Identification of a second neutrophil-chemoattractant cytokine generated during an inflammatory reaction in the rabbit peritoneal cavity *in vivo*

Purification, partial amino acid sequence and structural relationship to melanoma-growth-stimulatory activity

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The intraperitoneal injection of zymosan in the rabbit results in the generation of an inflammatory exudate containing oedema-forming and chemoattractant activities. Previous studies demonstrated the early appearance of the complement fragment C5a, followed by the generation of two mediators related to the cytokine interleukin-8 that were separable by cation-exchange h.p.l.c. *N*-Terminal amino acid sequencing identified one of these mediators as rabbit interleukin-8. This paper describes the purification of the second cytokine by cation-exchange, gel-filtration and reversed-phase h.p.l.c. The purified material had both oedema-forming and chemoattractant activity when assayed in rabbit skin *in vivo*. On SDS/PAGE a single 6–8 kDa band was observed and *N*-terminal amino acid sequencing of the reduced and alkylated protein positively identified 36 amino acids. This sequence revealed the rabbit homologue of melanoma-growth-stimulatory activity. The identification of these two cytokines *in vivo* will provide an opportunity to investigate the importance of their co-release in the inflammatory process.

INTRODUCTION

The presence of microbes in a tissue initiates an acute local inflammatory reaction that is essential for the neutralization and removal of the infective agent. One component of this reaction, microvascular plasma protein leakage, is important for the supply of complement proteins and antibodies to the extravascular interstitium [1,2]. A second component is the local accumulation of neutrophils that have the role of killing and phagocytosing the microbes.

We have been investigating the link between the inflammatory stimulus and these components of the inflammatory response *in vivo* using a reaction induced by zymosan in a rabbit model. Intraperitoneal injection of zymosan induced plasma protein leakage and neutrophil accumulation associated with the appearance of the chemotactic complement peptide C5a [3,4]. In a previous study, peritoneal exudate samples were tested in a rabbit skin bioassay for their ability to induce the accumulation of ¹¹¹In-labelled neutrophils and the leakage of ¹²⁵I-albumin [5]. It was found that the inflammatory activity in the exudate samples up to 2 h was completely blocked in the presence of an antibody to C5a. Activity in later samples (2–6 h) was, however, only partially blocked by the antibody. In further investigations [6], C5a was removed from 6 h peritoneal exudate using an affinity column, and the remaining activity was purified by h.p.l.c. techniques using the skin assay system to monitor each separation. Two peaks of activity were observed. One of these (rabbit permeability factor, RPF) was partially sequenced (*N*-terminal 31 residues) and found to be rabbit interleukin-8/neutrophil-activating protein-1 (IL-8/NAP-1) [6]. This paper

describes the purification, *N*-terminal amino acid sequencing and identification of the second peak of oedema-inducing and neutrophil-chemoattractant activity. The sequence reveals the rabbit homologue of melanoma-growth-stimulatory activity/neutrophil-activating protein-3/growth-regulated gene protein (MGSA/NAP-3/GRO) [7–9].

MATERIALS AND METHODS

Collection of inflammatory exudates

Exudate fluid was collected from the peritoneal cavities of 27 New Zealand White rabbits as described previously [6]. Briefly, a suspension of zymosan (500 mg in 75 ml of sterile saline, containing 40 µM-polymixin B sulphate to inhibit the effects of any endotoxin present) was injected into the peritoneal cavity of anaesthetized rabbits. After 6 h the animals were killed by an anaesthetic overdose and the exudate was removed into sodium EDTA (0.01 M, pH 7.4) or, when assays of unfractionated exudates were required, into heparin (10 units/ml), and kept on ice. Remaining exudate was washed out with 50 ml of sterile saline. After centrifugation to remove the accumulated cells and any remaining zymosan particles, the supernatant was heated to 60 °C for 30 min to inactivate any heat-labile proteases. C5a, which would otherwise have contributed to the bioassay for inflammatory activity, was removed by use of an affinity gel as previously described [6].

Rabbit skin bioassay for inflammatory mediators

Inflammatory activity in the peritoneal exudate, at various stages in the purification, was monitored by its ability to induce

Abbreviations used: RPF, rabbit permeability factor; IL-8, interleukin-8; NAP, neutrophil-activating protein; MGSA, melanoma-growth-stimulatory activity; GRO, growth-regulated gene; PBS, phosphate-buffered saline (0.15 M-NaCl in 0.01 M-sodium phosphate buffer at pH 7.4); TFA, trifluoroacetic acid.

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Fig. 1. Wide-pore reversed-phase h.p.l.c. of CM-1 and CM-2

Pooled bioactive fractions from sizing h.p.l.c. of CM-1 and CM-2 were applied separately to a Vydac (30 nm) C_{18} reversed-phase column in 0.1% TFA and eluted with linear acetonitrile gradients. (a) A_{214} , acetonitrile gradients and oedema-forming activity of fractions originating from cation-exchange h.p.l.c. peak CM-1. (b) A_{214} , acetonitrile gradients and oedema-forming activity of fractions originating from cation-exchange h.p.l.c. peak CM-2. In both (a) and (b), 2% of each fraction was freeze-dried for assay in rabbit skin (0.4% fraction/100 μ l per skin site). The horizontal broken line represents the weak response to prostaglandin E_2 (PGE_2) and anti-rabbit C5a antibody ($\alpha C5a$), which were routinely added to each sample (see the Materials and methods section). Each result represents the mean of triplicate injections in a single bioassay rabbit. ■, Bioactive fractions originating from CM-1; ▒, bioactive fractions originating from CM-2. The bioactive fractions in (a) were pooled for further h.p.l.c.

oedema formation [4] and neutrophil accumulation [10,11] in rabbit skin. Briefly, rabbits were anaesthetized, the dorsal fur was removed, and ^{125}I -albumin and ^{111}In -labelled neutrophils were injected intravenously as tracers. Test samples were mixed with potentiating doses of prostaglandin E_2 (3×10^{-10} mol/100 μ l per skin site) to facilitate measurement [12] and of goat anti-rabbit C5a IgG (10 μ l/100 μ l per skin site) to block responses to any remaining C5a [5,13]. Samples were injected intradermally into triplicate skin sites according to a balanced site plan, and after 30 min the animals were killed by an anaesthetic overdose. The skin was removed and individual sites were punched out (17 mm diameter) for counting of ^{125}I -albumin and ^{111}In -neutrophil tracers. Results were calculated in terms of microlitres of plasma and numbers of labelled cells per skin site by comparison with the counts in 1 ml of blood.

Samples of h.p.l.c. fractions used for bioassay were freeze-dried in the presence of BSA and reconstituted to 0.1% BSA in 500 μ l of phosphate-buffered saline (PBS), pH 7.4 [6].

Cation-exchange chromatography

The C5a-depleted exudate was adjusted to pH 5.0 with 0.15 M-HCl and centrifuged (3500 g, 10 min). The supernatant was mixed with carboxymethyl-Sephadex C25 equilibrated in 0.15 M-NaCl/0.01 M-sodium acetate, pH 5.0 (50 ml of exudate/ml of gel). The gel was washed with the pH 5.0 buffer and eluted with 5 vol. of 1.5 M-NaCl/0.05 M-sodium phosphate, pH 7.4. The eluate was diluted, adjusted to pH 5.5 and applied to a TSK 535CM h.p.l.c. column (0.75 cm $\times$ 15 cm) as described previously [6]. The column was eluted at 0.5 ml/min with a linear ammonium acetate gradient (0.1–1.0 M, pH 5.5, 40 min) followed by a steeper gradient to 2.0 M-ammonium acetate, pH 7.5. Fractions were collected every 2 min and samples were freeze-dried in preparation for testing inflammatory activity in the skin oedema bioassay. Two broad peaks of activity were revealed, similar to those observed previously [5]. These peaks were termed CM-1 and CM-2 (results not shown).

Size-exclusion h.p.l.c.

All fractions containing CM-1 activity were pooled, freeze-dried, dissolved in 0.1% trifluoroacetic acid (TFA), centrifuged to discard the pellet (3500 g, 10 min) and applied (two runs of 300 μ l) to a TSK G-2000SW h.p.l.c. column (0.75 cm $\times$ 60 cm, equilibrated in 0.1% TFA). This was eluted at 0.5 ml/min, and 1 min fractions were collected. Fractions active in the skin oedema bioassay were pooled and freeze-dried for reversed-phase h.p.l.c.

All fractions containing CM-2 activity were pooled and treated similarly.

Reversed-phase h.p.l.c.

Bioactive material from size-exclusion h.p.l.c. of CM-1 and CM-2 was applied separately to a wide-pore (30 nm) Vydac C_{18} column (0.4 cm $\times$ 25 cm) in 0.1% TFA. The column was eluted at 1 ml/min with acetonitrile gradients in 0.1% TFA (0 to 27% acetonitrile at 3%/min; 27 to 45% acetonitrile at 0.5%/min; 45 to 90% acetonitrile at 3%/min), and 1 ml fractions were collected.

Fractions from the Vydac column containing CM-1 oedema-forming activity (hatched shading, Fig. 1a) were pooled and applied to a 30 nm, 5 μ m Deltapak C_{18} column (0.2 cm $\times$ 15 cm) in 0.1% TFA. The column was eluted at 0.25 ml/min with acetonitrile gradients in 0.1% TFA (0 to 24% acetonitrile at 2.9%/min; 24 to 44% acetonitrile at 0.5%/min; 44 to 80% acetonitrile at 2.9%/min). Fractions were collected, each containing 4 drops (approx. 50 μ l/fraction), and made up to 500 μ l with 0.1% TFA.

SDS/PAGE analysis

Samples from the bioactive reversed-phase fractions were analysed under reducing conditions using 8–25% acrylamide Phast gels and silver staining as described previously [6].

Amino acid sequencing

Samples from the narrow-bore reversed-phase h.p.l.c. were reduced and alkylated using 4-vinylpyridine as described by Hawke & Yuan [14]. The N-terminal sequence was derived using an Applied Biosystems 477A pulse liquid sequencer with on-line phenylthiohydantoin amino acid analysis (model 120A).

Materials

The narrow-bore Deltapak reversed-phase column was from Millipore (Watford, Herts., U.K.). 4-Vinylpyridine was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). The low-

molecular-mass markers were from Bio-Rad (Watford, Herts., U.K.). All other equipment and reagents were as described previously [6].

RESULTS

Purification

Two vascular-permeability-increasing mediators with neutrophil-chemoattractant properties [5,6] were isolated from the 6 h C5a-depleted inflammatory exudate fluid. Bioactivity in the protein-rich exudate was purified by use of a soft gel cation-exchanger at pH 5.0. This was followed by cation-exchange h.p.l.c. in an ammonium acetate gradient at pH 5.5, which revealed two broad peaks of oedema-forming activity, termed CM-1 and CM-2 (results not shown). This is consistent with our previous observation when the effective salt gradient was maintained at pH 5.5 [5].

Fractions containing CM-1 activity were subjected to size-exclusion h.p.l.c. Fractions containing CM-2 activity were treated similarly, and in each case a single peak of oedema-forming activity, of approx. molecular mass 9 kDa, was observed [6].

Active fractions from the size-exclusion step were applied to a Vydac C₁₈ reversed-phase column. Activities derived from CM-1 and CM-2 were chromatographed separately, and in each case substantial purification of oedema-forming activity was achieved (Figs. 1a and 1b). The major peak of bioactivity originating in CM-2 was eluted at approx. 33% acetonitrile. Since this contained RPF/IL-8/NAP-1 [6] as indicated by SDS/PAGE analysis (results not shown), no further purification was attempted.

The major peak of activity originating in CM-1 was eluted from the Vydac C₁₈ column at approx. 30% acetonitrile. This activity, RPF2, was subjected to a second reversed-phase step using a narrow-bore Deltapak C₁₈ column. Two major peaks of absorbance, oedema-forming activity and neutrophil-chemoattractant activity, were observed (Fig. 2). The earlier peak (Fig. 2, ■) was eluted with a retention time of 42.5–43.2 min (corresponding to fractions 45–47; Fig. 3). The second peak (Fig. 2, ▨) was eluted with a retention time of 43.4–43.8 min (corresponding to fractions 49–50; Fig. 3). Fraction 48 represented a dip in the observed absorbance and inflammatory activities.

SDS/PAGE analysis

Fractions from the narrow-bore reversed-phase step were analysed using 8–25% polyacrylamide gels under reducing conditions followed by silver staining. Fractions 45–47 and 49–50 revealed a single band of 6–8 kDa, suggesting a protein with an apparent molecular size slightly smaller than that of the IL-8/NAP-1 standard (Fig. 3).

N-terminal sequence analysis

Amino acid sequencing was performed on a pool of fractions 45–47 from the narrow-bore reversed-phase column (RPF2-45–47). Fractions 49 and 50, which corresponded to the second major peak of absorbance and bioactivity originating in CM-1 (Fig. 2), were pooled for a separate run on the sequencer (RPF2-49–50). Fraction 48 was omitted from sequencing, since it contained a mixture of the two absorbance peaks, although no differences in mobility were observed between fractions 45–50 in the SDS/PAGE analysis (Fig. 3).

Fractions 45–47 contained approx. 200 pmol of material, of which 100 pmol, reduced and alkylated, was applied to the first sequencing run. The first 30 amino acids were positively identified (Fig. 4) before the signal decreased abruptly, residue 31 being

Fig. 2. Narrow-bore reversed-phase h.p.l.c. of CM-1

Pooled bioactive fractions from wide-pore reversed-phase h.p.l.c. of CM-1 were freeze-dried, applied to a 5 µm Deltapak (30 nm) C₁₈ reversed-phase column and eluted with linear acetonitrile gradients. (a) A₂₁₄ and acetonitrile gradients. (b) and (c) 2% of each fraction was freeze-dried and assayed for oedema-forming (b) and neutrophil chemoattractant (c) activities in the rabbit skin bioassay (0.4% fraction/100 µl per skin site). The horizontal-broken line shows the weak response to prostaglandin E₂ (PGE₂), added to each sample before bioassay. Each result represents the mean of triplicate injections in a single bioassay rabbit. The shaded areas (■, RPF2-45–47; ▨, RPF2-49–50) indicate two peaks of bioactivity. Fractions comprising each of these peaks were pooled and subjected separately to N-terminal amino acid sequence analysis.

Fig. 3. SDS/PAGE analysis of inflammatory mediators separated by reversed-phase h.p.l.c.

Fractions 45–52 from the narrow-bore reversed-phase h.p.l.c. (Fig. 2) were run under reducing conditions on an 8–25% acrylamide gradient gel. The gel was silver-stained. Standards used were low-molecular-mass markers (indicated by the bars) and human recombinant IL-8/NAP-1 (8.2 kDa; 25 ng/0.5 µl; approx. 40 times the limit of detection). Fractions 45–47 (RPF2-45–47) were pooled, as were fractions 49–50 (RPF2-49–50) and used for N-terminal amino acid sequence analysis. ■, ▨, Fractions corresponding to respective shaded regions in Fig. 2.

Fig. 4. N-Terminal amino acid sequence of RPF2 and comparison with related proteins

RPF2-45-47 and RPF2-49-50 sequences were obtained from approximately 100 pmol and 70 pmol respectively of purified material (see the Results section). The boxes show sequence similarity between RPF2 and the published sequences of human MGSA/NAP-3/GRO [7-9], hamster GRO [7], rabbit IL-8/NAP-1/RPF1 [6] and human IL-8/NAP-1 [21-23]. X, unidentified amino acids in RPF1; -, gap to optimize amino acid alignment.

only tentatively identified as proline (results not shown). Because a similar sequence was expected in fractions 49-50, extended cleavage cycles were incorporated into the sequencer programme to accommodate proline at residues 29 and 31. Although less material was loaded in the second run (approx. 70 pmol), 36 N-terminal amino acids were positively identified (Fig. 4). There was no difference between the two samples in their first 30 residues. Sequence similarities with human and hamster MGSA/NAP-3/GRO and, to a lesser extent, with rabbit and human IL-8/NAP-1, are also given in Fig. 4.

DISCUSSION

We have recently reported that peritoneal exudate collected from rabbits 6 h after an intraperitoneal challenge with zymosan contains a novel neutrophil-chemoattractant protein RPF (henceforth termed RPF1), which represents the rabbit homologue of human IL-8/NAP-1 [6]. In addition, a second neutrophil-chemoattractant activity was present which could be separated from RPF1 by cation-exchange h.p.l.c. and which, like RPF1, inhibited the binding of 125 I-human IL-8/NAP-1 to human neutrophils [5]. The present paper demonstrates that this activity, RPF2, can be purified from exudate using similar techniques to those described previously for rabbit IL-8/NAP-1 (RPF1). Both the bioassay and absorbance profiles from the second reversed-phase h.p.l.c. step suggested heterogeneity of this activity (RPF2-45-47 and RPF2-49-50). On SDS/PAGE and N-terminal amino acid sequence analysis, however, the two peaks appeared to be identical. The observed difference in the retention of these bioactive peaks may therefore represent either amino acid substitutions towards the C-terminus or, alternatively, chemical modification of amino acid residues during the purification process.

Examination of the amino acid sequence obtained indicates that RPF2 is the rabbit homologue of human MGSA/NAP-3/GRO (78% sequence similarity) and hamster GRO (72% similarity). The characteristic Cys-Xaa-Cys sequence close to the N-terminus indicates that rabbit MGSA/NAP-3/GRO (RPF2) is the second member (RPF1 being the first) of a family of rabbit proteins forming the equivalent of a human protein family which includes platelet factor 4, connective-tissue-activating protein, β thromboglobulin, IL-8/NAP-1 and NAP-2. MGSA was originally identified as a mitogenic factor for melanoma cells [15,16] and subsequently several squamous cell carcinomas and adenocarcinomas [8]. In addition, it has been postulated that MGSA may be involved in an auto-stimulatory feedback mechanism, as MGSA can induce MGSA mRNA expression in melanoma cells and in human umbilical vein endothelial cells [17]. More recently, Schroder *et al.* have demonstrated the generation of MGSA, together with IL-8/NAP-1, from lipopolysaccharide-stimulated human monocytes [9], and from IL-1- or tumour necrosis factor-stimulated human fibroblasts in culture [18] and in extracts of

human psoriatic skin [19]. The inflammatory activity of MGSA includes human neutrophil chemotaxis *in vitro* [9,18] and neutrophil accumulation in rat skin *in vivo* [20]. This unusual dual function of MGSA/NAP-3/GRO as both an inflammatory mediator and a growth factor presents intriguing possibilities for its physiological role in mediating the sub-acute phase of the inflammatory response. The presence of both IL-8/NAP-1 and MGSA/NAP-3/GRO subsequent to C5a may indicate a subtle change in the inflammatory response from that of recruitment of phagocytic cells and soluble components in response to the stimulus to the complex regulation of these and other cells, possibly via autocrine growth mechanisms in order to promote resolution of the inflammatory episode. In other types of inflammatory conditions, e.g. direct tissue injury, cell growth factors such as MGSA/NAP-3/GRO may be involved in regulating wound repair. It is also possible, however, that the mitogenic actions of MGSA/NAP-3/GRO represent a function of the protein that is separate from its chemoattractant properties or that requires different conditions to be invoked.

In conclusion, the results presented in this paper provide further evidence *in vivo* for a link between a specific particulate stimulus and an inflammatory response involving the progressive generation of C5a followed by the cytokines IL-8/NAP-1 and MGSA/NAP-3/GRO. Further investigation of the actions of MGSA/NAP-3/GRO in this and other models will clarify the precise role of this protein in the inflammatory response.

This work was supported by the National Asthma Campaign, the Wellcome Trust and Pfizer Central Research (Groton, CT, U.S.A.). The h.p.l.c. equipment was generously provided by the Steel Charitable Trust. We thank Dr. K. Matsushima and Dr. J. J. Oppenheim for generously providing human recombinant IL-8, and Miss C. J. Coker for typing this manuscript.

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Received 5 March 1991; accepted 19 April 1991