The present invention provides peptides having activity against intracellular infectious agents and in particular malarial parasites. The peptides have an amino acid sequence substantially corresponding to amino acids (132 to 157) or a part thereof. These peptides may also be used in the treatment of a subject infected with an intracellular infectious agent.
FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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The present invention relates to a peptide active against intracellular infectious agents and to a method of treatment using the peptide. The peptide is derived from human tumour necrosis factor alpha.

Tumour necrosis factor alpha (TNF) was first described as a factor found in the serum of BCG-treated mice which caused haemorrhagic regression of certain transplanted tumours and had cytolytic activity against several transformed cell lines in vitro (Carswell et al., 1975, PNAS 72: 3666-3670; Helson et al, 1975, Nature 258: 731-732). TNF was subsequently found to be produced by activated macrophages during the course of normal immune responses to many infectious agents. Sera containing TNF has been shown in vitro to be cytotoxic to cells infected with a number of different malarial parasites including *Plasmodium yoelii*, *P. berghei* and *P. falciparum* (Taverne et al., 1981 Infect. Immun. 33: 83-89; Haidaris et al., Infect. Immun. 42: 385-393) and to protect against *Klebsiella listeria* (Parant, 1980, Recent Results Cancer Res 75: 213) and *Plasmodium* (Clark et al., 1981 Infect. Immun. 32: 1058) in vivo in mice. TNF also has potent anti-microbial activity which is mediated by direct cytotoxicity of TNF for intracellularly infected cells (e.g. *Trypanosoma cruzi*, De Titto et al., 1986 J. Immunol. 137: 1342 and *Schistosoma mansoni*, (Czuprynski et al) 1988, J. Immunol. 140: 962) and also through TNF activation of neutrophil effector function (Djeu et al, 1986 J. Immunol. 137: 2980; Ferrante et al., 1989, Infect. Immun. 57: 3110; Ferrante et al., 1989, Infect. Immun. 57: 2115; Ferrante et al., Infect. Immun. 55: 1047).

In addition to its direct anti-parasite and anti-microbial activity, TNF has a direct effect on virus
infected cells. Pretreatment of cells in vitro with TNF has been shown to both inhibit the cytopathic effect of certain viruses and also to decrease the yield of virus from infected cells in a dose dependent manner (Mestan et al., 1986, Nature 323: 816). Further TNF has been shown to selectively kill virus-infected cells (Wong and Goeddel, 1986, Nature 323: 819). These studies all indicate the central role TNF plays in host responses to pathogens and indicate the potential therapeutic usefulness of TNF, particularly in the immunocompromised individual. Recent studies have, however, also indicated that overproduction of TNF is responsible for much of the cell and tissue damage observed in conditions such as cerebral malaria (Clark, 1987, Parasitol. Today 3: 300) and viral meningitis (Doherty et al., 1989, J. Immunol. 142: 3456). Further, clinical trials with TNF have shown it to be extremely toxic.

The present inventors have identified peptides of human TNF alpha which kill malarial parasites within infected blood cells in vitro and also reduces parasitemia in vivo. These peptides, which encompasses the peptide region 132-157 of human TNFa, would be expected to also be an effective agent against other intracellular infectious agents including other parasites, viruses and bacteria.

Accordingly, in a first aspect the present invention consists in a peptide active against intracellular infectious agents, the peptide having an amino acid sequence substantially corresponding to amino acid 132 to 157 of Figure 1 or a part thereof.

In a preferred embodiment of this aspect of the present invention the peptide has an amino acid sequence substantially corresponding to amino acids 132 to 157 of Figure 1.

In another preferred embodiment of this aspect of the
present invention the peptide has an amino acid sequence substantially corresponding to amino acids 132 to 150 of Figure 1.

As described in more detail hereunder, the peptide of the present invention have been shown to have activity against malarial parasites. Given the generalised activity of TNF against intracellular infectious agents, it is expected that the peptide of the present invention will also exhibit activity against other intracellular infectious agents apart from malarial parasites, such as microorganisms, viruses, mycoplasmas and other intracellular parasites. In particular, it is believed that the peptide of the present invention will be active against mycobacterial infections, such as tuberculosis and leprosy; Rickettsial infections, such as typhus, spotted fever, Q fever; Chlamydial infections; intracellular mycoplasmal infections; Listeria monocytogenes and Brucella infections; and against certain Salmonella infections.

In a second aspect the present invention consists in a method of treating a mammal infected with an intracellular infectious agent, the method comprising administering to the animal a composition comprising the peptide of the first aspect of the present invention in conjunction with a suitable pharmaceutical carrier.

In a preferred embodiment of this aspect of the present invention the intracellular infectious agent is selected from the group consisting of mycobacteria, viruses, and parasites, and in particular malarial parasites.

In the treatment of malaria peptide 305 or 347 may be coadministered with other anti-malarial drugs such as quinine, mefloquine, chloroquine and the like. It will also be appreciated that in certain therapies it may be useful to conjugate peptide 305 or 347 to another carrier
molecule which may or may not be a protein/peptide in nature.

As will be readily appreciated, the route of administration and the nature of the pharmaceutically acceptable carrier will depend on the nature of the infection and the mammal to be treated. It is believed that the choice of pharmaceutical carrier and route of administration is within the common general knowledge of a person skilled in the art.

In a third aspect the present invention consists in the use of a peptide having an amino acid sequence substantially corresponding to amino acid 132 to 157 of human TNF or a part thereof in the preparation of a medicament for the treatment of infection with an intracellular infectious agent.

In a preferred embodiment of this aspect of the present invention the peptide is peptide 305 or 347 as defined hereafter.

In a further preferred embodiment of this aspect of the present invention the intracellular infectious agent is selected from the group consisting of mycobacteria, viruses and parasites.

As the use of the peptide of the present invention obviates the need to use the entire TNF molecule, it is expected that the severe side effects associated with the therapeutic use of the whole TNF molecule will be avoided. This is confirmed by preliminary evidence which indicates that the peptide of the present invention is devoid of the toxicity associated with the whole TNF molecule. In addition, the peptide of the present invention being a non-specific mechanism for treatment of infection with intracellular infectious agents, would have certain advantages over a vaccine approach in that it is not dependent on stable virus/parasite phenotype and is thus not invalidated by antigenic modulation.
Armed with the information provided by the present application and with the aid of the co-ordinates of the crystalline structure of TNF at 2.6\text{\AA} as disclosed by Eck and Sprang, 1989 (J. Biol. Chem., 264; 17595-17605), a person skilled in the art would be able to design non-peptide structures which, in three dimensional terms, mimic the peptide of the present invention. It is believed that these non-peptide structures will also mimic the physiological effect of the peptide of the present invention. It is intended that such non-peptide structures are included within the scope of the present invention.

Accordingly, in a fourth 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 in which the compound is capable of killing malarial parasites within infected blood cells.

As will be understood by persons skilled in the art it may be possible to modify the peptide sequence of the peptide of the present invention at various points to confer advantages such as an increased potency or extended half-life in vivo.

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\alpha;

Figure 2 shows the effect of peptide 305 administered intravenously to mice infected with \textit{P}.\textit{chabaudi} (\textcircled{1} control 1, \textbullet control 2, \textsquare treated 1, \textcircled{2} treated 2);

Figure 3 shows the effect of peptide 305 administered intraperitoneally to mice infected with \textit{P}.\textit{chabaudi} (\textsquare control, \textbullet treated).

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.

PRODUCTION OF HUMAN TNF PEPTIDES TESTED FOR KILLING OF INTRACELLULAR INFECTIOUS AGENTS

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

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)

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

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 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)

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)

peptide 347

L S A E I N R P D Y L D F A E S G Q V Y F G I A L (132-157)

These peptides were synthesised using the following general protocol.
All peptides were synthesised using the Fmoc-polyamide method of solid phase peptide synthesis (Atherton et al., 1978, J. Chem. Soc. Chem. Commun., 13, 537-539). The solid resin used was PepSyn KA which is a polydimethyacrylamide 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>
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<tr>
<th>Amino acid</th>
<th>Protecting group</th>
<th>Coupling Method</th>
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</thead>
<tbody>
<tr>
<td>Arg</td>
<td>Mtr or Pmc</td>
<td>Either</td>
</tr>
<tr>
<td>Asp</td>
<td>OBut</td>
<td>Either</td>
</tr>
<tr>
<td>Cys</td>
<td>Acn (permanent)</td>
<td>Either</td>
</tr>
<tr>
<td>Glu</td>
<td>OBut</td>
<td>Either</td>
</tr>
<tr>
<td>His</td>
<td>Boc</td>
<td>OPfp only</td>
</tr>
<tr>
<td>Lys</td>
<td>Boc</td>
<td>Either</td>
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<tr>
<td>Ser</td>
<td>But</td>
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<tr>
<td>Thr</td>
<td>But</td>
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<tr>
<td>Tyr</td>
<td>But</td>
<td>Either</td>
</tr>
<tr>
<td>Asn</td>
<td>none</td>
<td>OPfp only</td>
</tr>
<tr>
<td>Gln</td>
<td>none</td>
<td>OPfp only</td>
</tr>
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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).

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).

EXAMINATION OF PEPTIDES FOR ABILITY TO KILL INTRACELLULAR INFECTIOUS AGENTS

Due to poor solubility peptides 308 and 309 were prepared as 50 X concentrated stock solutions containing 10% DMSO.

MALARIA GROWTH INHIBITION ASSAY

The effect of TNF peptides on malarial parasite growth in vitro (and in the absence of neutrophils) was carried out using human red blood cells infected with P.falciparum according to the method of Kumaratilake et al, 1990 Infect. Immun. 58 (in press). The results of these tests are set out in Tables 2 and 3.
TABLE 2

EFFECT OF PEPTIDES ON IN VITRO GROWTH OF P. FALCIPARUM

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>% INHIBITION OF GROWTH</th>
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<tr>
<td>275</td>
<td>0</td>
</tr>
<tr>
<td>301</td>
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<td>90</td>
</tr>
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<td>306</td>
<td>2</td>
</tr>
<tr>
<td>307</td>
<td>4</td>
</tr>
<tr>
<td>308</td>
<td>79 (all due to DMSO)</td>
</tr>
<tr>
<td>309</td>
<td>95 (all due to DMSO)</td>
</tr>
<tr>
<td>323</td>
<td>0</td>
</tr>
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</table>

TABLE 3

EFFECT OF PEPTIDE 305 ON IN VITRO GROWTH OF P. FALCIPARUM

<table>
<thead>
<tr>
<th>EXPERIMENT NO.</th>
<th>% INHIBITION OF GROWTH</th>
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<tr>
<td>1</td>
<td>88.5</td>
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<tr>
<td>2</td>
<td>90.0</td>
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<tr>
<td>3</td>
<td>84.4</td>
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<tr>
<td>mean+/−SD</td>
<td>87.6+/−2.37</td>
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MICROSCOPIC STUDIES

Effects of the peptides in the growth inhibition assays were confirmed microscopically by the appearance of crisis forms of the parasite.

The effect of peptide 347 of malarial parasite growth was assessed in a similar manner and the results are set out in Table 4.
TABLE 4

EFFECT OF PEPTIDE 347 on IN VITRO GROWTH OF P. FALCIPARUM

<table>
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<th>EXPERIMENT NUMBER</th>
<th>% INHIBITION OF GROWTH</th>
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<tr>
<td>5</td>
<td>89.4</td>
</tr>
<tr>
<td>2</td>
<td>91.9</td>
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Results of each experiment are the mean of 6 determinations. Identical results were achieved in assays performed in the presence of human AB serum thus indicating that serum factors will not hamper the effectiveness of this compound.

IN VIVO EFFECTS OF THE PEPTIDES

In separate experiments mice infected with P. chabaudi were injected with 1mg or 500 μg iv of peptide 305. P. chabaudi infected mice received a single injection of peptide. The mice were examined after treatment for the degree of parasitemia and for toxicity of the peptide.

MORPHOLOGICAL STUDIES ON PARASITES

Microscopic analysis of parasite infected red blood cells showed deterioration (i.e. crisis forms of the parasites) in the presence of peptide 305.

IN VIVO OBSERVATIONS

1mg of peptide 305 injected iv into mice infected with P. chabaudi appeared to control the parasitemia. 500 μg of peptide 305 administered daily to mice primed with P. vinkii did not show any evidence of toxicity.

EXPERIMENT 1

Four mice were infected with P. chabaudi and two days later blood smears were taken and the mice were found to be 1% parasitaemic.

9.5 mg of peptide 305 was dissolved in 4.5ml of Hanks Balanced Salt Solution. Two of the mice were treated at daily intervals for four days by i.v. injection of 0.5ml of peptide. Control mice received HBSS alone.
Blood smears were taken every day before treatment and 40000 RBC smear examined for parasite. The results are shown in Fig. 2.

EXPERIMENT 2

In experiment 2 mice were treated with 1mg of peptide 305 /day starting 3 days after infection for 4 days (i.e. until day 7 post-infection). The peptide in this experiment was administered i.p.

Blood smears were taken and examined as previously described. The results are shown in Fig. 3.

EFFECT OF PEPTIDE 305 ON INDUCTION OF ENDOTHELIAL CELL PROCOAGULANT ACTIVITY

Endothelial cell procoagulant activity is a sensitive measurement of a potentially lethal side-effect of TNF.

Peptide 305 is lacking in the ability to induce the expression of Tissue Factor (procoagulant activity) by endothelial cells.

Procoagulant induction by TNF on endothelial cells was assessed by the following method.

Bovine aortic endothelial cells (passage 10) were grown in RPMI-1640 containing 10% foetal calf serum (FCS), penicillin, streptomycin, and 2-mercaptoethanol at 37°C in 5% CO₂. For induction of procoagulant activity by TNF the cells were trypsinised and plated into 24-well Costar trays according to the protocol of Bevilacqua et al., 1986 (PNAS 83, 4533) TNF (100 units/culture) and peptide (100 µg 1ml) was added after washing of the confluent cell monolayer with HBSS. After 4 hours the cells were scrape harvested, frozen and sonicated. Total cellular procoagulant activity was determined by the recalcification time of normal donor platelet-poor plasma performed at 37°C, 100 microlitres of citrated platelet-poor plasma was added to 100 µl of cell lystate and 100 µl of calcium chloride (30mM) and the time taken for clot formation recorded.
RESULTS

<table>
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<th>TREATMENT</th>
<th>CLOTTING TIME (secs)</th>
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<tr>
<td>control</td>
<td>159.5 +/- 1.4</td>
</tr>
<tr>
<td>305</td>
<td>167.38 +/- 6.94</td>
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</table>

Results are the mean +/- standard deviation of quadruplicate measurements.

As can be seen from the results set out above peptide 305 shows marked activity against malarial parasites. In addition, it is to be noted that administration of peptide 305 did not appear to produce adverse side effects.
CLAIMS:-

1. A peptide active against intracellular infectious agents, the peptide having an amino sequence substantially corresponding to amino acids 132 to 157 of Figure 1 or a part thereof.

2. A peptide as claimed in claim 1 in which the peptide has an amino acid sequence substantially corresponding to amino acids 132 to 157 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 132 to 150 of Figure 1.

4. 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 3, in which the compound is capable of killing malarial parasites within ineffected blood cells.

5. A method of treating a mammal infected with an intracellular infectious agent, the method comprising administering to the animal a composition comprising a peptide as claimed in any one of claims 1 to 5 in conjunction with a suitable pharmaceutical carrier.

6. A method as claimed in claim 5 in which the intracellular infectious agent is selected from the group consisting of micobacteria, viruses, and parasites.

7. A method as claimed in claim 6 in which the intracellular infectious agent is malarial parasites.

8. A method as claimed in claim 7 in which the peptide is co-administered with an anti-malarial agent selected from the group consisting of quinine, mefloquine, and chloroquine.

9. The use of a peptide as claimed in any one of claims 1 to 3 in the preparation of a medicament for the treatment of infection with an intracellular agent.
VRSSRTPSD\textsuperscript{10}KPVAHVVP\textsuperscript{20}QAEGQLQWLN\textsuperscript{30}RRANALLANG\textsuperscript{40}
VELRDNLVV\textsuperscript{50}PSEGLYIYS\textsuperscript{60}QVLFKGGCP\textsuperscript{70}STHVLTLTHTI\textsuperscript{80}
SRIA\textsuperscript{90}VSYQTK\textsuperscript{90}VNLLSAIKSP\textsuperscript{100}CQRETPEGAE\textsuperscript{110}AKPWYEPIY\textsuperscript{120}
GGVFQLEKGD\textsuperscript{130}RLSAEINRP\textsuperscript{140}YLDFAESGQV\textsuperscript{150}YFGIAL\textsuperscript{157}
Fig 3

% parasitaemia

Days post Infection

- control
- 305

SUBSTITUTE SHEET
INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00142

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. 5
C07K 7/06, 7/08, 7/10, A61K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System | Classification Symbols
IPC | C07K 7/06, 7/08, 7/10, C07C 103/52
Chem. Abs. online | KEYWORDS: Tumor Necrosis Factor OR TNF
CAS online registry | PROTEIN SEQUENCE SEARCH

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category* | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 Relevant to Claim No 13

A | AU, B, 36014/84 (577810) (DAIPPON PHARMACEUTICAL CO LTD) 6 June 1985 (06.06.85) See whole document.
A | Patents Abstracts of Japan, C-599, page 128, JP,A, 1-37299 (TEIJIN LTD) 2 July 1989 (02.07.89)

* Special categories of cited documents: 10 "A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"E" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 4 July 1991 (04.07.91)
Date of Mailing of this International Search Report 19 July 91

International Searching Authority Australian Patent Office
Signature of Authorized Officer N. BLAM

Form PCT/ISA/210 (second sheet) (January 1985)
FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSearchABLE

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2. [ ] Claim numbers ..., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

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END OF ANNEX