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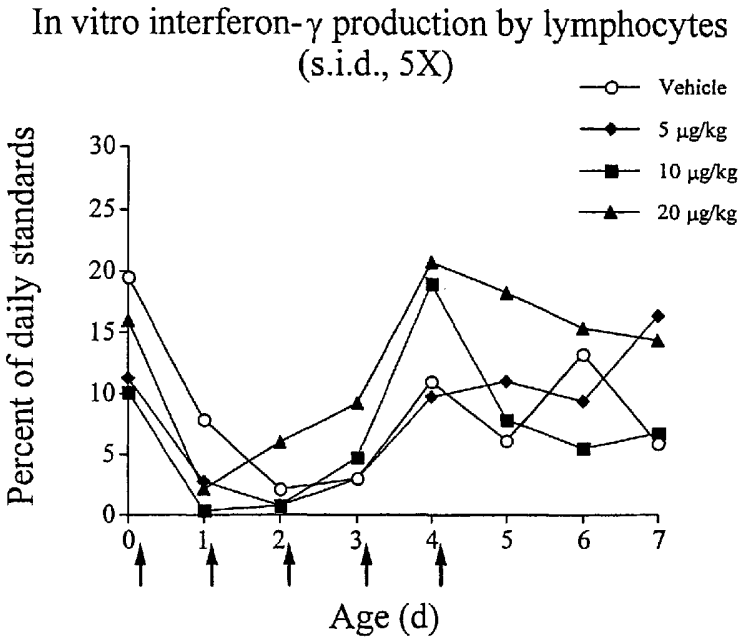
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(54) Title: METHODS FOR ENHANCING IMMUNE FUNCTIONS IN NEONATAL MAMMALS BY ADMINISTRATION OF IL-18



(57) Abstract: The present invention provides methods for increasing the production of interferon-υ in neonatal mammals in response to a stimulus by administration of interleukin-18 to neonatal mammals. The present invention further provides methods for enhancing the immunity in neonatal mammals and protecting neonatal mammals against infectious diseases caused by protozoal, bacterial, fungal or viral pathogens.

WO 2004/016278 A1

METHODS FOR ENHANCING IMMUNE FUNCTIONS IN NEONATAL MAMMALS BY ADMINISTRATION OF IL-18

Field Of The Invention

The present invention relates to methods for increasing the production of interferon-γ in young mammals in response to a stimulus by administration of interleukin-18. The methods of the present invention are useful for enhancing the function of the immune system in young mammals and protecting young mammals against infectious diseases caused by a protozoal, bacterial, fungal or viral pathogen.

Background Of The Invention

Neonatal mammals exhibit greatly reduced levels of interferon-γ relative to older mammals (Rajaraman, V., B. J. Nonnecke, R. L. Horst. J. Dairy Sci. 80:2380-90, 1997). Mammals typically do not exhibit normal immune competence until they approach puberty (Hauser, M. A., M. D. Koob, J. A. Roth. Am. J. Vet. Res. 47:152-153, 1986). Although exactly when mature capacity for interferon-γ is reached is not known, they are likely several weeks to months old. Interferon-γ is known to be an immune modulator (Steinbeck, M. J., J. A. Roth, M. L. Kaeberle. Cell Immun. 98: 137-144, 1986; Bielefeldt Ohmann, H., L. A. Babiuk. J. Interferon Res. 6: 123-136, 1986; Saulnier, D., S. Martinod, B. Charley. Ann. Rech. Vet. 22:1-9, 1991) and the relative lack of interferon-γ is associated with impaired immunity (Ishikawa, H., T. Shirahata, K. Hasegawa. J. Vet. Med. Sci. 56:735-738, 1994) and increased susceptibility to serious infections. (Chiang, Y.-W., J. A. Roth, J. J. Andrews. Am. J. Vet. Res. 51:759-762, 1990; Harp, J. A., R. E. Sacco. J. Parasitol. 82:245-9, 1996; Harp, J. A., W. M. Whitmire, R. Sacco. J. Parasitol. 80:67-72, 1994; Chen, W., J. A. Harp, A. G. Harmsen. Infect. Immun. 61:3928-32, 1993; Chen, W., J. A. Harp, A. G. Harmsen, E. A. Havell. Infect. Immun. 61:3548-51, 1993)

Summary Of The Invention

The present inventors have demonstrated for the first time that administration of interleukin-18 to neonatal mammals led to an increased capacity of lymphocytes from these mammals to produce interferon-γ in vitro in response to a stimulus.

Accordingly, one embodiment of the present invention provides a method for enhancing the capacity of young mammals to produce interferon-γ by administering interleukin-18 to the mammals.

In a preferred embodiment, interleukin-18 is administered to young calves to enhance the capacity of the young calves to produce interferon-γ.

Another embodiment of the present invention provides a method for protecting young mammals against infectious diseases by administering interleukin-18 to the mammals.

In a preferred embodiment, interleukin-18 is administered to young calves to protect the young calves against infectious diseases.

Brief Description Of The Drawings

Figure 1 depicts the effect of administration of recombinant bovine IL-18 to newborn calves. Each group is the mean of data from 6 calves administered injections s.i.d. for 5 days (see arrows).

Detailed Description Of The Invention

One embodiment of the present invention provides a method for enhancing the capacity of young mammals to produce interferon-γ by administering interleukin-18 to these mammals.

By "mammals" is meant cattle, swine, horses, sheep, goats, dogs and cats.

By "young mammals" is meant mammals of an age less than three months old, preferably less than one month old. Typically, mammals less than one month old do not exhibit normal levels of interferon-γ.

By "enhancing the capacity" to produce interferon-γ is meant an increased production of interferon-γ in response to a stimulus by about 35% to about 500%, relative to the average production of interferon-γ by young control mammals of the same age which have not been administered IL-18. The production of interferon-γ in response to a stimulus can be measured by *in vitro* and *ex vivo* assays which have been amply described in the art and in the example that follows (Rajaraman, V., B. J. Nonnecke, R. L. Horst. J. Dairy Sci. 80:2380-90, 1997).

In a preferred embodiment of the present invention, IL-18 is administered to young calves to enhance the capacity of the young calves to produce interferon-γ.

Interleukin-18 molecules suitable for use in the administration are preferably a mammalian IL-18 molecule, including human, murine, rat, bovine (Shoda, L. K., D. S. Zarlenga, A. Hirano, W. C. Brown. J Interferon Cytokine Res. 19:1169-77, 1999), porcine, equine, feline and canine IL-18. A most preferred IL-18 for administration to a mammal is the IL-18 from the same animal species as the mammal. For example, bovine IL-18 is preferred for administration to a young calf.

According to the present invention, an IL-18 molecule can be administered in the form of polypeptides, which can be readily obtained via extraction or purification from

natural sources, via organic chemical synthesis, via recombinant DNA technology, or via commercial sources.

Both full-length, wild-type IL-18 proteins and functional derivatives thereof can be used in the administration. A "functional derivative" of a wild type IL-18 molecule as used herein refers to a modified IL-18 protein, which differs in amino acid sequence from wild type IL-18 by a substitution, insertion or deletion of one or more amino acid residues, but nevertheless has substantially the same activity as the wild type IL-18 in enhancing the production of interferon-γ in young mammals.

Further according to the present invention, a nucleotide sequence encoding IL-18 or a functional derivative thereof can also be used in the administration. Preferably, such nucleotide sequence is provided in an expression vector which is capable of driving the expression of IL-18 molecules in young mammals.

The expression vector can be a plasmid or viral vector such as retroviral, adenoviral and adeno-associated viral vector. Typically, an expression vector includes a promoter sequence which is operably linked to the IL-18 coding sequence. Examples of suitable promoters include (human) cytomegalovirus immediate early promoter (Seed, B. et al., Nature 329, 840-842, 1987; Fynan, E.F. et al., Proc. Natl. Acad. Sci. 90, 11478-11482, 1993; Ulmer, J.B. et al., Science 259, 1745-1748, 1993), Rous sarcoma virus LTR (RSV, Gorman, C.M. et al., Proc. Natl. Acad. Sci. 79, 6777-6781, 1982; Fynan et al., supra; Ulmer et al., supra), the MPSV LTR (Stacey at el., J. Virology 50, 725-732, 1984), SV40 immediate early promoter (Sprague J. et al., J. Virology 45, 773, 1983), the metallothionein promoter (Brinster, R.L. et al., Nature 296, 39-42, 1982), the major late promoter of Ad2, the β-actin promoter (Tang et al., Nature 356, 152-154, 1992).

The expression vectors can also include other regulatory sequences, such as terminator and polyadenylation sequences. Sequences suitable for use include bovine growth hormone polyadenylation sequence, the SV40 polyadenylation sequence, and the human cytomegalovirus (hCMV) terminator and polyadenylation sequences.

The expression vectors can also include nucleotide sequences coding for other cytokines, which are appropriate for use in conjunction with IL-18 to enhance the immune function of neonatal mammals.

The IL-18 molecule, either in the form of polypeptide or in the form of an expression vector, can be administered together with other cytokines, which are appropriate for use in conjunction with IL-18 to enhance the immune function of neonatal mammals. Other cytokines which are appropriate for use in conjunction with IL-18 to enhance immune function of neonatal mammals might include but are not limited to: G-CSF, GM-CSF, IL-3, IL-7, IL-15, IL-17.

Other biologically active agents which can be administered in conjunction with IL-18 include, e.g., antiparasiticides, antibacterials, antifungals and antivirals, and the like.

According to the present invention, IL-18 and other appropriate biological agents can be admixed with a pharmaceutically acceptable carrier. Suitable pharmaceutical carriers include but are not limited to water, saline, adjuvant (such as oil emulsions, aluminium salts, derivatives of muramyl dipeptide, monophosphoryl lipid A, liposomes, QS21, MF-59, Iscoms, and the like), diluents, stabilizers (such as serum albumins, gelatins, saccharides including glucose, fructose, sucrose, maltose, lactose, trehalose, sorbitol, mannitol, maltitol, and lactitol), and buffers with phosphoric acid or succinic acid.

The compositions containing IL-18, a pharmaceutical carrier and any other appropriate biological agent may take any form that is suitable for oral, mucosal, or parenteral administration to neonatal mammals. For oral use, the compositions may be formulated as solutions, syrups, suspensions, tablets, capsules and the like. For parenteral use, the compositions according to the present invention may be formulated in a form suitable for injection such as suspensions, solutions, dispersions, emulsions, and the like. Preparation of the compositions according to the present invention is carried out by means conventional for the skilled person.

Preferred routes of administration are parenteral routes, e.g., intramuscular injection, intravenous injection, intradermal injection, subcutaneous injection, and mucosal routes, e.g. nasal drops, eye drops, (aerosol) sprays, and the like.

According to the present invention, administration of an IL-18-containing composition to mammals should begin shortly after birth, preferably, within one to two days after birth. The dosage and the number of times of the administration depends on the condition of the neonatal mammal (e.g., weight, response to the administration), the form of IL-18 in the composition (polypeptide or expression vector), and the route of administration. As a general rule, an IL-18-containing composition can be administered parenterally at a dose in the range of about 1 µg to 500 µg IL-18/kg per dose for about 1-4 times/day for about one to twenty-eight days. In a preferred embodiment, purified recombinant mammalian IL-18 proteins are given to neonatal mammals at 20 µg/kg/dose/day via subcutaneous injection for 5 days. A most preferred embodiment is a single injection of a dose of IL-18 in a formulation capable of providing a sustained effect of 14-28 days.

According to the present invention, administration of interleukin-18 to neonatal mammals increases the capacity of lymphocytes from these mammals to produce interferon-γ. Therefore, another embodiment of the present invention provides a method for protecting young mammals against infectious diseases by administering interleukin-18 to the mammals.

In a preferred embodiment, interleukin-18 is administered to young calves to protect the young calves against infectious diseases.

By "protecting" is meant enhancing the immunity and resistance against a disease, accelerating the recovery from a disease, or eliminating or alleviating the syndromes and symptoms of a disease.

Diseases against which the present method affords protection include, but are not limited to infectious diseases caused by a protozoal, bacterial, fungal or viral pathogen.

The type of IL-18 molecules appropriate for use in the administration, other biological active agents and pharmaceutical carriers which can be included in the administration, the schedule, dose and routes of administration are as described hereinabove.

The present invention is further illustrated by the following example.

EXAMPLE

Test Materials:

1. Compound Name: recombinant bovine IL-18
   Dosage Form: Injectable
   Potency: 1.107 mg/mL
   Formulation: Dialysis buffer

2. Compound Name: Dialysis buffer
   Dosage Form: Injectable
   Potency: N/A
   Formulation: 20 mM NaH_2PO_4, 500 mM NaC1, 0.1 mM EDTA, 25% glycerol (v/v), pH 8.04

Animals:

Species/breed:	Bovine/Holstein
Initial weight:	27-57 kg
Sex:	Male and female
Origin:	Calves born on site
Identification:	Ear tags
Pre-treatment	First Defense® bolus, ImmuCell Corp.

Management:

Housing: Individual calf hutches on a gravel base with straw bedding.

Feeding and watering method: Colostrum (5% of body weight twice daily) on day 1 & 2

Environmental control: Clean hutches 2x/week.

Design:

Treatment	Cytokine Dose	Day	Timing	No. Calves
T1	Vehicle control	0	SID-X1	4
T2	5 µg/kg SC	0, 1, 2, 3, 4	SID-X5	5
T3	10 µg/kg SC	0, 1, 2, 3, 4	SID-X5	5
T4	20 µg /kg SC	0, 1, 2, 3, 4	SID-X5	5

Experimental Procedure:

When a calf was born, it was given 1-2 quarts of colostrum and an oral commercial bolus of antibodies (against *E. coli* $K99^+$ and rotavirus). Calves born before 9 AM were bled and study day zero began that calendar date for that calf. Calves born after 9 AM were processed as normal except that study day zero began the next calendar day for purposes of completing the laboratory assays with blood samples. Test article injections were given subcutaneously ahead of the prescapular region of the neck. Alternating sides of the neck were used for injections on successive days. Leukograms were determined using a Coulter counter and CD45 vs. SSC on the flow cytometer. Peripheral blood mononuclear cells were isolated over Percoll density gradients (sp. gr. = 1.084) from calves and three lab control cows. Isolated cells were washed and adjusted to 4×10^6/mL in RPMI-1640 with antibiotic/antimycotic and 100 µL added to 100 µL culture media (RMPI-1640 with 25 mM HEPES, antibiotic/antimycotic and 10% heat inactivated FBS) with or without 2 µg/mL Con-A for 44 hours. Supernatants from these cultures were harvested and interferon-γ levels determined using the Bovigam™ bovine gamma interferon assay kit from CSL Veterinary adapted for a quantitative assay with recombinant bovine IFN-γ in a standard curve. Calf clinical scores were determined each morning on study days 0-7, 14 and 21. Scoring was as follows: (0) = normal, healthy; (1) = mildly ill; (2) = moderately ill; (3) = severely ill; (4) = moribund.

Data Analysis:

Assessment of test article efficacy was determined based upon comparisons of interferon-γ production between treatment groups. Data were analyzed using the MIXED procedure of PC-SAS version 6.12. The model included treatment, time and their interaction. Covariance within calves across time was modeled using the REPEATED statement with a spherical covariance structure to account for unequally spaced sampling times. Tests for significance were based upon the main treatment effect compared with the vehicle treatment group.

Results:

Figure 1 summarizes the ability of isolated mononuclear cells (lymphocytes + monocytes) to produce interferon-γ in response to mitogen stimulation. Consistent with published data, the ability of calf mononuclear cells to produce interferon-γ in response to mitogen stimulation was a fraction of adult capacity shortly after birth, this capacity further declined to a nadir at 2 days of age. Calves were still at less than 25% of adult capacity at 21 days of age. Calves that received 20 µg IL-18/kg, SC, 5X had the highest observed levels of induced interferon-γ production for a 5-day period beginning 3 days after the first injection (15.6% of adult capacity versus 7.9% for controls). This represents a nearly 100% increase relative to the average production of interferon-γ by young control calves of the same age which had not been administered IL-18. For this 5-day period, the effect of increasing mononuclear cell interferon-γ production was significant at $P<0.05$.

Accordingly, in accordance with the present invention, administration of recombinant bovine IL-18 at 20 µg/kg, SC, 5X, increased *ex vivo* interferon-γ production by mitogen-stimulated mononuclear cells.

What is claimed is:

1. A method for enhancing the capacity of a young mammal to produce interferon-γ comprising administering interleukin-18 to said mammal.

2. A method for protecting a young mammal against an infectious disease, comprising administering interleukin-18 to the mammal.

3. The method of claim 2, wherein the infectious disease is caused by a protozoal, bacterial, fungal or viral pathogen.

4. The method of claims 1, 2 or 3 wherein the young mammal is of an age of less than one month old.

5. The method of claim 4 wherein said interleukin-18 is an IL-18 from the same animal species as the mammal.

6. The method of claim 5, wherein said IL-18 is in the form of a recombinant protein.

7. The method of claim 5, wherein said IL-18 is in the form of a nucleotide sequence coding for said IL-18.

8. The method of claim 7, wherein said nucleotide sequence coding for said IL-18 is provided in an expression vector.

9. The method of claim 4, wherein said IL-18 is administered in conjunction with at least one other cytokine.

10. The method of claim 4, wherein said IL-18 is provided in a pharmaceutically acceptable carrier.

11. The method of claim 4, wherein said IL-18 is administered to said mammal via an oral, a mucosal, or a parenteral route.

12. The method of claim 4, wherein said IL-18 is first administered to said mammal within about two days after the birth of said mammal.

13. The method of claim 12, wherein said IL-18 is first administered to said mammal within 1 day after the birth of said mammal.

14. The method of claim 4, wherein the IL-18 protein is administered at about 1 to 500 μg/kg per dose, and one dose per day for about one to twenty-eight days.

15. The method of claim 4, wherein said young mammal is a young calf, and said IL-18 is bovine IL-18.

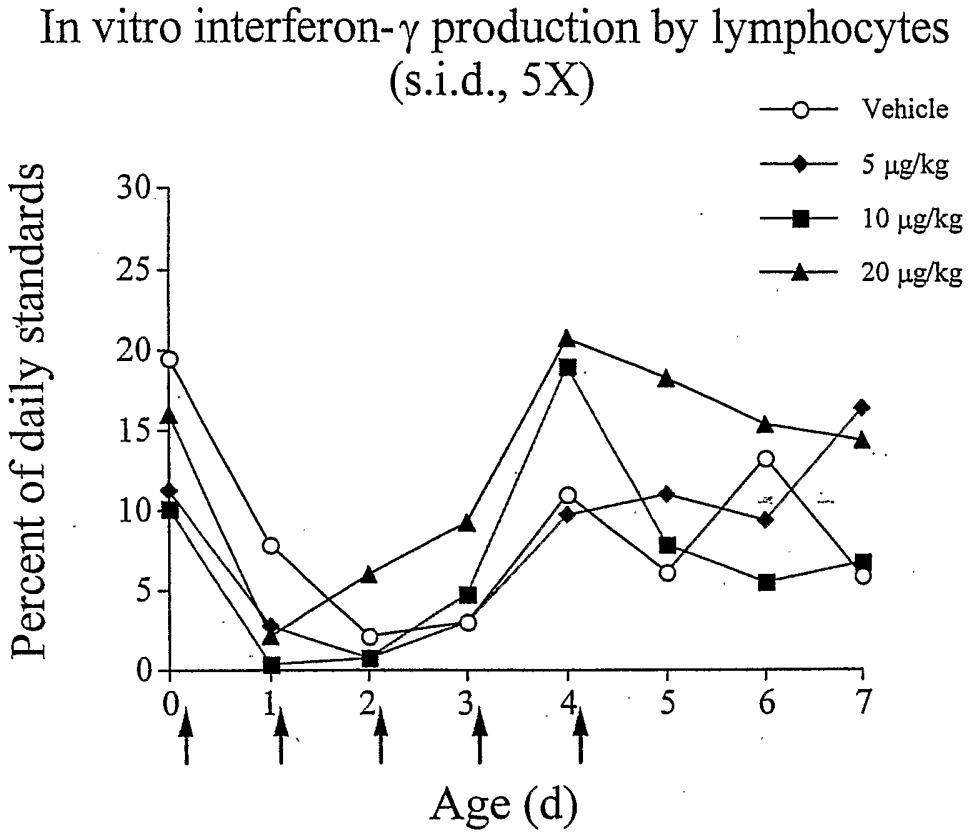

In vitro interferon-γ production by lymphocytes
(s.i.d., 5X)
Vehicle
5 µg/kg
10 µg/kg
20 µg/kg
Percent of daily standards
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Age (d)

FIGURE 1

INTERNATIONAL SEARCH REPORT

PCT/IB 03/03548

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/20 A61P31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SURI MANDHIR ET AL: "Prophylactic single and sequential cytokines (M-CSF, IL-12, IL-18) in combination with antibiotics (Ab) significantly enhances survival during experimental neonatal murine listeriosis: Implications for enhancing neonatal immunity." PEDIATRIC RESEARCH, vol. 53, no. 4 Part 2, April 2003 (2003-04), page 309A, XP0009020958 & ANNUAL MEETING OF THE PEDIATRIC ACADEMIC SOCIETIES'; SEATTLE, WA, USA; MAY 03-06, 2003 ISSN: 0031-3998 abstract ----- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"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 when the document is taken alone

"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 2003	28/11/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Winger, R

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 04271 A (WHITEHEAD STEPHEN S ; US HEALTH (US); COLLINS PETER L (US); MURPHY BRI) 18 January 2001 (2001-01-18)	1-14
Y	page 90, line 12 - line 31; claims 55-57	1-15
X	MASTROENI P ET AL: "Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent Salmonella typhimurium." INFECTION AND IMMUNITY. FEB 1999, vol. 67, no. 2, February 1999 (1999-02), pages 478-483, XP0002964670 ISSN: 0019-9567	1-3
Y	abstract; page 479, line 2	1-15
X	DUPRÉ L ET AL: "Immunostimulatory effect of IL-18-encoding plasmid in DNA vaccination against murine Schistosoma mansoni infection" VACCINE 08 JAN 2001 UNITED KINGDOM, vol. 19, no. 11-12, 8 January 2001 (2001-01-08), pages 1373-1380, XP0004313950 ISSN: 0264-410X	1-3
Y	abstract; page 1374, column 2	1-15
X	MUNETA YOSHIHIRO ET AL: "Expression of interleukin-18 by porcine airway and intestinal epithelium" JOURNAL OF INTERFERON AND CYTOKINE RESEARCH, vol. 22, no. 8, August 2002 (2002-08), pages 883-889, XP0009020960 & ISSN: 1079-9907	1
Y	abstract	1-15
Y	WO 01 93898 A (CHISARI FRANCIS V ; SMITHKLINE BEECHAM CORP (US); ESSER KLAUS M (US);) 13 December 2001 (2001-12-13) page 1 - page 2; examples 1,2	1-15
Y	EP 1 095 661 A (AZ UNIV AMSTERDAM) 2 May 2001 (2001-05-02) claims 1,5,7	1-15
Y	WO 99 66879 A (NAT JEWISH MED & RES CENTER) 29 December 1999 (1999-12-29) claims 84,88,94,98	1-15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	LA PINE TIMOTHY R ET AL: "Priming neonatal mononuclear cells with the Th-1 cytokines IL-18 and IL-12 corrects defective interferon-gamma production in response to group B streptococci" PEDIATRIC RESEARCH, vol. 51, no. 4 Part 2, April 2002 (2002-04), page 324A, XP0009020957 & ANNUAL MEETING OF THE PEDIATRIC SOCIETIES'; BALTIMORE, MD, USA; MAY 04-07, 2002 ISSN: 0031-3998 abstract ----	1-15

PCT/IB 03/03548

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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

Although claims 1-15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
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. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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.

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

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] 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 claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/IB 03/03548

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