneal exudate fluid collected 2 h following the intraperitoneal injection (50 ml) of a zymosan suspension in saline (10 mg ml^{-1}) (IPzym) or saline (IPsal). IPzym was material pooled from 2 rabbits and IPsal was pooled from 4 rabbits. Plasma samples were concentrated 50 fold, and exudate samples 250 fold, by use of CM-Sephadex.

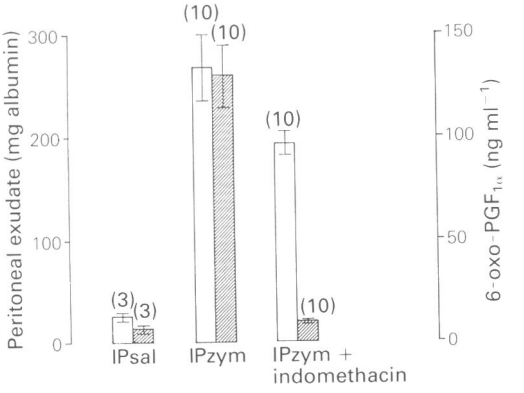

Figure 5 The effect of a cyclo-oxygenase inhibitor, indomethacin, on the concentration of 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) (hatched columns) and the protein extravasation (open columns) induced by intraperitoneal zymosan. Rabbits were injected intravenously with Evans blue dye followed 5 min later by an intraperitoneal injection (50 ml) of either a zymosan suspension in saline (10 mg ml^{-1}) (IPzym) or saline (IPsal). A third group of animals was given intravenous indomethacin (1.8 mg kg^{-1}) just before intraperitoneal zymosan and the same dose again 1 h later. After 2 h the total available exudate was collected into tubes containing EDTA and indomethacin for determination of total albumin and of prostaglandin concentration. Indomethacin treatment greatly reduced the generation of immunoreactive 6-oxo-$PGF_{1\alpha}$ in response to zymosan ($P < 0.001$) but had less effect (29%; $P < 0.05$) on albumin accumulation. Results are given as the mean ± s.e.mean for n (number in parentheses) rabbits.

induced peritoneal exudate fluids have no active vasodilator present as evidenced by the results in Figure 2 (i.e., the addition of exogenous prostaglandin is necessary for oedema formation in the rabbit skin test system). Further evidence is that zymosan-induced exudate did not increase skin blood flow as measured by use of the multiple site ^{133}Xenon clearance technique. Clearance from skin sites injected with exudate fluid obtained 2 h after intraperitoneal injection of zymosan was 5.0 ± 19.4% less than that from sites injected with saline ($n = 6$, a non-significant effect).

In rabbit skin, inhibition of prostaglandin synthesis by indomethacin suppresses zymosan-induced oedema by 70–90% (Williams & Jose, 1981). However, indomethacin had a much smaller, though significant, effect on oedema formation in the peritoneal cavity. Intravenous indomethacin (1.8 mg kg^{-1} given immediately before, and again 1 h after, the intraperitoneal injection of zymosan) virtually abolished the appearance of 6-oxo-PGF$_{1\alpha}$ in the exudate fluid ($P < 0.001$, Figure 5). Indomethacin reduced the total available exudate 2 h after intraperitoneal zymosan by 16% (from 39.3 ± 2.4 ml, $n = 10$, to 33.0 ± 1.4 ml, $n = 10$, $P < 0.05$) and the total recovery of extravasated albumin by 29% ($P < 0.05$, Figure 5). However, indomethacin had no significant effect on the generation of immunoreactive C5a (irC5a). Concentrations of irC5a were 583 ± 66 ng ml^{-1} without indomethacin ($n = 10$) and 597 ± 50 ng ml^{-1} in the indomethacin-treated animals ($n = 10$). When allowance is made for the difference in total exudate volumes, total irC5a was reduced by 17% (from 23.4 ± 3.4 μg without indomethacin to 19.5 ± 1.7 μg in the indomethacin-treated animals); this difference is not statistically significant.

These results show that inhibition of prostacyclin generation had no significant effect on C5a generation in the peritoneal cavity and, in contrast to experiments in the skin, had a relatively small effect on protein extravasation.

The time-course of the local response to intraperitoneal zymosan in terms of plasma protein leakage, neutrophil accumulation and the generation of chemical mediators

Figure 6 (a–e) shows the mean results of six experiments in which a suspension of zymosan was injected into the peritoneal cavity and 3 ml volumes of exudate were collected at the times indicated up to 12 h for subsequent analysis. Saline was injected into six animals to give control samples, but in this case insufficient volumes were available for collection after 4 h.

The maximal rate of extravasation of albumin-bound Evans blue dye in animals injected with zymosan occurred during the first hour; thereafter the concentration rose progressively but at a diminishing rate (Figure 6a). Little dye was apparent in control fluids obtained 1 h after injection of saline, but significant levels were present at 2 h and levels were approximately 50% of test levels at 4 h.

Figure 6b shows the appearance of PMNLs in exudates. Significant numbers of PMNLs were present in 2 and 4 h exudates from zymosan-injected animals (7.5 ± 2.0 × 10^5 PMNLs ml^{-1} and 5.5 ± 1.9 × 10^6 PMNLs ml^{-1}, respectively). Accurate estimates of the few PMNLs present before 2 h were difficult because of the preponderance of zymosan particles. The number of PMNLs accumulating in the zymosan-induced exudates increased slowly from 2 to 4 h but the rate of accumulation increased progressively, reaching a maximum later than 6 h. Some PMNLs were also detected in control exudates in 2/6 animals at 2 h (4 × 10^4 and 8.5 × 10^4 PMNLs ml^{-1}) and 3/6 animals at 4 h (1.1 × 10^6, 8.1 × 10^5 and 8 × 10^5 PMNLs ml^{-1}). The differences between test and control animals at 2 and 4 h were significant ($P < 0.01$ and $P < 0.05$, respectively).

Figure 6c shows the permeability-increasing activity in peritoneal exudate fluids as estimated by their ability to induce leakage of intravascular ^{125}I-albumin when mixed with PGE_2 and injected intradermally. Responses are plotted as the means of assays in six recipient animals. There was no significant difference between samples taken immediately after intraperitoneal injection of zymosan and saline. Permeability-increasing activity was present in zymosan-induced exudate fluid collected at 1 h. This activity remained high until 6 h and then fell to a low level at 12 h. A low level of activity was present in samples obtained after injection of saline. This activity was significantly different from that in sites injected with PGE_2 alone ($P < 0.05$) and remained constant up to 4 h.

Figure 6d shows the concentrations of immunoreactive C5a (irC5a) in exudate fluids. No irC5a was detected in any of the samples obtained after injection of saline (limit of detection 20 ng ml^{-1}). High levels of irC5a were detected in exudate fluids obtained 1, 2, 4 and 6 h after injection of zymosan. The peak value was 705 ± 133 ng irC5a ml^{-1} at 2 h and fell to 59.3 ± 8.1 ng ml^{-1} at 12 h. Intraperitoneal injection of zymosan had no detectable effect on the very low levels of irC5a found in circulating plasma (results not shown).

Figure 6e shows the concentrations of the PGI_2 metabolite, 6-oxo-PGF$_{1\alpha}$, in the peritoneal exudates. Low levels of 6-oxo-PGF$_{1\alpha}$ were found after injection of saline, possibly because of the presence of the cannula. Much higher levels of 6-oxo-PGF$_{1\alpha}$ were found 1 h after injection of zymosan. These rose to a maximum value of 161 ± 45 ng ml^{-1} at 2 h and fell to low values at 6 and 12 h.

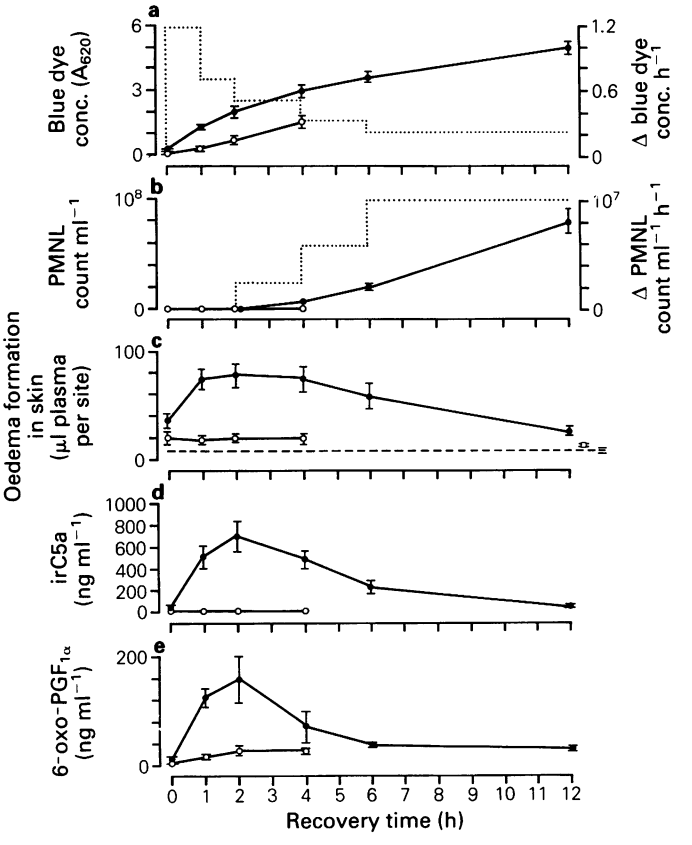

Figure 6 Kinetics of the inflammatory response and the generation of mediators induced by zymosan. Rabbits were injected intravenously with Evans blue dye followed by an intraperitoneal injection (50 ml) of either a zymosan suspension in saline (10 mg ml^{-1}) or saline. Fluid (IPzym, ●; or IPsal, ○) was withdrawn at timed intervals for analysis. No samples were recoverable in the saline-injected control animals after 4 h. (a) An estimate of the extravasated plasma protein content of exudate fluids was determined by measuring their absorbance at 620 nm. The rate (stippled line) of plasma protein extravasation induced by zymosan was obtained by calculating the difference in A_{620} values for successive samples and dividing by the time interval in hours. (b) The PMNL content of exudates was determined by light microscopy following dilution of (non-centrifuged) exudates in gentian violet stain. The rate (stippled line) of PMNL accumulation induced by zymosan was calculated as above and is plotted on a linear scale to be compared with the rate of plasma protein leakage in (a). Although not apparent in the figure there was a significant difference between PMNLs in zymosan- and saline-injected animals at 2 and 4 h (see Results). (c) The permeability-increasing activity of exudate fluids was determined by their ability to induce oedema formation when mixed with prostaglandin E_2 (PGE_2, 3×10^{-10} mol per 100 μl) and injected intradermally as described previously. The dashed horizontal line represents the skin plasma volume of sites injected with PBS alone. The skin plasma volume of sites injected with PGE_2 (3×10^{-10} mol) alone is also shown (□). Radioimmunoassay was used to measure (d) immunoreactive C5a (irC5a) and (e) 6-oxo-$PGF_{1\alpha}$ in exudate fluids. Each point represents the mean result of exudates obtained from 6 rabbits and vertical lines show s.e.mean.

Discussion

In our previous studies of the response to zymosan in rabbit skin (Williams, 1979; Williams & Jose, 1981) it was possible to measure the time-course of albumin extravasation accurately by giving multiple intradermal injections of zymosan at different intervals before an intravenous injection of ^{125}I-albumin and then measuring local isotope accumulation over a 30 min period. In the present study, using the peritoneal cavity, only one response could be monitored in each rabbit and radioisotopes were avoided because they

would interfere with the subsequent skin bioassay for permeability-increasing activity in the exudate samples and with radioimmunoassay. The time course of microvascular plasma protein leakage was monitored by measuring the concentration of albumin-bound Evans blue dye in exudate following an intravenous injection of dye and an intraperitoneal injection of zymosan. The concentration of dye at any time will be the resultant of several opposing factors. The major factors are dye-albumin extravasation, dilution by the intraperitoneal injection fluid, efflux and reabsorption of water by microvessels, lymphatic clearance of dye-albumin and systemic clearance of intravascular dye-albumin. The important point emerging from this part of the study is that protein extravasation, and by inference microvascular permeability, is high very early in the response to zymosan, i.e. at 15, 30, 60 and 120 min. This appears to be faster in onset than in the skin where little protein leakage occurs in the first 30 min. The presence of dye in samples from the saline-injected control animals was expected because of normal basal microvascular permeability to plasma proteins; however, superimposed upon this there may have been some low level inflammation caused by saline or by the indwelling cannula. Nevertheless, albumin extravasation two hours after injection of zymosan was more than 10 times that after injection of saline.

It was inferred from previous experiments in the skin that increased microvascular permeability induced locally by injection of zymosan results from the extravascular generation of C5a (Williams & Jose, 1981). The present study provides direct evidence for this inference. Zymosan-induced peritoneal exudate fluid was found to contain permeability-increasing activity which chromatographed as a single peak on Sephadex G-100, corresponding to the molecular weight previously determined for C5a in this system. Like C5a, the active substance in peritoneal exudate was basic as determined by use of the cation exchanger CM-Sephadex. Again like C5a, the substance had a long duration of action and a dependence on circulating neutrophils when tested in skin.

Identification of C5a in exudates was supported by Ouchterlony double radial immunodiffusion using a guinea-pig anti-rabbit C5a antiserum. Zymosan-induced exudate gave a single precipitin band showing co-identity with those obtained with rabbit purified C5a des Arg and zymosan-activated plasma (all test samples concentrated using CM-Sephadex). These observations were extended by radioimmunoassay for C5a. Immunoreactive C5a was detected in zymosan-induced exudates, with levels up to $705 \pm 133\ ng\ ml^{-1}$ at 2 h, whereas no detectable levels ($< 20\ ng\ ml^{-1}$) were present in control animals injected with saline. Because of the presence of plasma carboxypeptidase N in exudate fluids the C5a generated would be expected to be rapidly converted to the physiologically more stable C5a des Arg (Bokisch & Muller-Eberhard, 1970) which is active in increasing microvascular permeability (Jose *et al.*, 1981a).

No permeability-increasing activity was detected in Sephadex G-100 fractions corresponding to the low molecular weight mediators, histamine and bradykinin. Further, an antihistamine, mepyramine, only slightly suppressed responses to zymosan-induced exudate and an inhibitor of kinin formation had no effect. These results complement those obtained previously in the skin (Williams & Jose, 1981) and indicate that histamine and bradykinin do not have an important role in the response to zymosan. However, the presence of inactive metabolites in exudate has not been excluded, so that there remains a possible contribution of histamine and bradykinin to the zymosan-induced peritoneal response.

Another possible mediator of the response to zymosan, especially in the later phases at a time when C5a levels have decreased, is leukotriene B_4 (LTB_4) which is known to have chemoattractant and associated permeability-increasing activities (Ford-Hutchinson *et al.*, 1980; Goetzl & Pickett, 1980; Wedmore & Williams, 1981a). PMNLs release LTB_4 in response to opsonised zymosan (Palmer & Salmon, 1983) and LTB_4 has been found in inflammatory exudate fluids in the rat (Simmons *et al.*, 1983; Haworth & Carey, 1985) and in gouty effusions, but not in significant amounts in rheumatoid synovial fluid, in man (Rae *et al.*, 1982). LTB_4 may make some contribution to zymosan-induced plasma protein leakage in the peritoneal cavity and indeed we have detected low levels of immunoreactive LTB_4 in zymosan-induced exudate (peak concentrations of approx $6\ ng\ ml^{-1}$ at 6 h: Jose, Brain, Hellewell, Rampart & Williams, unpublished data). However, it is unlikely that LTB_4 contributes to the stable permeability-increasing activity in the zymosan-induced peritoneal exudates, as assayed in skin, because only low levels have been detected and LTB_4 is 50–100 times weaker than C5a des Arg, on a molar basis, as a permeability-increasing agent.

Platelet-activating factor (Paf) also increases microvascular permeability in the rabbit (Wedmore & Williams, 1981b; Humphrey *et al.*, 1982). Paf may contribute to zymosan-induced oedema in the peritoneal cavity. However, as Paf induces increased permeability by a PMN-independent mechanism (Wedmore & Williams, 1981b), the levels of active Paf in exudate samples, at least at 2 h, must be low because of the low activity in the skin of PMN-depleted rabbits (Figure 2). Paf is rapidly inactivated *in vivo* and lyso-Paf may be present in the exudates.

We were particularly interested in the contribution of prostaglandins to plasma protein leakage in the peritoneal cavity. In rabbit skin, zymosan-induced

oedema is effectively suppressed by inhibiting prostaglandin synthesis (Williams 1979; Williams & Jose, 1981) although prostaglandins alone are poor at inducing oedema (Williams & Morley, 1973; Williams & Peck, 1977). It was suggested from experiments in which blood flow and oedema formation were measured that endogenous prostaglandins enhance oedema because of their vasodilator activity (Williams & Peck, 1977). If this is the mechanism, then release of endogenous prostaglandins would be expected to be more important for plasma leakage in inflammatory reactions in regions of low basal blood flow (such as dorsal skin) than in regions of high basal blood flow (such as the peritoneal cavity). This is supported by the experiments described here. Indomethacin, at doses which virtually abolished prostaglandin formation, only partially suppressed zymosan-induced plasma protein leakage in the peritoneal cavity. Even in the absence of indomethacin, no vasodilator activity was detectable in zymosan-induced exudates. However, high levels of 6-oxo-$PGF_{1\alpha}$ were detected by radioimmunoassay indicating that the active vasodilator *in vivo* is the unstable PGI_2. This is consistent with observations showing that PGI_2 has vasodilator and oedema-potentiating activities in the rabbit similar to those of PGE_2 (Peck & Williams, 1978; Williams, 1979).

Our work in rabbit skin has led us to the conclusion that increased microvascular permeability induced by C5a and other chemoattractants results from the rapid accumulation of PMNLs (Wedmore & Williams, 1981a) although the precise mechanism underlying this is not clear. This can explain the inhibition of oedema in inflammatory responses in animals made neutropenic (Humphrey, 1955; Phelps & McCarty, 1966; Wedmore & Williams, 1981a; Issekutz, 1981). However, in the experiments described here the rate of plasma protein extravasation was maximal within the first hour after intraperitoneal zymosan, whereas the rate of PMNL accumulation in the exudate reached a maximum later than 6 h. Similar observations were made when rabbit purified C5a des Arg was injected intraperitoneally (Jose *et al.*, 1986). These results are in accord with observations in other model systems in which significant PMNL accumulation was observed only after the onset of other signs of inflammation (Hurley, 1964; Logan & Wilhelm, 1966; DeShazo *et al.*, 1972; Brune *et al.*, 1974). These kinetic differences have been used as evidence that plasma protein leakage and PMNL accumulation are mechanistically independent (Brune *et al.*, 1974). However, when the accumulation of radiolabelled PMNLs was monitored, the dissociation from increased vascular permeability was much less marked (Issekutz, 1981; Crawford *et al.*, 1982) and the maximal rate of PMNL accumulation after intradermal zymosan was found to be earlier, i.e. at 2–4 h (Colditz & Movat, 1984).

We propose the following explanation for these kinetic discrepancies. Increased microvascular permeability in response to C5a occurs very early (from 5–6 min; Wedmore & Williams, 1981a) when PMNLs are in close proximity to endothelial cells. Plasma proteins then pass relatively quickly into an inflammatory site, their progress limited principally by diffusion; whereas PMNLs take some time to traverse the perivascular basement membrane, connective tissue and, in the case of the peritoneum, the mesothelial layer before entering the cavity where their numbers are measured (see Forrest *et al.*, 1985). Thus, the extravascular histological detection of PMNLs will be slow in onset and their detection in cavity fluid even more so. However, measurements of tissue radioactivity following intravenous injection of labelled cells will include PMNLs at all stages of emigration and hence present an earlier appearance of these cells.

This investigation of the inflammatory response to intraperitoneal zymosan has provided direct pharmacological, biochemical and immunological evidence for the extravascular generationof C5a resulting in PMNL accumulation and PMNL-dependent oedema formation. C5-derived chemotactic activity has been detected in many different types of inflammatory reactions in many species (e.g. Snyderman *et al.*, 1971; Lo *et al.*, 1984; Ernst *et al.*, 1984) including synovial fluid from the rheumatoid joint (Ward & Zvaifler, 1971). Since decomplementation of rabbit and other species has been shown to inhibit many types of inflammatory reactions (Ward & Cochrane, 1965; Willoughby *et al.*, 1969; Cochrane *et al.*, 1970), we suggest that C5a generation can be an important factor controlling the ensuing inflammatory reaction. Mechanisms, similar to those described here, may operate in other types of reactions, triggered by other chemoattractants.

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