FIG. 1

```
27  ATGAAGGTCTCCGCGGCACGCCTCGCTGTCATCCTCATTGCTACTGCCCTCTGCGCTCCT   86
    M  K  V  S  A  A  R  L  A  V  I  L  I  A  T  A  L  C  A  P

87  GCATCTGCCTCCCCATATTCCTCGGACACCACACCCTGCTGCTTTGCCTACATTGCCCGC  146
    A  S  A  S  P  Y  S  S  D  T  T  P  C  C  F  A  Y  I  A  R

147 CCACTGCCCCCGTGCCCACATCAAGGAGTATTTCTACACCAGTGGCAAGTGCTCCAACCCA  206
    P  L  P  R  A  H  I  K  E  Y  F  Y  T  S  G  K  C  S  N  P

207 GCAGTCGTCTTTGTCACCCGAAAGAACCGCCAAGTGTGTGCCAACCCAGAGAAGAAATGG  266
    A  V  V  F  V  T  R  K  N  R  Q  V  C  A  N  P  E  K  K  W

267 GTTCGGGAGTACATCAACTCTTTGGAGATGAGCTAGG  303
    V  R  E  Y  I  N  S  L  E  M  S  *
```

FIG. 2

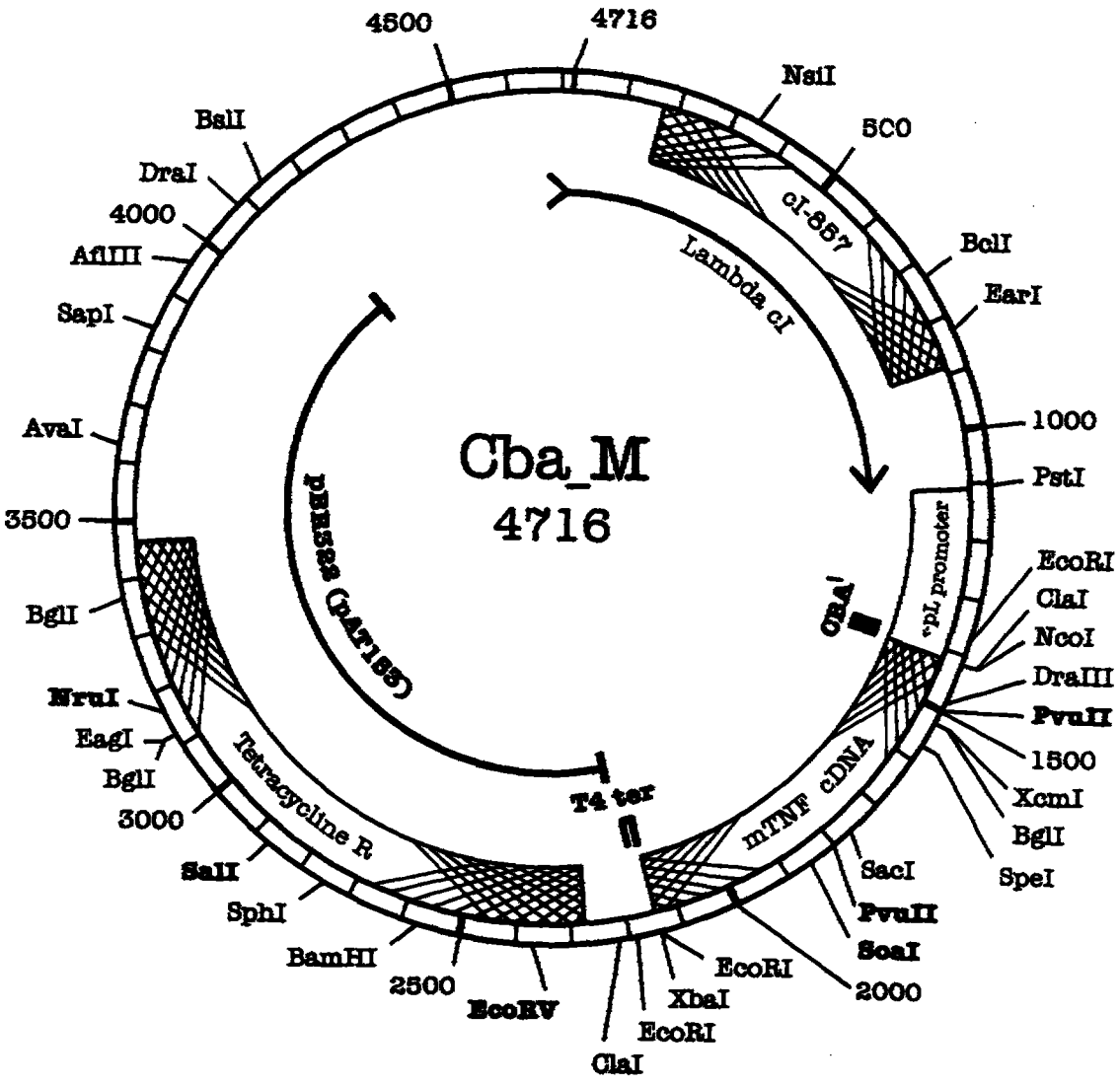
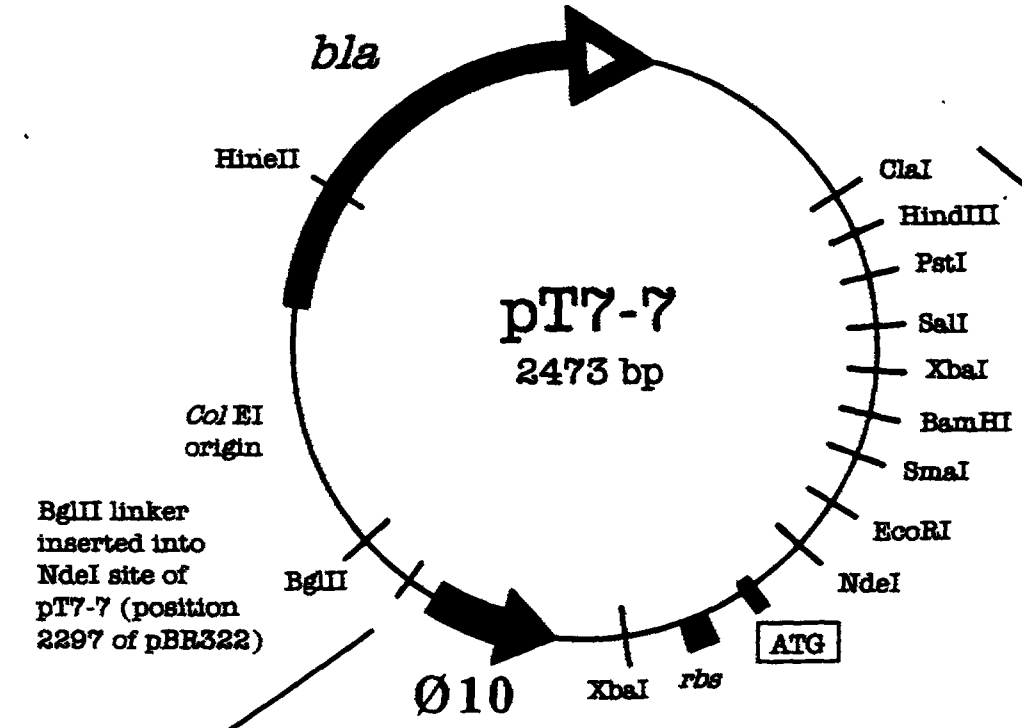

Cba_M
4716
4500
4716
NsiI
500
BclI
EarI
1000
PstI
EcoRI
ClaI
NcoI
DraIII
PvuII
1500
XcmI
BglI
SpeI
SacI
PvuII
ScaI
2000
EcoRI
XbaI
EcoRI
ClaI
2500
EcoRV
BamHI
SphI
SalI
3000
BglI
EagI
NruI
3500
BglI
AvaI
SapI
AflIII
4000
DraI
BalI
cI-857
Lambda cI
pL promoter
cba'
mTNF cDNA
T4 ter
Tetracycline R
pBR322 (pAT153)

FIG. 3

pT7-7

Contains T7 RNA polymerase promoter ø10 and the translation start site for the T7 gene 10 protein (T7 bp 22857 to 22972), inserted between the PvuII and the ClaI sites of pT7-5. Unique restriction sites for creation of fusion proteins (after filling in 5 ends) are:
Frame 0 : EcoRI
Frame 1 : NdeI, SmaI, ClaI
Frame 2 : BamHI, SalI, HindIII
SacI Site of original polylinker removed by deletion.
Note the additional XbaI site upstream of start codon.



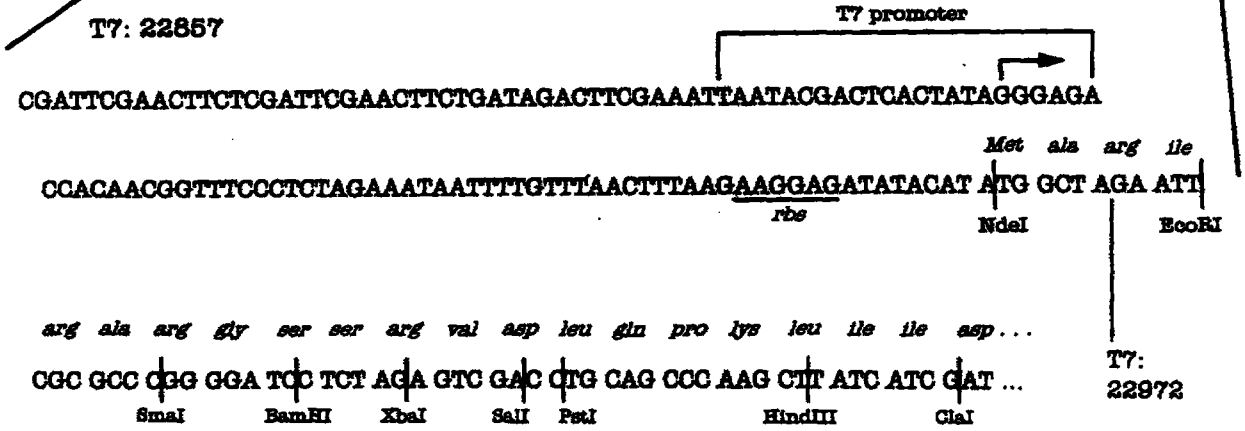
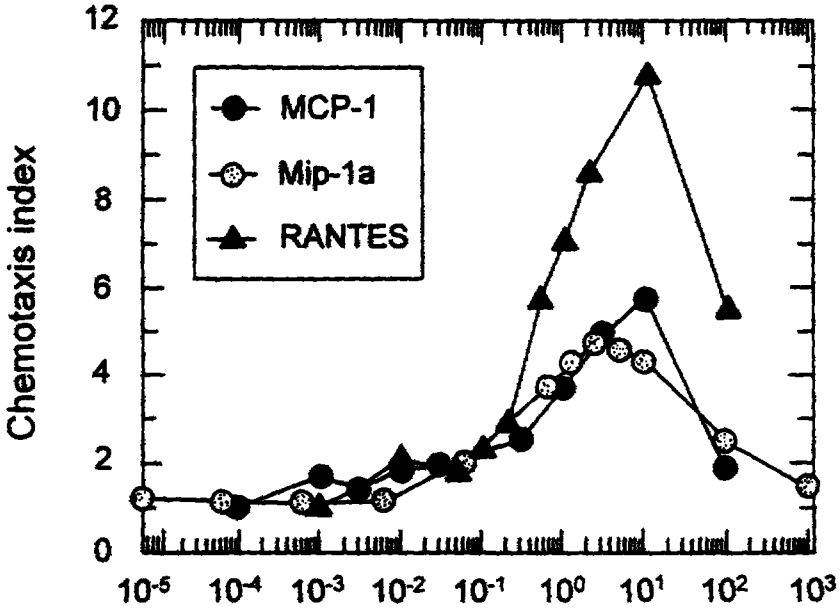


FIG. 4

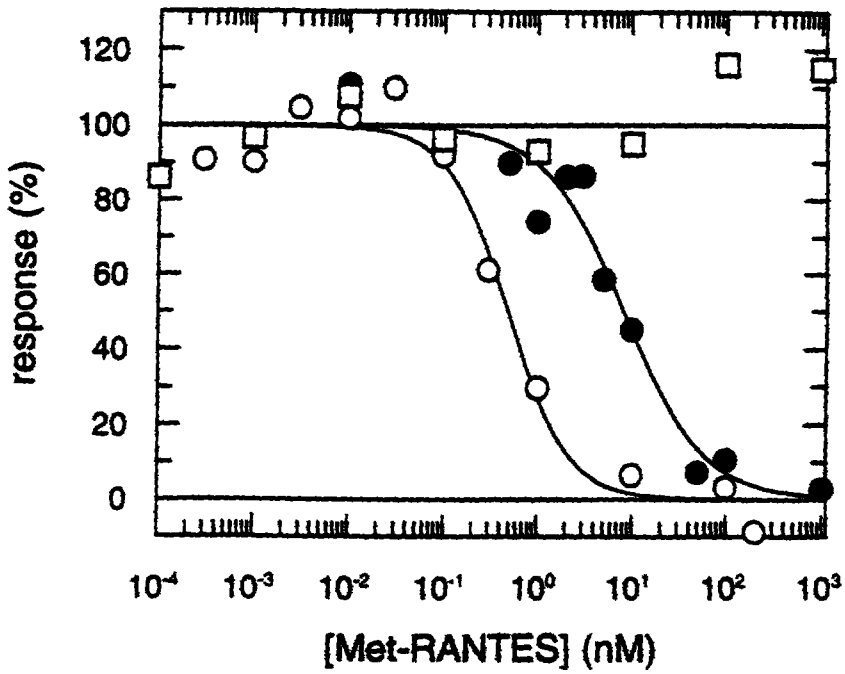
CC Chemokines induce chemotaxis in THP-1 cells.



	EC_{50} (nM)
MCP -1	1
MIP-1a	0.2
RANTES	0.68

FIG. 5

Inhibition of Chemokine induced Chemotaxis by Met-Rantes (GR 231774)

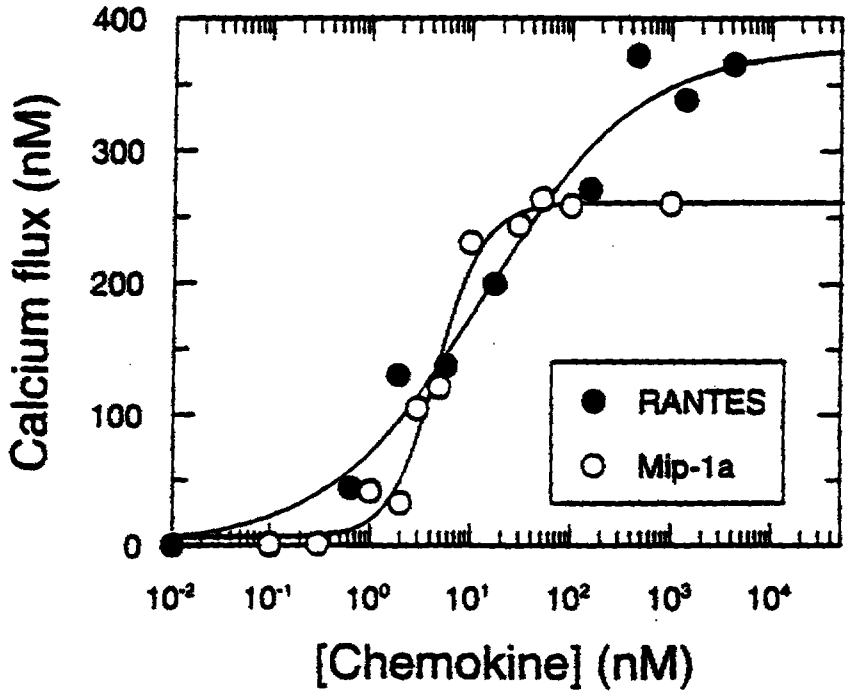


[Agonist] = 5 x EC_{50}

	IC_{50} (nM)
○ Mip-1α	0.49
● RANTES	8.18
□ MCP-1	-----

FIG. 6

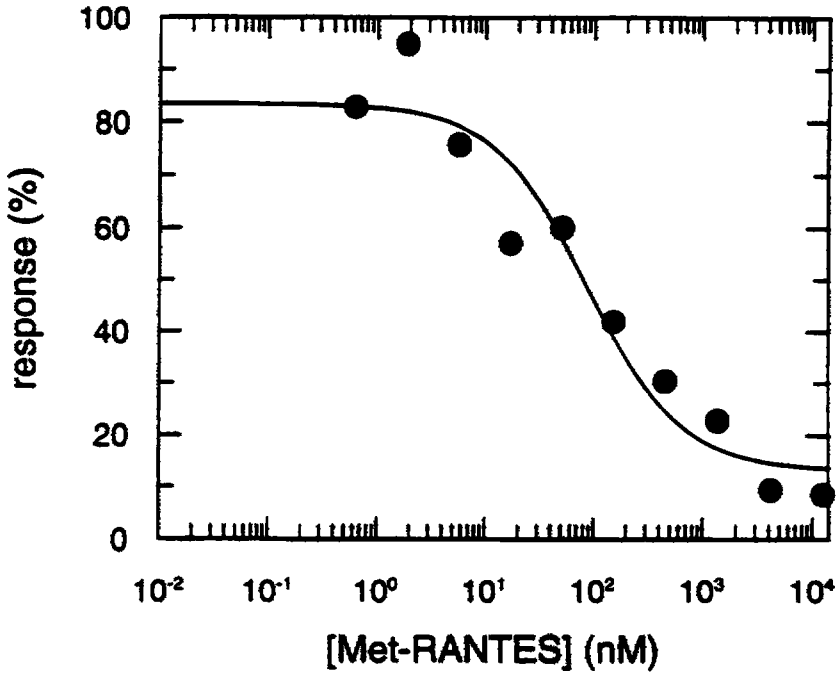
CC Chemokines induce Calcium flux in THP-1 cells.



	EC_{50} (nM)
Mip-1α	4.6
RANTES	13.21

FIG. 7

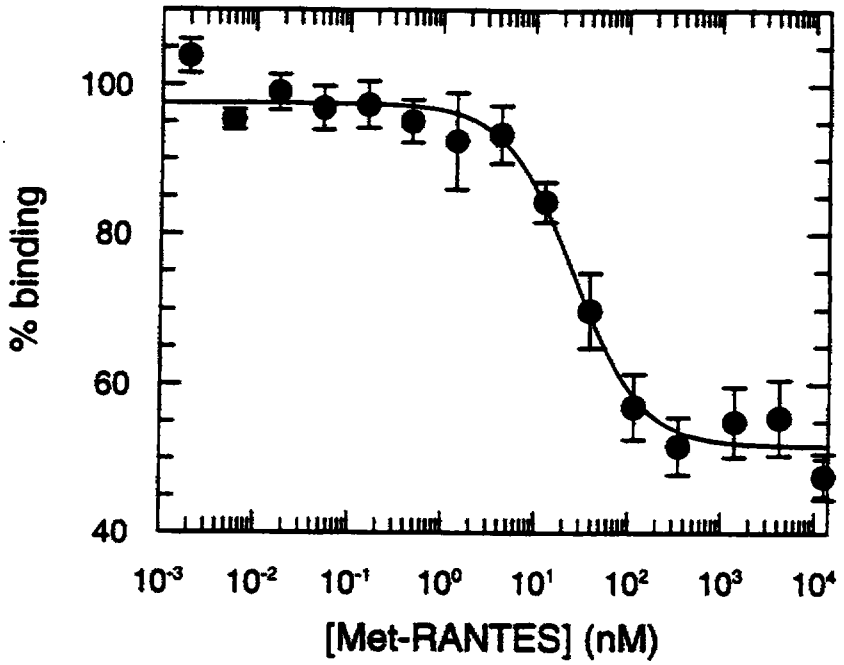
Inhibition of RANTES induced Calcium flux in THP-1 cells by Met-RANTES



RANTES at 5 x EC_{50} concentration

FIG. 8

Competion of ^{125}I-RANTES binding to THP-1 cells by Met-RANTES (GR 231774)



IC_{50} = 25 +/- 1.2 nM (n = 4 experiments)

[^{125}I]RANTES at 0.4 nM

FIG. 9

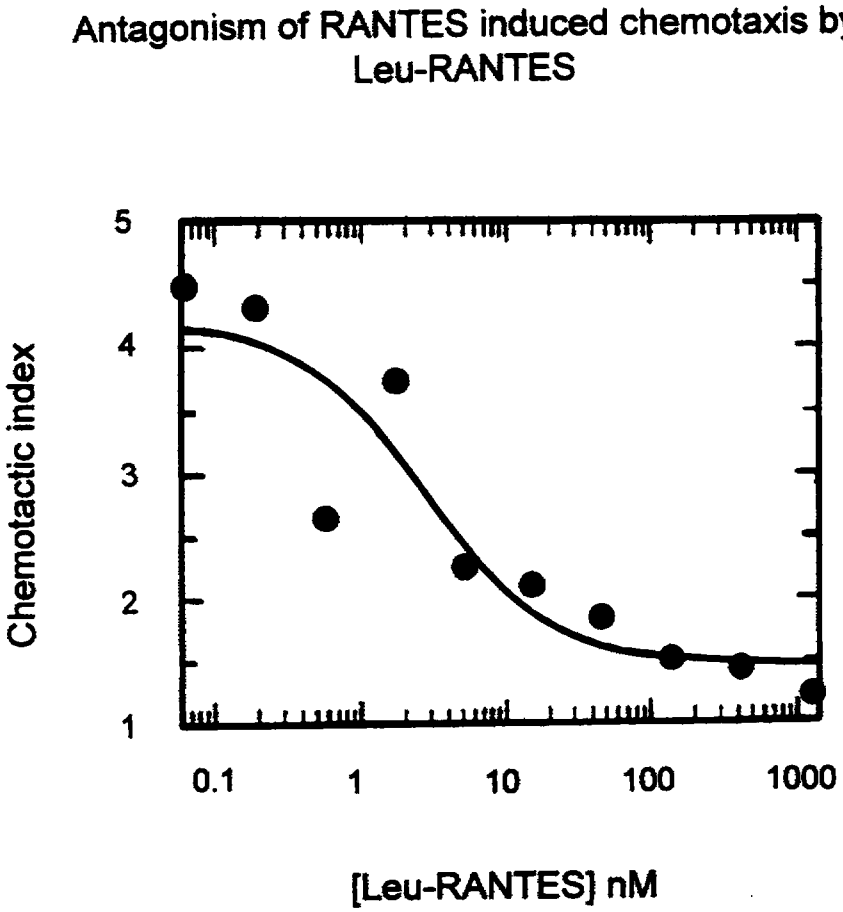
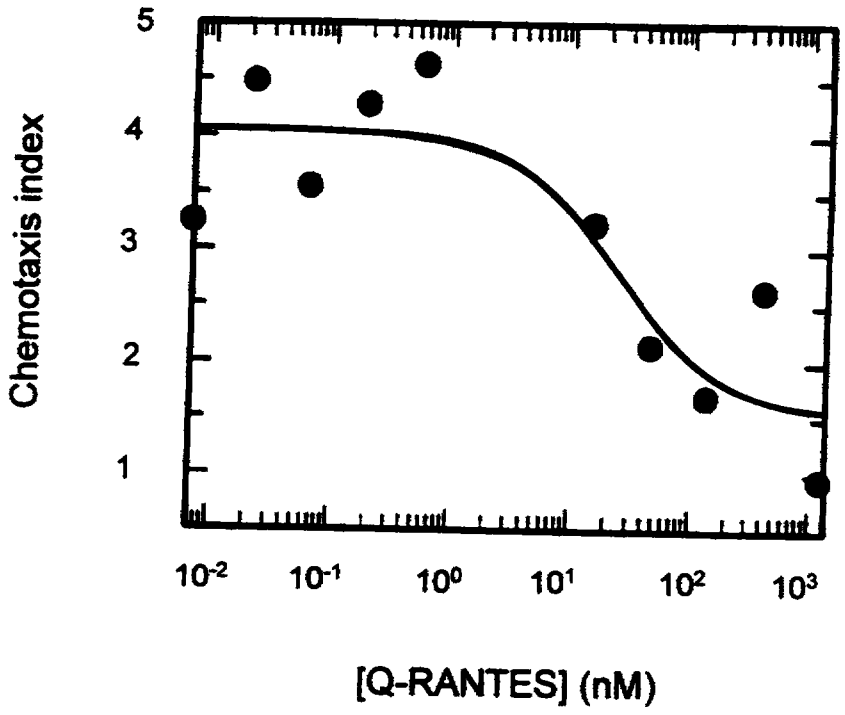
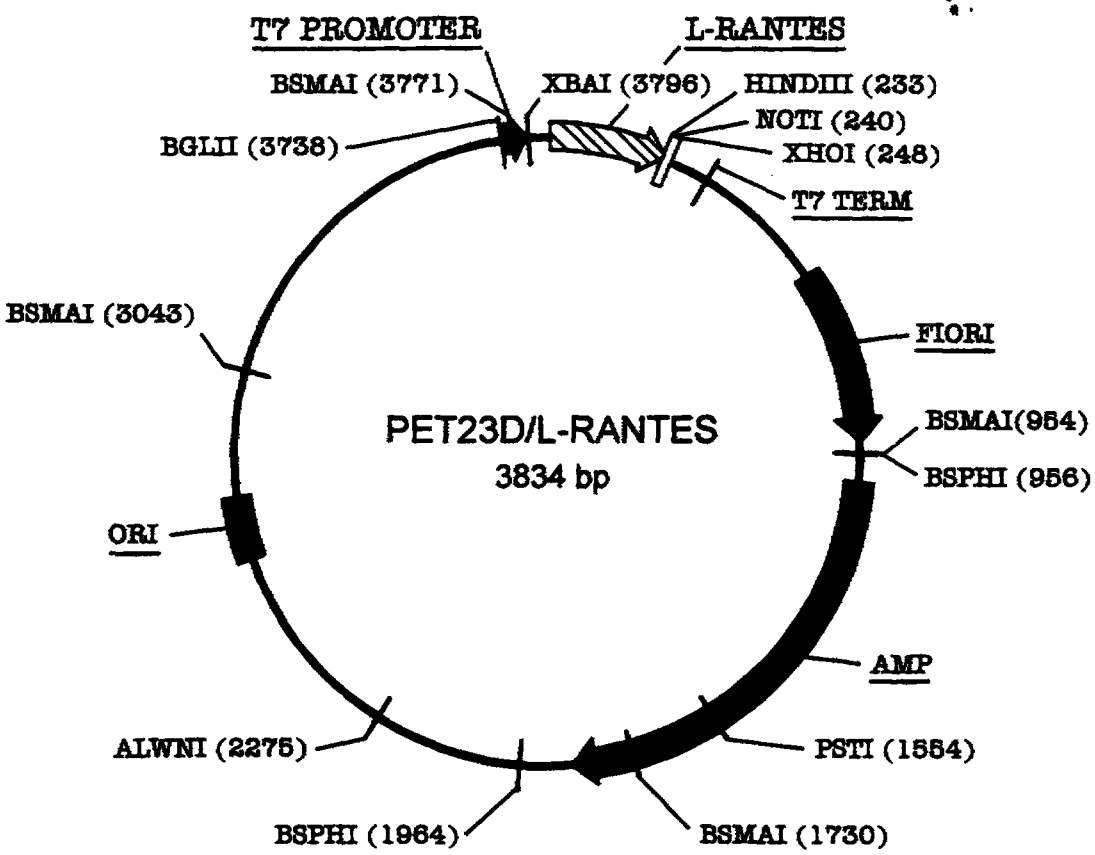

Antagonism of RANTES induced chemotaxis by Leu-RANTES
Chemotactic index
5
4
3
2
1
0.1
1
10
100
1000
[Leu-RANTES] nM

FIG. 10

Antagonism of RANTES induced chemotaxis of THP-1 cells by Glutamine-RANTES (Q-RANTES)






```
 +2   M  K  K   K  W  P  R   L  S  P   Y  S  S   D  T  T
  1  CATGAAAAAA AAATGGCCAA GGCTGTCCCC GTACTCCTCC GACACCACCC
     GTACTTTTTT TTTACCGGTT CCGACAGGGG CATGAGGAGG CTGTGGTGGG

 +2 P  C  C  F   A  Y  I   A  R  P  L   P  R  A   H  I  K
 51  CGTGCTGCTT TGCCTACATT GCCCGCCCAC TGCCCCGTGC CCACATCAAG
     GCACGACGAA ACGGATGTAA CGGGCGGGTG ACGGGGCACG GGTGTAGTTC

 +2  E  Y  F  Y   T  S  G   K  C  S   N  P  A  V   V  F  V
101  GAGTATTTCT ACACCAGTGG CAAGTGCTCC AACCCAGCAG TCGTCTTTGT
     CTCATAAAGA TGTGGTCACC GTTCACGAGG TTGGGTCGTC AGCAGAAACA

 +2   T  R  K   N  R  Q  V   C  A  N   P  E  K   K  W  V
151  CACCCGAAAG AACCGCCAAG TGTGTGCCAA CCCAGAGAAG AAATGGGTTC
     GTGGGCTTTC TTGGCGGTTC ACACACGGTT GGGTCTCTTC TTTACCCAAG

 +2 R  E  Y  I   N  S  L   E  M  S  *
                                    HINDIII NOTI    XHOI
                                    ------------- ----
201  GGGAGTACAT CAACTCTTTG GAGATGAGCT AAAGCTTGCG GCCGCACTCG
     CCCTCATGTA GTTGAGAAAC CTCTACTCGA TTTCGAACGC CGGCGTGAGC
```

FIG. 11

CLAIMS

1. A polypeptide having at least 40% amino acid sequence homology with RANTES and functioning as an antagonist to RANTES or to MIP-1α in one or more of the following:

(a) the chemotaxis of THP-1 cells in response to RANTES or to MIP-1α;

(b) the mobilisation of calcium ions in THP-1 cells due to the presence of RANTES or due to the presence of MIP-1α; and

(c) the binding of RANTES to receptors of THP-1 cells;

wherein the polypeptide acts as an antagonist to RANTES or to MIP-1α due to the presence of one or more N-terminal amino acids.

2. A polypeptide according to claim 1 wherein said one or more N-terminal amino acids are N-terminal to the sequence SPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S.

3. A polypeptide according to claim 1 or claim 2 wherein said one or more N-terminal amino acids comprise or consist of a methionine, a leucine, or a glutamine.

4. A polypeptide according to claim 3 wherein said methionine, leucine or glutamine is at the N-terminus of the polypeptide.

5. A polypeptide according to any preceding claim, wherein the polypeptide has the sequence:

(i) MSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR

* C L M S *

AMENDED SHEET

QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Met-RANTES")

(ii) LSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Leu-RANTES")

(iii) QSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Gln-RANTES")

or has a sequence which is substantially homologous with any of the above sequences.

6. A DNA or RNA sequence coding for a polypeptide according to any preceding claim.

7. A vector comprising a sequence according to claim 6.

8. A host cell comprising a vector according to claim 7.

9. A polypeptide according to any of claims 1 to 5 for use in therapy or diagnosis in respect of a human or non-human animal.

10. A polypeptide according to any of claims 1 to 5 for use in the treatment of a disease by inhibiting or reducing inflammation mediated by RANTES or MIP-1α.

11. A polypeptide according to claim 10 for use in the treatment of asthma, allergic rhinitis, atopic dermatitis, atheroma/atheroschleosis or rheumatoid arthritis.



12. A method for producing a polypeptide according to any of claims 1 to 5 comprising causing a host cell according to claim 8 to express said polypeptide.

13. A polypeptide substantially as hereinbefore described with reference to the accompanying examples.

AMENDED SHEET

* C L M S *

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RTMP Cop

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(57) Abstract

Modifications to RANTES can result in the modified polypeptide acting as a RANTES or MIP-1α antagonist. Such antagonists can be used in therapy to reduce inflammation. They are also useful in studying the properties of RANTES or of MIP-1α.

RANTES PEPTIDE AND FRAGMENTS AND COMPOSITIONS COMPRISING IT FOR TREATMENT OF INFLAMMATION

The present invention relates to derivatives of RANTES and their uses.

The protein known as RANTES was originally cloned by Schall T.J. *et al.*, (*J. Immunol.* **141** 1018-1025 (1988)) in Krensky's laboratory at Stanford University School of Medicine. The term RANTES is derived from the phrase "Raised on activation, normal T-cell derived and secreted" (relevant letters underlined). Its expression is inducible by antigen stimulation or mutagen activation of T-cells. The protein is a member of the chemokine superfamily (Schall T.J., *Cytokine* **3** 165-183 (1991); Oppenheim, J.J. *et al.*, *Ann. Rev. Immunol.* **9** 617-48 (1991)). The pure protein was first identified in 1992 in platelets (Kameyoshi *et al.*, *J. Exp. Med.* **176** 587-592 (1992)). It is a potent attractor for eosinophils, $CD4^+CD45RO^+$ T-cells, and also for monocytes. It has a sixty-eight amino acid sequence.

A receptor for RANTES has recently been cloned (Gao, J.L. *et al.*, *J. Exp. Med.* **177** 1421-7 (1993); Neote, K., *et al.*, *Cell* **72** 415-25 (1993)) - and this has been shown to bind chemokines in the rank order of potency of MIP-1α>RANTES.

The present invention provides polypeptides which are antagonists of RANTES and/or of MIP-1α.

Despite the considerable interest in cytokines generally and the work discussed above on RANTES and RANTES receptors in particular, prior to the present invention there has been no disclosure of the above antagonists or of the possible utilities of such antagonists.

According to the present invention there is provided a polypeptide having substantial amino acid sequence homology with RANTES and functioning as an antagonist to RANTES and/or MIP-1α in respect of one or more of the following:

(a) the chemotaxis of THP-1 cells in response to RANTES and/or in response to MIP-1α;
(b) the mobilisation of calcium ions in THP-1 cells due to the presence of RANTES and/or due to the presence of MIP-1α; and
(c) the binding of RANTES and/or of MIP-1α to receptors of THP-1 cells.

The polypeptides provided by the present invention are useful in further characterising RANTES and its effects - for example in studying RANTES induced chemotaxis, mobilisation of calcium ions and receptor binding. They are also useful in the characterisation of the binding of RANTES to its receptors. They are useful in studying MIP-1α for corresponding reasons.

Additionally, the polypeptides of the present invention are believed to be useful in the treatment of various diseases, as will be discussed later.

A preferred polypeptide of the present invention acts as an antagonist to RANTES and/or to MIP-1α due to the presence of one or more N-terminal amino acids (which are not present at the corresponding position in RANTES and which can therefore be regarded as additional N-terminal amino acids relative to those present at the N-terminus of RANTES). These N-terminal amino acids are preferably naturally occurring (L-) amino acids (which can be incorporated by using recombinant DNA techniques or by

peptide fusion techniques). However non-naturally occurring amino acids (e.g. D-amino acids) may be used. These may be incorporated by using chemical synthesis techniques.

There may be only one such additional amino acid, in which case it may be Leucine or Methionine, for example. Such polypeptides can be prepared by any suitable techniques (e.g. by using gene cloning techniques, chemical synthesis, etc.). In one embodiment of the present invention they are prepared by providing a larger polypeptide comprising a desired sequence and then using enzymatic cleavage to produce a polypeptide consisting of the desired sequence.

The polypeptides of the present invention may comprise more than one additional N-terminal amino acids e.g. they may include up to five, up to ten or up to twenty additional amino acids. In some cases over twenty additional N-terminal amino acids may be present.

Again, any suitable techniques can be used to prepare such polypeptides.

The various aspects of the present invention will now be discussed in further detail below.

The present inventors have discovered that using an *E. coli* expression system intended to express RANTES in a form corresponding to mature human RANTES (i.e. with the signal sequence removed) a polypeptide was expressed in which an additional N-terminal methionine was present (this was not cleaved from the remaining sequence by endogenous *E. coli* proteases). It was surprisingly found that the presence of this additional amino acid

substantially changed the characteristics of the polypeptide relative to those of RANTES. The methionylated polypeptide (referred to herein as methionylated RANTES or Met-RANTES) was found to act as an antagonist of RANTES and of MIP-1α in various assays and was not found to have any substantial agonist activity.

It should be appreciated that in many cases where N-terminal methionylation occurs in *E. coli*, it makes little or no difference to a polypeptide's properties or results in it merely having reduced levels of its previous biological activity. It was therefore totally unexpected that N-terminal methionylation would in the case of the present invention actually result in antagonistic activity.

In order to determine whether or not this effect was limited to the present of an N-terminal methionine, another polypeptide was produced, in which the N-terminal methionine was replaced with an N-terminal Leucine. Again, this was found to act as an antagonist of RANTES, as was a further polypeptide in which the N-terminal methionine was replaced with an N-terminal Glutamine.

In a preferred form, the polypeptide of the present invention has the sequence:

(i) MSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Met-RANTES")

(ii) LSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Leu-RANTES")

or

(iii) QSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Gln-RANTES")

or has a sequence which is substantially homologous with any of the above sequences. The polypeptide may be in a glycosylated or unglycosylated form.

The term "substantially homologous" when used herein includes amino acid sequences having at least 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% sequence homology with the given sequence (in order of preference). This term can include, but is not limited to, amino acid sequences having from 1 to 20, from 1 to 10 or from 1 to 5 single amino acid deletions, insertions or substitutions relative to a given sequence - provided that the resultant polypeptide acts as an antagonist to RANTES or to MIP-1α.

The polypeptide may be in substantially pure form. It may be isolated from naturally occurring polypeptides.

It should be noted that it is well known in the art that certain amino acids can be replaced with others resulting in no substantial change in the properties of a polypeptide. Such possibilities are within the scope of the present invention.

It should also be noted that deletions or insertions of amino acids can often be made which do not substantially change the properties of a polypeptide. The present invention includes such deletions or insertions (which may be, for example up to 10, 20 or 50% of the length of

the specific antagonists sequence given above). The present invention also includes within its scope fusion proteins in which the polypeptides of the present invention are fused to another moiety. This may be done, for example, for the purpose of labelling or for a medicinal purpose.

The present inventors have demonstrated that a polypeptide of the present invention can act as an antagonist to the effects of RANTES or of MIP-1α in chemotaxis, calcium mobilisation and receptor binding in THP-1 cells (a monocytic cell line). These cells are available from ATCC (American Tissue Culture Collection) and act as a good model system for studying RANTES because they show calcium responses and chemotactic responses to RANTES and MIP-1α, as well as other chemokines such as MCP-1. The polypeptide can also act as an antagonist of RANTES or of MIP-1α in chemotaxis, calcium mobilisation and receptor binding in these cells. MIP-1α was originally identified as part of a Macrophage Inflammatory Polypeptide fraction (which was split into MIP-1α and -β) (Obaru, K *et al.*, J Biochem 99:885-894 (1988). Sippel, PF *et al.*, J Immunol 142:1582-1590 (1989)). It shows chemotactic activity towards T-cells and monocytes. It has also been shown to be a potent inhibitor of stem cell proliferation.

Based upon these observations it is believed that the polypeptides of the present invention can be of utility in blocking the effects of RANTES and/or MIP-1α and can therefore be of use in therapy. A preferred use of the polypeptides of the present invention is in blocking the effects of RANTES and/or MIP-1α in the recruitment and/or activation of pro-inflammatory cells. The present invention may therefore be of utility in the treatment of

diseases such as asthma, allergic rhinitis, atopic dermatitis, atheroma/atheroschleosis and rheumatoid arthritis.

In addition to the polypeptides discussed above, the present invention also covers DNA sequences coding for such polypeptides (which may be in isolated or recombinant form), vectors incorporating such sequences and host cells incorporating such vectors which are capable of expressing the polypeptides of the present invention.

The polypeptides of the present invention can be produced by expression from prokaryotic or eukaryotic host cells, utilising an appropriate DNA coding sequence. Appropriate techniques are disclosed in Sambrook *et al.*, (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, Laboratory Press, USA. Alternatively, they may be produced by covalently modifying RANTES. This can be done, for example, by methionylating RNATES as its N-terminus.

The present invention will now be described by way of example only, with reference to the accompanying drawing, wherein:

FIGURE 1 shows a nucleotide sequence of a RANTES coding sequence which was cloned in *E. coli*, together with the amino acid sequence coded for by this nucleotide sequence.

FIGURE 2 shows a map of plasmid pCBA-M.

FIGURE 3 shows a map of plasmid pT7-7.

FIGURE 4 shows that various chemokines can induce chemotaxis in THP-1 cells.

FIGURE 5 shows that Met-RANTES can inhibit MIP-1α and RANTES induced chemotaxis in THP-1 cells.

FIGURE 6 shows that various chemokines can induce calcium flux in THP-1 cells.

FIGURE 7 shows that Met-RANTES can inhibit a RANTES induced calcium response in THP-1 cells.

FIGURE 8 shows competitive binding of Met-RANTES with RANTES to CCKR1 receptors.

FIGURE 9 shows that Leu-RANTES can act as an antagonist to RANTES-induced chemotaxis.

FIGURE 10 shows that Gln-RANTES can act as an antagonist to RANTES-induced chemotaxis.

FIGURE 11 shows the vector PET23D/L-RANTES.

EXAMPLE

(a) Cloning of Human RANTES coding sequence by PCR

Human RANTES was cloned from a human bone marrow λGT11 cDNA library (Clontech) by PCR. Briefly, total cDNA inserts in the bone marrow library were first amplified using λGT11 primers which flanked the *Eco*RI cloning site in a 100μl reaction containing 2μl of phage stock (10^6 pfus), 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dNTPs, 2.5 units AMPLITAQ™ (Perkin Elmer-Cetus) and 1μM of each primer (λGT11PCR-1 (forward primer) 5' GATTGGTGGCGACGACTCCT and λGT11PCR-2 (reverse primer) 5' CAACTGGTAATGGTAGCGAC) for 30 cycles of 95°C 2 min, 55°C 2 min and 72°C 5 min in a Techne PHC-2 thermal cycler. One tenth of the reaction mixture was then subjected to a 2nd round of PCR in a 100μl reaction now containing 1μM each of specific primers (RANTES-1 5' CCATGAAGGTCTCCGCGGCAC sense and RANTES-2 5' CCTAGCTCATCTCCAAAGAG antisense) based on the published RANTES sequence (Schall T.J. *et al.*, (*J. Immunol.* 141 1018-1025 (1988)) for 30 cycles of 95°C 2 min, 55°C 2 min and 72°C 2 min. PCR products were visualised on 3% Nu-Sieve (FMC) agarose gels stained with 0.5μg/ml ethidium bromide and bands migrating at the predicted size of RANTES cDNA (278bp) were gel purified by standard methods (Sambrook J. *et al.*, (1989) Molecular Cloning - A Laboratory Manual, Cold Spring Harbor, Laboratory Press, USA). Gel purified DNA was then rendered blunt-ended by sequential treatment with T4 polynucleotide kinase (New England Biolabs) according to the manufacturers' instructions, in a total volume of 50μl for 1 h at 37°C. After this time, 2.5μl of 2.5mM dNTPs and 1μl of *E. coli* DNA polymerase I Klenow fragment (New England Biolabs) were added and the incubation continued for a further 30 min at 37°C. The reaction mixture was then heat

inactivated at 70°C for 30 min and then extracted once with Tris-HCl pH 8.0 saturated phenol/chloroform (1:1 v/v). DNA was precipitated by addition of 10µl 3M sodium acetate pH 5.5, 1µl glycogen (20mg/ml) (Boehringer) and 250µl ethanol at -20°C. The DNA was recovered by centrifugation at 10,000 x g for 20 min at 4°C and washed with 70% ethanol. The final pellet was resuspended in sterile water at a concentration of 10ng/µl.

Blunt-ended PCR product (10ng) was ligated to 50ng of *Eco*RV digested, alkaline phosphatase treated pBluescript II SK- plasmid (Stratagene) in a 20µl volume using 2µl of T4 DNA ligase (400,000 units/ml) (New England Biolabs) for at least 16 h at 15°C. Ligation products were diluted to 100µl with 1 x TE (10mM Tris-HCl pH 8.0/1mM EDTA) and phenol/chloroform extracted as described previously. Ligation products were precipitated by the addition of 10µl 3M sodium acetate pH 5.5, 1µl glycogen (20mg/ml) and 250µl ethanol for 15 min at -70°C. DNA was recovered by centrifugation as described above and resuspended in 10µl of sterile water. 5µl of resuspended ligation products were then electroporated into electrocompetent *E. coli* strain XL-1 blue (40µl) using a Bio Rad Gene pulser according to the manufacturers' instructions. Following electroporation, 1ml of LB medium was added and cells were grown at 37°C for 1 h. After this time, 100µl aliquots of the culture medium were plated on LB plates containing 100µg/ml of ampicillin and grown up for 16 h at 37°C. Individual bacterial colonies were then picked into 5ml of LB medium containing 100µg/ml of ampicillin and grown overnight at 37°C. Small scale plasmid DNA preparations (mini-preps) were then made from 3ml of each culture using a WIZARD™ mini-prep DNA purification system (Promega) according to

the manufacturers' instructions. 3µl aliquots of mini-prep DNA was then digested with restriction enzymes *Hin*dIII and *Eco*RI (both from New England Biolabs) according to the manufacturers' instructions in a reaction volume of 15µl. Reaction products were analysed on 1% agarose gels containing 0.5µg/ml ethidium bromide. Mini-prep DNAs which yielded an insert size of approximately 280 bp were then subjected to DNA sequence analysis using T3 and T7 primers and Sequenase (USB) according to the manufacturers' instructions.

The pBluescript II SK- cloning vector was prepared as follows: 20µg of CsCl gradient purified plasmid was digested in a reaction volume of 100µl of 2 h at 37°C with 200 units of *Eco*RV (New England Biolabs) according to the manufacturers' instructions. After 2 h, the digested vector was treated with 10µl of calf intestinal alkaline phosphatase (20 units/ml) (Boehringer) for a further 30 min at 37°C. The reaction mixture was inactivated by heating at 68°C for 15 min and then extracted once with Tris-HCl pH 8.0 saturated phenol/ chloroform (1:1 v/v). Plasmid DNA was precipitated by addition of 10µl 3M sodium acetate pH 5.5 and 250µl ethanol at -20°C. The DNA was recovered by centrifugation at 10,000 x g for 20 min at 4°C, washed with 70% ethanol. The final pellet was resuspended in sterile water at a concentration of 50ng/ml.

Sequencing revealed that all clones obtained were identical to the published sequence except for a single base change at nucleotide 22 in the PCR sequence which would result in an Arg to Pro change in the proposed signal sequence of the RANTES propeptide.

This is illustrated in Fig 1, where the DNA coding sequence cloned is given, together with the corresponding amino acid sequence.

(b) Preparation of an expression vector for methionylated RANTES (a RANTES antagonist)

The construct containing the gene for RANTES was PCR'd using the primers

5'TTAATTAATTAAATCGATTCATATG.TCC.CCA.TAT.TCC.TCG.GAC.AC-3'

where the two underlined sections are a Cla1 and an Nde1 restriction site, respectively (the Nde1 site including part of an initiation methionine codon) and

5'-TACTGATATAAATCTAGACTAGCTCATCTCCAAAGAGTTG-3'

This fragment was then cleaved with Cla1 at the 5' end and Xba1 at the 3' end. The plasmid pCba-M, which is shown in Fig 2, was then cleaved with Xba1/Sal1. The large fragment was the cleaved with Sal1 and Cla1. Then a three-way ligation was carried out using the small Sal1/Xba1 fragment from the first digest, the Sal1/Cla1 fragment from the second digest, and the PCR fragment, to produce a construct similar to pCba-M where the mTNF gene was replaced by that for the secreted form of human RANTES starting with an initiating methionine. (The secreted form of human RANTES does not include the first twenty three amino acids of the amino acid sequence shown in Fig 1. It includes the remaining amino acids shown in Fig 1 and begins with the amino acids SPY...).

This NdeI/SalI fragment was then removed from this vector and put into the t7 expression plasmid pT7-7, which is shown in Fig 3. This fragment contains the gene of interest plus 600 bp of other material, but later experiments (not included herein) showed that the removal of the other material (vector sequences) had no effect on the expression levels.

The pT7 type expression vector is discussed by Studier FW, Rosenberg AH et al., in Meth Enzymol, *185*, 60-89, (1990). The construct was then transformed into *E. coli* BL21 (DE3) strain (F-ompT, hsd s_B (r_B^-, m_B^-) containing the LysS gene on plasmid pACYC184 (Chang and Cohen, J. Bact, 134, 1141, 1978). The expression vector requires the T7 polymerase to be induced in order for protein expression to take place. The T7 polymerase is induced in the cells by the addition of IPTG (isopropylthio-galactoside) to the medium.

(c) Demonstration that Met-RANTES does indeed show Antagonistic Activities

(i) Chemotaxis assay

In vitro chemotaxis was carried out using 96 well chambers (Neuro Probe MB series, Cabin John, MD 20818, USA) according to the manufacturers instructions. Chemotaxis induced by the CC chemokines, RANTES, MIP-1α and MCP-1 was assayed using the human monocytic cell-line, THP-1. $4x10^5$ THP-1 cells in 200 μl RPMI 1640 medium (Gibco) containing 2% inactivated fetal calf serum were incubated in each well of the upper chamber. 370 μl of RPMI 1640 medium (without FCS) containing the chemoattractant (i.e. the chemokine) in appropriate dilutions was placed in the lower chamber. For the inhibition of chemotaxis, the chemoattractant was kept at a constant concentration of 5x the EC_{50}, determined previously for each chemokine, and Met-RANTES was added at varying concentrations. The chambers were incubated for 1h at 37°C under 5% CO_2. The medium was removed from the upper chamber and replaced with PBS containing 20 mM EDTA, and the chambers incubated for 30 min at 4°C. The PBS was removed from the upper wells which were then wiped dry. The unit was centrifuged for 10 min at 1800 rpm to harvest the cells in the bottom chamber, and the supernatant removed by aspiration. The cells in the bottom chambers were measured using the Cell Titer 96™ Non-Radioactive Cell Proliferation Assay (Promega, Madison, USA) which monitors the conversion of terazolium blue into its formazan product. 100 μl of a 10% solution of the Dye in RPMI 1640 medium was added to each well, and the chamber incubated overnight at 37°C under 5% CO_2. 100 μl of Solubilisation solution was then added to each well, and the absorbance read at 590 nm after 4 h in a Thermomax microtitre plate reader (Molecular Devices, Palo Alto, CA).

The results are shown in Fig 4 and Fig 5.

(ii) Calcium Flux

The calcium flux induced by the chemokines RANTES and MIP-1α was measured according to Tsien R Y., Pozzan T., and Rink T J., ((1982) "Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator", *J Cell Biology* 94) but using Fura-*2/AM* (Fluka) instead of Quin2 as the fluorescent indicator. THP-1 cells were harvested at less than 10^6/ml to ensure that they were in the exponential phase. The cells were resuspended in Krebs-Ringer solution containing 0.2% Fura-*2/AM* and 1mg/ml BSA, at a concentration of 10^6/ml, and incubated at 37°C for 30 min in the absence of light. The cells were harvested by centrifugation and resuspended in Krebs-Ringer solution and kept on ice. 1 ml aliquots were incubated at 37°C for 2 min prior to use. The chemokines were added to the cell suspension under stirring. To study the inhibition by Met-RANTES, aliquots of the antagonist were added to the cells during the 2 min incubation at 37°C at varying concentration. The results are shown in Fig 6 and Fig 7.

(iii) Receptor Binding Assay

The competition assay was carried out in 96 well multiscreen filter-plates (Millipore, MADV N6500) which had been pretreated for 2 h with 50 mM HEPES buffer, pH 7.2 containing 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.5% BSA, (binding buffer). The assay was performed using either THP-1 cells or COS cells expressing the recombinant CC-CKR1 receptor (Neote, K., DiGregorio, Mak, J.Y., Horuk, R., and Schall, T.J., (1993) Molecular cloning, functional expression, and signalling characteristics of a C-C chemokine receptor, *Cell* 72 415-425; Gao, J.L., *et*

al, J. Exp. Med. 177 1421-7 (1993)). Each well contained 10^5 cells in a volume of 150 µl of binding buffer containing 0.4 nM [1^{125}]Mip-1a or 0.4 nm[1^{125}]RANTES (new England Nuclear, NEX 277) and varying concentrations of competing Met-RANTES. Assays were performed in triplicate. After 90 min incubation at 4°C, the cells were washed 4 times with 200 µl ice cold binding buffer containing 0.5 M NaCl which was removed by aspiration. The filters were dried, 3.5 ml Ultima Gold Scintillation fluid added (Packard) and counted on a Beckman LS5000 counter. The results are shown in Fig 8.

(d) Preparation of Leu-RANTES (also referred to here as L-RANTES) and demonstration of antagonism

The L-RANTES expression vector was made in two steps. First PCR was used to truncate the gene for human RANTES within the first cysteine codon and introduce unique restriction sites at either end of the gene. This PCR product was cloned into the T7 based *E. coli* expression vector pET23d (Novagen) using a SacI site introduced at the 5′ end of the gene and a BsmAI site designed to yield a HindIII compatable overhang at the 3′ end of the gene. Genes encoding N-terminal variants of human RANTES were then created by inserting oligonucleotides encoding the variants immediately 5′ to the RANTES coding sequence. For this purpose, the truncated RANTES clonein pET23d was digested with SacI, followed by T4 DNA polymerase to remove the protruding 3′ overhang left by SacI and then a second digestion with NcoI. Oligonucleotides encoding the peptide sequence MKKKWPRLSPYSSDTTP were then cloned into the digested vector. Expression of the pET23d/L-RANTES construct was carried out as described for the pT7-7 construct.

pET23d/RANTES NcoI	pET23d/RANTES SacI/T4
5′ C	C TGC TTT 3′
3′ GGTAC	G ACG AAA 5′

L-RANTES oligonucleotides

5′CATGAAAAAAAAATGGCCAAGGCTGTCCCCGTACTCCTCCGACACCACCCCGTG
3′ TTTTTTTTTACCGGTTCCGACAGGGGCATGAGGAGGCTGTGGTGGGGCAC

The pET23d/L-RANTES construct is shown in Figure 11 and is one of a series of expression vectors, which can be used to generate RANTES with different amino acids at the -1 position. These T7 expression vectors encode proteins with N-terminal sequences of MKKKWPR-X-RANTES. X may be either L, I, Q, E or G for example. Cleavage with endo-Arg-C will yield different X-RANTES proteins.

Purification of Leu-RANTES was carried out as follows:

4g *E. coli* cell paste were suspended in 15 ml 50 mM Tris-HCl buffer, pH 7.6, containing 1 mM dithiothreitol, 5 mM benzamidine-HCI, 0.1 mM phenylmethylsulfonyl fluoride and DNase (0.02 mg/ml). Cells were broken by three passages through a French Pressure cell, with 1 min sonication on ice after each passage. The resulting solution was centrifuged for 60 min at 10 000 x g. The pellets were dissolved in 2 ml 100 mM Tris-HCl buffer, pH 8.0, containing 6 M Guanidine-HCl and 1 mM dithiothreitol. The solution was heated for 60 min at 60°C to ensure monomerisation, cooled to room temperature and gel-filtered on a Superdex-200 16/60 column equilibrated in the same buffer. The fractions containing the recombinant RANTES construct (16 ml) were renatured by dropwise addition to 384 ml 100 mM Tris-HCl buffer, pH 8.0, containing 1 mM oxidised glutathione and 0.1 mM reduced glutathione, and stirred overnight. This solution was dialysed against 50 mM sodium acetate buffer, pH 4.5, and the applied to a HlLoad SP26/10 column equilibrated in the same buffer. The proteins were eluted by a linear gradient of 0 - 2 M NaCl in the same buffer. The fractions containing the renatured protein were dialysed against 3 x 5 liter 1% acetic acid and lyophilised.

To remove the KKKWPR hexapeptide from the fusion protein, the lyophilised powder was dissolved in water, and adjusted to 1 mg/ml 50 mM Tris-HCl buffer, pH 8.0. To 2 mls of this solution, 20 µg Endoproteinase Arg C(Boehringer Mannheim) was added and the solution incubated for 2 h at 37°C. The digested protein was separated from the starting material by reverse-phase HPLC using a Nucleosil-C_8 (10x250 mm) column equilibrated

in 0.1 % trifluoroacetic acid. The proteins were eluted with a gradient of 22.5-45% acetonitrile in 0.1 % trifluoroacetic acid, lyophilised and stored at -80°C.

The antagonist activities on RANTES induced chemotaxis of THP-1 cells was tested as described for the Met-RANTES protein.

The results are shown in Figure 9.

(e) Preparation of Gln-RANTES (sometimes referred to as Q-RANTES) and demonstration of antagonism

The procedure described in (d) above was repeated *mutatis mutandis* in order to prepare Gln-RANTES.

The antagonist activities of Gln-RANTES on RANTES induced chemotaxis of THP-1 cells was tested as described for the Met-RANTES protein. The results are shown in Figure 10.

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CLAIMS

1. A polypeptide having substantial amino acid sequence homology with RANTES and functioning as an antagonist to RANTES or to MIP-1α in one or more of the following:

(a) the chemotaxis of THP-1 cells in response to RANTES or to MIP-1α;

(b) the mobilisation of calcium ions in THP-1 cells due to the presence of RANTES or due to the presence of MIP-1α; and

(c) the binding of RANTES to receptors of THP-1 cells.

2. A polypeptide according to claim 1 wherein the polypeptide acts as an antagonist to RANTES or to MIP-1α due to the presence of one or more N-terminal amino acids.

3. A polypeptide according to claim 2 wherein said one or more N-terminal amino acids are N-terminal to the sequence SPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S.

4. A polypeptide according to claim 2 or claim 3 wherein said one or more N-terminal amino acids comprise or consist of a methionine, a leucine, or a glutamine.

5. A polypeptide according to claim 3 wherein said methionine or leucine is at the N-terminus of the polypeptide.

6. A polypeptide according to any preceding claim, wherein the polypeptide has the sequence:

(i) MSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR



QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Met-RANTES")

(ii) LSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Leu-RANTES")

(iii) QSPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S (sometimes referred to herein as "Gln-RANTES")

or has a sequence which is substantially homologous with any of the above sequences.

7. A DNA or RNA sequence coding for a polypeptide according to any preceding claim.

8. A vector comprising a sequence according to claim 7.

9. A host cell comprising a vector according to claim 8.

10. A polypeptide according to any of claims 1 to 6 for use in therapy or diagnosis in respect of a human or non-human animal.

11. A polypeptide according to any of claims 1 to 6 for use in the treatment of a disease by inhibiting or reducing inflammation mediated by RANTES or MIP-1α.

12. A polypeptide according to claim 11 for use in the treatment of asthma, allergic rhinitis, atopic dermatitis, atheroma/atheroschleosis or rheumatoid arthritis.

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13. A method for producing a polypeptide according to any of claims 1 to 6 comprising causing a host cell according to claim 9 to express said polypeptide.

14. A polypeptide substantially as hereinbefore described with reference to the accompanying examples.

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