

REPLACED SHEETS

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NEUTROPHIL STIMULATING PEPTIDES

The present invention relates to peptides having neutrophil stimulating activity, and to use of these peptides as therapeutic agents.

5 Tumour necrosis factor (TNF) was first identified as a factor found in the serum of Bacillus Calmette-Guerin treated mice which caused haemorrhagic regression of certain transplanted tumours and had cytolytic activity on several transformed cell lines in vitro (Carswell et al, 10 PNAS 72, 3666 - 3670; Helson et al, 1975, Nature 258, 731-732). TNF, a product of activated macrophages, has subsequently been shown to be a primary mediator in the pathology of endotoxic shock (Tracey et al 1986, Science 234, 470-474). In addition to its pathological effects 15 TNF also has a central role in host defenses against viral, bacterial and parasitic pathogens.

The cellular targets of TNF important in host defence include neutrophils, eosinophils, monocyte/macrophages and lymphocytes. Within this context TNF is a major mediator 20 of neutrophil activation. TNF stimulates enhanced phagocytosis (Shalaby et al 1985, J.Immunol., 135, 2069-2073), enhanced production of superoxide anions (Teujiimoto et al, 1986, Biochem. Biophys. Res. Commun., 137, 1094-1100), release of lysozyme and hydrogen peroxide 25 and causes neutrophil degranulation (Klebanoff et al, 1986, J.Immunol., 136, 4220-4225). Neutrophils also show enhanced microbicidal and tumouricidal activity when stimulated by TNF (Shalaby et al, 1985, J.Immunol., 135, 2069-2073; Djeu et al, 1986, J.Immunol., 137, 2980-2984; 30 Blanchard et al, 1989, J.Leuk. Biol., 45, 538-545). It has been hypothesized that the cytostatic effect of TNF is mediated by high local concentrations of hydrogen peroxide produced by neutrophils (Shau 1986, J.Immunol., 141, 234-240).

35 TNF pretreatment enhances the response of neutrophils

to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (F-met-leu-phe) and phorbol myristate acetate through specific receptors (Ferrante et al 1988, Int. Arch. Allergy Appl. Immunol., 86, 82-91). Neutrophils

5 accumulate at sites of inflammation, caused in part by the increased expression of complement receptors by TNF (Berger et al 1988, Blood 71, 151-158). Further TNF causes neutrophil emigration into skin (Cybulsky et al 1988, J. Immunol. 140, 3144-3149).

10 Neutrophil function is known to be depressed in a number of viral, bacterial and parasitic infections (Abramson and Mills, 1988, Rev. Infect. Dis., 10, 326-341; Ferrante et al, 1989, Immunol. Letts., 22, 301-6).

Depressed neutrophil function has, for example, been
15 described in Acquired Immune Deficiency Syndrome (Thorsen et al, 1989, AIDS, 3, 651-653; Ellis et al, 1988, J. Infect. Dis., 158, 1268-1276; Murphy et al, 1988, J. Infect. Dis., 158, 627-630). Clearly TNF, which appears to play an important role in neutrophil activation both
20 in vitro and in vivo as described above, given exogenously has the potential to overcome these neutrophil defects. The administration of TNF or indeed overproduction of TNF is, however, associated with severe side effects and the manifestation of pathology such as thrombocytopaenia,
25 lymphocytopaenia, hepatotoxicity, renal impairment and hypertension.

The present inventors have identified novel peptides derived from the primary amino acid sequence of human TNF which stimulate neutrophil activity. These peptides have
30 indicated that the region of amino acids 54 to 94 of human TNF has previously undiscovered neutrophil stimulating activity. This observation has important clinical applications as treatment with such peptides would be expected to restore depressed or aberrant neutrophil
35 activity, but would not be expected to cause the severe

side effects associated with the therapeutic use of the whole TNF molecule.

Accordingly, in a first aspect the present invention consists in a peptide having an amino acid sequence
5 substantially corresponding to amino acids 54 to 94 of Figure 1 or a part thereof, the peptide being characterized in that the peptide is capable of eliciting superoxide production by neutrophils and of priming neutrophils for an enhanced respiratory burst following
10 treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

In a preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 94 of Figure 1.

15 In a preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 63 to 83 of Figure 1.

In another preferred embodiment of the present invention the peptide has an amino acid sequence
20 substantially corresponding to amino acids 54 to 68 of Figure 1.

In yet another preferred embodiment of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 73 to 94 of
25 Figure 1.

In yet a further preferred embodiment of the present invention, the peptide has amino acid sequence substantially corresponding to amino acids 70 to 80 of
Figure 1.

30 As will be appreciated by those skilled in the art from the description which follows the present inventors have demonstrated that the region of human TNF from amino acid 54 to amino acid 94 plays an important functional role in the stimulation of neutrophils. Further, the
35 present inventors have produced 4 peptides namely peptides



304, 308, 309 and 395 (as referred to herein) which have neutrophil stimulating activity.

Armed with this information and with the aid of co-ordinates of the crystalline structure of TNF at 2.6 Å as disclosed by Eck and Sprang, 1989 (J. Biol. Chem., 264: 18795-17605), the person skilled in the art will be able to design non-peptide structures which, in 3 dimensional terms mimic the peptides of the present invention. It is believed that these non-peptide structures will also mimic the physiological effects of the peptides of the present invention. It is intended that such non-peptide structures are included within the scope of the present invention. Changes to the TNF molecule in these regions using e.g. site directed mutagenesis would also be expected to affect neutrophil activation. A schematic representation of the three dimensional structure of TNF α is shown in Figure 4.

Accordingly in a second aspect the present invention consists in a compound the three dimensional structure of which substantially corresponds to the three dimensional structure of the peptide of the first aspect of the present invention, the compound being characterized in that the compound is capable of eliciting superoxide production by neutrophils and of priming neutrophils for an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

In a preferred embodiment of this aspect of the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 63 to 83 of Figure 1.

In another preferred embodiment of this aspect of the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 54 to 68 of Figure 1.

In yet another preferred embodiment of this aspect of

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the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 73 to 94 of Figure 1.

5 In yet a further preferred embodiment of the aspect of the present invention the compound has a three dimensional structure substantially corresponding to the three dimensional structure of amino acids 70 to 80 of Figure 1.

10 In a further aspect, the present invention consists in a method of treating a subject having depressed neutrophil function, the method comprising administering to the subject a therapeutic amount of the peptide of the first aspect of the present invention.

15 In a preferred embodiment of the third aspect of the present invention the subject is suffering from acquired immune deficiency syndrome.

Peptide 308, through selective effects on neutrophil degranulation may be administered to individuals suffering from inflammatory syndromes e.g. rheumatoid arthritis, adult respiratory distress syndrome.

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples, and Figures in which:-

Figure 1 shows the amino acid sequence of human TNF;

Figure 2 shows the effects of peptides 304 (○), 308 (□) and 309 (△) on the fMLP induced human neutrophil response. Peptides were used at 100µg/10⁶ in the 20 min pre-incubation step,

Figure 3 shows the kinetics of the chemiluminescence response elicited by Peptide 395 (●—● ; 395 (50µg) + fMLP; ■—■ ; 395 (50µg); ▲—▲ HBSS + fMLP; ○—○ HBSS); and

35 Figure 4 is a representation of the TNFα monomer



showing the position of the neutrophil stimulating peptides.

Production of human TNF peptides tested for neutrophils stimulatory activity.

- 5 The following peptides were synthesised and are described using the I.U.P.A.C. one-letter code abbreviations for amino acid residues with the TNF sequence region indicated in brackets.

peptide 275

- 10 A K P W Y E P I Y L (111-120)

peptide 301

V R S S S R T P S D K P V A H V V A (1-18)

peptide 302

L R D N Q L V V P S E G L Y L I (43-58)

- 15 peptide 303

L S A I K S P C Q R E T P E G A (94-109)

peptide 304

L F K G Q G C P S T H V L L T H T I S R I (63-83)

peptide 305

- 20 L S A E I N R P D Y L D F A E S G Q V (132-150)

peptide 306

V A H V V A N P Q A E G Q L (13-26)

peptide 307

A E G Q L Q W L N R R A N A L L A N G (22-40)

- 25 peptide 308

G L Y L I Y S Q V L F K G Q G (54-68)

peptide 309

H V L L T H T I S R I A V S Y Q T K V N L L (73-94)

peptide 323

- 30 T I S R I A V S Y Q T (79-89)

These peptides were synthesised using the following general protocol.

All peptides were synthesised using the Fmoc-polyamide method of solid phase peptide synthesis

- 35 (Atherton et al, 1978, J. Chem. Soc. Chem. Commun., 13,



537-539). The solid resin used was PepSyn KA which is a polydimethacrylamide gel on kieselguhr support with 4-hydroxymethylphenoxyacetic acid as the functionalised linker (Atherton et al, 1975, J. Am. Chem. Soc., 97, 6584-6585).

The carboxy terminal amino acid is attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification.

All Fmoc-groups are removed by piperidine/DMF wash and peptide bonds are formed either via pentafluorophenyl active esters or directly by BOP/NMM/HOBt (Castro's reagent) except for certain amino acids as specified in Table 1.

Side chain protection chosen for the amino acids are removed concomitantly during cleavage with the exception of Acn on cysteine which is left on after synthesis

TABLE 1

	<u>Amino acid</u>	<u>Protecting group</u>	<u>Coupling Method</u>
	Arg	Mtr or Pmc	Either
20	Asp	OBu	Either
	Cys	Acn (permanent)	Either
	Glu	OBu	Either
	His	Boc	OPfp only
	Lys	Boc	Either
25	Ser	But	BOP only
	Thr	But	BOP only
	Tyr	But	Either
	Asn	none	OPfp only
	Gln	none	OPfp only

30 Cleavage and Purification

Peptide 302. Peptide is cleaved from the resin with 95% TFA and 5% thioanisole (1.5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A)

35 Peptide 304. Peptide is cleaved from the resin with



95% TFA and 5% phenol (5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

5 Peptide 308. Peptide is cleaved from the resin with 95% TFA and 5% water (1.5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

10 Peptide 309. Peptide is cleaved from the resin with 95% TFA and 5% thioanisole and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

15 In addition, the following synthetic fragments of peptide 309 were synthesized. These peptides had the following amino acid sequence with the TNF sequence region indicated in brackets.

Peptide 393

L T H T I S R I A (76-84).

Peptide 394

S R I A V S Y Q T K V N L L (81-94).

20 Peptide 395

P S T H V L L T H T I (70-80).

Peptide 396

A V S Y Q T K V N L L (84-94).

Effect of TNF peptides on neutrophil function.

25 Chemiluminescence assay.

This assay examined the effect of TNF peptides on priming for a neutrophil F-met-leu-phe response as described by Ferrante et al, 1988, (Int. Arch. Allergy Appl. Immunol, 86, 82-91). Purified human neutrophils
30 were pretreated with peptide for 20 minutes before the addition of f-met-leu-phe. The lucigenin dependent chemiluminescence response, which reflects superoxide production, was then measured. The results obtained are set out in Table 2 and are expressed as mV of lucigenin
35 dependent chemiluminescence and represent the maximal cell



activity attained.

In addition, the effects of peptide 304, 308 and 309 are shown graphically in Figure 2.

This experiment was repeated with peptides 304, 308
5 and 309. The results obtained as shown in Table 3.

The experiment was also conducted using peptides 393, 394, 395 and 396. Of these peptides only peptide 395 was able to stimulate the neutrophil respiratory burst (Table 4). The effect of peptide 395 was dose dependent
10 as shown by the results of 3 experiments (Table 5). The kinetics of the chemiluminescence response elicited by peptide 395 is shown in Figure 3. Peptide 395 displays improved solubility over peptide 309.

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TABLE 2

Peptide	Concentration $\mu\text{g}/10^6$ cells)				
	0	1	10	100	500
275	1.02	0.99	0.69	0.43	0.80
301	0.34	0.93	0.74	0.55	1.10
302	0.37	0.16	0.18	0.29	
303	0.37	0.23	0.17	0.22	
304	0.37	0.18	0.43	2.56	2.76
305	0.37	0.27	0.36	0.24	
306	0.37	0.27	0.35	0.23	
307	0.37	0.35	0.37	0.42	
323	0.37	0.23	0.17	0.47	
308	0.37	0.91	4.80	49.52	
309	0.37	0.38	0.98	13.44	

Results are expressed as mV of lucigenin dependent chemiluminescence and represent peak of response i.e. the maximal cell activity attained.

TABLE 3

Peptide	Peptide concentration ($\mu\text{g}/10^6$ cells)		
	0	10	100
304	0.04	0.36	0.64
304 + fMLP	0.71	0.91	6.97
308	0.04	1.00	11.76
308 + fMLP	0.42	2.74	28.56
309	0.04	0.31	0.69
309 + fMLP	0.42	2.46	12.84

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Table 4 Comparisons of 309 and its subpeptides on neutrophil respiratory burst

Treatment (100 ug peptide)	Chemiluminescence (mV)
Diluent	0.58
309	4.70
393	0.31
394	0.33
395	5.32
396	0.70

Table 5 Effect of 395 on neutrophil respiratory burst

Treatment	Chemiluminescence (mV)		
	Exp. 1	Exp. 2	Exp. 3
Diluent	0.58	0.68	0.38
fMLP	1.53	3.53	1.96
1 μ g 395	3.25	0.89	0.03
1 μ g 395 + fMLP	3.36	4.55	0.29
10 μ g 395	4.92	3.97	0.64
10 μ g 395 +fMLP	7.31	9.10	2.34
50 μ g 395	8.01	10.81	
50 μ g 395 + fMLP	12.58	22.09	
100 μ g 395	2.36	19.14	5.26
100 μ g 395 + fMLP	5.29	18.10	10.59
100 μ g 309	5.98	6.68	1.24
100 μ g 309 + fMLP	27.44	22.77	6.69

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Effect on Superoxide Formation

The effect of peptides 308 and 309 on superoxide formation was examined by the cytochrome reduction assay, according to the procedure of Ferrante, 1989 (Infection and Immunity), 57: 2115-2122). The results, expressed as n moles of $O_2/5 \times 10^5$ cells as set out in Table 6.

TABLE 6

Peptide	Peptide concentration ($\mu g/5 \times 10^5$ cells)		
	0	10	100
308	0.270	2.78	4.892
308 + fMLP	2.757	5.00	6.729
309	0.270	0.62	2.30
309 + fMLP	2.757	3.87	5.14

Effect of TNF peptides on neutrophil random migration

Migration of cells is an important property by which cells reach infection sites. Their accumulation at these sites is also dependent on the capacity of inflammatory mediators to inhibit their migration out of the sites. The present inventors have examined TNF and peptide 304, 308 and 309 for their effect on the migration of neutrophils.

In these experiments neutrophils were pre-treated with the peptide or TNF and then examined their ability to migrate out of wells in agarose as described by Ferrante et al, 1988, (Arch. Allergy Appl. Immunol. 86:82-91). The results are shown in Table 7. The results show that TNF was only partially migration inhibitory at 100 units/ 10^6 cells. Both peptides 308 and 309 were potent migration inhibitors, however, peptide 304 was found to be chemokinetic (it stimulated cell migration).

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TABLE 7

Treatment	Inhibition of Migration ($\mu\text{g}/10^6$ cells)		
	0	10	100
5 TNF	ND	ND	4%
304*	-16%	-43%	-883%
308	0	0	100%
309	0	0	100%

10 *Peptide 304 was found to stimulate (chemokinetic)
Chemotactic properties of TNF and peptides
 The chemotactic properties of TNF and peptides 304, 308 and 309 were examined using the following method:
 3ml of molten 2% agarose was mixed with 3ml of
 15 2x concentrated medium 199 containing foetal calf serum (10%) and poured into Petri dishes. Sets of 3 wells of 2.5mm diameter, each 3mm apart, were cut in the agarose. 5 μ l of neutrophils (2×10^5
 20 cells) were added to the inner well, with chemotactic agent or control medium added to the outer wells. Migration at various time intervals was then measured. The results of these experiments are shown in Table 8.

TABLE 8

Agent*	Migration distance (mm) at			
	1.5h		2.5h	
	None	Agent	None	Agent
30 fMLP	0.50	1.46	0.66	2.45
TNF	0.50	0.48	0.66	0.69
304	0.48	0.47	0.68	0.72
308	0.50	0.66	0.63	1.41
309	0.50	0.53	0.63	0.68

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- * To the chemotactic well was added 5ml of 1×10^{-7} MfMLP, of either peptide 304; peptide 308 and peptide 309 or 10^3 U/ml of TNF α

Effect of TNF Peptides on Neutrophil Degranulation

- 5 The conditions of measuring degranulation were as described by Ferrante A, 1989, (Infect and Immunity 57, 3110-3115). In these studies $100 \mu\text{l}$ of neutrophils ($10^7/\text{ml}$) were incubated for 20min at 37°C after which $10 \mu\text{l}$ of cytochalasin B was added. After 10 min
- 10 incubation the volume of cell suspension was made up to 1 ml with Hanks Balanced Salt Solution (HBSS). The cell-free supernatants were collected and analysed for enzyme levels after a further incubation at 37°C . β -Glucuronidase activity was measured
- 15 fluorimetrically by using 4-methylumbelliferyl- β -D-glucuronide as substrate. This involved incubating $50 \mu\text{l}$ of 2.5 mM substrate in 0.1 M citric acid -sodium phosphate buffer, pH 4.5, at 37°C for 3 h. The reaction was stopped by adding 1.5 ml
- 20 of 0.2 M glycine-sodium hydroxide buffer, pH 10.7 and the fluorescence of the liberated 4-methylumbelliferone was quantitated by using excitation and emission wavelengths of 336 and 446 nm, respectively. Vitamin B₁₂ binding protein was measured using ^{57}Co -vitamin B₁₂. This
- 25 assay is based on the principle that the binding protein binds to the ^{57}Co -Vitamin B₁₂ and as a result the radioactive vitamin B₁₂ does not bind to charcoal. The resultant radioactivity in the supernatant can then be equated to the concentration of vitamin B₁₂-binding
- 30 protein in the sample. The results of these experiments are set out in Table 9 A and B.

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Table 9 Effect of TNF Peptides on Neutrophil Degranulation

Neutrophils were treated with $100\mu\text{g}/10^6$ cells of 304, 305 or 308+fMLP (in the presence of CytoB).

A.

Treatment	β -glucuronidase release (%)			
	Exp. 1	Exp. 2	Exp. 3	Exp.
HBSS	3.63	1.84	2.72	6.23
HBSS + fMLP	23.62	41.41	40.19	27.54
304	3.63	3.14	2.52	9.82
304 + fMLP	26.95	36.43	35.34	36.65
Control peptide	-	-	-	13.41
Control peptide + fMLP	-	-	-	35.69
308	0.8	0.65	2.76	0.72
308 + fMLP	17.57	28.86	17.86	18.20

B.

Treatment	Vitamin B ₁₂ Binding Protein			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
HBSS	9.21	9.27	9.67	4.80
HBSS + fMLP	28.85	27.91	45.31	27.33
304	11.40	10.82	13.06	8.42
304 + fMLP	43.76	35.60	59.15	37.12
Control peptide	-	-	-	7.49
Control peptide + fMLP	-	-	-	38.62
308	2.00	2.08	5.70	2.25
308 + fMLP	35.81	27.59	26.55	21.51



The effects of TNF α peptides on stimulation of neutrophil respiratory burst, degranulation, migration inhibition, chemokinesis and chemotaxis were investigated. As can be seen from the results set out above only
5 peptides 304, 308 and 309 were found to prime human neutrophils for the respiratory burst associated with f-met-leu-phe treatment, i.e. in a manner analogous to that of TNF α . Together these peptides comprise the primary amino acids sequence region of amino acids 54 to
10 94 of human TNF α . Peptide 308 is a particularly potent primer of neutrophils in this assay.

It is to be noted, however, that peptide 323 which has a sequence which corresponds to amino acids 79 to 89 of human TNF was not found to be capable of priming
15 neutrophils for the respiratory burst associated with f-met-leu-phe treatment. The reason for the lack of neutrophil stimulating activity of this peptide has not as yet been elucidated, however, one hypothesis for the lack of activity of this peptide may be that peptide 323 does
20 not include the amino acid residues which bind to the TNF receptor on the neutrophils.

Peptides 308 and 309 have also been found to be potent inhibitors of neutrophil migration whilst peptide 304 has been found to be chemokinetic. Peptide 308 has
25 also been found to be strongly chemotactic.

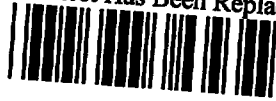
The effects of TNF peptides 304 and 308 on degranulation of neutrophils (Table 9) showed that peptide 308 decreased the release of the contents of both the specific and the azurophilic granules as measured by the
30 release of Vitamin B₁₂ binding protein and β -glucuronidase release respectively. This effect of peptide 308 was still apparent following stimulation with fMLP. In contrast, peptide 304 had no effect on neutrophil degranulation in the absence of fMLP. In the
35 presence of fMLP peptide 304 enhanced release from



specific granules but not azurophilic granules.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments
5 without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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CLAIMS:-

1. A peptide having amino acids sequence substantially corresponding to amino acids 54 to 94 of Figure 1, or a part thereof, wherein the peptide is capable of priming neutrophils for a superoxide production and an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.
2. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 52 to 94 of Fib. 1.
3. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 68 of Figure 1.
4. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 73 to 94 of Figure 1.
5. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 70 to 80 of Figure 1.
6. A compound having a three dimensional structure which substantially corresponds to the three dimensional structure of the peptide as claimed in any one of claims 1 to 5, in which the compound is capable of priming neutrophils for a respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.
7. A method of treating a subject having depressed neutrophil function the method comprising administering to the subject an effective therapeutic amount of the peptide as claimed in any one of claims 1 to 5.
8. Method as claimed in claim 7 in which the subject is suffering from acquired immune deficiency syndrome.
9. A method as claimed in claim 7 in which the subject is suffering from cancer.
10. A method of treating a subject suffering from an inflammatory syndrome comprising administering to the

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subject an effective therapeutic amount of peptide as claimed in claim 3.

11. A method as claimed in claim 10 in which the inflammatory syndrome is rheumatoid arthritis or adult respiratory distress syndrome.

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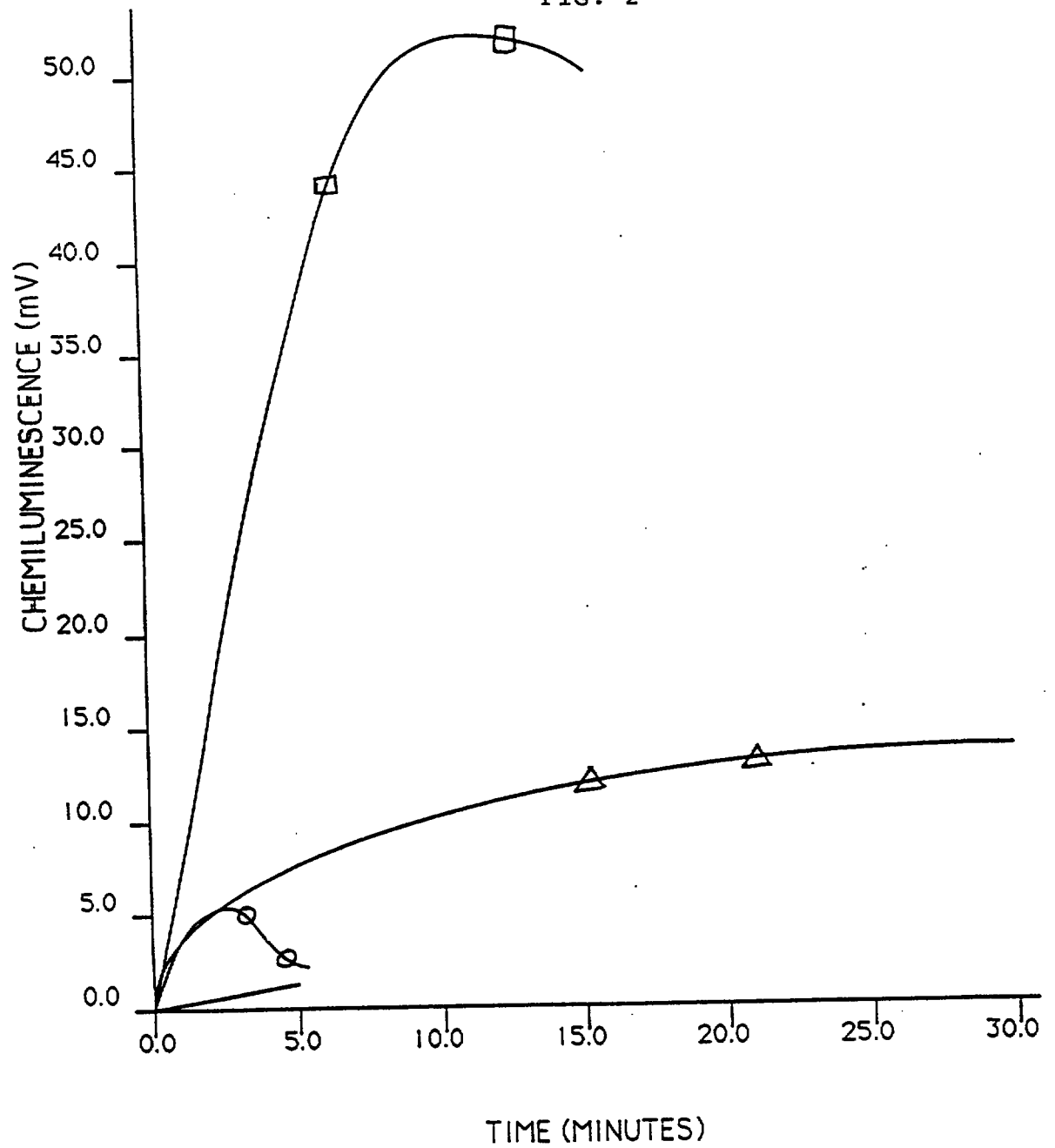
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FIG. 1

VRSSSRTPSD¹⁰KPVAHVVANP²⁰QAEGQLQWLN³⁰RRANALLANG⁴⁰
VELRDNQLVV⁵⁰PSEGLYLIYS⁶⁰QVLFKGQGCP⁷⁰STHVLLTHTI⁸⁰
SRIAVSYQTK⁹⁰VNLLSAIKSP¹⁰⁰CQRETPEGAE¹¹⁰AKPWYEPIYL¹²⁰
GGVFQLEKGD¹³⁰RLSAEINRPD¹⁴⁰YLDFAESGQV¹⁵⁰YFGHIAL¹⁵⁷

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FIG. 2



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FIG. 3

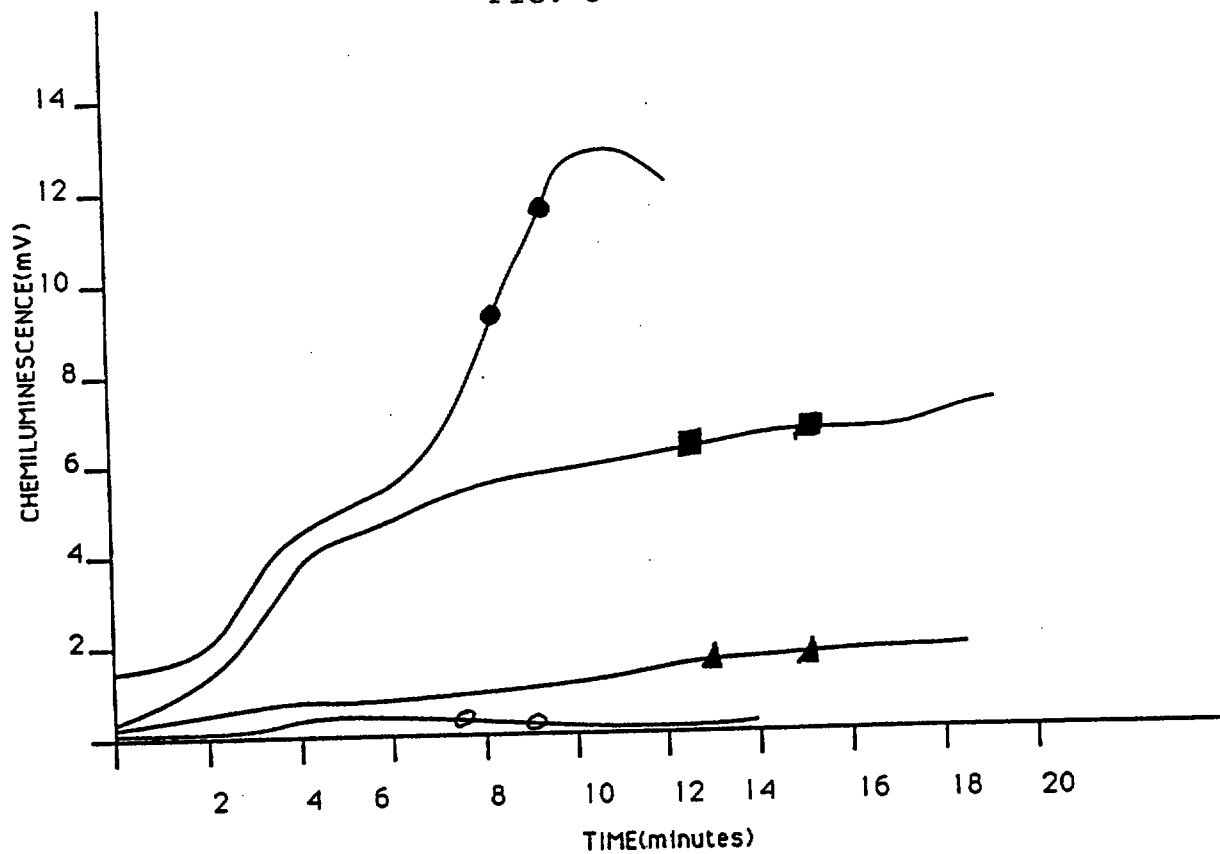
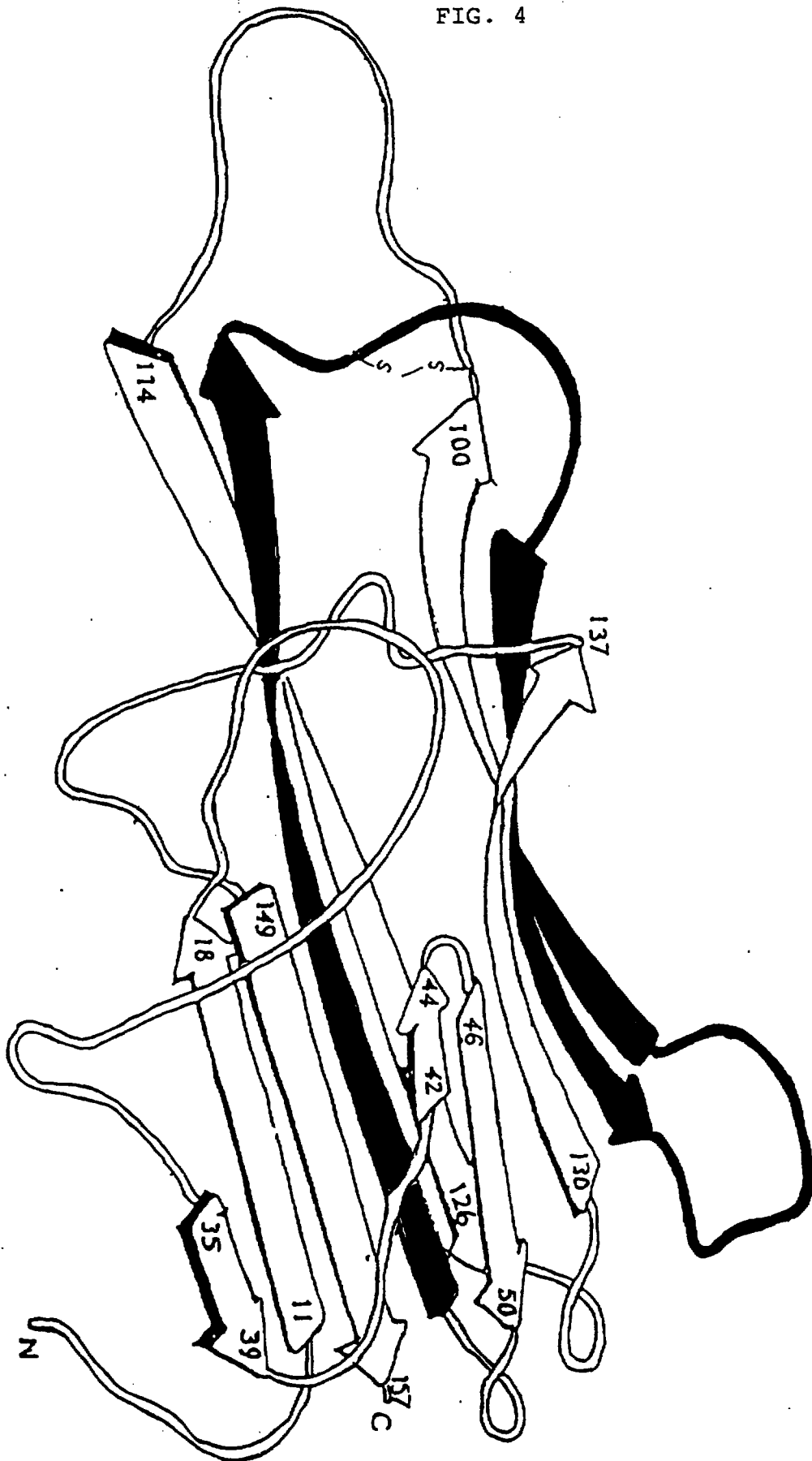


FIG. 4



ABSTRACT

NEUTROPHIL STIMULATING PEPTIDES

The present invention provides peptides capable of stimulating neutrophils. In particular, the peptides prime neutrophils for a respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine. The peptides have an amino acid sequence substantially corresponding to amino acids 54 to 94 of Figure 1 or a part thereof. These peptides may also be used in the treatment of a subject having depressed neutrophil function.



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CLAIMS

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1. A peptide having amino acids sequence substantially corresponding to amino acids 54 to 94 of Figure 1, or a part thereof, wherein the peptide is capable of priming neutrophils for a superoxide production and an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.
2. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 94 of Figure 1.
3. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 54 to 68 of Figure 1.
4. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 73 to 94 of Figure 1.
5. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 70 to 80 of Figure 1.
6. A compound having a three dimensional structure which substantially corresponds to the three dimensional structure of the peptide as claimed in any one of claims 1 to 5, in which the compound is capable of priming neutrophils for a respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.
7. A pharmaceutical formulation comprising a peptide as claimed in any one of claims 1 to 5 and any acceptable pharmaceutical carrier or diluent.

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8. A peptide as claimed in any one of claims 1 to 5 or a compound as claimed in claim 6 for use in medicine.

9. Use of a peptide as claimed in any one of claims 1 to 5 or a compound as claimed in claim 6 in the preparation of an agent for the treatment or prophylaxis of diseases characterised by depressed neutrophil function.

10. Use as claimed in claim 9 in which the subject is suffering from either acquired immune deficiency syndrome or cancer.

11. Use of a peptide as claimed in claim 3 in the preparation of an agent for the treatment of a subject suffering from an inflammatory syndrome.

12. Use as claimed in claim 11 in which the inflammatory syndrome the subject is suffering from is rheumatoid arthritis or adult respiratory distress syndrome.

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side effects associated with the therapeutic use of the whole TNF molecule.

Accordingly, in a first aspect the present invention consists in a peptide which primes neutrophils for superoxide production and an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine, wherein the peptide is of the general formula:-

10 $X_1-X_2-X_3-X_4-X_5$ in which

X_1 is null or A1-A2-A3-A4-A5-A6-A7

in which A1 is Leu, Ile, Val or Met

A2 is Phe, Tyr, Trp or His

15 A3 is Lys, Arg or His

A4 is Gly or Ala

A5 is Gln or Asn

A6 is Gly or Ala

A7 is Cys

20 X_2 is null or A8-A9-A10

in which A8 is Pro or N α -alkylamino acid

A9 is Ser or Thr

A10 is Thr or Ser

X_3 is A11-A12-A13-A14-A15-A16-A17-A18

25 in which A11 is His, Lys or Arg

A12 is Val, Ile, Leu or Met

A13 is Leu, Ile, Val or Met

A14 is Ile, Leu, Val or Met

A15 is Thr or Ser

30 A16 is His, Lys or Arg

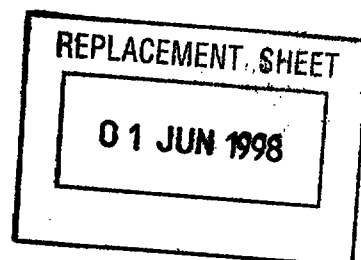
A17 is Thr or Ser

A18 is 16, Leu, Val or Met

X_4 is null or A19-A20-A21

in which A19 is Ser or Thr

35 A20 is Arg, Lys or His



A21 is Ile, Leu, Val or Met

X5 is null or A22-A23-A24-A25-A26-A27-A28-A29-A30-A31-A32

in which A22 is Ala or Gly

5 A23 is Val, Ile, Leu or Met

A24 is Ser or Thr

A25 is Tyr, Phe, Trp or His

A26 is Glu or Asp

A27 is Thr or Ser

10 A28 is Lys, Arg or His

A29 is Val, Ile, Leu or Met

A30 is Asn or Gln

A31 is Leu, Ile, Val or Met

A32 is Leu, Ile, Val or Met

15 with the proviso that:

if X2 is null then X1 is null

if X4 is null then X5 is null.

In a preferred embodiment of the present

invention X1 is null, X2 is A8-A9-A10, X4 is null and

20 X5 is null. It is further preferred that A8 is Pro,

A9 is Ser, A10 is Thr, A11 is His, A12 is Val, A13 is

Leu, A14 is Leu, A15 is Thr, A16 is His, A17 is Thr and

A18 is Ile, or A8 is Pro, A9 is Ser, A10 is Thr, A11 is

His, A12 is Val, A13 is Leu, A14 is Ile, A15 is Thr,

25 A16 is His, A17 is Thr and A18 is Ile.

In another preferred embodiment of the present

invention X1 is A1-A2-A3-A4-A5-A6-A7, X2 is A8-A9-A10,

X4 is A19-A20-A21 and X5 is null. It is further

preferred that A1 is Leu, A2 is Phe, A3 is Lys, A4 is

30 Gly, A5 is Gln, A6 is Gly, A7 is Cys, A8 is Pro, A9 is

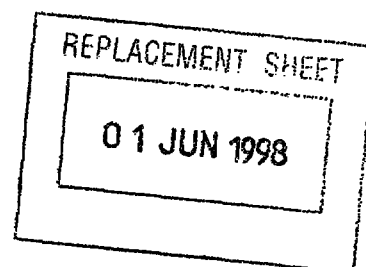
Ser, A10 is Thr, A11 is His A12 is Val, A13 is Leu, A14

is Leu, A15 is Thr, A16 is His, A17 is Thr, A18 is Ile,

A19 is Ser, A20 is Arg and A21 is Ile.

In yet another preferred embodiment of the present

35 invention X1 and X2 are null, X4 is A19-A20-A21 and X6



is A22-A23-A24-A25-A26-A27-A28-A29-A30-A31-A32. It is further preferred that A11 is His, A12 is Val, A13 is Leu, A14 is Leu, A15 is Thr, A16 is His, A17 is Thr, A18 is Ile, A19 is Ser, A20 is Arg, A21 is Ile, A22 is Ala, 5 A23 is Val, A24 is Val, A25 is Ser, A26 is Tyr, A27 is Glu, A28 is Lys, A29 is Val, A30 is Asa, A31 is Leu and A32 is Leu.

In a second aspect the present invention consists in a peptide which primes neutrophils for superoxide 10 production and an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine, wherein the peptide is of the general formula:

Y1-Y2-Y3-Y4-Y5-Y6-Y7-Y8-Y9-Y10-Y11-Y12-Y13-Y14-Y15
15 in which Y1 is Gly or Ala
Y2 is Leu or Ile or Val or Met
Y3 is Tyr or Phe or Trp or His
Y4 is Leu or Ile or Val or Met
Y5 is Ile or Leu or Val or Met
20 Y6 is Tyr or Phe or Trp or His
Y7 is Ser or Thr
Y8 is Gln or Asn
Y9 is Val or Ile or Leu or Met
Y10 is Leu or Val or Ile or Met
25 Y11 is Phe or Tyr or Trp or His
Y12 is Lys or Arg or His
Y13 is Gly or Ala
Y14 is Asn or Gln
Y15 is Gly or Ala

30 As will be appreciated by those skilled in the art from the description which follows the present inventors have demonstrated that the region of human TNF from amino acid 54 to amino acid 94 plays an important functional role in the stimulation of neutrophils. Further, the 35 present inventors have produced 5 peptides namely peptides

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304, 308, 309, 395, 419 (as referred to herein) which have neutrophil stimulating activity.

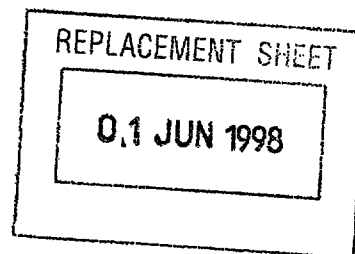
Armed with this information and with the aid of co-ordinates of the crystalline structure of TNF at 2.6 Å as disclosed by Eck and Sprang, 1989 (J. Biol. Chem., 264: 18795-17605), the person skilled in the art will be able to design non-peptide structures which, in 3 dimensional terms mimic the peptides of the present invention. It is believed that these non-peptide structures will also mimic the physiological effects of the peptides of the present invention. It is intended that such non-peptide structures are included within the scope of the present invention. Changes to the TNF molecule in these regions using eg. site directed mutagenesis would also be expected to affect neutrophil activation. A schematic representation of the three dimensional structure of TNF α is shown in Figure 5.

Accordingly in a third aspect the present invention consists in a compound the three dimensional structure of which substantially corresponds to the three dimensional structure of the peptide of the first or second aspects of the present invention, the compound being characterized in that the compound is capable of eliciting superoxide production by neutrophils and of priming neutrophils for an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.

In a further aspect, the present invention consists in a method of treating a subject having depressed neutrophil function, the method comprising administering to the subject a therapeutic amount of the peptide of the first aspect of the present invention.

In a preferred embodiment of this aspect of the present invention the subject is suffering from acquired immune deficiency syndrome.

Peptide 308, through selective effects on neutrophil



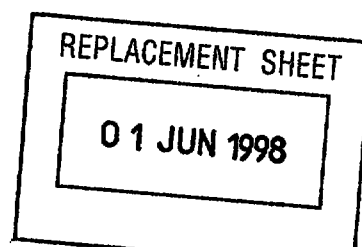
degranulation may be administered to individuals suffering from inflammatory syndromes e.g. rheumatoid arthritis, adult respiratory distress syndrome.

It will be appreciated by those skilled in the art
5 that a number of modifications may be made to the peptide of the present invention without deleteriously effecting the biological activity of the peptide. This may be achieved by various changes, such as insertions, deletions and substitutions (e.g., sulfation, phosphorylation,
10 nitration, halogenation), either conservative or non-conservative (e.g., W-amino acids, desamino acids) in the peptide sequence where such changes do not substantially altering the overall biological activity of the peptide. By conservative substitutions the intended
15 combinations are:-

G, A; V, I, L, M; D, E; N, Q; S, T; K, R, H; F, Y, W, H; and P, α -alkylamino acids.

It may also be possible to add various groups to the peptide of the present invention to confer advantages such
20 as increased potency or extended half-life in vivo, without substantially altering the overall biological activity of the peptide.

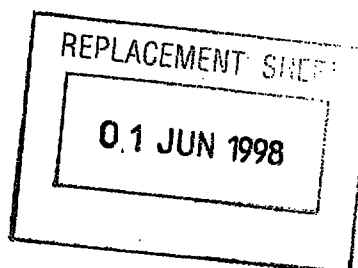
The term peptide is to be understood to embrace peptide bond replacements and/or peptide mimetics, i.e.
25 pseudopeptides, as recognised in the art (see for example: Proceedings of the 20th European Peptide Symposium, edt. G. Jung. E. Bayer, pp. 289-336, and references therein), as well as salts and pharmaceutical preparations and/or formulations which render the bioactive peptide(s)
30 particularly suitable for oral, topical, nasal spray, ocular pulmonary, I.V., subcutaneous, as the case may be, delivery. Such salts, formulations, amino acid replacements and pseudopeptide structures may be necessary and desirable to enhance the stability, formulation,
35 deliverability (e.g., slow release, prodrugs), or to



improve the economy of production, and they are acceptable, provided they do not negatively affect the required biological activity of the peptide.

Apart from substitutions, three particular forms of peptide mimetic and/or analogue structures of particular relevance when designating bioactive peptides, which have to bind to a receptor while risking the degradation by proteinases and peptidases in the blood, tissues and elsewhere, may be mentioned specifically, illustrated by the following examples: Firstly, the inversion of backbone chiral centres leading to D-amino acid residue structures may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation while not impairing activity. An example is given in the paper "Tritriated D-ala¹-Peptide T Binding", Smith, C.S. et al, Drug Development Res. 15, pp. 371-379 (1988). Secondly, cyclic structure for stability, such as N to C interchain imides and lactames (Ede et al in Smith and Rivier (Eds) "Peptides: Chemistry and Biology", Escom, Leiden (1991), p268-270), and sometimes also receptor binding may be enhanced by forming cyclic analogues. An example of this is given in "Conformationally restricted thymopentin-like compounds", U.S. pat. 4,457,489 (1985), Goldstein, G. et al. Finally, the introduction of ketomethylene, methylsulfide or retroinverse bonds to replace peptide bonds, i.e. the interchange of the CO and NH moieties may both greatly enhance stability and potency. An example of the latter type is given in the paper "Biologically active retroinverse analogues of thymopentin", Sisto A. et al in Rivier, J.E. and Marshall, G.R. (eds.) "Peptides, Chemistry, Structure and Biology", Escom, Leiden (1990), p.722-773.

The peptides of the invention can be synthesized by various methods which are known in principle, namely by chemical coupling methods (cf. Wunsch, E.: "Methoden der

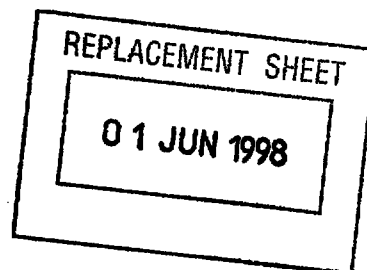


organischen Chemie", Volume 15, Band 1 + 2, Synthese von Peptiden, Thieme Verlag, Stuttgart (1974), and Barrany, G.; Merrifield, R.B: "The Peptides", eds. E. Gross, J. Meienhofer., Volume 2, Chapter 1, pp. 1-284, Academic Press (1980)), or by enzymatic coupling methods (cf. Widmer, F., Johansen, J.T., Carlsberg Res. Commun., Volume 44, pp. 37-46 (1979), and Kullmann, W.: "Enzymatic Peptide Synthesis", CRC Press Inc., Boca Raton, Florida (1987), and Widmer, F., Johansen, J.T. in "Synthetic Peptides in Biology and Medicine:", eds., Alitalo, K., Partanen, P., Vattieri, A., pp. 79-86, Elsevier, Amsterdam (1985)), or by a combination of chemical and enzymatic methods if this is advantageous for the process design and economy.

It is intended that such modifications to the peptide of the present invention which do not result in a decrease in biological activity are within the scope of the present invention.

Moreover, the three-dimensional structure and function of the biologically active peptides can be simulated by other compounds, some not even peptidic in nature, but which mimic the activity of such peptides. This field of science is summarised in a review by Goodman, M. (1990). (Synthesis, spectroscopy and computer simulations in peptide research. Proc. 11th American Peptide Symposium published in Peptides-Chemistry, Structure and Biology pp 3-29. Ed Rivier, J.E. and Marshall, G.R. Publisher ESCOM.)

As will be recognized by those skilled in the art, armed with the disclosure of this application, it will be possible to produce peptide and non-peptide compounds having the same three-dimensional structure as the peptide of the present invention. These "functionally equivalent structures" or "peptide mimics" will react with antibodies raised against the peptide of the present invention and



may also be capable of stimulating macrophages and/or neutrophils. It is intended that such "peptide mimics" are included within the scope of the present invention.

Accordingly, in a fifth aspect the present invention consists in a compound the three-dimensional structure of which is similar as a pharmacophore to the three-dimensional structure of the peptide of the first aspect of the present invention, the compound being characterized in that it reacts with antibodies raised against the peptide of the first aspect of the present invention and that the compound is capable of activating macrophages and/or neutrophils.

More detail regarding pharmacophores can be found in Bolin et al. p 150, Polinsky et al. p 287, and Smith et al. p 485 in Smith and Rivier (Eds) "Peptides: Chemistry and Biology", Escom, Leiden (1991).

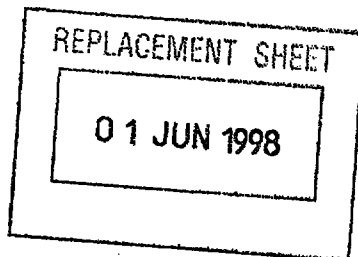
In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples, and Figures in which:-

Figure 1 shows the amino acid sequence of human TNF; Figure 2 shows the effects of peptides 304 (○), 308 (□) and 309 (△) on the fMLP induced human neutrophil response. Peptides were used at $100\mu\text{mg}/10^6$ in the 20 min pre-incubation step,

Figure 3 shows the kinetics of the chemiluminescence response elicited by Peptide 395 (●—● ; 395 (50mg) + fMLP; ■—■ ; 395 (50mg); ▲—▲ HBSS + fMLP; ○—○ HBSS);

Figure 4 shows stimulation of superoxide production by peptide 419; and

Figure 5 is a representation of the TNF α monomer showing the position of the neutrophil stimulating peptides.



Production of human TNF peptides tested for neutrophils stimulatory activity.

The following peptides were synthesised and are described using the I.U.P.A.C. one-letter code abbreviations for amino acid residues with the TNF sequence region indicated in brackets.

Peptide 275

A K P W Y E P I Y L (111-120)

Peptide 301

10 V R S S S R T P S D K P V A H V V A (1-18)

Peptide 302

L R D N Q L V V P S E G L Y L I (43-58)

Peptide 303

L S A I K S P C Q R E T P E G A (94-109)

15 Peptide 304

L F K G Q G C P S T H V L L T H T I S R I (63-83)

Peptide 305

L S A E I N R P D Y L D F A E S G Q V (132-150)

Peptide 306

20 V A H V V A N P Q A E G Q L (13-26)

Peptide 307

A E G Q L Q W L N R R A N A L L A N G (22-40)

Peptide 308

G L Y L I Y S Q V L F K G Q G (54-68)

25 Peptide 309

H V L L T H T I S R I A V S Y Q T K V N L L
(73-94)

Peptide 323

T I S R I A V S Y Q T (79-89)

30 Peptide 393

L T H T I S R I A (76-84).

Peptide 394

S R I A V S Y Q T K V N L L (81-94).

Peptide 395

35 P S T H V L L T H T I (70-80).

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Peptide 396

A V S Y Q T K V N L L (84-94).

Peptide 419

P S T H V L I T H T I

5 Peptide 462

K G Q G Cys (Acm) P S T H V L L T H T

These peptides were synthesised using the following general protocol.

All peptides were synthesised using the

- 10 Fmoc-polyamide method of solid phase peptide synthesis
(Atherton et al, 1978, J. Chem. Soc. Chem. Commun., 13,

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537-539). The solid resin used was PepSyn KA which is a polydimethylacrylamide gel on kieselguhr support with 4-hydroxymethylphenoxyacetic acid as the functionalised linker (Atherton et al, 1975, J. Am. Chem. Soc., 97, 6584-6585).

The carboxy terminal amino acid is attached to the solid support by a DCC/DMAP-mediated symmetrical-anhydride esterification.

All Fmoc-groups are removed by piperidine/DMF wash and peptide bonds are formed either via pentafluorophenyl active esters or directly by BOP/MMM/HOBt (Castro's reagent) except for certain amino acids as specified in Table 1.

Side chain protection chosen for the amino acids are removed concomitantly during cleavage with the exception of Acn on cysteine which is left on after synthesis

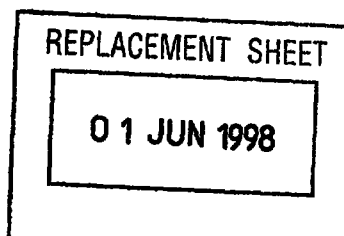
TABLE 1

	<u>Amino acid</u>	<u>Protecting group</u>	<u>Coupling Method</u>
	Arg	Mtr or Pmc	Either
20	Asp	OBu	Either
	Cys	Acn (permanent)	Either
	Glu	OBu	Either
	His	Boc	OPfp only
	Lys	Boc	Either
25	Ser	But	BOP only
	Thr	But	BOP only
	Tyr	But	Either
	Asn	none	OPfp only
	Gln	none	OPfp only

30 Cleavage and Purification

Peptide 302. Peptide is cleaved from the resin with 95% TFA and 5% thioanisole (1.5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A)

35 Peptide 304. Peptide is cleaved from the resin with



95% TFA and 5% phenol (5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

Peptide 308. Peptide is cleaved from the resin with 95% TFA and 5% water (1.5 h) and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

Peptide 309. Peptide is cleaved from the resin with 95% TFA and 5% thioanisole and purified on reverse phase C4 column. (Buffer A - 0.1% aqueous TFA, Buffer B - 80% ACN 20% A).

~~In addition, the following synthetic fragments of peptide 309 were synthesized. These peptides had the following amino acid sequence with the TNF sequence region indicated in brackets.~~

~~Peptide 393~~

~~L T H T I S R I A (76-84).~~

~~Peptide 394~~

~~S R I A V S Y Q T K V N I L L (81-94).~~

20 ~~Peptide 395~~

~~P S T H V L L T H T I (70-80).~~

~~Peptide 396~~

~~A V S Y Q T K V N I L L (84-94).~~

Effect of TNF peptides on neutrophil function.

25 Chemiluminescence assay.

This assay examined the effect of TNF peptides on priming for a neutrophil F-met-leu-phe response as described by Ferrante et al, 1988, (Int. Arch. Allergy Appl. Immunol, 86, 82-91). Purified human neutrophils were pretreated with peptide for 20 minutes before the addition of f-met-leu-phe. The lucigenin dependent chemiluminescence response, which reflects superoxide production, was then measured. The results obtained are set out in Table 2 and are expressed as mV of lucigenin dependent chemiluminescence and represent the maximal cell

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activity attained.

In addition, the effects of peptide 304, 308 and 309 are shown graphically in Figure 2.

This experiment was repeated with peptides 304, 308
5 and 309. The results obtained as shown in Table 3.

The experiment was also conducted using peptides 393, 394, 395 and 396. Of these peptides only peptide 395 was able to stimulate the neutrophil respiratory burst (Table 4). The effect of peptide 395 was dose dependent
10 as shown by the results of 3 experiments (Table 5). The kinetics of the chemiluminescence response elicited by peptide 395 is shown in Figure 3. Peptide 395 displays improved solubility over peptide 309.

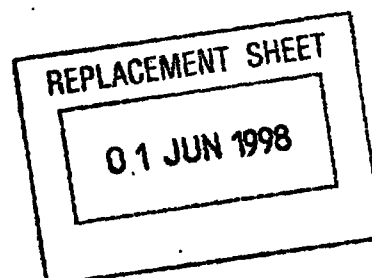


TABLE 2

Peptide	Concentration $\mu\text{g}/10^6$ cells)				
	0	1	10	100	500
275	1.02	0.99	0.69	0.43	0.80
301	0.34	0.93	0.74	0.55	1.10
302	0.37	0.16	0.18	0.29	
303	0.37	0.23	0.17	0.22	
304	0.37	0.18	0.43	2.56	2.76
305	0.37	0.27	0.36	0.24	
306	0.37	0.27	0.35	0.23	
307	0.37	0.35	0.37	0.42	
323	0.37	0.23	0.17	0.47	
308	0.37	0.91	4.80	49.52	
309	0.37	0.38	0.98	13.44	

Results are expressed as mV of lucigenin dependent chemiluminescence and represent peak of response i.e. the maximal cell activity attained.

TABLE 3

Peptide	Peptide concentration ($\mu\text{g}/10^6$ cells)		
	0	10	100
304	0.04	0.36	0.64
304 + fMLP	0.71	0.91	6.97
308	0.04	1.00	11.76
308 + fMLP	0.42	2.74	28.56
309	0.04	0.31	0.69
309 + fMLP	0.42	2.46	12.84

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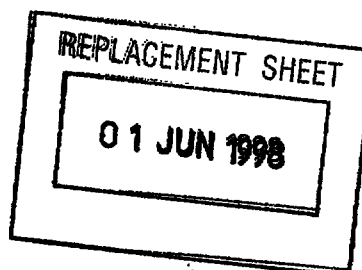
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Table 4 Comparisons of 309 and its subpeptides on neutrophil respiratory burst

Treatment (100 ug peptide)	Chemiluminescence (mV)
Diluent	0.58
309	4.70
393	0.31
394	0.33
395	5.32
396	0.70

Table 5 Effect of 395 on neutrophil respiratory burst

Treatment	Chemiluminescence (mV)		
	Exp. 1	Exp. 2	Exp. 3
Diluent	0.58	0.68	0.38
fMLP	1.53	3.53	1.96
1µg 395	3.25	0.89	0.03
1µg 395 + fMLP	3.36	4.55	0.29
10µg 395	4.92	3.97	0.64
10µg 395 + fMLP	7.31	9.10	2.34
50µg 395	8.01	10.81	
50µg 395 + fMLP	12.58	22.09	
100µg 395	2.36	19.14	5.26
100µg 395 + fMLP	5.29	18.10	10.59
100µg 309	5.98	6.68	1.24
100µg 309 + fMLP	27.44	22.77	6.69



Effect on Superoxide Formation

The effect of peptides 308 and 309 on superoxide formation was examined by the cytochrome reduction assay, according to the procedure of Ferrante, 1989 (Infection and Immunity), 57: 2115-2122). The results, expressed as n moles of $O_2/5 \times 10^5$ cells as set out in Table 6.

TABLE 6

Peptide	Peptide concentration ($\mu g/5 \times 10^5$ cells)		
	0	10	100
308	0.270	2.78	4.892
308 + FMLP	2.757	5.00	6.729
309	0.270	0.62	2.30
309 + FMLP	2.757	3.87	5.14

~~Effect of TNF peptides on neutrophil random migration~~

Migration of cells is an important property by which cells reach infection sites. Their accumulation at these sites is also dependent on the capacity of inflammatory mediators to inhibit their migration out of the sites. The present inventors have examined TNF and peptide 304, 308 and 309 for their effect on the migration of neutrophils.

In these experiments neutrophils were pre-treated with the peptide or TNF and then examined their ability to migrate out of wells in agarose as described by Ferrante et al, 1988, (Arch. Allergy Appl. Immunol. 86:82-91). The results are shown in Table 7. The results show that TNF was only partially migration inhibitory at 100 units/ 10^6 cells. Both peptides 308 and 309 were potent migration inhibitors, however, peptide 304 was found to be chemokinetic (it stimulated cell migration).

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The effect of peptide 419 on superoxide formation was also assessed in a like manner and the results shown in Figure 4.

In Socher et al., 1987 (PNAS:84:8829-8833) a peptide having some similarity to the peptides of the present invention is disclosed. The disclosed peptide is a fragment of TNF from residues 65-79. This peptide was synthesized by the present applicant and is referred to herein as "peptide 462".

Peptide 462 was assayed for its ability to prime neutrophils for a respiratory burst in comparison with peptide 419 using the protocol set out above. The results obtained are set out in Table 7.

TABLE 7

<u>Treatment</u>	<u>Chemiluminescence mean + sem (mV)</u>
HBSS	4.1 ± 0.3
462 (1mg)	3.1 ± 0.5
462 (10mg)	4.8 ± 0.7
462 (100mg)	4.8 ± 0.5
419 (100mg)	18.6 ± 0.5

Effect of TNF peptides on neutrophil random migration

Migration of cells is an important property by which cells reach infection sites. Their accumulation at these sites is also dependent on the capacity of inflammatory mediators to inhibit their migration out of the sites. The present inventors have examined TNF and peptide 304, 308 and 309 for their effect on the migration of neutrophils.

In these experiments neutrophils were pre-treated with the peptide or TNF and then examined their ability to migrate out of wells in agarose as described by Ferrante et al, 1988, (Arch. Allergy Appl. Immunol. 86:82-91). The results are shown in Table 7. The results show that TNF was only partially migration inhibitory at 100 units/10⁶ cells. Both peptides 308 and 309 were potent migration inhibitors, however, peptide 304 was found to be chemokinetic (it stimulated cell migration).

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TABLE 8

Treatment	Inhibition of Migration ($\mu\text{g}/10^6$ cells)		
	0	10	100
5 TNF	ND	ND	4%
304*	-16%	-43%	-883%
308	0	0	100%
309	0	0	100%

- 10 *Peptide 304 was found to stimulate (chemokinetic)
Chemotactic properties of TNF and peptides
 The chemotactic properties of TNF and peptides 304,
 308 and 309 were examined using the following method:
 3ml of molten 2% agarose was mixed with 3ml of
 15 2x concentrated medium 199 containing foetal calf
 serum (10%) and poured into Petri dishes. Sets of 3
 wells of 2.5mm diameter, each 3mm apart, were cut in
 the agarose. 5 μ l of neutrophils (2×10^5
 20 cells) were added to the inner well, with chemotactic
 agent or control medium added to the outer wells.
 Migration at various time intervals was then measured.
 The results of these experiments are shown in Table 9.

TABLE 9

Agent*	Migration distance (mm) at			
	1.5h		2.5h	
	None	Agent	None	Agent
30 fMLP	0.50	1.46	0.66	2.45
TNF	0.50	0.48	0.66	0.69
304	0.48	0.47	0.68	0.72
308	0.50	0.66	0.63	1.41
309	0.50	0.53	0.63	0.68

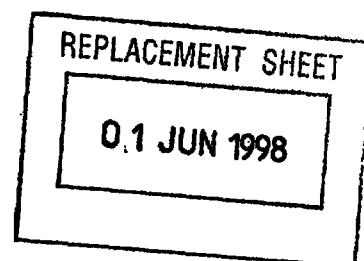
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- * To the chemotactic well was added 5ml of 1×10^{-7} MfMLP, of either peptide 304; peptide 308 and peptide 309 or 10^3 U/ml of TNF α

Effect of TNF Peptides on Neutrophil Degranulation

- 5 The conditions of measuring degranulation were as described by Ferrante A, 1989, (Infect and Immunity 57, 3110-3115). In these studies 100 μ l of neutrophils (10^7 /ml) were incubated for 20min at 37 °C after which 10 μ l of cytochalasin B was added. After 10 min
- 10 incubation the volume of cell suspension was made up to 1 ml with Hanks Balanced Salt Solution (HBSS). The cell-free supernatants were collected and analysed for enzyme levels after a further incubation at 37°C. β -Glucuronidase activity was measured
- 15 fluorimetrically by using 4-methylumbelliferyl- β -D-glucuronide as substrate. This involved incubating 50 μ l of 2.5 mM substrate in 0.1 M citric acid -sodium phosphate buffer, pH 4.5, at 37°C for 3 h. The reaction was stopped by adding 1.5 ml
- 20 of 0.2 M glycine-sodium hydroxide buffer, pH 10.7 and the fluorescence of the liberated 4-methylumbelliferone was quantitated by using excitation and emission wavelengths of 336 and 446 nm, respectively. Vitamin B₁₂ binding protein was measured using ⁵⁷Co-vitamin B₁₂. This
- 25 assay is based on the principle that the binding protein binds to the ⁵⁷Co-Vitamin B₁₂ and as a result the radioactive vitamin B₁₂ does not bind to charcoal. The resultant radioactivity in the supernatant can then be equated to the concentration of vitamin B₁₂-binding
- 30 protein in the sample. The results of these experiments are set out in Table 10 A and B.



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Table 10 Effect of TNF Peptides on Neutrophil Degranulation

Neutrophils were treated with $100\mu\text{g}/10^6$ cells of 304, 305 or 308 + fMLP (in the presence of CytoB).

A.

Treatment	β -glucuronidase release (%)			
	Exp. 1	Exp. 2	Exp. 3	Exp.
HBSS	3.63	1.84	2.72	6.23
HBSS + fMLP	23.62	41.41	40.19	27.54
304	3.63	3.14	2.52	9.82
304 + fMLP	26.95	36.43	35.34	36.65
Control peptide	-	-	-	13.41
Control peptide + fMLP	-	-	-	35.69
308	0.8	0.65	2.76	0.72
308 + fMLP	17.57	28.86	17.86	18.20

B.

Treatment	Vitamin B ₁₂ Binding Protein			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
HBSS	9.21	9.27	9.67	4.80
HBSS + fMLP	28.85	27.91	45.31	27.33
304	11.40	10.82	13.06	8.42
304 + fMLP	43.76	35.60	59.15	37.12
Control peptide	-	-	-	7.49
Control peptide + fMLP	-	-	-	38.62
308	2.00	2.08	5.70	2.25
308 + fMLP	35.81	27.59	26.55	21.51

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The effects of TNF α peptides on stimulation of neutrophil respiratory burst, degranulation, migration inhibition, chemokinesis and chemotaxis were investigated. As can be seen from the results set out above only peptides 304, 308 and 309 were found to prime human neutrophils for the respiratory burst associated with f-met-leu-phe treatment, i.e. in a manner analogous to that of TNF α . Together these peptides comprise the primary amino acids sequence region of amino acids 54 to 94 of human TNF α . Peptide 308 is a particularly potent primer of neutrophils in this assay.

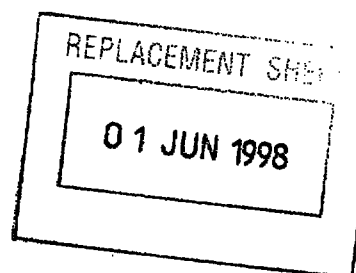
It is to be noted, however, that peptide 323 which has a sequence which corresponds to amino acids 79 to 89 of human TNF was not found to be capable of priming neutrophils for the respiratory burst associated with f-met-leu-phe treatment. The reason for the lack of neutrophil stimulating activity of this peptide has not as yet been elucidated, however, one hypothesis for the lack of activity of this peptide may be that peptide 323 does not include the amino acid residues which bind to the TNF receptor on the neutrophils.

Peptides 308 and 309 have also been found to be potent inhibitors of neutrophil migration whilst peptide 304 has been found to be chemokinetic. Peptide 308 has also been found to be strongly chemotactic.

The effects of TNF peptides 304 and 308 on degranulation of neutrophils (Table 10) showed that peptide 308 decreased the release of the contents of both the specific and the azurophilic granules as measured by the release of Vitamin B₁₂ binding protein and β -glucuronidase release respectively. This effect of peptide 308 was still apparent following stimulation with fMLP. In contrast, peptide 304 had no effect on neutrophil degranulation in the absence of fMLP. In the presence of fMLP peptide 304 enhanced release from

specific granules but not azurophilic granules.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments
5 without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.





CLAIMS:-

1. A peptide which primes neutrophils for superoxide production and an enhanced respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine,
5 wherein the peptide is of the general formula:-

$X_1-X_2-X_3-X_4-X_5$ in which

X1 is null or A1-A2-A3-A4-A5-A6-A7

- 10 in which A1 is Leu, Ile, Val or Met
A2 is Phe, Tyr, Trp or His
A3 is Lys, Arg or His
A4 is Gly or Ala
A5 is Gln or Asn
15 A6 is Gly or Ala
A7 is Cys

X2 is null or A8-A9-A10

- in which A8 is Pro or α -alkylamino acid
A9 is Ser or Thr
20 A10 is Thr or Ser

X3 is A11-A12-A13-A14-A15-A16-A17-A18

- in which A11 is His, Lys or Arg
A12 is Val, Ile, Leu or Met
A13 is Leu, Ile, Val or Met
25 A14 is Ile, Leu, Val or Met
A15 is Thr or Ser
A16 is His, Lys or Arg
A17 is Thr or Ser
A18 is 16, Leu, Val or Met

30 X4 is null or A19-A20-A21

- in which A19 is Ser or Thr
A20 is Arg, Lys or His
A21 is Ile, Leu, Val or Met

X5 is null or A22-A23-A24-A25-A26-A27-A28-A29-A30-A31-

35 A32

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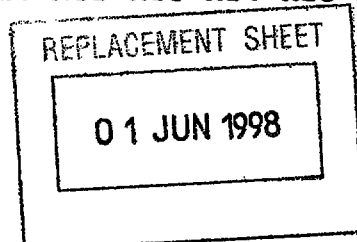


in which

5 A22 is Ala or Gly
 A23 is Val, Ile, Leu or Met
 A24 is Ser or Thr
 A25 is Tyr, Phe, Trp or His
 A26 is Glu or Asp
 A27 is Thr or Ser
 A28 is Lys, Arg or His
 A29 is Val, Ile, Leu or Met
 A30 is Asn or Gln
10 A31 is Leu, Ile, Val or Met
 A32 is Leu, Ile, Val or Met

with the proviso that:-

- if X2 is null then X1 is null,
 if X4 is null then X5 is null.
- 15 2. A peptide as claimed in claim 1 in which X1 is null,
 X2 is A8-A9-A10, X4 and X5 are null.
3. A peptide as claimed in claim 2 in which A8 is Pro,
 A9 is Ser, A10 is Thr, A11 is His, A12 is Val, A13 is Leu,
 A14 is Leu, A15 is Thr, A16 is His, A17 is Thr and A18 is
20 Ile.
4. A peptide as claimed in claim 2 in which A8 is Pro,
 A9 is Ser, A10 is Thr, A11 is His, A12 is Val, A13 is Leu,
 A14 is Ile, A15 is Thr, A16 is His, A17 is Thr and A18 is
 Ile.
- 25 5. A peptide as claimed in claim 1 in which X1 is
 A1-A2-A3-A4-A5-A6-A7, X2 is A8-A9-A10, X4 is A19-A20-A21
 and X5 is null.
6. A peptide as claimed in claim 5 in which A1 is Leu,
 A2 is Phe, A3 is Lys, A4 is Gly, A5 is Gln, A6 is Gly, A7
30 is Cys, A8 is Pro, A9 is Ser, A10 is Thr, A11 is His A12
 is Val, A13 is Leu, A14 is Leu, A15 is Thr, A16 is His,
 A17 is Thr, A18 is Ile, A19 is Ser, A20 is Arg and A21 is
 Ile.
7. A peptide as claimed in claim 1 in which X1 and X2
35 are null, X4 is A19-A20-A21 and X6 is A22-A23-A24-A25-A26-
 A27-A28-A29-A30-A31-A32.





8. A peptide as claimed in claim 7 in which A11 is His, A12 is Val, A13 is Leu, A14 is Leu, A15 is Thr, A16 is His A17 is Thr, A18 is Ile, A19 is Ser, A20 is Arg, A21 is Ile, A22 is Ala, A23 is Val, A24 is Val, A25 is Ser, A26 is Tyr, A27 is Glu, A28 is Lys, A29 is Val, A30 is Asa, A31 is Leu and A32 is Leu.
9. A peptide which primes neutrophils for superoxide production and an enhanced respiratory burst following treatment with
- 10 N-formyl-L-methionyl-L-leucyl-L-phenylalanine, wherein the peptide is of the general formula:
- Y1-Y2-Y3-Y4-Y5-Y6-Y7-Y8-Y9-Y10-Y11-Y12-Y13-Y14-Y15
- in which Y1 is Gly or Ala
- Y2 is Leu or Ile or Val or Met
- 15 Y3 is Tyr or Phe or Trp or His
- Y4 is Leu or Ile or Val or Met
- Y5 is Ile or Leu or Val or Met
- Y6 is Tyr or Phe or Trp or His
- Y7 is Ser or Thr
- 20 Y8 is Gln or Asn
- Y9 is Val or Ile or Leu or Met
- Y10 is Leu or Val or Ile or Met
- Y11 is Phe or Tyr or Trp or His
- Y12 is Lys or Arg or His
- 25 Y13 is Gly or Ala
- Y14 is Asn or Gln
- Y15 is Gly or Ala
10. A compound having a three dimensional structure which substantially corresponds to the three dimensional
- 30 structure of the peptide as claimed in any one of claims 1 to 9, in which the compound is capable of priming neutrophils for a respiratory burst following treatment with N-formyl-L-methionyl-L-leucyl-L-phenylalanine.
11. A method of treating a subject having depressed
- 35 neutrophil function the method comprising administering to

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the subject an effective therapeutic amount of the peptide as claimed in any one of claims 1 to 9.

12. Method as claimed in claim 11 in which the subject is suffering from acquired immune deficiency syndrome.

5 13. A method as claimed in claim 11 in which the subject is suffering from cancer.

10 14. A method of treating a subject suffering from an inflammatory syndrome comprising administering to the subject an effective therapeutic amount of peptide as claimed in claim 3.

15. A method as claimed in claim 14 in which the inflammatory syndrome is rheumatoid arthritis or adult respiratory distress syndrome.

