The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 1991

The sequential generation of neutrophil chemoattractant proteins in acute inflammation in the rabbit in vivo. Relationship between C5a and proteins with the characteristics of IL-8/neutrophil-activating protein 1. FREE

P D Collins; ... et. al

J Immunol (1991) 146 (2): 677–684.
https://doi.org/10.4049/jimmunol.146.2.677

0022-1767/91/1462-0677$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 146, 677-684, No. 2, January 15, 1991
Printed in U.S.A.

THE SEQUENTIAL GENERATION OF NEUTROPHIL CHEMOATTRACTANT PROTEINS IN ACUTE INFLAMMATION IN THE RABBIT IN VIVO

Relationship between C5a and Proteins with the Characteristics of IL-8/Neutrophil-Activating Protein 1[1]

PAUL D. COLLINS,[2] PETER J. JOSE, AND TIMOTHY J. WILLIAMS

From the Department of Applied Pharmacology, National Heart and Lung Institute, London SW3 6LY, UK

An in vivo experimental peritonitis model was investigated in the rabbit using zymosan as the inflammatory stimulus. After an i.p. injection of zymosan, exudate was removed at intervals and tested in the back skin of assay rabbits. Assay rabbits received i.v. injections of ^{125}I-albumin and ^{111}In-neutrophils, and the local accumulation of each label was measured in response to intradermal injections of exudate samples mixed with a potentiating dose of PGE_2. When peritoneal exudate samples were tested in the presence of a specific anti-C5a antibody, virtually all the edema-inducing and neutrophil chemoattractant activity was abolished in samples taken up to 2 h after the zymosan injection. Later samples, however, contained increasing levels of a non-C5a component. In C5a-depleted 6-h exudate two peaks of inflammatory activity were separated using cation exchange HPLC. Evidence is presented that C5a itself is unable to stimulate the production of these activities. Both peaks of activity appear related to IL-8/NAP-1 as they inhibited the binding of ^{125}I-IL-8/NAP-1 to human neutrophils.

Injection of *Bordetella pertussis* organisms or zymosan particles intradermally in the rabbit, to simulate a local microbial infection, induces an acute inflammatory reaction characterised by microvascular plasma protein leakage, increased blood flow, and neutrophil accumulation (1-4). Using ^{125}I-albumin to monitor plasma protein leakage, it was shown that the edema response can be suppressed by inhibiting PG synthesis (3). Further, the edema reaction can be mimicked by intradermal injection of plasma after exposure to the bacteria or zymosan, provided that the plasma is injected together with a vasodilator PG that potentiates edema (3, 5). Purification of zymosan-activated plasma showed that the active constituent is C5a (3) and that C5a increases microvascular permeability by a neutrophil-dependent mechanism (6). Thus, circumstantial evidence suggested that 1) the microbial stimuli activate complement in extravascular tissue fluid to generate C5a, which increases microvascular permeability by triggering a rapid neutrophil/endothelial cell interaction, and 2) that plasma protein leakage is enhanced by locally generated vasodilator PG (3).

Further experiments, in which zymosan was injected i.p., provided direct evidence that C5a (7, 8) and a PG (8) were present in the inflammatory exudate. It was noted, however, that a small amount of permeability-increasing activity was separable from C5a in zymosan-induced peritoneal exudate (8). We have discovered subsequently that this second activity appears some time after the first appearance of C5a and that the activity increases with time, reaching high levels by 6 h after zymosan injection. We describe the generation of this second activity, how it may be linked to C5a generation and its resolution into two clearly separable peaks of inflammatory activity. Evidence for a relationship between the two peaks of activity and IL-8/NAP-1[3] is presented.

MATERIALS AND METHODS

Male, specific pathogen-free, NZW rabbits weighing 2.5 to 4.0 kg were used in all experiments.

Generation of exudate fluid in rabbit peritoneal cavity. Rabbits were anesthetized with an i.v. injection of Sagatal (sodium pentobarbitone, 30 mg/kg) followed by maintenance doses as required. Evans blue dye (2.5% w/v in sterile saline, 0.5 ml/kg) was injected i.v. to monitor plasma protein extravasation into the peritoneal cavity. A 14-gauge polyethylene catheter was inserted into the cavity through which a suspension of zymosan (10 mg/ml) in 50 ml sterile pyrogen-free saline was injected. In some experiments zymosan was replaced by purified rabbit C5a des Arg (60 nM). Control animals received 50 ml of sterile pyrogen-free saline. All peritoneal injections routinely contained polymixin B sulfate (40 μM) to negate the effects of any endotoxin present. At predetermined times after the i.p. injection, 3 ml samples of exudate were removed via the peritoneal catheter into heparin (10 U/ml exudate). After immediate centrifugation (1950 × *g* for 10 min at 2°C) to remove cells and particulate matter the supernatant was placed on ice. A 100-μl aliquot was removed for measurement of Evans blue dye concentration. A 200-μl aliquot was removed into sodium EDTA (10 mM final concentration) to prevent in vitro complement activation and stored at −20°C for subsequent determination of C5a concentration by RIA. The remainder was tested in the rabbit skin bioassay for inflammatory mediators.

Measurement of plasma protein extravasation in peritoneal cavity. Plasma protein extravasation was assessed by measuring the i.p. accumulation of i.v. Evans blue dye (which binds to plasma

Received for publication August 20, 1990.
Accepted for publication October 26, 1990.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported by Roche Products Ltd, UK; the Wellcome Trust, UK; the National Asthma Campaign, UK; and the Medical Research Council, UK. Some of the work described in this paper was carried out whilst the authors were in the Section of Vascular Biology, MRC Clinical Research Centre, Harrow, Middlesex, HA1 3UJ, UK.
[2] Address correspondence and reprint requests to Dr. P. D. Collins, Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse St., London SW3 6LY, UK.

[3] Abbreviations used in this paper: MDNCF, human recombinant monocyte-derived neutrophil chemotactic factor; NAP-1, neutrophil activating protein 1; ZAP, zymosan-activated plasma; MGSA, melanoma growth stimulatory activity.

albumin) using spectrophotometric analysis at 620 nm. Results are expressed as the exudate: plasma blue dye ratio.

Preparation of C5a, anti-C5a, and immunoabsorbents. Rabbit C5a was purified, as the des Arg metabolite, from zymosan-activated serum by 1) batch-wise cation exchange, 2) ethanol precipitation of unwanted proteins, 3) gel permeation, and 4) cation exchange HPLC as described previously (3, 7, 9). This preparation of C5a was used to immunize a goat using CFA and, for booster injections, incomplete adjuvants. For use as a neutralising agent in the rabbit skin assay, the IgG fraction of this antibody was prepared by use of *n*-octanoic acid to precipitate unwanted proteins (10). This was followed by ammonium sulfate precipitation of the IgG and dialysis of the pellet, resuspended in a volume equal to that of the original serum, against 0.15 M sodium chloride. This antibody binds rabbit C5a and C5a des Arg.

In a second preparation of C5a des Arg, which was used for i.p. and intradermal injections, step 2 above was replaced by affinity chromatography. The affinity gel consisted of guinea-pig anti-rabbit C5a IgG (7) covalently attached to protein A Sepharose CL-4B by use of dimethylpimelimidate (11). Elution of C5a from this gel was achieved by use of 0.2 M glycine:HCl buffer pH 2.5.

In some experiments, ZAP was used as a source of C5a des Arg for intradermal injection (3). Rabbit heparinized plasma was incubated with zymosan (1 mg/ml, 37°C for 30 min), centrifuged to remove the zymosan, and chromatographed on columns of Sephadex G-25M in PBS to remove low m.w. substances.

Measurement of C5a concentration in peritoneal exudate samples. Immunoreactive C5a was measured by a modification of an RIA previously described (7, 12). Briefly, exudate samples were mixed with an equal volume of 22% polyethylene glycol 6000, containing 1% protamine sulfate, and centrifuged to precipitate high m.w. proteins including C5. The supernatant was mixed with ^{125}I-rabbit C5a des Arg and goat anti-rabbit C5a antibody in a competitive binding assay. Antibody-bound ligand was precipitated using a donkey anti-goat IgG second antibody and counted in a gamma-counter. Concentrations were determined from standard curves constructed by use of a spline fit program.

Preparation of ^{111}In-neutrophils. ^{111}In-labeled rabbit neutrophils were prepared using a technique previously described (4). Briefly, blood from a donor rabbit, collected into acid citrate dextrose and mixed with hydroxyethyl starch, was centrifuged twice to remove first RBC and then platelets. Neutrophils were harvested from a discontinuous Percoll-plasma gradient and incubated with $^{111}InCl_3$ (50 to 200 μCi) chelated to 2-mercaptopyridine-N-oxide. Labeled cells were washed in autologous platelet-poor plasma and 2.5 to 5.0 $\times$ 10^7 cells injected i.v. in 3 ml.

Measurement of inflammatory activity in peritoneal exudate fluid: rabbit skin bioassay. The in vivo inflammatory activity of peritoneal exudate fluid was assessed in a rabbit skin bioassay as previously described (2, 13).

Samples of exudate (100 μl) were injected intradermally into the shaved dorsal skin of anesthetized rabbits in the presence of a potentiating dose of PGE_2 (3×10^{-10} mol/100 μl). Exudates were tested in the presence and absence of an excess of a goat anti-rabbit C5a antibody (10 μl of IgG fraction/100 μl). Intradermal injection of ZAP or purified rabbit C5a des Arg was used as a positive control for the neutralizing activity of the antibody. In some assays the exudates were depleted of C5a, before intradermal testing. This was achieved by incubation of 50 ml exudate with 2.5 ml of the anti-C5a affinity gel described above (equilibrated in PBS) for 30 min.

Edema formation and neutrophil accumulation were monitored by the local accumulation of i.v. administered ^{125}I-albumin and ^{111}In-neutrophils, respectively. Thirty min after intradermal injections, the animal was killed by an anesthetic overdose, the skin was removed and injection sites punched out (17-mm diameter) for measurements in a gamma-counter.

In some experiments the effects of an anti-histamine (mepyramine, 3×10^{-9} mol/100 μl) or a platelet-activating factor antagonist (WEB 2086, 10^{-7} mol/100 μl) on inflammatory activity were tested by co-injection with zymosan-induced exudate depleted of C5a. Histamine (10^{-8} mol/100 μl) and platelet-activating factor (10^{-9} mol/100 μl) were used as positive controls for antagonist activity. The stability of inflammatory activity in exudate fluid depleted of C5a was tested by incubation at 37°C for 30 min (with and without 10% rabbit plasma), 60°C for 30 min and 100°C for 10 min. Samples were cooled on ice and centrifuged before intradermal testing. The effect of pH on the stability of the inflammatory activity was tested by the addition of hydrochloric acid or sodium hydroxide, incubation at pH 4.0 or 10.0 for 2 h at 0°C, and neutralization before intradermal testing. The small changes in salt concentration resulting from these procedures did not alter the skin bioassay responses.

Biochemical characterization of zymosan-induced exudate. For cation exchange chromatography, a pool of 6-h zymosan-induced exudate (80 ml) and cavity wash fluid (235 ml, recovered after the injection of 75 ml of sterile saline into the previously drained peritoneal cavity of each animal) was obtained from four rabbits. After removal of cells and particulate matter by centrifugation the fluid was incubated at 60°C for 30 min (see *Results*) followed by further centrifugation and depleted of C5a by incubation with the affinity gel described above (2.5 ml gel/50 ml fluid, 30 minutes, room temperature). The C5a-depleted supernatant was adjusted to pH 5.0 by the addition of 0.15 M hydrochloric acid and after 20 min the precipitated material was removed by centrifugation (5300 $\times$ *g* for 10 min). The pH 5.0 supernatant was then incubated with CM-Sephadex C-25 (1 ml swollen gel in 10 mM sodium acetate pH 5.0 containing 0.15 M sodium chloride per 50-ml sample) for 15 h at 4°C. After removal of the supernatant, bound material was eluted from the gel (6 ml) in a total volume of 30 ml of 0.1 M sodium phosphate pH 7.4 containing 1.5 M sodium chloride. The eluate was filtered (0.2 μ) and dialyzed twice (Spectra/por membrane, molecular mass cut off 3.5 kDa) against 1.5 liters of 0.08% trifluoroacetic acid at 4°C. The dialysate was lyophilized, reconstituted in 10 mM sodium phosphate pH 5.5 and, after centrifugation, applied to a carboxymethyl HPLC column (TSK 535CM, 7.5 $\times$ 150 mm in 10 mM sodium phosphate pH 5.5). The column was eluted at 0.5 ml/min with a two-stage linear sodium chloride gradient (0 to 1.0 M NaCl over 40 min, 1.0 to 1.5 M NaCl over 10 min) in 10 mM sodium phosphate pH 5.5 and 2-min fractions were collected. Each 1-ml fraction was desalted into 0.08% trifluoroacetic acid by application to columns (6 ml) of Sephadex G-10 and elution of the exclusion peak (>700 Da) in a volume of 1.5 ml.

For testing in the rabbit skin bioassay 250 μl of each fraction was lyophilized in the presence of BSA giving 0.1% BSA when reconstituted with 500 μl PBS. For in vitro binding studies 250 μl of each fraction was lyophilized in the presence of BSA giving 0.2% BSA when reconstituted with 500 μl PBS.

Radio-iodination of IL-8/NAP-1[3]. IL-8/NAP-1 (human rMDNCF) was iodinated using Iodogen reagent as described by Besemer et al. (14). Briefly 5 μg IL-8/NAP-1 was incubated for 10 min at room temperature with 500 μCi of Na^{125}I in 20 μl 0.2 M sodium phosphate pH 7.4 in a tube coated with 10 μg Iodogen. After the addition of 500 μl of PBS containing 2 mg/ml gelatin, 0.25 mM NaI and 28 mM L-tyrosine, radiolabeled IL-8/NAP-1 was purified by gel filtration on columns of Sephadex G-25M equilibrated in PBS containing 2 mg/ml gelatin. The specific radioactivity of ^{125}I-IL-8/NAP-1 was 53 μCi/μg protein.

Human neutrophil isolation. Human venous blood (50 ml) was collected into acid citrate dextrose and the RBC sedimented by addition of an equal volume of hydroxyethyl starch. After 30 min the leukocyte-rich supernatant was removed and centrifuged (280 $\times$ *g* for 7 min). The cell pellet was resuspended in 55% Percoll in PBS, applied to a discontinuous Percoll gradient comprising 70% Percoll over 81% Percoll in PBS, and centrifuged (600 $\times$ *g* for 25 min). The resulting neutrophil band routinely contained >98% neutrophils. After three washes with PBS containing 0.2% BSA and 0.1% sodium azide the number of neutrophils was determined using a Coulter (Coulter Electronics, Hialeah, FL) counter and the cells resuspended at a concentration of 1.3 $\times$ 10^7 cells/ml.

Binding of ^{125}I-IL-8/NAP-1 to human neutrophils. For binding studies 2 $\times$ 10^5 neutrophils were incubated for 60 min at 0°C with 1 nM ^{125}I-IL-8/NAP-1 and either IL-8/NAP-1, human rC5a, purified rabbit C5a des Arg, or CM-HPLC fractions purified from 6-h zymosan-induced rabbit peritoneal exudate (see above), in a total volume of 25 μl of PBS/0.2% BSA/0.1% sodium azide. After the addition of 1 ml of ice cold PBS/BSA/azide, centrifugation (5300 $\times$ *g* for 2 min at 0°C), and aspiration of the supernatant, cell pellets were counted in a gamma-counter.

Statistical analysis. Where appropriate statistical significance was determined on $\log_{10}$ transformed data using two-way analysis of variance, the statistical difference between groups being examined using the Newman-Keuls procedure. A $p < 0.05$ was considered to be significant.

Materials. Evans blue dye and polyethylene glycol 6000 were from BDH Chemicals Ltd, Poole, UK. Sodium pentobarbitone BP and mepyramine maleate were from May and Baker Ltd., Dagenham, UK. BSA (low endotoxin), protamine sulfate (grade II), zymosan A (from *Saccharomyces cerevisiae*), polymixin B sulfate, PGE_2, histamine diphosphate, Freund's adjuvants, and mercaptopyridine-N-oxide (1-hydroxypyridine-2-thione, sodium salt) were from Sigma Chemical Company, Poole, UK. Platelet-activating factor (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine) was from Bachem, Saffron Walden, UK. Hydroxyethyl starch (Hespan) was from Du Pont Ltd., Stevenage, UK. Heparin sodium BP (0.3% cresol as preservative) was from Paines and Byrne Ltd, Greenford, UK. $^{111}InCl_3$, ^{125}I-human serum albumin, and Na^{125}I were from Amersham International Plc, Little Chalfont, UK. Iodogen radio-iodination reagent

and Spectra/por dialysis membrane were from Life Science Laboratories Ltd, Luton, UK. Saline (sterile, pyrogen-free) was from Boots Ltd., Nottingham, UK. Donkey anti-goat IgG was from Nordic Immunological Laboratories, Maidenhead, UK. Percoll, Sephadex G-25M (PD-10) columns, Sephadex G-10, Protein-A-Sepharose CL-4B, and CM Sephadex C-25 were from Pharmacia, Milton Keynes, UK. Catheters (14 g arterioveine) were from Vygon, Ecouen, France. The TSK 535CM HPLC column was from LKB Ltd., Milton Keynes, UK. IL-8/NAP-1 (human rMDNCF) was a gift from Dr. K. Matsushima and Dr. J. J. Oppenheim, National Cancer Institute, Frederick, MD. Human rC5a was a gift from Dr. H. J. Showell, Pfizer Central Research, Groton, CT. WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno (3,2f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl]-1-(4-morpholinyl)-1-propanone) was a gift from Dr. H. Heuer, Boehringer Ingelheim KG, Ingelheim am Rhein, FRG. Rabbits were from Froxfield Farm, Froxfield, UK and Hacking and Churchill, Huntingdon, UK.

RESULTS

Time course of generation of inflammatory activity in zymosan-induced peritoneal exudate fluid. The i.p. injection of zymosan induced microvascular plasma protein leakage, as shown in Table I. Exudate:plasma ratios of i.v. injected Evans blue dye were three to four times higher in the zymosan-injected animals when compared with the saline-injected controls. Approximately 30 ml of peritoneal fluid was recovered at 6 h from each of six animals injected with zymosan, whereas fluid was recoverable from only four (2 h) and two (6 h) of seven saline-injected animals. Zymosan-induced exudates contained approximately 100 nM C5a whereas the saline-injected controls contained barely detectable levels of C5a.

The time course for the appearance of inflammatory activity in zymosan-induced exudate fluid was investigated by injecting exudate samples intradermally into the backs of assay rabbits and measuring the local accumulation of i.v. administered ^{125}I-albumin and ^{111}In-neutrophils over a 30-min period. All samples were tested in the presence of a potentiating dose of PGE_2 (2) in this and all subsequent experiments.

Figure 1 shows the results of a time course experiment. The *open symbols* in Figure 1 show that peritoneal exudate fluid collected as early as 5 min and up to 6 h after i.p. zymosan induced both edema formation (*upper panel*) and neutrophil accumulation (*lower panel*) when injected intradermally. To investigate the contribution of the complement fragment C5a to this activity, intradermal injections of these exudate fluids were also carried out in the presence of an excess of a specific anti-rabbit C5a antibody. In the presence of this antibody edema formation and neutrophil accumulation were virtually abolished in exudate removed from the peritoneal cavity at times up to 2 h. In contrast, samples collected at four and six h after zymosan retained inflammatory activity in the presence of the antibody (Fig. 1, *closed symbols*). The total antibody-resistant activity is indicated by the *shaded area* in Figure 1. The time-course for generation of activity measured in terms of neutrophil accumulation and edema induction were similar, but not identical. The apparent differences were not further investigated in this study.

In the experiment illustrated in Figure 1, intradermal injection of zymosan-activated rabbit plasma (260 nM C5a) resulted in edema formation and neutrophil accumulation that was totally inhibited by the anti-C5a antibody. This confirms that the antibody was used in excess because the exudates contained a lower C5a concentration (91 nM at 6 h in this experiment) than the activated plasma. The goat antibody used was specific for rabbit C5a in so far as it did not inhibit responses to histamine, bradykinin, platelet-activating factor, leukotriene B_4, FMLP, or human C5a (data not shown).

The inflammatory activity in 2- and 6-h zymosan-induced peritoneal exudates was investigated in further experiments, as shown in Figure 2. Zymosan-induced peritoneal exudate samples were collected at 2 and 6 h from donor animals. The samples from each donor were injected intradermally into the skin of individual recipient animals and edema formation and neutrophil accumulation, in the absence and presence of the anti-C5a antibody, was assessed as described above. The data demonstrate that both 2- and 6-h exudates contained inflammatory activity (Fig. 2, *open columns*: edema, *upper panel*; neutrophil accumulation, *lower panel*). When tested in the presence of the anti-C5a antibody (Fig. 2, *shaded columns*), however, the inflammatory response to 2-h exudate was substantially attenuated ($p < 0.05$) while 6-h exudate induced edema formation and neutrophil accumulation that was not significantly affected by the antibody. Peritoneal fluid taken from control animals injected i.p. with saline and polymixin B contained negligible inflammatory activity (see legend to Fig. 2). These results demonstrate that while the predominant inflammatory mediator present in 2-h zymosan-induced peritoneal exudate was C5a there was the subsequent generation of inflammatory activity that was C5a independent.

The possibility that C5a induces the generation of the second activity in vivo was investigated using the physiologically more stable des Arg form. Purified rabbit C5a

TABLE I

Measurement of microvascular plasma protein leakage and exudate C5a concentrations after intraperitoneal injection[a] of saline, zymosan[b], and purified rabbit C5a des Arg[c]

Time of Exudate Collection	Saline		Zymosan		C5a des Arg	
	2 h	6 h	2 h	6 h	2 h	6 h
No. of exudates[d]	4/7	2/7	7/7	6/6	4/4	4/4
Volume of exudate available at 6 h (ml)[e]	ND	19.0	ND	29.7 ± 2.2	ND	17.4 ± 4.6
Exudate: plasma ratio of Evans blue dye concentration[e]	0.04 ± 0.03	0.08	0.17 ± 0.04	0.27 ± 0.03	0.10 ± 0.01	0.22 ± 0.04
Immunoreactive C5a (nM)[e]	0.1	0.1	98 ± 10	94 ± 7	48 ± 5	10 ± 1

[a] All i.p. injections were 50 ml in saline containing 40 μM polymixin B.
[b] 500 mg zymosan.
[c] 60 nM C5a des Arg.
[d] No. of fluids obtainable/number of animals injected.
[e] Mean ± SEM.

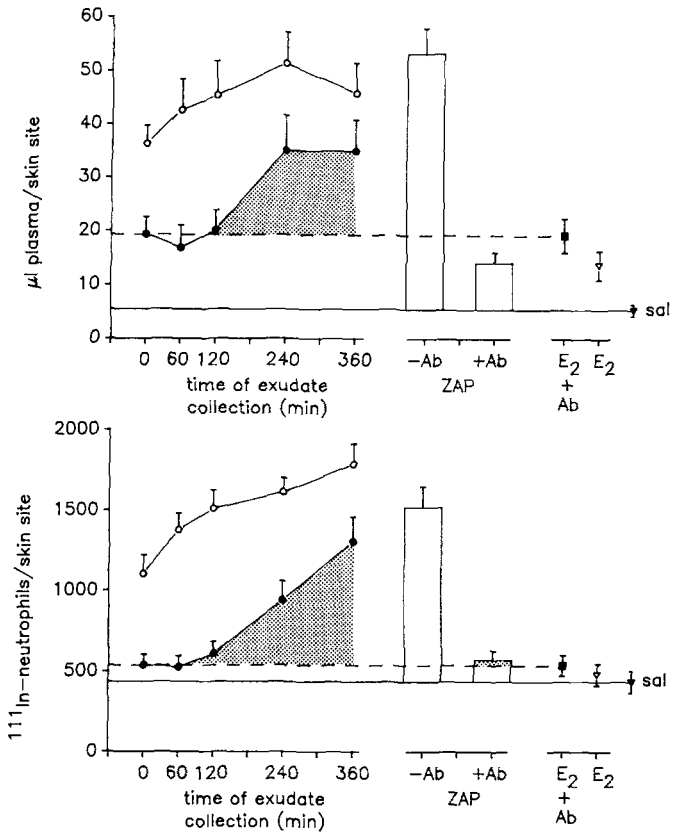

Figure 1. Time course for the generation of C5a-independent inflammatory activity in the rabbit peritoneum in response to zymosan. Exudate samples were collected 5, 60, 120, 240, and 360 min after i.p. injection of zymosan (500 mg in 50 ml saline containing 40 μM polymixin B). After centrifugation exudate fluids were mixed with goat anti-rabbit C5a antibody (10 μl/site), or saline for the control, and the vasodilator PGE_2 (3×10^{-10} mol/site) and injected intradermally (100 μl/site) in assay rabbits. Edema formation (*upper panel*) and neutrophil accumulation (*lower panel*) were monitored over 30 min (see *Materials and Methods*). The *horizontal solid line* represents the response to intradermal injection of saline (the only sites to which PGE_2 was not added) and the dashed line the response to saline + PGE_2 + antibody. The response to saline + PGE_2 in the absence of antibody is shown near the right-hand margin. Responses to exudate + PGE_2 are shown as *open symbols* and responses to exudate + PGE_2 + antibody as *closed symbols*. *Shaded areas* show *antibody-resistant responses*. The columns show the positive control in which the responses to zymosan-activated plasma (ZAP, 2.6×10^{-11} mol C5a des Arg/site) + PGE_2 were greatly suppressed by the anti-C5a antibody. Results are the mean ± SEM of six replicate injection sites.

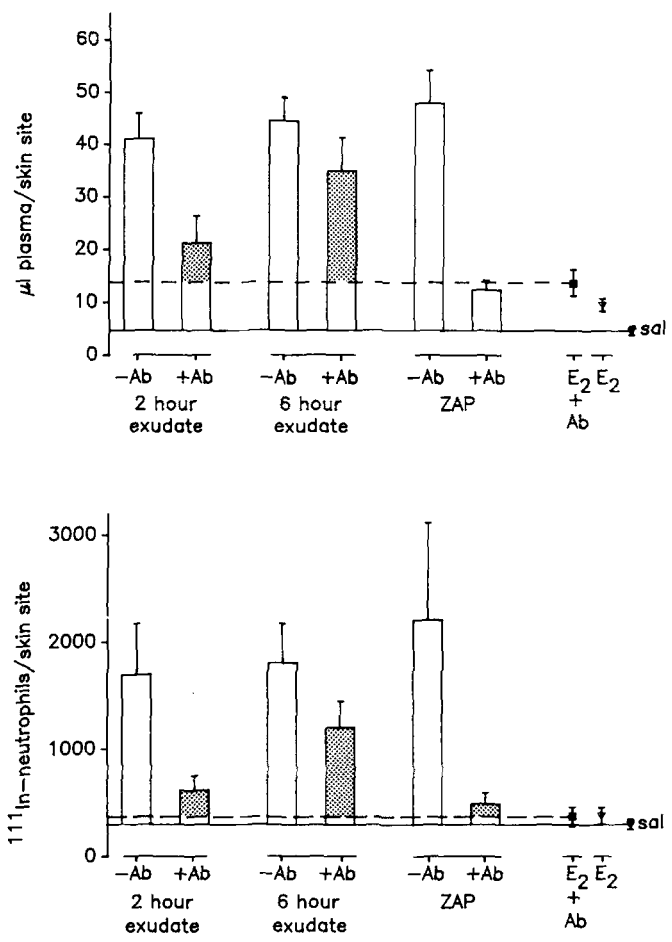

Figure 2. Detection of C5a-independent inflammatory activity in peritoneal exudate fluid. Exudate fluids, collected 2 and 6 h after i.p. injection of zymosan, were tested for activity in the skin bioassay for edema formation and neutrophil accumulation as described in the legend to Figure 1. Responses to saline, saline + PGE_2 (3×10^{-10} mol/site), and saline + PGE_2 + anti-C5a antibody (10 μl/site) are depicted as in Figure 1. The *columns* show responses to exudate (or ZAP, 2.6×10^{-11}mol C5a des Arg site, as the positive control) + PGE_2 in the absence and presence of antibody. *Shaded areas* represent antibody-resistant activity. Results are the mean ± SEM of exudates obtained from 2 h ($n = 7$) and 6 h ($n = 6$) peritoneal responses tested in six to seven assay rabbits. The same batch of ZAP was tested in each ($n = 7$) assay rabbit. Fluids were also collected where possible at 2 and 6 h from rabbits given i.p. saline containing polymixin B (see *Materials and Methods*, and Table I). These fluids induced negligible responses when assayed as above. Responses in rabbit skin to i.p. saline and polymixin B fluids obtained at 2 h were: edema 2.2 ± 0.6 μl ($n = 4$) and ^{111}In-neutrophils 103 ± 261 ($n = 4$). For 6-h samples edema responses were 2.2 μl ($n = 2$) and ^{111}In-neutrophils 239 ($n = 2$). These control samples were assayed in the presence of PGE_2 and values for responses to intradermal saline + PGE_2 have been subtracted.

des Arg (60 nM in 50 ml sterile saline containing polymixin B) was injected i.p. C5a des Arg induced a peritoneal inflammatory response as indicated by the higher exudate: plasma blue dye ratios when compared to the saline-injected controls (Table I). Samples of exudate fluid were collected at 2 and 6 h and assayed for inflammatory activity in rabbit skin. Figure 3 shows the results from four experiments. Peritoneal exudate fluid induced by C5a des Arg collected after 2 h induced edema formation and neutrophil accumulation (Fig. 3, *open columns*) that was totally inhibited by co-injection with anti-C5a antibody (*shaded columns*). The 6-h exudate contained no detectable activity.

These results, while demonstrating that intraperitoneal administration of the pre-formed inflammatory mediator C5a des Arg can produce an inflammatory reaction in the peritoneal cavity, show that it is unable in this model to induce the generation of the second inflammatory activity.

Characterization of C5a-independent inflammatory activity induced by zymosan in vivo. The inflammatory activity of 6-h zymosan-induced peritoneal exudate, depleted of C5a by use of an affinity gel, was stable to incubation at 60°C for 30 min but was abolished at 100°C for 10 min (Fig. 4). Activity was stable (0°C for 2 h) at pH 4 and relatively stable at pH 10 (Fig. 4). The C5a-independent edema-forming activity was distinguishable from histamine and platelet-activating factor as antagonists to these mediators (mepyramine and WEB 2086, respectively) had no effect on C5a-independent edema formation while responses to specific agonists were blocked (Fig. 5). Incubation of exudate with 10% rabbit plasma had no effect on activity, whereas the activity of bradykinin was inhibited by such treatment (Fig. 5).

Although the inflammatory activity was stable at 37°C for 30 min (Fig. 5), we observed that activity in some

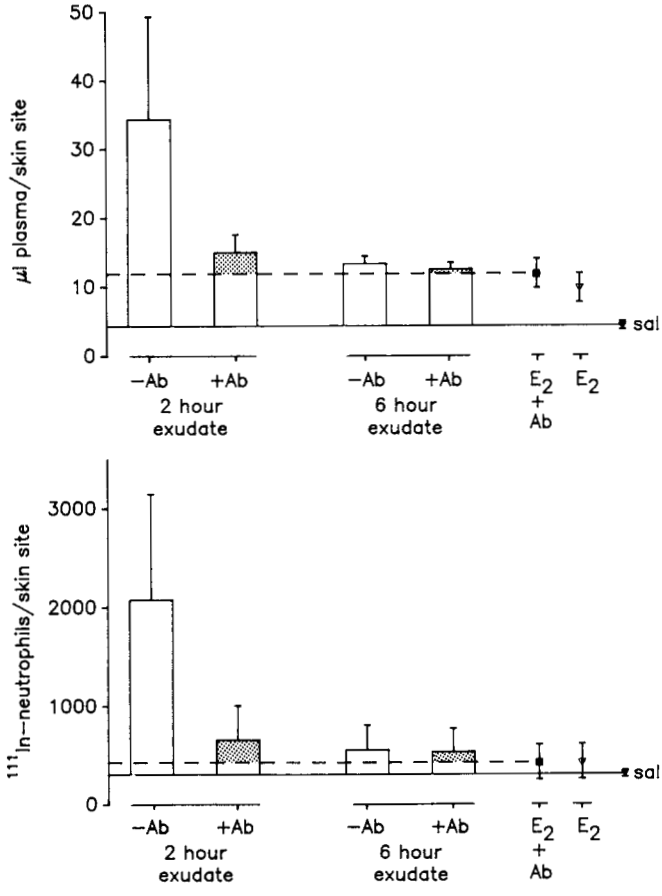

Figure 3. Inflammatory activity in peritoneal exudates collected 2 and 6 h after the i.p. injection of purified rabbit C5a des Arg (60 nM in 50 ml saline containing polymixin B). After centrifugation exudate fluids were mixed with goat anti-rabbit C5a antibody (10 μl/site), or saline for the control, and PGE_2 (3×10^{-10} mol/site) before testing in the skin bioassay for edema formation and neutrophil accumulation as described in the legend to Figure 1. Responses to saline, saline + PGE_2, and saline + PGE_2 + antibody are shown as in Figure 1. The *columns* show responses to exudates + PGE_2 in the absence and presence of anti-C5a antibody. The *shaded areas* represent the very low levels of antibody-resistant activity. Results are the mean ± SEM of exudates obtained from $n = 4$ peritoneal responses tested in four assay rabbits.

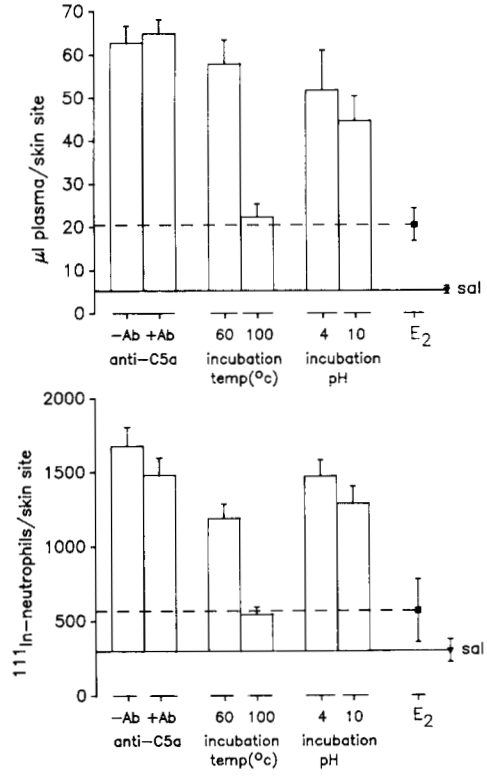

Figure 4. Physical characterization of inflammatory activity in 6-h zymosan-induced peritoneal exudate fluid depleted of C5a. Samples were treated as follows. *a*, Kept at 0°C for 2 h and then tested with and without anti-C5a antibody; *b*, incubation at 60°C (30 min) or 100°C (10 min) followed by cooling to 0°C and centrifugation; *c*, incubation at pH 4.0 or 10.0 (2 h, 0°C) followed by neutralization and centrifugation. Samples were then injected intradermally in 100 μl volumes in the presence of PGE_2 (3×10^{-10} mol/site). Edema formation (*upper panel*) and neutrophil accumulation (*lower panel*) were monitored over 30 min. The *horizontal solid line* represents the response to intradermal injection of saline (the only sites to which PGE_2 was not added) and the dashed line the response to saline + PGE_2. Note that the exudate was depleted of C5a and that addition of anti-C5a antibody (10 μl/site, second column only) did not significantly alter responses. Results are expressed as the mean ± SEM of six replicate injections for each sample.

zymosan-induced exudates was slightly reduced when the in vitro incubation was continued for 2 h (data not shown). Therefore, to protect against possible enzymatic degradation of bioactivity during purification, C5a-depleted exudates were heated to 60°C for 30 min before chromatography.

Partial purification of 6-h zymosan-induced C5a-independent inflammatory activity. Preliminary experiments showed that both C5a and the C5a-independent activity was retained by carboxymethyl Sephadex at pH 5.5 and below. Therefore the 6-h zymosan-induced exudates and cavity wash fluids, depleted of C5a using an anti-C5a affinity gel, were incubated with the soft gel cation exchanger at pH 5.0. More than 94% of the total protein content was discarded in the gel supernatant. Gel-bound material was eluted, applied to a carboxymethyl HPLC column and eluted with a sodium chloride gradient (Fig. 6*a*). De-salting of successive fractions, lyophilization and reconstitution with PBS allowed inflammatory activity to be measured in an in vivo bioassay. Figure 6*b* shows two peaks of bioactivity eluting at 0.5 M NaCl (peak 1) and 0.9 M NaCl (peak 2) each spread over several successive fractions. Both peaks induced edema formation and ^{111}In-neutrophil accumulation.

Inhibition by CM-HPLC fractions of ^{125}I-IL-8/NAP-1 binding to human neutrophils in vitro. Human neutrophils have been shown to possess specific receptors for IL-8/NAP-1 (14, 15), therefore, in our studies all CM-HPLC fractions were tested for their ability to inhibit the binding of ^{125}I-IL-8/NAP-1 to human neutrophils in vitro. Figure 6*c* shows that the fractions comprising the two peaks of in vivo inflammatory activity inhibited the binding of 1 nM ^{125}I-IL-8/NAP-1 to human neutrophils. A peak inhibition of 53% was observed for peak 1 (fraction 20) and 62% for peak 2 (fraction 28). This inhibition of binding was dilution dependent (data not shown). In addition minor peaks of inhibitory activity, not associated with in vivo bioactivity at the concentration of fraction used, were observed in fractions 25 and 35. Binding of ^{125}I-IL-8/NAP-1 was inhibited in a dose-dependent manner by IL-8/NAP-1 (*inset* to Fig. 6*c*). Specificity of the assay was verified by the inability of rabbit C5a des Arg or human rC5a, at concentrations up to 10^{-6} M, to inhibit binding (Fig. 6*c* and *inset*, respectively).

DISCUSSION

Intraperitoneal injection of zymosan in the rabbit stimulates microvascular plasma protein leakage into the

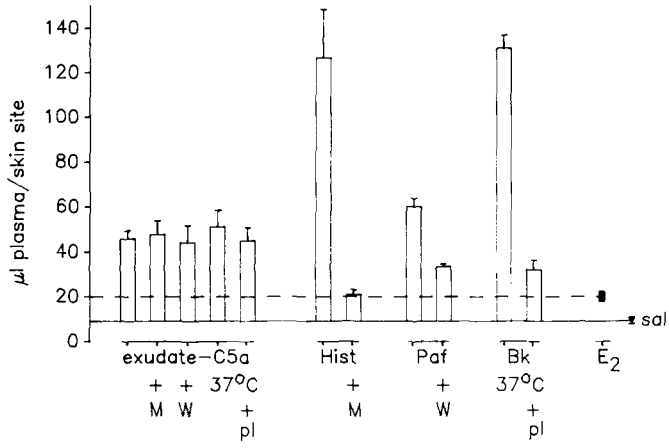

Figure 5. Pharmacologic characterization of inflammatory activity in zymosan-induced peritoneal exudate fluid depleted of C5a. Exudate + PGE_2 (3×10^{-10} mol/site) was injected in 100 μl volumes in the absence or presence of mepyramine (*M*, 3×10^{-9} mol/site) or WEB 2086 (*W*, 10^{-7} mol/site). Histamine (*H*, 10^{-8} mol/site) + PGE_2 was injected in the absence and presence of mepyramine, and platelet-activating factor (*Paf*, 10^{-9} mol/site) + PGE_2 in the absence and presence of WEB 2086. Exudate or bradykinin (*Bk*, 10^{-10} mol/site), was incubated (37°C for 30 min) in the absence and presence of 10% rabbit plasma (*pl*) and then injected intradermally together with PGE_2. Responses to saline and saline + PGE_2 are depicted as in Figure 4. Note that the exudate was depleted of C5a and that no anti-C5a antibody was used in this assay. Results are the mean ± SEM of six replicate sites for each sample.

cavity (7, 8). In an attempt to analyze the chemical mediators responsible, we injected zymosan-induced exudate fluid into rabbit back skin and measured the local leakage of i.v. injected ^{125}I-albumin. As edema responses to zymosan in the rabbit are neutrophil dependent (6), we also assayed exudates in terms of the accumulation of i.v. injected ^{111}In-neutrophils. In vivo assays were facilitated by the addition of the vasodilator PGE_2 to the exudate samples to potentiate edema responses. This was necessary as the exudate contained no detectable stable vasodilator, although high levels of the metabolite of prostacyclin, 6-oxo-PGF1α, were present (8).

In previous experiments we demonstrated high concentrations of the complement-derived neutrophil chemoattractant C5a (assumed to be in its des Arg form) in zymosan-induced exudate (7). Further, we showed that C5a and C5a des Arg increase microvascular permeability by a neutrophil-dependent process (6, 16). In the experiments described in this report, zymosan-induced exudate was assayed in rabbit skin in the presence and absence of a specific antibody to rabbit C5a. This procedure revealed that the low level of edema-inducing activity separable from C5a as originally detected in 2-h exudate (8) was present in higher levels in 6-h exudate ($p < 0.05$). The 6-h exudates induced marked edema formation and neutrophil accumulation when assayed together with an excess of the anti-C5a antibody.

Further experiments were carried out with 6-h zymosan-induced peritoneal exudates depleted of C5a using immunoabsorbance with solid-phase anti-C5a antibody. When assayed in the skin for its edema-inducing effect, the non-C5a activity was unaffected by an H_1 anti-histamine and an antagonist of platelet-activating factor. The activity was also stable in fresh plasma, unlike that of kinins. Levels of leukotriene B_4 in exudates, as measured by RIA, were well below the concentrations necessary for edema-inducing activity (H. Yarwood, S. J. Foster, and T. J. Williams, unpublished observations). In terms of edema-inducing and neutrophil-chemoattractant activity in vivo, the non-C5a activity was unstable at 100°C, but stable at 60°C and also stable on ice at pH 4 and 10.

After a soft gel cation exchange step to remove the majority of unwanted proteins, cation-exchange HPLC of C5a-depleted 6-h exudate resolved the bioactivity into two discrete peaks, both able to induce edema formation and ^{111}In-neutrophil accumulation in rabbit skin.

The relationship between the mediators present in these peaks and human IL-8/NAP-1 was investigated in vitro. Binding studies revealed that CM-HPLC fractions containing in vivo bioactivity were able to inhibit the binding of ^{125}I-IL-8/NAP-1 to human neutrophils in vitro.

IL-8/NAP-1 (17–19) is the name recently ascribed to an 8-kDa human protein, identified by several groups and variously described as MDNCF (20), neutrophil-activating factor (21), monocyte-derived neutrophil activating peptide (22), lymphocyte-derived neutrophil activating peptide (23), endothelial-derived neutrophil-activating peptide (24), granulocyte chemotactic protein (25), fibroblast-derived neutrophil activating protein (26), and neutrophil chemotactic factor (27). As the nomenclature implies, this peptide is secreted by numerous cells in response to IL-1, TNF, and, in some cases, LPS and PHA. The biologic activities of IL-8/NAP-1 include neutrophil chemotaxis (20, 21), elevation of cytosolic calcium (28, 29), and degranulation (28, 30) in vitro and both edema formation and neutrophil accumulation in vivo (31–33). There are numerous N-terminal processed variants of this protein, ranging from 79 to 70 amino acids (34, 35), which retain biologic activity in vitro. Purification of CM-HPLC peak 2 to homogeneity and subsequent amino acid sequence analysis revealed a partial peptide sequence (31 N-terminal residues, 3 of which were unidentified) with considerable homology (75%) to the 77 amino acid extended form of human IL-8/NAP-1 (36).

Recently Schroder et al. (37) identified a 6- to 8-kDa peptide, NAP-3, which was secreted along with IL-8/NAP-1 from LPS-stimulated human monocytes. This peptide, identical to MGSA (38) and the product of the human GRO gene (39), shows 43% homology with human IL-8/NAP-1. In vivo MGSA induces neutrophil accumulation in rat skin (40), and in vitro is a neutrophil chemoattractant with a similar ED_{50} (though smaller maximal response) to that of human IL-8/NAP-1 (37). NAP-3 and human IL-8/NAP-1 show a high degree of cross-desensitisation with respect to neutrophil enzyme release in vitro suggesting that these mediators may share a common receptor (37). In further studies (P. J. Jose, P. D. Collins, J. M. Perkins, B. C. Beaubien, N. P. Totty, J. Hsuan, and T. J. Williams, unpublished observations) we have identified the activity in CM-HPLC peak 1, and it appears to represent a rabbit protein related to human NAP-3/MGSA/GRO. The small inhibitory activity on human ^{125}I-IL-8/NAP-1 binding observed in fractions 25 and 35 may represent minor amounts of other N-terminal variants of these rabbit proteins.

Although other cytokines, e.g., IL-1 or TNF, may contribute to the inflammatory response in the peritoneal cavity, it is unlikely that these proteins are responsible for the activity detected when the exudate was tested in the skin. Rabbit IL-1 is unstable at 60°C (41) and rabbit TNF unstable at less than pH 6 (42).

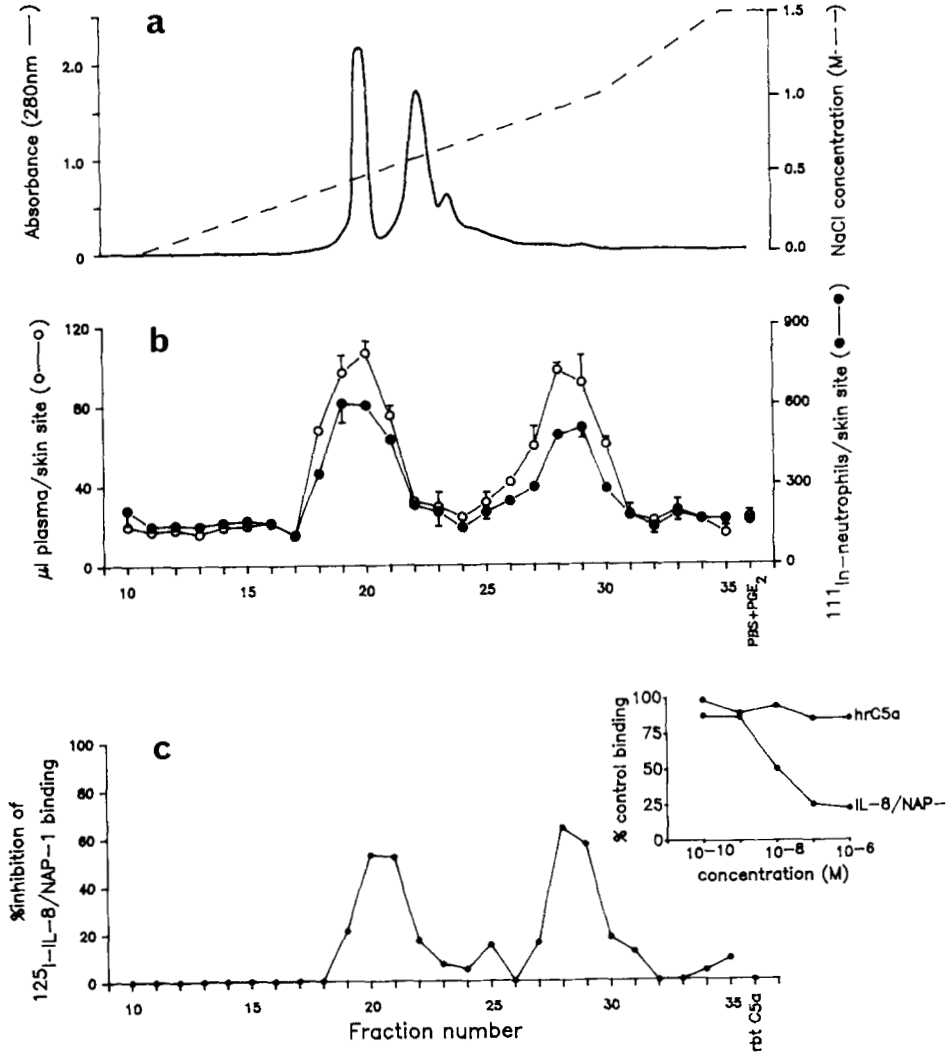

Figure 6. Cation exchange HPLC of 6-h zymosan-induced peritoneal exudate depleted of C5a. *a*, Absorbance profile at 280 nm (*solid line*) and sodium chloride gradient (*dashed line*). More than 94% of the exudate proteins were removed by use of a soft gel cation exchange before HPLC. *b*, Inflammatory activity of sequential fractions in rabbit skin in vivo. Each fraction was treated as described in *Materials and Methods* and injected intradermally in a volume of 100 μl together with PGE_2 (3×10^{-10} mol/100 μl) in triplicate skin sites. Edema formation (*open symbols*) and ^{111}In-neutrophil accumulation (*closed symbols*) was measured over 30 min. In this experiment 1.4×10^7 ^{111}In-neutrophils were injected i.v. Responses to intradermally injected PBS + 0.1% BSA and PGE_2 are shown near the right-hand margin. *c*, Inhibition of binding of ^{125}I-IL-8/NAP-1 to human neutrophils by sequential fractions. Purified rabbit C5a des Arg (10^{-6} M) did not affect binding of ^{125}I-IL-8/NAP-1 as shown near the right-hand margin. The effects of unlabeled IL-8/NAP-1, and human rC5a, over the concentration range 10^{-6} to 10^{-10} M on ^{125}I-IL-8/NAP-1 binding, is shown in the *inset*. Non-specific binding (<5%) was subtracted from all values before calculation of binding relative to the control, specific, binding of 1nM ^{125}I-IL-8/NAP-1 (16 to 21%). Each *point* represents the mean of three measurements.

Whether zymosan itself or an endogenous intermediate mediator is responsible for triggering rabbit IL-8/NAP-1 production has yet to be established. Other studies have shown that unidentified neutrophil chemoattractants can be generated in vivo in response to endotoxin (43, 44). As polymixin B was routinely co-injected with zymosan, it is unlikely that endotoxin was the stimulus for rabbit IL-8/NAP-1 production in our studies. It may be that C5a stimulates the generation of rabbit IL-8/NAP-1 in vivo. To test this possibility the physiologically more stable form of C5a, C5a des Arg, was injected into the rabbit peritoneal cavity. This induced an inflammatory response, but no significant non-C5a activity was detected in the exudates. It is feasible that the endogenous stimulus for rabbit IL-8/NAP-1 production is intact C5a. This is unlikely as C5a would be rapidly converted to C5a des Arg in the exudate in situ. Other soluble products of complement activation, such as C3 fragments, may also be involved. We shall test these possibilities by infusing intact C5a and other products into the peritoneal cavity in future experiments.

We propose the following working hypothesis to account for the sequence of events leading to rabbit IL-8/NAP-1 secretion. Zymosan activates the low level of complement in extravascular tissue fluid in the cavity. A small amount of C5a is generated that stimulates neutrophils to attach to microvascular endothelial cells and emigrate. This interaction results in plasma protein leakage, thus supplying more complement to the extravascular space. Further activation results in more C5a generation. The stimulus for rabbit IL-8/NAP-1 production may be an interaction between iC3b on opsonized zymosan particles and CR3 receptors on macrophages. Such an interaction has been suggested to stimulate other macrophage functions (45). If such opsonisation is a prerequisite for IL-8/NAP-1 production, this has the interesting consequence that IL-8/NAP-1 generation would be dependent on the initial phase of C5a-induced plasma protein leakage in vivo. Further investigation is necessary to establish the exact source and stimulus for rabbit IL-8/NAP-1 production in this and other types of inflammatory reactions. We believe that these observations provide an insight into the relationship between complement-derived and cell-derived neutrophil chemoattractant proteins in vivo.

Acknowledgments. The authors thank T. L. Buckley for guidance on statistical analysis and C. J. Coker for assistance in the preparation of this manuscript.

REFERENCES

1. **Williams, T. J., and M. J. Peck.** 1977. Role of prostaglandin-mediated vasodilatation in inflammation. *Nature 270:530.*

2. **Williams, T. J.** 1979. Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br. J. Pharmacol. 65:517.*
3. **Williams, T. J., and P. J. Jose.** 1981. Mediation of increased vascular permeability after complement activation: histamine-independent action of rabbit C5a. *J. Exp. Med. 153:136.*
4. **Rampart, M., and T. J. Williams.** 1988. Evidence that neutrophil accumulation induced by interleukin-1 requires both local protein biosynthesis and neutrophil CD18 antigen expression in vivo. *Br. J. Pharmacol. 94:1143.*
5. **Jose, P. J., M. J. Peck, C. Robinson, and T. J. Williams.** 1978. Characterization of a histamine-independent vascular permeability-increasing factor generated on exposure of rabbit plasma to zymosan. *J. Physiol. 281:13.*
6. **Wedmore, C. V., and T. J. Williams.** 1981. Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature 289:646.*
7. **Jose, P. J., M. J. Forrest, and T. J. Williams.** 1983. Detection of the complement fragment C5a in inflammatory exudates from the rabbit peritoneal cavity using radioimmunoassay. *J. Exp. Med. 158:2177.*
8. **Forrest, M. J., P. J. Jose, and T. J. Williams.** 1986. Kinetics of the generation and action of chemical mediators in zymosan-induced inflammation of the rabbit peritoneal cavity. *Br. J. Pharmacol. 89:719.*
9. **Haslett, C., P. J. Jose, P. C. Giclas, T. J. Williams, and P. M. Henson.** 1989. Cessation of neutrophil influx in C5a-induced acute experimental arthritis is associated with loss of chemoattractant activity from the joint space. *J. Immunol. 142:3510.*
10. **Steinbuch, M., and R. Audran.** 1969. The isolation of IgG from mammalian sera with the aid of caprylic acid. *Arch. Biochem. Biophys. 134:279.*
11. **Schneider, C., R. A. Newman, D. R. Sutherland, U. Asser, and M. F. Greaves.** 1982. A one-step purification of membrane proteins using a high efficiency immunomatrix. *J. Biol. Chem. 257:10766.*
12. **Jose, P. J., I. K. Moss, R. N. Maini, and T. J. Williams.** 1990. Measurement of the chemotactic complement fragment C5a in rheumatoid synovial fluids by radioimmunoassay: role of C5a in acute inflammatory phase. *Ann. Rheum. Dis. 49:747.*
13. **Nourshargh, S., M. Rampart, P. G. Hellewell, P. J. Jose, J. M. Harlan, A. J. Edwards, and T. J. Williams.** 1989. Accumulation of ^{111}In-neutrophils in rabbit skin in allergic and non-allergic inflammatory reactions in vivo: inhibition by neutrophil pretreatment in vitro with a monoclonal antibody recognising the CD18 antigen. *J. Immunol. 142:3193.*
14. **Besemer, J., A. Hujber, and B. Kuhn.** 1989. Specific binding, internalization, and degradation of human neutrophil activating factor by human polymorphonuclear leukocytes. *J. Biol. Chem. 264:17409.*
15. **Samanta, A. K., J. J. Oppenheim, and K. Matsushima.** 1989. Identification and characterisation of specific receptors for monocyte-derived neutrophil chemotactic factor (MDNCF) on human neutrophils. *J. Exp. Med. 169:1185.*
16. **Jose, P. J., M. J. Forrest, and T. J. Williams.** 1981. Human C5a des Arg increases vascular permeability. *J. Immunol. 127:2376.*
17. **Baggiolini, M., A. Walz, and S. L. Kunkel.** 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest. 84:1045.*
18. **Larsen, C. G., A. O. Anderson, E. Appella, J. J. Oppenheim, and K. Matsushima.** 1989. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science 243:1464.*
19. **Westwick, J., S. W. Li, and R. D. Camp.** 1989. Novel neutrophil-stimulating peptides. *Immunol. Today 5:146.*
20. **Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim, and E. J. Leonard.** 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. USA 84:9233.*
21. **Walz, A., P. Peveri, H. Aschauer, and M. Baggiolini.** 1987. Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem. Biophys. Res. Commun. 149:755.*
22. **Schroder, J-M., U. Mrowietz, E. Morita, and E. Christophers.** 1988. Identification of different charged species of a human monocyte derived neutrophil activating peptide (MONAP). *Biochem. Biophys. Res. Commun. 152:277.*
23. **Schroder, J.-M., U. Mrowietz, and E. Christophers.** 1988. Purification and partial biological characterization of a human lymphocyte-derived peptide with potent neutrophil-stimulating activity. *J. Immunol. 140:3534.*
24. **Schroder, J-M., and E. Christophers.** 1989. Secretion of novel and homologous neutrophil-activating peptides by LPS-stimulated human endothelial cells. *J. Immunol. 142:244.*
25. **Van Damme, J., J. Van Beeumen, G. Opdenakker, and A. Billiau.** 1988. A novel, NH_2-terminal sequence-characterized human monokine possessing neutrophil chemotactic, skin-reactive, and granulocytosis-promoting activity. *J. Exp. Med. 167:1364.*
26. **Schroder, J-M., M. Sticherling, H. H. Henneicke, W. C. Preissner, and E. Christophers.** 1990. IL-1α or tumor necrosis factor-α stimulate release of three NAP-1/IL-8-related neutrophil chemotactic proteins in human dermal fibroblasts. *J. Immunol. 144:2223.*
27. **Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks.** 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS, and IL-1β. *Science 243:1467.*
28. **Peveri, P., A. Walz, B. Dewald, and M. Baggiolini.** 1988. A neutrophil-activating factor produced by human mononuclear phagocytes. *J. Exp. Med. 167:1547.*
29. **Thelen, M., P. Peveri, P. Kernen, V. Von Tscharner, A. Walz, and M. Baggiolini.** 1988. Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. *FASEB J. 2:2702.*
30. **Willems, J., M. Joniau, S. Cinque, and J. Van Damme.** 1989. Human granulocyte chemotactic peptide (IL-8) as a specific neutrophil degranulator: comparison with other monokines. *Immunology 67:540.*
31. **Foster, S. J., D. M. Aked, J. M. Schroder, and E. Christophers.** 1989. Acute inflammatory effects of a monocyte-derived neutrophil-activating peptide in rabbit skin. *Immunology 67:181.*
32. **Colditz, I., R. Zwahlen, B. Dewald, and M. Baggiolini.** 1989. In vivo inflammatory activity of neutrophil-activating factor, a novel chemotactic peptide derived from human monocytes. *Am. J. Pathol. 134:755.*
33. **Rampart, M., J. Van Damme, L. Zonnekyn, and A. G. Herman.** 1989. Granulocyte chemotactic protein/interleukin-8 induces plasma leakage and neutrophil accumulation in rabbit skin. *Am. J. Pathol. 135:1.*
34. **Van Damme, J., J. Van Beeumen, R. Conings, B. Decock, and A. Billiau.** 1989. Purification of granulocyte chemotactic peptide/interleukin-8 reveals N-terminal sequence heterogeneity similar to that of β-thromboglobulin. *Eur. J. Biochem. 181:337.*
35. **Yoshimura, T., E. A. Robinson, E. Appella, K. Matsushima, S. D. Showalter, A. Skeel, and E. J. Leonard.** 1989. Three forms of monocyte-derived neutrophil chemotactic factor (MDNCF) distinguished by different lengths of the amino-terminal sequence. *Mol. Immunol. 26:87.*
36. **Beaubien, B. C., P. D. Collins, P. J. Jose, N. F. Totty, M. D. Waterfield, J. Hsuan, and T. J. Williams.** 1990. A novel neutrophil chemoattractant generated during an inflammatory reaction in the rabbit peritoneal cavity in vivo: purification, partial amino acid sequence and structural relationship to interleukin 8. *Biochem. J. 271:797.*
37. **Schroder, J-M., N. L. M. Persoon, and E. Christophers.** 1990. Lipopolysaccharide-stimulated human monocytes secrete, apart from neutrophil-activating peptide 1/interleukin 8, a second neutrophil-activating protein. *J. Exp. Med. 171:1091.*
38. **Richmond, A., E. Balentien, H. G. Thomas, G. Flaggs, D. E. Barton, J. Spiess, R. Bordoni, U. Francke, and R. Derynck.** 1988. Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to β-thromboglobulin. *EMBO J. 7:2025.*
39. **Anisowicz, A., L. Bardwell, and R. Sager.** 1987. Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. *Proc. Natl. Acad. Sci. USA 84:7188.*
40. **Moser, B., I. Clark-Lewis, R. Zwahlen, and M. Baggiolini.** 1990. Neutrophil-activating properties of the melanoma growth-stimulatory activity. *J. Exp. Med. 171:1797.*
41. **Simon, P. L., and W. F. Willoughby.** 1981. The role of subcellular factors in pulmonary immune function: physicochemical characterisation of two distinct species of lymphocyte-activating factor produced by rabbit alveolar macrophages. *J. Immunol. 126:1534.*
42. **Shirai, T., H. Yamaguchi, H. Ito, C. W. Todd, and R. B. Wallace.** 1985. Cloning and expression in Escherichia coli of the gene for human tumour necrosis factor. *Nature 313:803.*
43. **Cunha, F. Q., and S. H. Ferreira.** 1986. The release of a neutrophil chemotactic factor from peritoneal macrophages by endotoxin: inhibition by glucocorticoids. *Eur. J. Pharmacol. 129:65.*
44. **Issekutz, A. C., P. Megyeri, and T. B. Issekutz.** 1987. Role for macrophage products in endotoxin-induced polymorphonuclear leukocyte accumulation during inflammation. *Lab. Invest. 56:49.*
45. **Ding, A., S. D. Wright, and C. Nathan.** 1987. Activation of mouse peritoneal macrophages by monoclonal antibodies to Mac-1 (complement receptor type 3). *J. Exp. Med. 165:733.*