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(54) Title: EQUINE GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

(54) Titre: GM-CSF EQUIN

SEQUENCE OF EQUINE GM-CSF GENE
SEQUENCE DU GÈNE GM-CSF EQUIN

```
1   ATG TGG CTG CAG AAC CTG CTT CTT CTG GGC ACT GTG GTT TAC AGC ATG CCC GCA CCC ACC
    TAC ACC GAC GTC TTG GAC GAA GAA GAC CCG TGA CAC CAA ATG TCG TAC GGG CGT GGG TGG
 1  M   W   L   Q   N   L   L   L   L   G   T   V   V   Y   S   M   P   A   P   T

61  CGC CAA CCC AGC CCT GTC ACT CGG CCC TGG CAG CAT GTG GAT GCC ATC AAG GAG GCC CTG
    GCG GTT GGG TCG GGA CAG TGA GCC GGG ACC GTC GTA CAC CTA CGG TAG TTC CTC CGG GAC
21  R   Q   P   S   P   V   T   R   P   W   Q   H   V   D   A   I   K   E   A   L

121 AGC CTT CTG AAC AAC AGT AGT GAC ACT GCT GCT ATC ATG AAT GAA ACA GTA GAA GTC GTC
    TCG GAA GAC TTG TTG TCA TCA CTG TGA CGA CGA TAG TAC TTA CTT TGT CAT CTT CAG CAG
41  S   L   L   N   N   S   S   D   T   A   A   I   M   N   E   T   V   E   V   V

181 TCT GAA ACG TTT GAC GCC GAG GAG CTG ACA TGC CTG CAG ACT CGC CTC AAG CTG TAC AAA
    AGA CTT TGC AAA CTG CGG CTC CTC GAC TGT ACG GAC GTC TGA GCG GAG TTC GAC ATG TTT
61  S   E   T   F   D   A   E   E   L   T   C   L   Q   T   R   L   K   L   Y   K

241 CAG GGC TTG CGG GCC AGC CTC ATC AAG CTC GAA GGC CCC TTG ACC ATG ATG GCC AGC CAC
    GTC CCG AAC GCC CCG TCG GAG TAG TTC GAG CTT CCG GGG AAC TGG TAC TAC CGG TCG GTG
81  Q   G   L   R   G   S   L   I   K   L   E   G   P   L   T   M   M   A   S   H

301 TAC AAG CAG CAC TGC CCC CCC ACC CTG GAA ACT TCC TGT GCA ACC CAG ATG ATC ACC TTC
    ATG TTC GTC GTG ACG GGG GGG TGG GAC CTT TGA AGG ACA CGT TGG GTC TAC TAG TGG AAG
101 Y   K   Q   H   C   P   P   T   L   E   T   S   C   A   T   Q   M   I   T   F

361 AAA AGT TTC AAA AAG AAC CTG AAG GAT TTT CTG TTT GAG ATC CCG TTT GAC TGC TGG AAC
    TTT TCA AAG TTT TTC TTG GAC TTC CTA AAA GAC AAA CTC TAG GGC AAA CTG ACG ACC TTG
121 K   S   F   K   K   N   L   K   D   F   L   F   E   I   P   F   D   C   W   K

421 CCA GCC CAG AAG TAA
    GGT CGG GTC TTC ATT
141 P   A   Q   K
```

(57) Abstract: The invention concerns equine GM-CSF, the nucleotide sequence coding for the equine GM-CSF gene and its amino acid sequence, as well as it use as adjuvant in equine vaccination and as non-specific immunity stimulating agent. The invention also concerns equine GM-CSF or vectors expressing GM-CSF in vivo.

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(57) **Abrégé:** L'invention est relative au GM-CSF équin, la séquence nucléotidique codant pour le gène GM-CSF équin et sa séquence en acides aminés, ainsi que son utilisation comme adjuvant en vaccination équine et comme stimulant non spécifique de l'immunité. Compositions contenant le GM-CSF équin ou des vecteurs exprimant le GM-CSF in vivo.

# Equine GM--CSF

The present invention relates to the nucleotide sequence of the gene encoding the horse GM-CSF cytokine, to expression vectors containing it and to its use as an adjuvant in equine vaccination and as a nonspecific stimulant of immunity.

The documents cited herein are incorporated by way of reference into the present application.

The first discovery of a granulocyte-macrophage colony-stimulating factor (or GM-CSF) was in 1977 (Burgess A.W. *et al*. J. Biol. Chem. 1977. **252**. 1998-2003). This is murine GM-CSF, purified from mouse lung culture supernatants.

The biological activities of GM-CSF have been demonstrated by studies on murine and human GM-CSFs (Clark S. C. *et al*. Science 1987 .**230**.1229; Grant S. M. *et al*. Drugs 1992.**53**.516).

GM-CSF has many physiological roles (Dy M. in "Les cytokines" [Cytokines] Cavaillon J-M., 1996, ed. Masson, Paris, France, 43-56). In particular, GM-CSF stimulates the production, development and formation of colonies of granulocytes, of macrophages, of eosinophils and of megakaryocytes. GM-CSF in particular induces macrophagic cytotoxicity and stimulates antibody-dependent cytotoxic activity (ADCC) and the recruitment of leukocytes at the sites of inflammation.

GM-CSFs of various animal species have already been demonstrated.

The sizes of the nucleotide sequences encoding the known GM-CSFs of various species range from 381 to 432 nucleotides. The human and murine nucleotide sequences

have a degree of homology of 69%. The degree of homology is 54% at the amino acid sequence level (Cantrell M.A. *et al.* Proc. Natl. Acad. Sci. USA 1985. **82**. 6250-6254). However, this homology does not allow any cross-activity between the two human and murine species (Metcalf D. *et al.* Blood 1986. **67**. 37-45).

The administration of heterologous GM-CSF, i.e. GM-CSF originating from a species other than that treated, does not make it possible to obtain an optimum adjuvant effect, in particular stimulation of the activity of the hematopoietic cells and a substantial increase in the immune response.

Up until now, it has not been possible to demonstrate equine GM-CSF. However, this cytokine is of considerable value for therapeutic and vaccinal applications for use in horses.

The applicant has succeeded in isolating and sequencing the equine GM-CSF gene. This gene was isolated after polymerase chain reaction (PCR) using the oligonucleotides described in the examples.

The equine GM-CSF gene is 432 nucleotides in size (SEQ ID N° 8 and figure 1) and encodes a 144 amino acid protein (SEQ ID N° 9 and figure 1). The protein encoded by this gene exhibits at least 75% homology with the GM-CSF polypeptide sequences of other animal species.

A subject of the present invention is therefore an isolated DNA fragment encoding equine GM-CSF, e.g. a fragment comprising SEQ ID N° 8. A subject of the present invention is also the DNA fragment having, or essentially consisting of, this sequence.

A subject of the present invention is also an isolated DNA fragment encoding the amino acid sequence SEQ ID N° 9.

The invention covers the equivalent nucleotide sequences of equine or synthetic origin, i.e. the nucleotide sequences encoding a protein with equivalent functionality and specificity in horses. The nucleotide sequences which differ by virtue of the degeneracy of the genetic code will, of course, be included. In particular, the sequences which are equivalent are the DNA sequences which exhibit greater than or equal to 90%, in particular 92%, preferably to 95%, homology with the sequence SEQ ID N° 8.

The subject of the invention is also DNA fragments comprising a nucleotide sequence encoding equine GM-CSF, e.g. according to SEQ ID N° 8, or a sequence encoding the amino acid sequence SEQ ID N° 9, this nucleotide sequence being combined, by fusion, with the nucleotide sequence encoding at least one immunogene or at least one immunologically active fragment or at least one epitope of an immunogen. The DNA fragment does not then comprise a stop codon between the sequence encoding GM-CSF and the combined immunogene sequence. For example, if reference is made to SEQ ID N° 8, the coding sequence inserted ends at nucleotide 432 and does not include the stop codon.

The subject of the present invention is also the isolated equine GM-CSF protein or polypeptide, e.g. as encoded by the nucleotide sequence SEQ ID N° 8 or by the equivalents of the latter as defined above.

A subject of the present invention is also the equine GM-CSF protein having the amino acid sequence SEQ ID N° 9.

The equine GM-CSF protein is 144 amino acids in size. However, the present invention also comprises the proteins, protein fragments and polypeptides of equine or synthetic origin which are greater than, equal to or less than these 144 amino acids in size, and also the

recombined proteins (proteins having one or more substitutions, deletions or additions) and fusion proteins as long as their biological activity (for the part common with GM-CSF) is substantially equivalent to that of the natural equine GM-CSF protein *in vivo* in the horse, and that their species specificity is not modified. The sequences included as equivalents are the amino acid sequences encoded by any of the equivalent nucleotide sequences defined above.

A subject of the present invention is also a pure preparation of equine GM-CSF protein.

A subject of the present invention is also the expression vectors comprising, as an insert, one of the DNA fragments or nucleotide sequences defined above, in particular the equine GM-CSF gene (SEQ ID N° 8) or an equivalent thereof as defined above, and also any nucleotide sequence encoding any amino acid sequence as defined above. Similarly, the vector may also comprise a nucleotide sequence encoding at least one immunogen or at least one immunologically active fragment or at least one epitope of an immunogen, which may or may not be combined by fusion as described above.

The nucleotide sequence may be inserted into conventional *in vitro* expression systems of viral, such as Baculovirus, origin, in particular propagated on insect cells or cells of prokaryotic (for example *Escherichia coli*) or eukaryotic origin, in particular yeasts, especially *Saccharomyces cerevisiae*, mammalian eukaryotic cells, especially hamster cells (for example hamster ovary or CHO cells) and horse cells. The invention therefore also covers these expression systems transformed with a sequence according to the invention, the equine GM-CSF proteins thus produced and their use as a vaccinization adjuvant and nonspecific stimulant of immunity.

Preferably, the sequence according to the invention is introduced into *in vivo* expression vectors under conditions which allow the expression, in horses, of a functional equine GM-CSF protein along with, optionally, a nucleotide sequence encoding at least one immunogen or at least one immunologically active fragment or at least one epitope of an immunogen. These expression vectors may be plasmids, or viral vectors, such as poxviruses, for example the vaccinia virus and avipoxes (canarypox, fowlpox), including species-specific poxviruses (swinepox, raccoonpox and camelpox), adenoviruses and herpesviruses, such as equine herpesviruses.

The term "plasmid" is intended to cover any DNA transcription unit in the form of a polynucleotide sequence comprising the equine GM-CSF gene sequence and the elements required for its expression *in vivo*. The circular plasmid form, which may or may not be supercoiled, is preferred. The linear form also enters into the context of this invention.

Each plasmid comprises a promoter capable of ensuring, in host cells, the expression of the gene inserted under its control. It is, in general, a strong eukaryotic promoter and, in particular, an early promoter of the cytomegalovirus, CMV-IE, of human or murine origin, or optionally of another origin such as rat or guinea pig. More generally, the promoter is either of viral origin or cellular origin. As a viral promoter other than CMV-IE, mention may be made of the SV40 virus early or late promoter or the Rous sarcoma virus LTR promoter. It may also be a promoter of a virus from which the gene originates, for example the gene's own promoter. As a cellular promoter, mention may be made of the promoter of a gene of the cytoskeleton, such as for example the desmin promoter or the actin promoter. When several genes are present in the same plasmid, they may be within the same

transcription unit or in two different units.

The plasmids may also comprise other transcription regulation elements, such as for example stabilizing sequences such as an intron, preferably intron II of the rabbit β-globin gene (van Ooyen *et al.* Science, 1979, **206**: 337-344), a signal sequence of the protein encoded by the tissue plasminogin activator gene (tPA; Montgomery *et al.* Cell. Mol. Biol. 1997, **43**: 285-292) and a polyadenylation (polyA) signal, in particular from the bovine growth hormone (bGH) gene (US-A-5 122 458) or from the rabbit β-globin gene.

The invention also covers the immunogenic compositions and the vaccines comprising the equine GM-CSF protein according to the invention, at least one immunogenic or vaccinal preparation of an equine pathogen, and an excipient or vehicle which is acceptable from a veterinary point of view. The notion of immunogenic preparation covers herein any preparation which is capable, once administered to the horse, of inducing an immune response directed against the equine pathogen in question, this response being increased by the presence of the GM-CSF protein. It is preferably a vaccinal preparation capable of inducing effective protection or a certain degree of protection against this pathogen, this degree of protection being herein increased by the presence of the equine GM-CSF protein. The immunogenic and vaccinal preparations targeted in the invention cover all known types, such as inactivated preparations, attenuated live preparations, subunit preparations and recombinant preparations (using an *in vivo* expression vector, in particular of viral or plasmid origin). As seen above, the GM-CSF protein may be added as it is to the immunogenic or vaccinal preparation, so as to form, in the presence of an excipient or vehicle which is acceptable from a veterinary point of view, an immunogenic composition or a vaccine ready to be administered. It is also possible

to envision combining the GM-CSF protein with a sustained-release system designed to release the protein gradually.

According to a more advantageous mode of the invention, it is, however, preferred to express the GM-CSF protein *in vivo* using an *in vivo* expression vector as described above. In this case, it is also preferred that the immunogenic or vaccinal preparation is also of the recombinant type, based on the use of an *in vivo* expression vector, of the same type or of a different type. It is also possible to envision using the same *in vivo* expression vector, comprising and expressing at least one immunogen of an equine pathogen and the equine GM-CSF protein.

The advantages of using GM-CSF with vaccinations are, in particular, the decrease in the dose of immunogen, of vector or of DNA used. In addition, in some animals which do not respond when a conventional vaccine is administered, the use of GM-CSF makes it possible to stimulate the immune response and to increase it up to a protective level.

The present invention therefore preferably covers the immunogenic compositions and the vaccines comprising:

- an *in vivo* expression vector containing a nucleotide sequence encoding an equine GM-CSF, under conditions which allow the expression, in the horse, of a functional equine GM-CSF protein,
- at least one *in vivo* expression vector containing at least one nucleotide sequence encoding at least one equine immunogen, it being understood that this vector or some or all of these vectors (when there are several vectors encoding different immunogens) may also constitute the GM-CSF vector (the vector comprises at least one GM-CSF sequence and one immunogen sequence), and
- a vehicle or excipient which is acceptable from a

veterinary point of view

According to the preferred mode of the invention, the invention covers the immunogenic compositions and the vaccines of the DNA type, comprising a plasmid encoding and expressing the equine GM-CSF according to the invention and at least one other plasmid encoding and expressing an equine immunogen or an immunologically active fragment derived from the latter. Examples of plasmid constructs which can be used in the invention, containing an equine immunogen, are given in patent application WO-A-9803198. The invention also covers the DNA vaccines comprising a plasmid encoding and expressing simultaneously the equine GM-CSF and at least one equine immunogen.

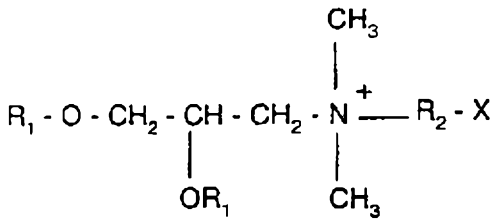
The invention is directed toward all equine pathogens. Mention may be made more particularly of: equine herpesvirus type 1 or type 4 (and preferably, the invention envisions the combination of both), equine flu virus, tetanus, *Borrelia burgdorferi*, Eastern, Western or Venezuelan equine encephalitis, and rabiesvirus. For the subunit vaccines and the recombinant vaccines the equine immunogens are preferably chosen from the group comprising the gB, gC and gD glycoproteins of equine herpesvirus type 1 or type 4, hemagglutinin (HA) and the nucleoprotein (NP) of the equine flu virus, subunit fragment C of the tetanus toxin, the OspA protein of *Borrelia burgdorferi*, the E2 and C genes of Eastern, Western or Venezuelan encephalitis, and the G gene of rabiesvirus.

A subject of the present invention is also compositions which are nonspecific stimulants of immunity i.e. which can be used as a general stimulant of immunity in horses. These compositions are administered both in the presence and in the absence of a declared pathological condition, in general independently of any vaccine, in order to reinforce the horse's immune defenses. These

compositions comprise GM-CSF according to the invention, in all the forms described above, the protein or recombinant, preferably recombinant (viral or plasmid *in vivo* expression vector), and an excipient or vehicle which is acceptable from a veterinary point of view. The characteristics of these vectors have already been described.

The compositions which are nonspecific stimulants, the immunogenic compositions and the vaccines according to the invention may also comprise one or more adjuvants of immunity, in particular chosen from those conventionally used in equine vaccination against the pathogen(s) (valences) in question. The stimulant compositions and the conventional (inactivated, attenuated live, subunit) immunogenic compositions and vaccines may thus comprise, as a conventional adjuvant, compounds of the carbomer type or alumina hydroxide, or be formulated in the form of an oil-in-water emulsion. For the stimulant compositions and the immunogenic compositions and vaccines which are recombinant and based on a viral expression vector, mention may be made of oil-in-water emulsions.

According to a preferred mode of the invention, for the stimulant compositions of the plasmid type and the immunogenic compositions and vaccines of the plasmid type, the plasmid encoding and expressing the equine GM-CSF, the plasmid encoding and expressing GM-CSF and at least one equine immunogen and also the mixtures of plasmids containing the latter and at least one plasmid encoding an equine immunogen, may advantageously be formulated in an original manner with a cationic lipid containing a quaternary ammonium salt, of formula:



in which $R_1$ is a saturated or unsaturated, linear aliphatic radical having 12 to 18 carbon atoms, $R_2$ is another aliphatic radical containing 2 or 3 carbon atoms, and X is a hydroxyl or amine group.

It is preferably DMRIE (N-(2-hydroxyethyl) - N, N - dimethyl - 2,3 - bis (tetradecyloxy) - 1 - propanammonium; WO-A-9634109), preferably coupled to a neutral lipid, DOPE (dioleoylphosphatidylethanolamine), so as to preferentially form DMRIE-DOPE. Preferably, the mixing of the recombinant vector with this adjuvant takes place extemporaneously and the mixture thus formed is preferably given time to complex, for example for a period of time ranging from 10-60 minutes, in particular about 30 minutes before it is administered to the animal.

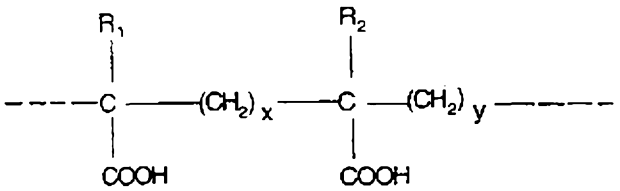
When DOPE is present, the DMRIE:DOPE molar ratio preferably ranges from 95:5 to 5:95, more particularly 1:1.

The plasmid: DMRIE and/or DMRIE-DOPE adjuvant weight ratio may range in particular from 50:1 to 1:10, in particular from 10:1 to 1:5, preferably from 1:1 to 1:2.

According to another advantageous mode of the invention, for the stimulant compositions of the recombinant type and the immunogenic compositions and vaccines of the recombinant type (viral vector or plasmid), use may be made, as an adjuvant, of acrylic or methacrylic acid polymers or copolymers of maleic

anhydride and an alkenyl derivative. Acrylic or methacrylic acid polymers which are crosslinked, in particular with polyalkenyl ethers of sugars or polyalcohols, are preferred. These compounds are known under the name carbomer (Pharmeuropa vol. 8, N°2, June 1996). Those skilled in the art may also refer to US-A-2 909 462 (incorporated by way of reference) which describes such acrylic polymers crosslinked with a polyhydroxylated compound having at least 3 hydroxyl groups, preferably no more than 8, the hydrogen atoms of at least three hydroxyls being replaced with unsaturated aliphatic radicals having at least 2 carbon atoms. The preferred radicals are those containing from 2-4 carbon atoms e.g. vinyls, allyls and other ethylenically unsaturated groups. The unsaturated radicals may, themselves, contain other substituents, such as methyl. The products sold under the name Carbopol® (BF Goodrich, Ohio, USA) are particularly suitable. They are crosslinked with an allyl sucrose or with allyl pentaerythritol. Among these products, mention may be made of Carbopol® 974P, Carbopol ® 934P and Carbopol ® 971P.

Among the copolymers of maleic anhydride and an alkenyl derivative, preference is given to the EMAs® (Monsanto) which are copolymers of maleic anhydride and ethylene, which may be linear or crosslinked, for example crosslinked with divinyl ether. Reference may be made to J. Fields *et al.*, Nature, **186:** 778-780, June 4, 1960 (incorporated by way of reference). In terms of their structure, the acrylic or methacrylic acid polymers and the EMAs® are preferably made up of basic units of the following formula:



in which:

- $R_1$ and $R_2$, which may be identical or different, represent H or $CH_3$
- x = 0 or 1, preferably x = 1
- y = 1 or 2, with x + y = 2.

For the EMAs® ,x = 0 and y = 2. For the carbomers, x = y = 1.

Dissolving these polymers in water produces an acid solution which will be neutralized, preferably to physiological pH, so as to give the adjuvant solution into which the vaccine per se will be incorporated. The carboxylic groups of the polymer are then partly in the $COO^-$ form.

Preferably, a solution of carbomer or of EMA® is prepared in distilled water, preferably in the presence of sodium chloride, the solution obtained being at acid pH. This stock solution is diluted by adding it to the required amount (for obtaining the desired final concentration), or a considerable part thereof, of water loaded with NaCl, preferably physiological saline (9 g/l NaCl), all at once or in several steps, with concomitant or subsequent neutralization (pH 7.3 to 7.4), preferably with NaOH. This solution at physiological pH will be used as it is for mixing with the immunogenic or vaccinal preparation, in particular conserved in a lyophilized, liquid or frozen form.

The concentration of polymer in the final vaccinal composition will be from 0.01% to 2% W/V, more particularly from 0.06 to 1% W/V, preferably from 0.1 to 0.6% W/V.

Another subject of the invention is a method for stimulating the immunity and/or for immunizing and/or for vaccinating the equine species, in which a

stimulant, immunogenic and/or vaccinal composition according to the invention is administered to an animal of the equine species, in particular a horse. The administration is preferably carried out parenterally, such as intramuscularly, intradermally or subcutaneously. One or more administrations may be carried out. In particular, in the case of vaccination, an administration is carried out each time the vaccine is administered.

The amount of DNA used in the stimulant compositions and the immunogenic compositions and vaccines according to the present invention is between approximately 10 $\mu$g and approximately 2000 $\mu$g, and preferentially between approximately 50 $\mu$g and approximately 1000 $\mu$g, for a given plasmid. Those skilled in the art possess the competence required to precisely define the effective dose of DNA to be used for each therapeutic protocol or vaccination protocol.

If a live vector is used, the doses may be between $10^4$ and $10^{10}$ pfu (plaque-forming units), preferably between $10^6$ and $10^8$ pfu.

For a composition containing the GM-CSF protein, the doses may be between 1 $\mu$g and 5 mg, preferably between 50 $\mu$g and 1 mg.

The dose volumes may in particular be between 0.5 and 5 ml, preferably between 2 and 3 ml.

The invention will now be described in greater detail using embodiments taken by way of nonlimiting examples and referring to the drawing in which:

**Figure 1:** Sequences of the equine GM-CSF gene and protein

**Figure 2:** Restriction map of the pJP097 plasmid

**SEQ ID sequence listing for the constructs of the present invention**

| | |
|---|---|
| **SEQ ID N° 1** | Oligonucleotide JP705 |
| **SEQ ID N° 2** | Oligonucleotide JP706 |
| **SEQ ID N° 3** | Oligonucleotide JP729 |
| **SEQ ID N° 4** | Oligonucleotide JP730 |
| **SEQ ID N° 5** | Oligonucleotide JP731 |
| **SEQ ID N° 6** | Oligonucleotide JP734 |
| **SEQ ID N° 7** | Oligonucleotide JP735 |
| **SEQ ID N° 8** | Sequence of the equine GM-CSF gene (see figure 1) |
| **SEQ ID N° 9** | Sequence of the horse GM-CSF protein (see figure 1) |

**EXAMPLES**

All the plasmid constructs were prepared using standard molecular biology techniques (cloning, digestion with restriction enzymes, synthesis of a single-stranded complementary DNA, polymerase chain reaction, extension of an oligonucleotide with a DNA polymerase, etc) described by Sambrook J. *et al.* (Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor Laboratory. Cold Spring Harbor. New York. 1989). All the restriction fragments used for the present invention, and also the diverse polymerase chain reaction (PCR) fragments, were isolated and purified using the "Geneclean®" kit (BIO101 Inc. La Jolla, CA).

**EXAMPLE 1: Preparation of the total RNA of horse lymphocytes stimulated *in vitro* with mitogenes**

Horse blood was collected in a tube containing EDTA, via a blood sample taken from the jugular vein. The mononuclear cells were harvested by centrifugation on a Ficoll gradient, and then placed in culture in a 60mm-diameter Petri dish. The horse mononuclear cells in culture were then stimulated with either

concanavalin A (conA) (final concentration of approximately 5 µg/ml) or with phytohemagglutinin (PHA)(final concentration of approximately 10 µg/ml). After stimulation, the "ConA" and "PHA" lymphoblasts were harvested by scraping the culture dishes and the total RNA of these cells was extracted using the "mRNA isolation kit for White Blood Cells" kit (Boehringer Mannheim/Roche Cat # 1 934 325).

**Example 2: Isolation of the gene encoding equine GM-CSF**

The oligonucleotides JP075 and JP076 were synthesized and have the following sequences:

JP705 (SEQ ID N° 1) (20 mer)
5' TGGGCACTGTGGYCTGCAGC 3'
JP706 (SEQ ID N° 2) (17 mer)
5'AGCATGTGRATGCCATC 3'

These oligonucleotides were used with the 5'/3'RACE kit (Boehringer Mannheim/Roche Cat # 1 734 792) so as to generate the 3'RACE clones 6S4, 6W6 and 6W7. The 3' consensus sequence established from these 3 clones was used to synthesize the oligonucleotides JP729, JP730 and JP731 which should be used to generate the corresponding 5'RACE clones:

JP729 (SEQ ID N° 3) (21 mer)
5' AGCTCCCAGGGCTAGCTCCTA 3'
JP730 (SEQ ID N° 4) (21 mer)
5' CCCTGTTTGTACAGCTTCAGG 3'
JP731 (SEQ ID N° 5) (21 mer)
5' TGTTGTTCAGAAGGCTCAGGG 3'

The corresponding 5'RACE clones obtained were the clones 7D2 and 7D10. The consensus sequences generated from the 3'RACE clones and the 5'RACE clones were used to amplify the entire sequence of the equine GM-CSF gene according to the reverse transcriptase technique

followed by a PCR. The total RNA extracted from the horse lymphocytes stimulated with ConA or with PHA (example 1) was used as a matrix for synthesizing the first strand of complementary DNA. This first strand of complementary DNA was produced by extension of the oligonucleotide p(dT)15 (Boehringer Mannheim/Roche Cat # 814 270). The single-stranded complementary DNA obtained was then used as a matrix for a PCR reaction with the following oligonucleotides:

JP734 (SEQ ID N° 6) (44 mer)
5'CATCATCATGTCGACGCCACCATGTGGCTGCAGAACCTGCTTCT 3'
and JP735 (SEQ ID N°7) (41 mer)
5'CATCATCATGCGGCCGCTACTTCTGGGCTGCTGGCTTCCAG 3'

so as to amplify a PCR fragment of approximately 500 base pairs (bp). This fragment was purified by agarose gel electrophoresis (= fragment A).

**Example 3: Construction of the pJP097 plasmid and sequence of the equine GM-CSF gene**

Fragment A (example 2) was digested with NotI and SalI and the NotI-SalI fragment thus obtained was ligated with the pVR1012 plasmid (Hartikka J. *et al*. Human Gene Therapy. 1996. **7**.1205-1217), digested beforehand with NotI and SalI, so as to give the pJP097 plasmid (5334 bp, figure 2). The NotI-SalI fragment cloned on this plasmid was entirely sequenced. This sequence (SEQ ID No. 8), which encodes a 144 amino acid protein (SEQ ID No. 9), is the horse GM-CSF cytokine (=equine GM-CSF) represented on figure 1.

**Example 4: *in vitro* biological activity of the product of the equine GM-CSF gene**

CHO-K1 cells (hamster ovary cells, available from the American Type Culture Collection strain library under the accession number CCL-61) are cultured in minimum essential medium or MEM (Gibco-BRL) in 60 mm-diameter Petri dishes and transfected with 5 µg of pJP097

plasmid, complexed beforehand with 10 µl of LipofectAmine PLUS® (Cat# 10964-013, Gibco-BRL, Cleveland, OH, USA). The conditions for formation of the DNA/LipofectAmine® complexes and for transfection of the cells were those recommended by the supplier (Gibco-BRL). 48 hours after transfection, the culture supernatants are harvested and frozen.

Bone marrow cells taken from pigs are cultured in a semi-solid Methocult medium (Cat# H4230 from StemCell Technologies). 10 µl of the supernatant of the cells transfected with the pJP097 plasmid are then added or not added (negative control). Two independent transfections were carried out with the pJP097 plasmid, which were given the codes pJP097 T1 and pJP097 T2. Each supernatant (10 µl diluted tenfold) is tested in parallel on 3 culture dishes. The negative control consists of a CHO culture supernatant. After culturing for 14 days, the dishes are examined for the formation of macrophage colonies, and the possible colonies are counted.

The supernatants of CHO cells transfected with the pJP097 plasmid gave the following results:

| Plasmid/super-natant dilution | number of dishes | mean number of colonies per dish | Standard deviation |
|---|---|---|---|
| Control | 3 | 0 | 0 |
| pJP097 T1 (eGM-CSF) | 3 | 12 | 2 |
| pJP097 T2 (eGM-CSF) | 3 | 15 | 0 |

These results show that the product of the equine GM-CSF gene expressed by the pJP097 plasmid has activity of the GM-CSF type on cells *in vitro*.

**Example 5: Preparation of the plasmids according to the invention**

For the preparation of the plasmids intended for the vaccination of horses, any technique which makes it possible to obtain a suspension of purified plasmids may be used. These techniques are well known to those skilled in the art. The plasmids are produced by culturing *Escherichia coli* K12 bacteria transformed with the plasmids according to the invention. Mention may be made in particular of the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in Sambrook J. *et al.* (Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Reference may also be made to patent applications WO-A-95/21250 and WO-A-96/02658, which describe methods for producing, on an industrial scale, plasmids which can be used for vaccination. For the needs of vaccine manufacture, the plasmids are resuspended so as to obtain highly concentrated solutions (> 2 mg/ml) compatible with storage. To do this, the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0).

**Example 6: Manufacture of the vaccines according to the invention and administration**

The pJP097 plasmid stock is diluted in TE buffer, in physiological saline or in PBS buffer, and mixed with diverse vaccinal plasmids expressing protective immunogens. These plasmids may, for example, be those cited in the examples of PCT patent application WO 98/03198.

The horses are vaccinated with doses of 100 µg, 250 µg

or 500 µg per plasmid.

The various mixtures of "immunogenic" plasmids and of the "equine GM-CSF" pJP097 plasmid thus obtained are coadministered intramuscularly (syringe + needle) in the muscles of the neck or of the breast. In this case, the vaccinal doses are injected in a volume of 2 ml.

The intramuscular injections may also be carried out using a liquid-jet injection apparatus (needle-free) which propels a dose of for example 0.5 ml. If necessary, several successive administrations can be carried out in the same animal in order to inject volumes greater than 0.5 ml. The successive shots are then carried out in a spatially shifted manner, so that the injection areas are separated by approximately 1 to 2 centimeters.

The injections may also be carried out intradermally using a liquid-jet injection apparatus (needle-free) delivering a dose of 0.2 ml at 5 points (0.04 ml per injection point) (for example the "PIGJET®" apparatus, Endoscoptic, Laon, France).

Horses are typically vaccinated with two injections of mixtures of plasmids according to the invention, given 4-5 weeks apart.

**Example 7: Formulation of the plasmids according to the invention**

The mixture of "immunogenic" plasmids and of the pJP097 plasmid is diluted in TE buffer, in physiological saline or in PBS buffer, so as to obtain a concentration of 1 mg/ml. A 0.75 mM DMRIE-DOPE solution is prepared by taking a DMRIE-DOPE lyophilisate up with a suitable volume of sterile $H_2O$.

The plasmid DNA/lipid complexes are formed by diluting,

in equal parts, the 0.75 mM DMRIE-DOPE solution with the 1 mg/ml DNA solution. The DNA solution is gradually introduced, using a 26G crimped needle, along the wall of the flask containing the cationic lipid solution, so as to avoid the formation of foam. As soon as the two solutions have been mixed, gentle stirring is carried out. A composition is finally obtained, which comprises 0.375 mM of DMRIE-DOPE and 500 µg/ml of DNA.

It is desirable for all of the solutions used to be at room temperature for all of the operations described above. The DNA/DMRIE-DOPE complexation is left to develop at room temperature for 30 minutes, before immunizing the animals as described in example 6.

It should be clearly understood that the invention defined by the appended claims is not limited to the particular embodiments indicated in the description above, but encompasses the variants which depart neither from the context nor from the spirit of the present invention.

## Claims

1. An isolated DNA fragment comprising the nucleotide sequence SEQ ID No. 8.

2. An isolated DNA fragment encoding the amino acid sequence SEQ ID No. 9.

3. An isolated DNA fragment having the nucleotide sequence SEQ ID No. 8.

4. The DNA fragment as claimed in claim 1, combined, by fusion, with the nucleotide sequence encoding at least one immunogen or at least one immunologically active fragment of an immunogen, in particular of an equine immunogen.

5. An isolated equine GM-CSF polypeptide.

6. An isolated equine GM-CSF polypeptide having the amino acid sequence SEQ ID No. 9.

7. A fusion protein encoded by a fragment as claimed in claim 4.

8. An *in vitro* expression system comprising, as an insert, and expressing *in vitro*, a DNA fragment as claimed in claim 1.

9. An *in vivo* expression vector comprising a DNA fragment as claimed in claim 1, under conditions which allow the expression, in horses, of a functional equine GM-CSF protein.

10. The vector as claimed in claim 9, the vector being a plasmid.

11. The vector as claimed in claim 9, the vector being

a viral vector.

12. The vector as claimed in claim 11, the viral vector being chosen from the group consisting of poxviruses, adenoviruses and herpesviruses.

13. The vector as claimed in claim 12, the poxvirus being chosen from the group consisting of the vaccinia virus, canarypox, fowlpox, swinepox, raccoonpox and camelpox.

14. Equine immunogenic composition or vaccine, comprising the equine GM-CSF protein, an immunogenic or vaccinal preparation against an equine pathogen, and an excipient or vehicle which is acceptable from a veterinary point of view.

15. The composition or vaccine as claimed in claim 14, in which the immunogenic or vaccinal preparation is chosen from the group consisting of an inactivated preparation, an attenuated live preparation, a subunit preparation and a recombinant preparation.

16. An equine immunogenic composition or vaccine, comprising an *in vivo* expression vector as claimed in one of claims 9 to 13, an immunogenic or vaccinal preparation against an equine pathogen, and an excipient or vehicle which is acceptable from a veterinary point of view.

17. The composition or vaccine as claimed in claim 16, in which the immunogenic or vaccinal preparation is chosen from the group consisting of an inactivated preparation, an attenuated live preparation, a subunit preparation and a recombinant preparation.

18. An equine immunogenic composition or vaccine,

comprising a plasmid expressing an equine GM-CSF protein, a plasmid expressing an immunogen of an equine pathogen, and a vehicle or excipient which is acceptable from a veterinary point of view.

18. A nonspecific stimulant composition comprising the equine GM-CSF protein, and an excipient or vehicle which is acceptable from a veterinary point of view.

19. A nonspecific stimulant composition comprising an *in vivo* expression vector as claimed in one of claims 9 to 13, and an excipient or vehicle which is acceptable from a veterinary point of view.

SEQUENCE LISTING

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<141> patent filing date

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20 25 30

Val Asp Ala Ile Lys Glu Ala Leu Ser Leu Leu Asn Asn Ser Ser Asp
35 40 45

Thr Ala Ala Ile Met Asn Glu Thr Val Glu Val Val Ser Glu Thr Phe
50 55 60

Asp Ala Glu Glu Leu Thr Cys Leu Gln Thr Arg Leu Lys Leu Tyr Lys
65 70 75 80

Gln Gly Leu Arg Gly Ser Leu Ile Lys Leu Glu Gly Pro Leu Thr Met
85 90 95

Met Ala Ser His Tyr Lys Gln His Cys Pro Pro Thr Leu Glu Thr Ser
100 105 110

Cys Ala Thr Gln Met Ile Thr Phe Lys Ser Phe Lys Lys Asn Leu Lys
115 120 125

Asp Phe Leu Phe Glu Ile Pro Phe Asp Cys Trp Lys Pro Ala Gln Lys
130 135 140

**Figure 1**

**Sequence of the equine GM-CSF gene**

```
   1 ATG TGG CTG CAG AAC CTG CTT CTT CTG GGC ACT GTG GTT TAC AGC ATG CCC GCA CCC ACC
     TAC ACC GAC GTC TTG GAC GAA GAA GAC CCG TGA CAC CAA ATG TCG TAC GGG CGT GGG TGG
   1 M  W  L  Q  N  L  L  L  L  G  T  V  V  Y  S  M  P  A  P  T

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     GCG GTT GGG TCG GGA CAG TGA GCC GGG ACC GTC GTA CAC CTA CGG TAG TTC CTC CGG GAC
  21 R  Q  P  S  P  V  T  R  P  W  Q  H  V  D  A  I  K  E  A  L

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 101 Y  K  Q  H  C  P  P  T  L  E  T  S  C  A  T  Q  M  I  T  F

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     TTT TCA AAG TTT TTC TTG GAC TTC CTA AAA GAC AAA CTC TAG GGC AAA CTG ACG ACC TTC
 121 K  S  F  K  K  N  L  K  D  F  L  F  E  I  P  F  D  C  W  K

 421 CCA GCC CAG AAG TAA
     GGT CGG GTC TTC ATT
 141 P  A  Q  K
```

**Figure 2**

**Restriction map of the pJP097 plasmid**