

5

REPLACED SHEETS		SG
☒	Description : paged 4, 9-10, 13 to paged	
☒	Claim(s) : paged 36, 38-39 to paged	
☒	Drawing(s) : paged 6/13 to paged	
☐	Abstract : paged to paged	
DATE OF RECEIPT		

NOVEL CHEMOTACTIC FACTOR

10 This invention relates to a novel chemotactic factor, to the purification and characterisation of this factor from natural sources, and to the preparation of this factor as a synthetic or recombinant product. The invention further extends to analogues of the novel chemotactic factor, as well as to mutants, fragments and derivatives thereof. The invention also extends to antibodies to this factor (both polyclonal and monoclonal), to pharmaceutical compositions comprising the factor or the antibodies thereto, and to the therapeutic or diagnostic use of the factor or the antibodies thereto.

20 Leukocyte recruitment is vital for most inflammatory and immunologically-mediated responses. Chemotactic factors (CFs) released locally mediate directed migration of leukocytes and thus play an important role in the accumulation of these cells. Numerous CFs have been described including activated complement components (C5a, C3b), products of the activated coagulation cascade (kinins, thrombin, fibrino-peptides), bacterial and viral products, products of platelets, fibroblasts and neutrophils as well as the low molecular weight mediators leukotriene B4 and platelet activating factor (PAF) (reviews 1,2).

30 The delayed-type hypersensitivity (DTH) reaction is a widely studied example of cellular immunity. It is characterised by accumulation, over 4-48 hr, of leukocytes at the site of intradermally-injected antigen in a sensitised subject. Polymorphonuclear leukocytes (PMNs) and monocytes are dominant

in the lesion. The development of the reaction is considered to be due to the integrated release of numerous cytokines which not only control blood flow and vascular permeability but also determine the composition of the inflammatory infiltrate. It has been suggested that there may be a selective advantage in having a variety of factors (as evidenced by the number of CFs with activity towards, for example, PMNs) released under specific conditions and acting in particular micro-environments (3). Furthermore, the leukocyte composition of the inflammatory infiltrate depends on the temporal stage of the lesion and the nature of the stimulus. Thus activated lymphocytes may produce factors which are distinct from those derived from monocytes. Ward *et.al.* (4) and Altman (5) originally described the induction of CF when antigen was added to sensitised lymphocytes and the activity was attributed to a 12.5 kD protein (called lymphocyte-derived CF:LDCF). LDCF appears at the site of antigen challenge prior to macrophage influx (6) and has been extracted from DTH lesions (7). Miura *et.al.* (8) showed that activated Lyt 1+2-T lymphocytes from murine spleen were primarily responsible for the production of a macrophage CF but this factor has not been characterised and its relationship to the recently-described interleukin 8 family of monocyte-derived CFs (reviews 3,9) is unknown.

It has been previously proposed that fibrin, formed as a consequence of activated cell-mediated immunity (CMI), may enmesh infiltrating macrophages and maintain them at the inflammatory site (10). The generation of procoagulant by human monocytes reacting with microbial antigens is a close *in vitro* correlate of DTH reactions in man (11) and parallels the capacity of different mouse strains to develop DTH (12). Studies suggest that fibrin deposition at extravascular sites is mediated by macrophage procoagulant activity induced by an apparently unique T-cell derived product, macrophage procoagulant inducing factor (MPIF; 13-15).

Preliminary studies describing the characterisation of murine MPIF indicated activity associated with two heparin-binding peptides (α and β) with

pIs of 8.5 and 9.0 respectively (14). On the basis of studies with other recombinant cytokines, depletion of activity with antibodies (Ab) to some cytokines and a variety of bioassays, MPIF was proposed to be a newly described factor. Furthermore, chromatographic fractions highly enriched for MPIF induced strong responses characterised by an intense infiltrate of PMNs after 4 hrs and of both PMNs and mononuclear cells after 24 hr when injected intradermally into rat skin. In addition, skin sections showed significant interstitial fibrin deposition at 4 hr with maximal deposition at 24 hr post injection (15,16). The histopathological changes induced by MPIF $\alpha\beta$ were reminiscent of those described for skin reactive factor, a poorly characterised factor (17) distinct from macrophage migration inhibition factor (MIF) (18) which provokes DTH-like reactions when injected into normal skin. These results, also confirmed by *in vitro* experiments, indicated that a chemotactic activity was associated with MPIF $\alpha\beta$ (16), thereby suggesting multiple biological functions of MPIF.

It has now been discovered that MPIF is distinct from a chemotactic cytokine contained in the MPIF preparation previously described, and in one aspect the present invention relates to the purification and characterisation of this chemotactic factor.

Proteases appear to play a central role in chemotaxis. They are implicated in the generation of chemotactic factors derived from extracts of DTH reactions from guineapig skin (25,26), and serine proteases are apparently important in chemotaxis of neutrophils and macrophages (27,28), particularly along a substratum (29). It has been suggested that proteases bound to macrophages may cleave lymphocyte-derived CF precursor resulting in its activation (30). It has also been shown that chemotaxis of neutrophils in response to complement fragments or bacterial factors involves activation of a cell-bound serine esterase which apparently exists as a proenzyme and which is activated by chemotaxis. Heparin inhibits some types of chemotaxis and may act by suppressing the protease activity (31).

Accordingly, one aspect of the present invention is directed to a mammalian peptide having chemotactic properties, and having an apparent molecular weight of approximately 10 kD. The peptide of the present invention has chemotactic activity for neutrophils, monocytes/macrophages and/or other mammalian cells and also has apparent proteolytic activity which appears to play a role in leukocyte chemotaxis.

The peptide of this invention is referred to herein as chemotactic protein CP-10. It is to be understood, however, that the present invention extends to all analogues, mutants, fragments, derivatives, and functional homologues of CP-10 and to CP-10 molecules from all mammals such as from mice, humans and livestock animals. Use, therefore, of the term "CP-10" is meant to encompass all such forms of the molecule including single or multiple amino acid deletions, substitutions and insertions and/or any single or multiple deletions, substitutions and/or insertions of associated molecules such as carbohydrates, lipids or other peptides. All CP-10 molecules and derivatives, mutants, fragments and/or functional homologues thereof may be prepared by recombinant or chemical synthesis or may be naturally occurring.

The purification of CP-10 from a MPIF preparation, and in particular the purification of murine CP-10 from a murine MPIF preparation, is described in detail herein. In general terms, a solution containing the chemotactic factor may be purified by chromatography and affinity procedures and the desired compound isolated and, if required, fragments or derivatives prepared therefrom. The chemotactic peptide of this aspect of the invention may be purified from any solution containing the peptide, including cell-extracts, pre-purified cell extract, cell supernatant or culture filtrate of stimulated lymphoid cells or other mammalian cells or of permanent cell lines. The purification procedures used may in general be selected from ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, reversed phase chromatography or affinity chromatography employing heparin, Cu^{2+} , Zn^{2+} or antibodies to CP-10, or any



combinations thereof using open or high performance liquid chromatography systems. By appropriate purification methods, the chemotactic peptide of this invention, particularly murine CP-10, has now been obtained in essentially pure form.

5

N-terminal sequence analysis of the intact CP-10 molecule and tryptic and cyanogen bromide peptides has yielded a partial sequence with no potential glycosylation sites. By combining this partial N-terminal sequence with new data obtained by cDNA sequencing, the full length amino acid sequence of murine CP-10 has been determined to be:

10

15

Pro-Ser-Glu-Leu-Glu-Lys-Ala-Leu-Ser-Asn-Leu-Ile-Asp-Val-Tyr-His-Asn-Tyr-Ser-Asn-Ile-Gln-Gly-Asn-His-His-Ala-Leu-Tyr-Lys-Asn-Asp-Phe-Lys-Lys-Met-Val-Thr-Thr-Glu-Cys-Pro-Gln-Phe-Val-Gln-Asn-Ile-Asn-Ile-Glu-Asn-Leu-Phe-Arg-Glu-Leu-Asp-Ile-Asn-Ser-Asp-Asn-Ala-Ile-Asn-Phe-Glu-Glu-Phe-Leu-Ala-Met-Val-Ile-Lys-Val-Gly-Val-Ala-Ser-His-Lys-Asp-Ser-His-Lys-Glu

20

The calculated molecular weight of full length murine CP-10 has been determined to be 10,163D and the isoelectric point has been determined to be approx. 5.5.

25

30

No apparent amino acid sequence similarity is apparent with other CFs, interleukins, CSFs, interferons or tumor necrosis factors. Approx. 50% amino acid sequence similarity of CP-10 with two human calcium-binding proteins (CaBPs) recently identified in cells of myeloid origin and variously referred to as the cystic fibrosis (C.F.) antigen (19), MRP-8 or P8 and, MRP14 or P14 (20,21) respectively, is evident (see Fig.1 and Australian Patent Application No. 79309/87). The full length sequence of murine CP-10 reveals 69.6% homology with MRP8 at the cDNA level and 54.8% homology at the amino acid level (Fig.1a). These proteins are members of a family of highly conserved CaBPs of low molecular weight (all approx. 10 kD) including S-100 α and β proteins, calcyclin and intestinal CaBP (review 22). None of the S-100-related proteins contains a signal sequence but a conserved hydrophobic domain present in the N terminus of all the proteins (including CP-10) may

function as an internal signal sequence. Little is known about the function of MRP8 and 14; both are expressed in circulating neutrophils and monocytes but not in normal tissue macrophages (21,23). In chronic inflammatory conditions such as rheumatoid arthritis however, macrophages in affected tissues express both MRP8 and 14 whereas in acute inflammatory lesions only MRP14 is expressed (21). The proteins are also present in high levels in serum from cystic fibrosis patients (24). Interestingly, MRP8 and 14 were isolated from cultures of stimulated human mononuclear cells as part of a complex using a MAb directed against human MIF (21). However neither protein has MIF activity and their true biological function is unknown although by analogy with calmodulin they may be involved in intracellular signalling.

CP-10 of the present invention has also been shown to be a unique chemotactic factor on the basis of the following further experimental evidence. ³²P-labelled oligonucleotide probes complementary to the mRNA of CP-10 were chemically synthesised based on the amino acid sequences of:

1. Ile¹² - Tyr¹⁸
2. Tyr²⁹ - Met³⁶
3. Gln⁴⁶ - Asn⁵² (See Fig.1)

Positive clones reacting with all 3 probes were identified from a cDNA library from activated spleen cells from A/J mice in the vector λgt 10 by *in situ* plaque hybridisation. Re-screening at low plaque density with probe 3 (probe No.3 corresponds to the "hinge" or variant region) allowed selection of 2 positive clones. Probes 1 and 2 are prepared according to amino acid sequences in the highly conserved regions of CP-10 and CFa/MRP8 in the N-terminal portion of the molecules. Results indicate that the cDNA library contains sequences coding for the murine equivalent of CFa/MRP8 in the N-terminal region of the molecule which are similar to those coding for CP-10. In addition, sequences coding for the "hinge" regions of CFa/MRP8 (probe 4) and CP-10 (probe 3) indicate that the proteins have distinct differences.

Earlier studies showed that *in vivo* skin reactions elicited with guinea pig skin reactive factor (probably related to CP-10) was suppressed by serine protease inhibitors and implicated involvement of proteolytic activity in cell recruitment in DTH reactions (32). Chemotactic activity of CP-10 is inhibited by heparin and heparin fragments with high affinity for antithrombin III. Although CP-10 is a heparin-binding protein, it is not yet known whether heparin inhibits via direct binding to CP-10 or by interacting with a cell membrane component essential for chemotaxis. It is suggested that CP-10 has proteolytic activity and that this activity plays a role in leukocyte chemotaxis.

Because of its chemotactic activity, CP-10 or its analogues, or CP-10-derived peptides with chemotactic activity may be useful if administered locally (e.g. around a tumor, site of infection) to attract neutrophils and/or monocytes to the site, particularly in immuno-compromised patients (i.e. those with severe combined immunodeficiencies, AIDS, tumors, burns etc.) or any patient who is allergic to skin test antigens (Type IV hypersensitivity).

In addition, CP-10 may contribute to inflammatory pathology in various conditions including those with strong involvement of T-cells. These include, for example, infection (bacterial, parasitic, fungal, viral), malignancy, kidney and other transplants, skin disorders (e.g. atopic dermatitis, psoriasis), granulomatous disease (e.g. sarcoidosis), lung diseases (e.g. idiopathic pulmonary fibrosis, ARDS, asthma), autoimmune diseases characterised by chronic inflammation (e.g. rheumatoid arthritis, SLE, diabetes), myocardial infarction (CP-10 may contribute to the reperfusion damage associated with release of inflammatory mediators from neutrophils), and thrombosis (neutrophil products may be involved in recanalisation of blocked vessels).

Accordingly, CP-10 or analogues, mutants, fragments or derivatives of CP-10 may be used to alter the inflammatory capacity or metabolism of mammalian cells. Similarly, these compounds or antibodies or antagonists

thereto, particularly antibodies to the chemotactic domain of CP-10, may be useful anti-inflammatory agents.

Since CP-10 is associated with delayed-type hypersensitivity reactions and is responsible for similar histopathology when injected intradermally it has potential therapeutical applications. First, a composition comprising an antibody to CP-10 may be administered to a patient undergoing a delayed-type hypersensitivity reaction, for example one with a contact allergy to heavy metals, such as gold, or to agents such as poison ivy. Secondly, a non-functional antagonist may be used for this purpose. Alternatively, CP-10 or peptides thereof may be used in patients who are immunocompromised - such as those with AIDS, or those undergoing immunosuppressive therapy, to attract phagocytes to, and to activate phagocytes at sites of infection such as the pneumocystis - infected lung.

In addition, as a number of chronic inflammatory conditions are characterised by a localised high concentration of phagocytic cells, the administration of antibodies against CP-10 may inhibit inflammation and therefore provide the basis for their therapeutic use. This would apply to such conditions as autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosis, coeliac disease, multiple sclerosis, Guillian Barre syndrome, autoimmune thyroiditis, rejecting grafts, tumours, etc. In general, monoclonal antibodies or non-functional analogues or antagonists of CP-10 may be used in diseases currently treated using steroid therapy.

Occupancy of receptors of chemoattractants on phagocytes initiates cellular responses that are essential for normal host defence and wound healing. Intracellular signals generated by chemoattractant receptor occupancy are capable of regulating gene expression, such as of the oncogenes co-fos and c-myc, to produce distinct modulatory signals. All chemoattractants known to date interact initially with a receptor molecule on the cell surface which initiates these events. Non-functional analogues or antagonists of CP-10 may

suppress chronic inflammation by inhibiting CP-10 receptor binding. Binding of CP-10 to its receptor would be the trigger for the cellular responses including chemotaxis.

5 Thus, in a further aspect of the present invention, there is provided a pharmaceutical composition comprising CP-10, or an analogue, mutant, fragment or derivative thereof, or an antibody thereto in association with a pharmaceutically acceptable carrier or diluent. The active component of such a pharmaceutical composition may of course either be derived from natural
10 sources or be prepared by synthetic or recombinant DNA techniques. Antibodies may be prepared by known techniques as described in greater detail hereinafter.

15 Cystic fibrosis (C.F.) is the most common lethal genetic syndrome among white children and is also the cause of much of the chronic progressive pulmonary disease encountered in children. Early features of lung disease in C.F. are of an inflammatory nature and are non-infective. The majority of cells infiltrating the lung are PMNs and many appear to be dead or degenerating. C.F. antigen comprises about 20% of the total cytoplasmic
20 protein of neutrophils and is responsible for calcium binding to yield the typical turbidity characteristic of C.F. saliva. C.F. antigen (MRP8, 14) is also present in inflammatory macrophages. The function of these proteins is unclear although they may be involved in the killing of pathogens. CP-10 may be involved in recruitment and activation of PMNs in the lung in C.F.

25 Furthermore, it has been suggested that the inflammatory lung disease, critical in patients with cystic fibrosis, may be mediated by proteases. These patients have low levels of serum protease inhibitors (indicating binding with active proteases) and tissue damage similar to that believed to occur in
30 lungs of patients with emphysema by proteolytic enzymes, is proposed (33).

This Sheet Has Been Replaced





Accordingly, in view of the proteolytic activity of CP-10, therapeutic intervention by CP-10, by analogues of CP-10 or of the active site of the protease, by specific protease inhibitors or by antibodies to CP-10 or to the active site may be possible. These reagents may be useful, not only to control inflammation and tissue damage in inflammatory conditions, but also in cystic fibrosis and emphysema.

As broadly described above, the present invention extends to analogues, mutants, fragments and derivatives of CP-10. Suitable analogues include chemically modified CP-10-like peptides, and the term "analogues" is used herein to include any functional chemical equivalent of CP-10 or fragments thereof which has CP-10-like properties and activity. Analogues contemplated herein include, but are not limited to modifications to side chains, incorporation of non-natural amino acids and/or their derivatives during peptide synthesis, and the use of cross-linkers or other conformational constraints during synthesis. Suitable mutants include, for example, mutants of the murine CP-10 sequence set out above or the human or other mammalian equivalent thereof, in which single or multiple amino acids are added or deleted or replaced by different amino acids. Fragments of CP-10 include, for example, fragments of the murine CP-10 sequence set out above or the human or other mammalian equivalent thereof, comprising at least 10 and preferably at least 20 consecutive amino acids. Particularly preferred fragments are those based on or corresponding to the amino acid "hinge" or "variant" region of the murine CP-10 sequence between amino acids Pro⁴² and Arg⁵⁵. Derivatives of CP-10 include, for example, derivatives of the murine CP-10 sequence set out above or the human or other mammalian equivalent thereof, wherein amino and/or hydroxyl functions are glycosylated.

The present invention further extends to homo- and hetero-dimers of CP-10 wherein the two CP-10 moieties or one CP-10 moiety and a heterologous peptide are bound together, for example through an intermolecular S-S bridge or by non-covalent binding. Once again, the CP-10

moiety may, for example, be murine CP-10 having the sequence set out above or the human or other mammalian equivalent thereof.

5 The present invention also extends to antibodies, both polyclonal and monoclonal, or fragments thereof or specific binding moieties based on the structure of immunoglobulin, reacting with CP-10 as well as antagonists of CP-10. In particular, in this aspect the invention includes such antibodies directed to murine CP-10 having the sequence set out above or to the human or other mammalian equivalent thereof, or to peptide epitopes thereof. The antibodies
10 may also be directed to the analogues, mutants, fragments or derivatives of CP-10 as described above.

Such antibodies or antagonists may be used to alter the chemotactic activity of CP-10, to alter the proteolytic activity of CP-10, to purify CP-10 by
15 affinity chromatography, or they may be used as diagnostic reagents to detect CP-10 in body fluids such as urine, blood, serum, plasma, in cultures of mammalian cells, or in tissue sections.

The chemotactic factor CP-10 of this invention, or analogues,
20 mutants, fragments or derivatives thereof, as described herein may be synthesised by solid phase peptide synthesis or other known chemical techniques for peptide synthesis. Alternatively, "recombinant" CP-10 or mutants, fragments or derivatives may be prepared by use of the recombinant DNA techniques which are now well known and widely used. Both chemical
25 synthesis and preparation by recombinant DNA techniques provide means whereby analogues, mutants, fragments and derivatives can be readily prepared, as well as CP-10 itself. Preparation of these compounds by recombinant DNA techniques will usually comprise the steps of:

- 30 (a) isolating a DNA coding for the desired compound or a fragment thereof from a cDNA or a genomic DNA library of murine or human

cells and optionally mutating it, or chemically synthesising such a DNA,

- (b) incorporating the DNA into an appropriate expression vector,
- (c) transferring the obtained hybrid vector into a recipient host,
- (d) selecting the transformed host from untransformed hosts by culturing under conditions under which only the transformed host survives,
- (e) culturing the transformed host under conditions which allow expression of the heterologous polypeptide, and
- (f) isolating the desired compound, mutant, fragment or derivative thereof,

and, if required, derivatizing the obtained compound, mutant or fragment thereof.

Purification of murine CP-10 from a murine MPIF preparation, and techniques for production of recombinant CP-10 are described above. CP-10 of human or other mammalian origin may be identified by techniques which are well known in the art.

For example, by purification of stimulated human lymphoid cells or of cell lines of culture supernatants or other human cells to homogeneity using procedures similar to those described using open and high performance liquid chromatography or by affinity of human CP-10 to antibodies to murine CP-10 which may cross-react with the human equivalent. Confirmation of amino acid sequence by N-terminal sequence analysis of the intact molecule and of fragments thereof may be carried out as described for murine CP-10. Alternatively, positive clones may be identified from human cDNA libraries on

the basis of hybridisation with oligonucleotide probes prepared from the murine CP-10 sequence, particularly according to the amino acid sequence of the novel "hinge" region sequence of murine CP-10 as described herein. Preparation of human CP-10 by recombinant DNA techniques using DNA from such positive clones is described above.

Comparative testing of the chemotactic activity of full length murine CP-10 and a peptide corresponding to the "hinge" region of CP-10 (CP-10₄₂₋₅₅) has revealed that the smaller peptide has similar chemotactic potency to full CP-10.

Accordingly a further aspect of the invention relates to a chemotactic peptide of amino acid sequence Pro-Gln-Phe-Val-Gln-Asn-Ile-Asn-Ile-Glu-Asn-Leu-Phe-Arg(-Tyr) and derivatives, functional homologues, mutants or substitutions thereof. This peptide has chemotactic activity for neutrophils, monocytes/macrophages or other mammalian cells.

The following Examples and accompanying drawings illustrate the steps and procedures used in the purification and characterisation of murine CP-10 in accordance with this invention. In the drawings:

Fig.1 is a comparison of amino acid sequences of CP-10 and various S-100 proteins.

Alignment of the amino acid sequences (single letter amino acid code) was achieved by introducing a minimal number of short gaps into individual sequences. Conserved regions between at least three of the S-100 proteins are boxed. Regions of absolute similarity are shaded. Regions typical for calcium binding domains of S-100 proteins (position 19-32 and 62-73) and the N-terminus are highly conserved in all S-100 proteins (22).

This Sheet Has Been Replaced



Fig.1A is a comparison of the amino acid and nucleotide sequences of full length murine CP-10 and hMRP8.

Fig.2 shows a C₄-RP-HPLC profile of CP-10.

5 Con A stimulated spleen cell supernatant was concentrated and purified by cation exchange, Cu⁺⁺ affinity and Cu⁺⁺ PolyCatA mixed batch MPLC as described in steps 3.1, 3.3.1, 3.2.1, 3.3.2 and applied to reversed-phase HPLC on a Bakerbond C₄-WP HPLC column. Aliquots of 1 min fractions were assayed for chemotactic activity. Absorbance at 214 nm
10 (-) and chemotactic activity of peak fractions in 0.1% (lighter boxes) and 0.5% (darker boxes) aliquots is shown.

Fig.3 shows a size exclusion HPLC profile of CP-10.

15 A preparation of CP-10, purified on reversed phase HPLC (C₄-RP-HPLC, step 3.2.3) was subjected to gel filtration HPLC on a TSK G3000SW column and tested for chemotactic activity as described in step 3.2.4. Aliquots of fractions (0.5%) are used at 1:2 (darker boxes) and 1:4 (lighter boxes) dilution. Reference proteins chromatographed under the same conditions provide a m.wt. calibration curve; 1: OVA (44 kD), 2: soybean trypsin
20 inhibitor (20.1 kD), 3: cytochrome C (12.4 kD), 4: aprotinin (6.5 kD).

Fig.4 shows C₈-RP-HPLC profiles of CP-10.

25 Homogeneous CP-10 obtained by rechromatography of material with chemotactic activity from S.E. HPLC (Fig.2) on analytical C₈-RP-HPLC (*) is injected onto microbore C₈-RP-HPLC. Peaks A and B are collected manually and each subjected to chemotaxis assay (step 1). Hatched bars represent chemotactic activity of peaks A and B.

Fig.5 shows gel electrophoresis of CP-10.

30 Aliquots of CP-10 preparations, which were homogeneous as judged by HPLC on an Aquapore RP300 HPLC column (step 3.2.6) were subjected to Tricine-SDS PAGE on a 16.5% polyacrylamide gel under reducing conditions

(5% β -mercaptoethanol) as described (35). Electrophoresis was carried out for 18 h at 90 V and gels were silverstained according to Heukeshoven and Dernick (36).

The Figure represents mounted photographs of two gels; lanes 1-3, dilutions of molecular weight marker proteins (14.4 - 90.0 kD, Pharmacia); lane 1, CP-10 purified as described in steps 3.1-3.2.3, 3.2.5 (C_8 -RP.HPLC) and 3.2.6; lane 2, CP-10 purified as described in steps 3.1-3.2.4 and 3.2.6; lanes 4-6, dilutions of molecular weight marker peptides (2.4-17.2 kD, Pharmacia).

Fig.6 is a graphic illustration of the microbore RP-HPLC separation of peptides of Cm-CP-10 derived from digestion with trypsin.

Fig.7 shows the partial amino acid sequence of murine CP-10 obtained by chemical sequencing and the various peptides used in obtaining this sequence.

Fig.8 shows the C_8 RP-HPLC of CP-10 and titration of chemotactic activity.

Chemotactic protein eluting from SE-HPLC was applied to a C_8 Aquapore HPLC cartridge and eluted with a gradient of CH_3CN (—). Homogenous CP-10 was collected manually according to the absorbance at 214 nm (—) and diluted serially for chemotaxis assays using murine neutrophils. Mean values (●) of a representative experiment are shown (inset to the figure). Chemotactic activity of 5% EAMS (○) is given for comparison.

Fig.9 illustrates the chemotactic response of murine and human cells to CP-10 and a derived peptide, CP-10₄₂₋₅₅.

Homogeneous CP-10 obtained from C_8 -RP.HPLC (step 3.2.6) and synthetic CP-10₄₂₋₅₅ were diluted 10-fold between 10^{-7} and 10^{-15} M into RPMI 1640 containing 0.1% BSA and tested for chemoattractant activity. Murine PMN (Panel A), human PMN (Panel B) and murine mononuclear cells or a murine monocytoid cell line, WEHI 265 (Panel C) were used as effector cells. The chemotactic response to native CP-10 (—●—) and CP-10₄₂₋₅₅ (--○--) was

compared to the chemotactic activity of 5% EAMS (panel A,X) or 10^{-7} M FMLP (panel C,X). In addition, murine macrophages were tested for their response to CP-10₄₂₋₅₅ (panel C, --●--).

Fig.10 shows overexpression of mCP10 in *E.coli* using pGEX expression system.

EXAMPLE 1

Step 1. Measurement of bioactivity

Aliquots of chromatography fractions without organic solvents are diluted directly with RPMI 1640 (Gibco, Grand Is, NY) before assay. To remove organic solvent prior to assay, fractions (5-100 μ l) are freeze-dried in the presence of 2 μ g of albumin (Calbiochem; 5 x crystalline) and reconstituted to the original volume with RPMI 1640. Samples to be stored before testing are kept at -70°C , preferably in the solvent used for elution.

CP-10 associated MPIF activity is measured as described by J. Ryan and C. Geczy, (16). Chemotaxis of mouse polymorphonuclear leukocytes (PMN) is measured as described by Falk et al. (34) Briefly, PMN are obtained from the peritoneal cavity 1 day after ip injection with thioglycollate broth. Chemotaxis is assayed in a 48 - well microchemotaxis chamber (Neuro Probe Inc., Bethesda, MD). The upper wells contain cells (2.5×10^4) in 50 μ l RPMI 1610 containing 5% heated (56°C , 30 min) fetal calf serum (FCS) or 0.1% bovine serum albumin (BSA; Calbiochem). The lower wells contain separated fractions diluted in RPMI 1640 with 5% FCS or 0.1% BSA. The upper and lower chambers are separated with a PVP-free polycarbonate membrane (Nucleopore, 5 μ m; Neuroprobe). The chamber is incubated for 1.5-2 hr at 37°C after which the filter is removed, air dried and stained. Migrating cells are quantitated over 5 microscope fields (x40 objective) using Image Analysis (Wild-Leitz, Lane Cove, Australia).

Step 2. Preparation of the CP10-containing supernatant

Spleen cells from QS-mice are isolated and cultured for 24 hr with Sepharose-4B bound Concanavalin A (Pharmacia, North Ryde, Australia) and the cell supernatant is harvested as described earlier by C J. Ryan and C. Geczy, (14). Prior to storage at -70 °C supernatants are adjusted to 0.1 µg/ml DNase, 0.1 µg/ml RNase (Boehringer Mannheim, North Ryde, Australia), 0.01% Tween-20 (Sigma) and 0.25 mM phenylmethylsulphonyl fluoride (PMSF, Sigma).

Batches of 5-6 l supernatant are concentrated to 150-200 ml and dialysed against 0.05 M NaCl in buffer A (0.02 M Tris -HCl, 0.01% Tween-20, 0.25 mM PMSF, pH: 6.5) using a spiral ultrafiltration cartridge (mol. weight cut off: 10,000D, Amicon Scientific, Australia).

Step 3. Isolation and Purification of CP10

Step 3.1. Heparin-Sepharose affinity chromatography

The concentrated supernatant is centrifuged at 10,000 g, 4 °C for 30 min and loaded at a flow rate of 18 ml/h, 4 °C (peristaltic Minipuls 3-pump, Gilson) onto a column (16 x 160 mm) of Heparin Sepharose 6B (Pharmacia) equilibrated in buffer A. The column is washed with 0.05 M NaCl and with 0.2 M NaCl in buffer A until the absorbance at 280 nm (UV-detector 115, Gilson) reaches background levels and bound material is eluted with a NaCl gradient (total vol, 100 ml) of 0.2-M NaCl in buffer B (0.02 M Tris-HCl, pH: 6.8) at a flow rate of 7 ml/h. Fractions of 2.5 ml are collected and aliquots of individual fractions assayed at 5% and 2.5% (v/v) for bioactivity. CP-10 associated with macrophage procoagulant inducing factor (MPIF) elutes in two peaks between 0.6 and 0.9 M NaCl (14,16).

Step 3.2. High performance liquid chromatography (HPLC)

Nonmetallic "Waters 650 Advanced Protein Purification Systems" equipped either with a Waters 484 Tunable Absorbance Detector or a Waters 490 Programmable Multiwavelength Detector and a Gilson 202 Fraction Collector are used for all MPLC and HPLC separations. Unless otherwise stated, samples were loaded onto columns by using an HPLC pump and one of the four solvent lines of the HPLC system. After sample load the same solvent line is used to pump equilibration buffer through the HPLC system until the absorbance reaches the baseline level of the solvent in use.

10 Step 3.2.1. Zinc-, copper-affinity chromatography

Step 3.2.1.1. Preparation of Zn^{++} and Cu^{++} affinity medium pressure liquid chromatography (MPLC) columns

15 Suspensions of iminodiacetic acid (IDAA) immobilized on TSK-HW 65F gel particles (Pierce, Rockford, IL, USA) are incubated with solution, of 1mg/ml $ZnCl_2$ or 1 mg/ml $CuSO_4$ for 30 min at r/t and fines are removed from the gel slurry as described by the manufacturers (Fractogel TSK catalog, E. Merck, F.R.G.). Gel-bound chelates of zinc and copper in H_2O are packed at constant
20 flow (3 ml/min) into individual glass columns (10 x 100 mm; Waters Advanced purification -1 glass columns, Millipore/Waters, Lane Cove, Australia) and the columns are washed subsequently with each 200 ml H_2O and 2 M NaCl in buffer B at 3ml/min.

25 Step 3.2.1.2. Cu^{++} -affinity MPLC

The pooled fractions of Step 3.1. are adjusted to 2M NaCl and pumped at a flow rate of 3 ml/min onto a Zn^{++} - and a Cu^{++} -affinity column in sequence (equilibrated at 2M NaCl in buffer B), beginning with the Zn^{++} -affinity
30 column. When the absorbance at 280 nm reaches baseline levels, the columns are disconnected and bound material eluted separately with a ternary gradient of 0.1 M to 1.0 M NaCl and 0.00 M to 0.05 M imidazole in Buffer B at a flow

rate of 3.25 ml / min. Fractions are collected every minute and aliquots of individual fractions tested at 5% and 2.5% for bioactivity. MPIF-associated chemotactic activity elutes only from the Cu^{++} -affinity MPLC column at a buffer composition of 0.02 M imidazole, 0.1 M NaCl in buffer B.

5

Step 3.2.2. High resolution cation exchange HPLC

Pooled fractions from step 3.2.1.2. are diluted four fold with H_2O and loaded at 1.5 ml/min onto a Bakerbond Wide-Pore CBX HPLC column (4.6 x 250 mm, JT. Baker, Phillipsburg N.J., USA) equilibrated with 0.05 M NaCl in 0.02 M Tris-HCl, pH 6.5. Bound proteins are eluted with a 0.05-0.5 M gradient of NaCl in 0.02 M Tris-HCl pH 6.5-7.0.

The absorbance of the eluate is monitored at 280 nm and 1 min fractions collected automatically. Aliquots assayed at 5% and 10% (v/v) revealed bioactivity between 0.25 and 0.35 M NaCl.

Step 3.2.3. Reversed-phase HPLC on butyl wide pore silica

Pooled material from step 3.2.2. (having specific activity) is adjusted to 0.1% TFA and loaded onto a Bakerbond C4-wide pore HPLC column (4.6 x 250 mm), equilibrated in 80% Solvent C and 20% solvent D; solvent C is 0.1% TFA in water and solvent D is 80% acetonitrile, 0.1% TFA in water. After 5 min at this solvent composition proteins are eluted with a three-step gradient of solvent D in solvent C ranging from 20% to 40% D in 10 min followed by 40% to 50% D in 20 min and 50% to 100% D in 20 min. Aliquots (0.5%) of individual 1 min fractions are assayed for chemotactic activity and reveal peak activity eluting with 49.4% solvent D (39.5% acetonitrile) as part of a complex peak eluting with 52% solvent D (41.6% acetonitrile). The absorbance profile and chemotactic activity of relevant fractions is shown in Figure 2.

Step 3.2.4. Size exclusion HPLC

Peak fractions of step 3.2.3. are concentrated to 50 μ l, using a "Speed Vac" Concentrator (Savant), adjusted to 40% acetonitrile, 0.1% TFA in water and injected via a Model 9125 Rheodyne sample injector (Rheodyne, CA., USA) onto a TSKG 3000 SW high performance gel filtration column (7.5 x 300 mm, LKB) equilibrated with 40% acetonitrile, 0.1% TFA in water at 0.5 ml/min. Fractions are collected manually according to the absorbance profile of the eluate at 214 nm and 0.5% aliquots of individual fractions diluted to 50, 25 and 10% are tested for chemotactic activity.

As shown in Figure 3 CP-10 is eluted with an apparent molecular weight of 10.3 kD, when compared to standard molecular weight markers aprotinin (6.5 kD), cytochrome C (12.4 kD), soybean trypsin inhibitor (20.1 kD) and ovalbumin (44 kD).

Step 3.2.5. Reversed-phase HPLC on octadecyl wide-pore silica

As an alternative to the separation on size exclusion HPLC (3.2.4.) pooled material from step 3.2.3. is diluted 4 x with aqueous 0.1% TFA and loaded onto a Bakerbond C₁₈-wide pore HPLC column (4.6 x 100 mm; JT. Baker), equilibrated at 1 ml/min in 68% solvent C and 32% solvent D. A two step gradient of solvent D in solvent C is applied 10 min after loading the sample, starting with 32-59% D in 10 min followed by 59%-70% D in 30 min. CP10 elutes with 49.7% acetonitrile in 0.1% aqueous TFA (62.1% solvent D), with a purity of approximately 90% as judged by SDS-Tricine PAGE (35) and silverstaining (36).

Step 3.2.6. Reversed-phase HPLC on octyl wide-pore silica

Analytical and microbore reversed-phase C₈ HPLC is performed on Aquapore RP300 Cartridges (4.6 x 100 mm and 2.1 x 100 mm, 7 μ m silica, Applied

Biosystems, Foster City, CA, USA) equilibrated in 100% solvent C.

Preparations of CP10 from step 3.2.4. or 3.2.5. are diluted 4 fold with solvent C and loaded in multiple 1 ml injections via the Rheodyne injector onto the HPLC cartridge.

5

The flow rate for loading a sample onto the analytical cartridge is 1.5 ml/min and onto the microbore cartridge is 0.15 ml/min. A gradient of 1%/min acetonitrile in solvent C over 60 min is started 5 min after the last injection.

10

The flow rate on the analytical cartridge is kept at 1 ml/min, on the microbore cartridge at 0.1 ml/min and eluting material is collected manually according to the absorbance at 214 nm.

15

Fig. 4 shows that CP-10 elutes as a symmetrical peak with retention times of 47.7 min and 49.2 min (equivalent to 47.5% acetonitrile) from the analytical and the microbore C_8 -HPLC cartridges respectively. This preparation has chemotactic activity in the range 10^{-12} - 10^{-13} M.

20

Analysis on silverstained (36) SDS-Tricine PAGE under reducing conditions (5% β -mercaptoethanol) of CP-10 purified in steps 3.2.5. and 3.2. 6. reveals a predominant band at 10kD (more than 95%) and a minor band at 65 kD. Material purified in steps 3.2.4. and 3.2.6. reveal the same predominant band at 10-kD and a minor band of 14 kD as shown in Fig. 5.

25

Step 3.3. Alternative method of purifying CP10 from concentrated supernatants of Con A stimulated murine spleen cells

Step 3.3.1. Cation exchange HPLC on Poly (Aspartic Acid) - Silica

30

The crude supernatant of step 1 is centrifuged at 10,000g, 4 °C for 30 min and proteins are separated on a column (10 x 300 mm, Advanced Purification-1 glass column, Millipore/Waters) of Poly CAT A (polyaspartic acid) silica (Poly LC, Columbia, M.D., USA). The column is packed using a slurry of the silica

particles (15-20 μm diameter, 30 nm pore size) in 100% isopropanol under constant flow as described in step 3.2.1.1.

5 For chromatography of CP10 the column is equilibrated with 0.02 M Tris, pH6.5 at 3 ml/min and washed with this buffer after loading of the sample until the absorbance 280 nm reaches baseline levels.

Bound material is eluted at 3 ml/min using a gradient of 0-0.5 M NaCl in Tris-HCl, pH 6.5-7.0 over 40 min and the absorbance monitored at 280 nm.
10 Fractions are collected automatically every min and assayed for bioactivity.

Material eluting in the range from 0.17-0.33 M NaCl was pooled for further purification.

15 **Step 3.3.2. Mixed mode HPLC on IDAA-Cu⁺⁺ TSK/Poly Cat A-Silica**

The pooled material from step 3.3.1. is adjusted to 2 M NaCl and separated on IDAA-Cu⁺⁺ affinity HPLC as described in 2.2.2. Proteins eluting with 0.02 M imidazole/0.1 M NaCl are collected manually and diluted
20 fourfold with buffer B for mixed mode HPLC.

A 10 x 100 mm Advanced purification 1- glass column (Millipore/Waters) is packed under constant flow with a 1:1 mixture of IDAA-Cu⁺⁺TSK65F and Poly Cat A-silica in 3 M sodium acetate as packing solvent.
25 The column is equilibrated at 2.25 ml/min with buffer B and the sample is loaded at this flow rate following the procedure described in 3.2.1.1.

The column is washed with 0.6 M NaCl in buffer B (5 min at 2.25 ml/min), buffer B (5 min at 2.5 ml/min), 0.02 M imidazole/0.06 M NaCl in buffer B (10 min at 2.5 ml/min) and buffer B (10 min at 2.5 ml/min). Bound
30 material is eluted with a binary gradient of 0.06 -1.0 M NaCl and 0.0-0.05 M imidazole over 40 min at a flow rate of 2.5 ml/min. Fractions are collected

every minute and aliquots assayed at 2.5% and 5% for CP-10-associated bioactivity.

Proteins eluting in the range 0.24 M NaCl/0.01 M imidazole and 0.4 M NaCl/0.02 M imidazole were adjusted to 0.1% TFA for chromatography on C4-RP-HPLC (step 3.2.3.).

Step 3.4. Cyanogen bromide cleavage and peptide mapping on RP - HPLC

Homogeneous CP-10, obtained as manually collected fractions from Step 3.2.6 - (approx. 2.5µg) in 48% acetonitrile in buffer C, 0.02% Tween 20 is lyophilized to near dryness (10µl) and resuspended to 100µl with 70% formic acid. Cyanogen bromide (2.5mg) is added under nitrogen, the tube sealed and incubated in the dark at 25 °C for 20h. The peptide mixture is diluted 50 fold with H₂O and injected in 1 ml portions onto an Aquapore RP300 cartridge (2.1 x 100mm, ABI), equilibrated at 0.5ml/min with 100% solvent C. Elution of peptides is achieved at 0.1ml/min as described in step 3.2.6 and peptides are collected manually according to the absorbance at 214 nm.

Step 3.5. Trypsin digestion and peptide mapping on RP HPLC

Step 3.5.1. Preparation of murine S-carboxymethyl-CP-10 (Cm-CP-10)

CP-10 (5µg) in 120 µl 35% aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid (F₃AcOH; 99% pure, Pierce Chemical Co., Rockford III, USA) and 0.02% Tween 20 (Pierce) is concentrated to approximately 10 µl by centrifugal centrifugation (Savant, Ind. Hickville, NY), diluted to 160 µl with 7.5 M guanidine.HCl (Sequal grade, Pierce) containing 0.2 M Tris.HCl buffer, pH 8.5, 0.002 M EDTA and 0.02% (v/v) Tween 20 and then reduced with dithiothreitol (Calbiochem La Jolla, Ca. USA; 0.015 M) at 40 °C for 4.5

h. Alkylation is achieved by the addition of iodacetic acid (puriss grade, Fluka, Buchs, Switzerland; final concentration, 0.05 M) to the mixture and incubation continued for 30 min at 25 °C in the dark. The reaction is halted by addition of 25 µl of 2-mercaptoethanol. Cm-CP-10 is recovered from the mixture using a reversed-phase high-performance liquid chromatography (RP-HPLC) procedure previously described by Simpson et al (37). The Cm-CP-10 containing fraction (60 µl) is adjusted to 0.02% (v/v) with respect to Tween 20 and then diluted to 1 ml with 1% (w/v) NH_4HCO_3 (containing 0.02% (v/v) Tween 20) prior to enzymatic digestion.

Step 3.5.2. Trypsin digestion

Cm-CP-10 (4 µg) in 1 ml of 1% (w/v) NH_4HCO_3 , pH 7.8 containing 0.001 M CaCl_2 and 0.02% (v/v) Tween 20 is digested with 0.5 µg trypsin (treated with tosylphenylethylchloro-methane; Worthington Biochemical Co. NJ, USA) for 16 hrs at 37 °C.

Step 3.5.3. Purification of polypeptides by high-performance liquid chromatography instrumentation

Peptide mixtures resulting from trypsin digestion are fractionated by reversed-phase HPLC on a Hewlett-Packard liquid chromatograph (model 1090A)-fitted with a diode-array detector (model 1040A) as described (37) employing a low-pH (F_3 AcOH, pH2.1) mobile phase and a gradient of acetonitrile.

Reversed-phase fractionation of these digests, however, results in a complex pattern of peptide-containing peaks (Fig. 6). All of the peptide fractions from the first dimension RP-HPLC are subjected to a second chromatographic step using the same chromatographic support and acetonitrile gradient but a different mobile phase (e.g., unbuffered sodium chloride or 20 mM sodium phosphate, pH 7.0). For some peptides a third chromatographic

step is necessary before a homogeneous peptide is isolated. In the latter situation, an ODS-hypersil column and a different organic solvent (methanol) are used (37).

5 Column support used for the purification of Cm-CP-10 and derived peptides is Brownlee RP-300 (300 nm pore size, 7 μ m particle diameter, octylsilica packed into a stainless steel column 100 x 2.1 mm i.d; Brownlee Laboratories, Santa Clara, USA).

10 **Step 3.5.4. Peptide nomenclature**

The following prefixes are used to denote the origin of various peptides: T, Trypsin, CN, Cyanogen bromide. Peptides are numbered in the order of their positions in the final sequence.

15 **Step 3.6. Amino acid sequence analysis**

Automated Edman degradation of protein and peptides is performed using Applied Biosystems sequencers (models 470A and 477A) equipped with on-line
20 phenylthiohydantion (Pth) amino acid analyzer (model 120A). Total Pth-amino acid derivatives from the sequencer are injected onto the liquid chromatograph using a modified sample transfer device as described (38). Polybrene (39) is used as a carrier.

25 Partial amino-acid sequence of CP-10 and trypsin and cyanogen bromide-derived peptides is given in Fig. 7.

Step 4. Demonstration of proteolytic activity of CP10

30 **Step 4.1. Degradation of Ovalbumin by 125 I-CP10**

Step 4.1.1. Radioiodination of CP10

Because homogeneous CP-10 is poorly soluble in aqueous buffers the iodination with protocol Enzymobeads (BioRad Australia) as suggested by the manufacturers is modified as follows:

A preparation of CP10, purified to apparent homogeneity as described in Steps 3.1 to 3.2.6. and with a mass of approximately 2 μg is iodinated with 1 m Ci NaI^{125} (Amersham Australia) using 0.05/0.1 ml of the Enzymobead Radioiodination Reagent (single reaction vial; Bio Rad).

The RP-HPLC purified peptide is freeze dried in a "Speed-Vac" Concentrator, reconstituted into 0.05 ml of 60% acetonitrile in Solvent C and adjusted to 0.2 M sodium phosphate buffer, pH 7.2 in a total volume of 0.1 ml. Enzymobeads and NaI^{125} are added and the reaction is started with 0.025 ml β -D-glucose (1% in 0.2 M phosphate buffer, pH 7.2). After 15 min the mixture is transferred to a conditioned (as instructed by the manufacturer) 5 ml- C_4 WP-SPE cartridge (J T.Baker) and washed with 25 ml of Solvent C. Bound material is eluted with 3 x 0.8 ml of 60% acetonitrile in Solvent C and injected in 1 ml-aliquots onto an equilibrated (100% Solvent C) Bakerbond C_{18} -WP HPLC column (4.6 x 100 mm J T Baker). Bound material is eluted with a gradient of 1%/min acetonitrile in solvent C over 60 min., and fractions are collected every minute into tubes (Nunc immunotubes, Medos, Victoria, Australia) containing 10 μg ovalbumin (OVA) and then freeze dried. Aliquots (0.01 ml) of individual fractions are counted in a γ -counter (LKB) and analysed by SDS-Tricine PAGE and autoradiography to confirm the molecular weight of the radiolabelled protein. ^{125}I -CP10 is stored in aliquots of approx. 4×10^5 cpm in the presence of 0.01 mg OVA in 0.15 M NaCl, 0.02 M Tris pH: 7.2 (TBS) at -80°C .

Step 4.1.2. Assessment of proteolytic activity of ^{125}I -CP10

Radioiodinated CP10 in the presence of ovalbumin is analysed for proteolytic activity by S.E. HPLC and SDS-Tricine-PAGE, silverstaining and autoradiography. Chromatography and calibration of the TSK 3000 SW column (7.5 x 300 mm, LKB) is performed as described in step 3.2.4.

Prior to injection onto the S.E. HPLC column an aliquot of ^{125}I -CP10 (approx. 4×10^6 cpm equiv. to 1.5% of the total preparation, step 4.1.1.), is adjusted to 40% acetonitrile in solvent C. One half of this sample is injected onto the HPLC column after 2 h at r/t, the eluate is collected manually according to the absorbance at 214 nm and radioactivity of the load and individual fractions quantitated in a γ -counter.

Gel sample buffer, (0.05 ml/0.05 M Tris, 4% SDS, 12% [w/v] glycerol, 0.002% bromophenol blue, pH 6.8) is added to the second half of the sample and to individual fractions and the volume is reduced to 0.05 ml in a Speed-Vac concentrator. The samples are adjusted to 5% β -mercaptoethanol and incubated for 1 h at 56 °C prior to electrophoresis according to Schagger and von Jagow (35).

Molecular weight marker proteins (14.4 - 90.0 kD, 2.6 - 17.2 kD; Pharmacia) and OVA (subjected to S.E. HPLC under the same conditions as described above) are loaded in parallel lanes onto the same gel. After electrophoresis the gel is silverstained and autoradiographed for 55 h using a Kodak X-Omatfilm.

Analysis on S.E. HPLC reveals three peaks with apparent molecular weights of I:15kD, II:12.5kD, III:11.5kD containing I:22.8%, II:21.5% and III:6.8% of the injected radioactivity. No absorbance is seen in the elution position of ovalbumin. Analysis of peak I on SDS-Tricine PAGE shows a single band at 44 kD by silverstain and a single band at 10 kD by autoradiography. Peak II and

III reveal multiple bands ranging from 67 - 2.6 kD by silverstaining; only minor bands at 44 kD are visible, the equivalent fraction not subjected to size exclusion reveal bands ranging from 90 - 14.5 kd with a predominant band in the position of ovalbumin.

5

Quantitation of autoradiography of this lane using a Hoefer GS-300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, Ca) and a GS-370 Data System for the integration of peaks reveals 18.7% of the radioactivity (gaussian integration of peak area) in the migration position of CP10 and 11.7% in the migration of OVA. The predominant radioactivity (52.5%) is associated with bands ranging from 30 to 14.4 kD.

10

::

The distribution of radioactive bands is similar in the lane loaded with peak II from the S.E. HPLC: 13.1% and 6% of the radioactivity is associated with the positions of CP-10 and OVA respectively, while the major portion is distributed over the molecular weight range from 30-14.4 kD. Although there is a discrepancy between the elution position of proteins on S.E. HPLC and the band pattern on SDS-Tricine PAGE, these results indicate degradation of OVA in the presence of ^{125}I -CP-10.

15

20

EXAMPLE 2

1. Method of chemotaxis measurement.

Triplicate samples (in RPMI 1640 and 0.1% serum albumin) to be tested are pipetted into the low wells of a Neuro Probe 48 well micro-chemotaxis chamber (Neuro Probe Inc. MD, USA) in a total vol. of approx. 25 μ l. Wells are covered with a Nuclepore PC membrane (PVP free, 5 μ M) and upper chambers filled with cell suspension ($1-1.5 \times 10^4$ cells in 50 μ l RPMI 1640 containing 0.1% albumin). Chambers are incubated for various times depending on the cells being tested (90 min. for neutrophils, 3hr for macrophages) at 37 °C in an atmosphere of 5% CO₂ in air. Membranes are removed and wiped free of excess cells on the "cell" side of the membrane.

25

30

Cells are fixed in methanol and stained with Wright's stain or Coomassie blue. Destained membranes are placed on microscope slides and held in place with a thin layer of microscopic immersion oil. Cells which have migrated through the pores of the membrane are quantitated by measuring the total cell area per 5 microscope fields using an Image Analysis system. Alternatively the number of cells migrated/5 fields is counted manually. The chemotactic activity was tested in the following cell types: mouse macrophages and neutrophils; human neutrophils and mononuclear cells.

2. Preparation of CP-10 peptide.

A synthetic peptide of the sequence proline-glutamine-phenylalanine-valine-glutamine-asparagine-isoleucine-asparagine-isoleucine-glutamic acid-asparagine-leucine-phenylalanine-arginine-tyrosine comprising the unique "hinge" region of CP-10 from amino acids Pro⁴²-Arg⁵⁵ was prepared by standard techniques. A C-terminal tyrosine was added so that it could be used as a radioiodinated tracer.

Amino acid code - P⁴² Q F V Q N I N I E N L F R⁵⁵ Y

3. Preparation of CP-10 and CP-10₄₂₋₅₅ Test Solution.

Purified CP-10 and CP-10₄₂₋₅₅ were stored in 35% acetonitrile/0.1% TFA in water and were diluted to 0.1% BSA in RPMI 1640 to yield 10-fold dilutions between 10⁻⁸ 10⁻¹³M.

4. Preparation of murine macrophages.

Murine macrophages are elicited by ip injection of thioglycollate broth 3 days before harvesting by peritoneal lavage and cells are tested in chemotaxis assays as outlined above. Alternatively, a murine monocytoid cell line, WEHI 265, grown in continuous cell culture can be used as effector cells.

5. Measurement of Vascular and Dermatological Changes in Rat Skin. Purified CP-10 and CP-10₄₂₋₅₅ were injected subcutaneously into Sprague-Dawley rats. Small sections of skin from treated rats were removed and subjected to microscopic examination after fixing and staining with Haemotoxyline Eosin.

RESULTS

Effect on murine cells.

Full length CP-10 was chemotactic for mouse neutrophils to 10^{-14} M with peak activity at 10^{-13} M (Figure 8).

CP-10₄₂₋₅₅ was chemotactic for mouse neutrophils to 10^{-12} M with peak activity at 10^{-11} M.

Full length CP-10 was found to be chemotactic for murine macrophages to 10^{-13} M with peak activity between 10^{-11} - 10^{-12} M. CP-10₄₂₋₅₅ was chemotactic for murine macrophages with peak activity at 10^{-11} M.

Effect on human cells.

Full length CP-10 was found to be chemotactic to human blood neutrophils to at least a concentration of 10^{-13} M. Peak activity was at 10^{-11} M.

CP-10₄₂₋₅₅ was chemotactic for human blood neutrophils to at least a concentration of 10^{-11} M. Peak activity was at 10^{-10} M.

Full length CP-10 was found to be chemotactic for human peripheral blood mononuclear cells to at least a concentration of 10^{-11} M. Peak activity was not determined.

CP-10₄₂₋₅₅ was found to be chemotactic for human peripheral blood mononuclear cells to a concentration of at least 10^{-11} M. Peak activity was not

determined. (A comparison of the activities of full length CP-10 and CP-10₄₂₋₅₅ on human and mouse cells is shown in Figure 9.)

Determination of full length cDNA sequence of murine CP-10

5 The cDNA sequence of murine CP-10 is given in Fig.1a. The mCP10 cDNA was derived from a Sepharose-Concanavalin A-activated spleen cell library prepared using cells from A/J mice.

10 The mCP10 cDNA isolate is a λ gt10 recombinant with a 430bp EcoRI insert. The insert was subcloned in both orientations into the 2.7kb vector pUC119 to yield recombinants 10/pUC119 (mCP10 cDNA 3' to 5' with respect to universal sequencing primer) and 12/pUC 119 (mCP10 cDNA 5' to 3' with respect to universal sequencing primer). The DNA sequence of mCP-10 was determined on both strands.

15 The cDNA sequence of CP-10 is given (Fig.1a) together with human MRP8 for comparison. These proteins show 69.6% homology at the DNA level. The full length amino acid sequence of CP-10 is given as

20 P S E L E K A L S N¹⁰ L I D V Y H N Y S N²⁰ I Q G N H H A L Y K³⁰ N
D F K K M V T T E⁴⁰ C P Q F V Q N I N I⁵⁰ E N L F R E L D I N S D N⁶⁰
A - I N F E E F⁷⁰ L A M V I K V G V A⁸⁰ S H K D S H K E⁸⁸

25 The amino acid sequence deduced from the cDNA sequence is in full agreement with sequence pro¹-lys⁷⁶ obtained by amino acid sequencing of the N-terminal, tryptic and cyanogen bromide peptide fragments derived from purified CP-10. Sequence val⁷⁷-glu⁸⁸ representing the C-terminal end of the molecule is new data obtained by cDNA sequencing.

30 Thus CP-10 is an 88 amino acid protein with a calculated mass of 10,163D. Isoelectric point as determined by computer analysis of amino acid sequence was 5.5. In addition to the amino acid sequence differences between

mCP-10 and hMRP8, the cDNA sequences reveal an alignment gap in the CP-10 sequence between his⁸⁸ and lys⁸³ when compared with the MRP8 amino acid sequence:

5 mCP-10 -A S H⁸² * * * * K⁸³ D S H K E
 hMRP8 -A A H⁸² K K S H E⁸⁷ E S H K E

MRP8 has an additional 4 amino acids (12 base pairs) not present in CP-10.
 (see Fig.1a).

10 Vascular and dermatological changes in rat skin.

Examination of microscopic sections of rat skin following administration of full length CP-10 or peptide corresponding to CP-10₄₂₋₅₅ revealed accumulation of neutrophils and increased vascular permeability revealing the potent chemotactic activity of these peptides. Native CP-10 induced a
 15 response over 24hr which comprised neutrophils and mononuclear cells and was similar to that of a delayed-type hypersensitivity reaction. CP-10₄₂₋₅₅ induced a shorter-lived response which was optimal after 6-8hr indicating differences in the abilities of these agents to attract phagocytic cells *in vivo*.

20

EXAMPLE 3

Expression of Recombinant CP-10

CP-10 has been overexpressed in *E.coli* using the pGEX expression system (40). Two different CP-10 overexpression constructs have been
 25 produced: pCP12 contains the CP-10 coding region cloned into the *Bam* HI site of pGEX-2T; pCP10 contains a Factor Xa cleavage site followed by the CP-10 coding region cloned into the *Eco* RI site of pGEX-IN. Both constructs have been verified by double-stranded DNA sequencing.

30

The level of CP-10 overexpression in this system has been evaluated by purifying fusion proteins from crude cell-free lysates by affinity chromatography on immobilised glutathione and analysed using SDS-PAGE in

accordance with the manufacturer's directions (Glutagene, Trade Mark of AMRAD Corporation Limited). Figure 10 shows expression of these fusion proteins as follows:

- 5 lane 1: affinity purified GST expressed from pGEX-IN;
 lane 2: affinity purified GST-Xa-mCP10 fusion protein expressed
 from pCP10;
 lane 3: affinity purified GST expressed from pGEX-2T;
 lane 4: affinity purified GST-T-mCP10 fusion protein expressed
10 from pCP12.

It is estimated that the yield of the two GST/CP-10 fusion proteins is ~ 5µg/ml culture. The GST/CP-10 fusion protein containing the thrombin cleavage site has been successfully cleaved with thrombin to release r-CP-10 and will be purified from cleavage products by methods similar to those described above.

REFERENCES:

1. Snyderman R, Pike ML, *Ann. Rev. Immunol.* 1: 257, 1984
2. Ryan, GB, Majno J, *Am. J. Pathol* 86: 185, 1977
3. Wolpe SD, Cerami A, *Faseb J.* 3: 2565, 1989
4. Ward PA, Remold HG, David JR, *Cell Immunol.* 1: 162, 1970
5. Altman LC, Kirchner H, *Immunology* 26: 393, 1974
6. Postlethwaite A, Snyderman R, *J. Immunol.* 114: 274, 1975
7. Cohen S, Ward P, Yoshida T, Burek CL, *Cell Immunol.* 9: 363, 1973
8. Miura K, Shimokawa Y, Honda M, Hayashi H, *Cell Immunol.* 75: 383, 1983.
9. Westwick J, Li SW, Camp RD, *Immunol. Today* 10: 146, 1989
10. Hopper KE, Geczy CL, Davies W, *J. Immunol.* 126: 1052, 1981
11. Geczy CL, Meyer P, *J. Immunol.* 128: 331, 1982
12. Geczy CL, Farram E, et al. *J. Immunol.* 130: 2743, 1983
13. Geczy CL, Hopper K, *J. Immunol.* 126 :1059, 1981
14. Ryan J, Geczy CL, *Immunol* 137: 2864, 1986
15. Ryan J, Geczy CL, *Immunol. & Cell Biol.* 65 :127, 1987
16. Ryan J, Geczy CL, *J. Immunol.* 141: 2110, 1988
17. Bennett B, Bloom BR, *Proc Natl. Acad Sci USA* 59: 756, 1968
18. Geczy CL, Friedrich W, de Weck AL, *Cell Immunol.* 19: 65, 1975
19. Dorin JR, Novak M, et al. *Nature* 326: 614, 1987
20. Andersson KB, et al. *Scand. J. Immunol.* 28: 241, 1988
21. Odink K, Cerletti N, et al. *Nature* 330: 80, 1989
22. Kligmann D, Hilt DC, *Trends in Biochem. Sci.* 13: 437, 1988
23. Hogg N, Allen C, Edgeworth J, *Eur. J. Immunol.* 19 :1053, 1989
24. van Heyningen V, Hayward C, et al. *Nature* 315: 513, 1985
25. Ishida M, Honda M, Hayashi H, *Immunology* 35: 167, 1978
26. Hayashi H, Honda M, Yoshimura T, In "Macrophage Biology" (Reichard S, Kojima M, Eds) Alan R Liss Inc, p. 231, 1985
27. Becker EL, Ward PA, *J. Exp. Med.* 125 :1021, 1967
28. Ward PA, Becker EL, *J. Immunol.* 105: 1057, 1970

29. Wilkinson PC, Bradley GR, Immunology 42: 637, 1981
30. Postlethwaite AE, Kang AH, J. Immunol. 123: 561, 1979
31. Matzner Y, Marx G, Drexler R, Eldor A, Thromb. Haemost. 52 :134,1984
32. Ceczy CL, Molecular Immunol. 17: 601, 1980
33. Kuzemko JA, The Lancet Feb 26: 448, 1983
31. Falk W, Goodwin RH, Leonard EJ, J. Immunol. Methods. 33: 239, 1980
35. Schagger H, von Jagow G, Anal Biochem 166: 368, 1987
36. Heukeshofen J, Dernick R, Electrophoresis 9: 28, 1988
37. Simpson IRJ, Moritz RL, Rubira MR, Van Snick J, Biochem. 176: 107, 1988
38. Begg GS, Simpson RJ, In "Techniques in Protein Chemistry", Hugli TE (Ed) Academic Press, Orlando FL., USA. 79, 1989
39. Klapper DG, Wilde CE, Capra JD, Anal. Biochem. 85: 126, 1978
40. Smith DB, Johnson KS, Gene 67: 31, 1988



CLAIMS:

1. The chemotactic protein CP-10, in essentially pure form, said protein having an apparent molecular weight of approximately 10 kD and having chemotactic activity for neutrophils, monocytes/macrophages and/or other mammalian cells, or an analogue, mutant, fragment, derivative or functional homologue of CP-10.
2. A product of claim 1, which is of a mouse, human, livestock animal, or other mammalian origin.
3. A product of claim 1, which comprises or includes the amino acid sequence:
Pro-Ser-Glu-Leu-Glu-Lys-Ala-Leu-Ser-Asn-Leu-Ile-Asp-Val-Tyr-His-Asn-Tyr-Ser-Asn-Ile-Gln-Gly-Asn-His-His-Ala-Leu-Tyr-Lys-Asn-Asp-Phe-Lys-Lys-Met-Val-Thr-Thr-Glu-Cys-Pro-Gln-Phe-Val-Gln-Asn-Ile-Asn-Ile-Glu-Asn-Leu-Phe-Arg-Glu-Leu-Asp-Ile-Asn-Ser-Asp-Asn-Ala-Ile-Asn-Phe-Glu-Glu-Phe-Leu-Ala-Met-Val-Ile-Lys-Val-Gly-Val-Ala-Ser-His-Lys-Asp-Ser-His-Lys-Glu
or an analogue, mutant, fragment, derivative, or functional homologue thereof.
4. A product of claim 1, which comprises or includes the sequence:
Pro-Gln-Phe-Val-Gln-Asn-Ile-Asn-Ile-Glu-Asn-Leu-Phe-Arg(-Tyr)
or an analogue, mutant, fragment, derivative or functional homologue thereof.
5. A product which is a homo-dimer comprising two CP-10 moieties as claimed in any of claims 1 to 4 bound together through an intermolecular S-S bridge or by non-covalent binding.
6. A product which is a hetero-dimer comprising a CP-10 moiety as claimed in any of claims 1 to 4 bound to a heterologous peptide through an intermolecular S-S bridge or by non-covalent binding.

This Sheet Has Been Replaced





7. A product of claim 5 or claim 6 wherein the, or each, CP-10 moiety has or includes the amino acid sequence set out in claim 3 or claim 4.
8. An antibody or antagonist to a product of any of claims 1 to 7, or a fragment thereof.
9. An antibody of claim 8, which is a polyclonal antibody.
10. An antibody of claim 8, which is a monoclonal antibody.
11. A pharmaceutical composition comprising a product of any of claims 1 to 7, or an antibody or antagonist of any of claims 8 to 10, in association with a pharmaceutically acceptable carrier or diluent.
12. Use of a product of any of claims 1 to 7, or an antibody or antagonist of any of claims 8 to 10, for the alteration of the inflammatory capacity or metabolism of mammalian cells.
13. Use of a product of any of claims 1 to 7, or an antibody or antagonist of any of claims 8 to 10, in the control of inflammation and/or tissue damage in inflammatory conditions, cystic fibrosis or emphysema.
14. Use according to claim 13, wherein said inflammation is caused by a delayed-type hypersensitivity reaction, or by autoimmune disease.
15. Use of an antibody or antagonist of any of claims 8 to 10, to alter the chemotactic activity of CP-10, to alter the proteolytic activity of CP-10, to purify CP-10 by affinity chromatography, or as a diagnostic reagent to detect CP-10 in body fluid, mammalian cell cultures or tissue sections.



16. A process for the preparation of a product of claim 1, in essentially pure form, which comprises purification of a CP-10 containing material by chromatography and/or affinity procedures.
17. A process of claim 16, wherein the CP-10 containing material is selected from cell extracts, pre-purified cell extracts, cell supernatant or culture filtrate of stimulated lymphoid cells or other mammalian cells or of permanent cell lines.
18. A DNA sequence coding for a compound of claim 1.
19. A DNA sequence coding for a compound having or including the amino acid sequence:
Pro-Ser-Glu-Leu-Glu-Lys-Ala-Leu-Ser-Asn-Leu-Ile-Asp-Val-Tyr-His-Asn-Tyr-Ser-Asn-Ile-Gln-Gly-Asn-His-His-Ala-Leu-Tyr-Lys-Asn-Asp-Phe-Lys-Lys-Met-Val-Thr-Thr-Glu-Cys-Pro-Gln-Phe-Val-Gln-Asn-Ile-Asn-Ile-Glu-Asn-Leu-Phe-Arg-Glu-Leu-Asp-Ile-Asn-Ser-Asp-Asn-Ala-Ile-Asn-Phe-Glu-Glu-Phe-Leu-Ala-Met-Val-Ile-Lys-Val-Gly-Val-Ala-Ser-His-Lys-Asp-Ser-His-Lys-Glu
or an analogue, mutant, fragment, derivative or functional homologue thereof.
20. A DNA sequence coding for a compound having or including the amino acid sequence:
Pro-Gln-Phe-Val-Gln-Asn-Ile-Asn-Ile-Glu-Asn-Leu-Phe-Arg(-Tyr)
or an analogue, mutant, fragment, derivative or functional homologue thereof.
21. A DNA sequence having or including the CP10 nucleotide sequence as set out in Figure 1A, a degenerate variant thereof or a fragment thereof comprising at least 15 nucleotides.
22. A vector comprising a DNA sequence of any of claims 18 to 21, operatively linked to an expression control sequence.

This Sheet Has Been Replaced





23. A host cell transformed with a vector of claim 22.
24. A process for the preparation of a compound of claim 1, which comprises:
- (a) preparation of a DNA sequence of any of claims 18 to 21,
 - (b) incorporation of said DNA sequence into a vector of claim 22,
 - (c) transforming a host cell of claim 23 with said vector,
 - (d) culturing said host cell under conditions which allow expression of said DNA sequence, and
 - (e) isolating the expressed peptide.
25. An isolated oligonucleotide for use as a probe for diagnostic or analytical purposes, in *in situ* hybridisation, or as a primer in amplification of nucleotide sequences by polymerase chain reaction (PCR) technology, having a nucleotide sequence corresponding to a fragment of the CP10 nucleotide sequence as set out in Figure 1A, or a degenerate variant thereof.
26. Use of an isolated oligonucleotide of claim 25, as a probe for diagnostic or analytical purposes, in *in situ* hybridisation, or as a primer in amplification of nucleotide sequences by polymerase chain reaction (PCR) technology.

This Sheet Has Been Replaced

