(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 February 2008 (14.02.2008)

PCT

(10) International Publication Number
WO 2008/017568 A1

(51) International Patent Classification:
A61K 39/145 (2006.01) *A61P 35/00* (2006.01)
C12N 15/79 (2006.01) *A61P 31/00* (2006.01)
A61K 48/00 (2006.01)

(21) International Application Number: PCT/EP2007/057224

(22) International Filing Date: 13 July 2007 (13.07.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06118717.5 10 August 2006 (10.08.2006) EP

(71) Applicant (*for all designated States except US*): INTERNATIONAL INVESTMENT AND PATENTS SA; 18 Avenue de la Porte-Neuve, L-2227 Luxembourg (LU).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): GERLONI, Mara [IT/US]; 2933 Arianne Drive, San Diego, US92117 (US).

(74) Agent: PISTOLESI, Roberto; Via Turati 32, I-20121 Milano, (IT).

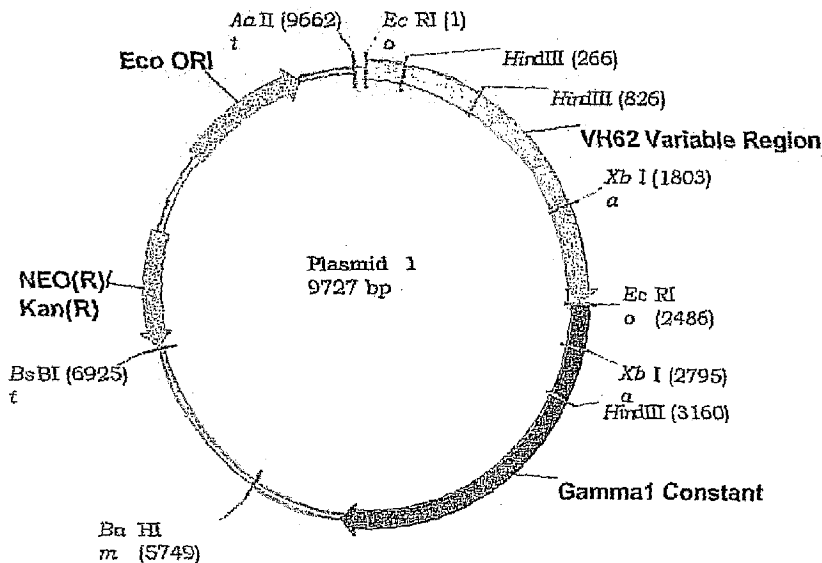
(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: PLASMIDS WITH IMMUNOLOGICAL ACTION

Map of plasmid 1



1-2483bp Segment encoding the variable region Vh62
2484-5077 bp Segment encoding the constant region γ1
6943-7737bp Segment encoding the gene for resistance to neomycin
9324-9267bp *E. coli origin of replication*

(57) Abstract: Recombinant plasmids usable for the transfection of eukaryotic and prokaryotic cells are described; such plasmids have a length comprised between 7 and 12 kbases and comprise a sequence encoding the heavy chain of an immunoglobulin; in particular, they may be used: -in a process of transfection of prokaryotic or eukaryotic cells (ex vivo) which can be inoculated into higher organisms in order to induce a prophylactic or therapeutic immune response; -in a protocol of direct inoculation (in vivo) in higher organisms in genie immunization methodologies with the aim of evoking prophylactic or therapeutic immune responses.

WO 2008/017568 A1

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

Title

Plasmids with immunological action

Description

The object of the present invention is represented by a recombinant plasmid usable for the transfection of eukaryotic and prokaryotic cells, having a length comprised between 7 and 12 kbases and comprising a sequence encoding the heavy chain of the immunoglobulin. A further object of the invention is represented by the use of the aforesaid plasmid for the preparation of a pharmaceutical formulation, or of a vaccine or a therapeutic treatment, for inducing an immune response in a human or animal organism.

In particular, described and claimed is a sequence of bases that are used for the construction of plasmids which may be used:

- in a process of transfection of prokaryotic or eukaryotic cells (*ex vivo*) which can be inoculated into higher organisms in order to induce a prophylactic or therapeutic immune response (in particular by means of the plasmid 1 having the sequence SEQ ID NO. 1);
- in a protocol of direct inoculation (*in vivo*) into higher organisms in genic immunization methodologies in order to evoke prophylactic or therapeutic immune responses (in particular by means of the plasmid 2 having the sequence SEQ ID NO.2).

A description is also given of a profile of pathologies which may be treated with the plasmids described.

STATE OF THE ART

DNA of plasmidic origin may be used for the transfection of prokaryotic and eukaryotic cells through known methods. The plasmids constructed for 5 this purpose are generally constituted by a skeleton which has inserted units of genetic material encoding a certain protein which may or may not be provided with its own biological activity.

The plasmids may be of commercial origin, into 10 which a part bearing the specificity is introduced into a structural construct that is already known and used, or generated autonomously, that is, by assembling fragments of selected genetic material on the basis of a certain profile to be reconstructed at the organism 15 for which the plasmid is destined.

The synthesis of a plasmid is an operation of fundamental importance for the purpose of obtaining the desired cellular properties. The plasmids determine the efficiency of the transfection and of the synthesis 20 of the transgenic protein, and also the safety of the transfection, and therefore in the final analysis the resultant efficacy of the proteic expression of the transfected cell.

A plasmid is a vector of genetic data which 25 influence the cellular cycle of the host cell and, consequently, the life cycle of the organism which hosts these transfected cells: the more data introduced into the plasmid, the more risks are run in the course of the transfection.

30 A plasmid is characterized by the specificity of the data contained: the less complex it is, the easier the synthesis thereof and the safer the use thereof.

A plasmid exercises the capacity of transfecting a cellular population more or less spontaneously

depending on the type of cell and the experimental conditions of contact/incubation in which transfection is carried out. The more selective a plasmid is for a specific cellular population, the more it is usable in conditions of safety.

The conditions in which transfection is carried out are decisive for the success thereof: the homogeneity and the concentration of the cells to be transfected, the incubation time and conditions, the possibility of monitoring the phenomenon with specific and selective methods constitute an extremely important corollary for the success of the operation.

In the specific case in which a plasmid is used for transfecting cells to be injected *in vivo* in patients, such cells are to be handled with extreme caution inasmuch as they must be re-inserted into the patient, and risky and fatal collateral phenomena cannot be risked: the less handling is necessary, the greater the safety of the method and the more reproducible the result.

From the above it inevitably follows that the use of a plasmid of reduced dimensions, especially if combined with a method with limited handling, is a condition to be preferred in transfection used for prophylactic and therapeutic purposes.

The use of a plasmid encoding immunogenic epitopes in an autologous transfection method is one of the systems that can be used for inducing a specific immune response in some pathologies characterized by the appearance of infecting agents, or by spontaneous or induced cellular mutation (carcinogenesis), with modification of the apoptotic course of a selected cell or cellular line.

WO 90/09804 describes immunoglobulins genetically engineered for expressing a predefined peptide epitope in the variable region or in the bond domain of the immunoglobulin.

5 WO 00/61766 describes tumoral antigens derived from telomerasis that can be used for generating a response mediated by T-cells against telomerasis and consequently against the tumour itself.

WO 00/64488 describes a plasmid encoding chimeric 10 heavy chain of an immunoglobulin, the pN$\gamma_1 V_H$62 plasmid, which is obtained by subcloning the murine V_H62 gene into the pNγ_1 plasmid, containing a sequence encoding a human γ_1 costant region. This plasmid can be modified by introduction of heterologous epitopes in any of the 15 complementarity determining regions of the variable region. Therefore, its use in a method for inducing an immunoresponse is disclosed.

However, the pN$\gamma_1 V_H$62 plasmid contains portions that will be harmful if this plasmid would be injected into 20 humans. Furthermore, the dimensions of this plasmid are such that a very low transfection yield is obtained.

DESCRIPTION OF THE INVENTION

The present inventors have now developed plasmids comprising a sequence encoding the heavy chain of 25 immunoglobulin that do not present the drawbacks of the pN$\gamma_1 V_H$62 plasmid when used for inducing an immunoresponse *in vivo* or *ex vivo* in a human or animal organism. In particular, the plasmids developed have a better safety profile and show an increase yield when 30 transfected into cells.

These plasmids have a length comprised between 7 and 12 kbases, preferably between 8 and 12 kbases, more preferably between 9 and 11 kbases and, even more

preferably, between 9 and 10 kbases and are able to express the heavy chain of immunoglobulin when they are transfected into lymphocytes.
According to a preferred embodiment, these plasmids
5 express a chimeric heavy chain of immunoglobulin, preferably comprising a murine variable region and a human constant region, preferably Igγ1.
The murine variable region is preferably the V_H region from hybridoma 62 derived from splenocytes of an adult
10 hyperimmunised mouse (Zanetti et al. *J. Immunol.,* 1983, 131:2452), hereinafter referred to as V_H62 region. , The V_H62 hybridoma 62 secretes a monoclonal antibody with anti-tyroglobulin activity
The Igγ1 region gene is preferably cloned from the
15 vector pNγ1.
The plasmids may further contain a promoter specific for lymphocytic cells, preferably of around 50 bp, or of viral origin, preferably the CMV promoter.
The plasmids of the invention preferably also comprise
20 the polyadenylation sequence AATAAA.
It is preferred that the plasmids of the invention do not express resistance to betalactamic antibiotics and in particular to ampicillin, and/or do not comprise a replication origin of SV40.
25 Accordingly, the plasmids preferably contain a replication origin of *Escherichia coli*, preferably PBR322. Furthermore, the plasmids preferably express resistance to neomycin.
Preferred plasmids of the invention are plasmids 1 and
30 2.
The plasmid 1 has a length of 9727 bp (SEQ ID NO. 1 and Figure 1) and the plasmid 2 of 10004 bp (SEQ ID NO. 2

and Figure 2). Both the plasmids described encode a chimeric heavy chain of immunoglobulin.

The sole structural difference between the two plasmids is determined by the specific promoter, which in the case of the plasmid 1 is a specific promoter for lymphocytic cells of 50 bp, and in the case of the plasmid 2 is a viral promoter with dimension of 742 bp.

The skeleton of the plasmids is represented by pSV2neo, a DNA of bacterial origin containing the gene for resistance to neomycin and the origin of replication PBR322.

The genetic sequence encoding the heavy immunoglobulinic chain is composed of:

- a murine variable region (V_H62) of around 2.5 kb, originally cloned from a mouse hybridoma (hybridoma 62) secreting a monoclonal antibody with anti-tyroglobulin activity (Sollazzo, et. al., *Eur. J. Immunol.*, 1989);
- a human Igγ1 constant region deriving from the pNγ1 vector (Hybritech Corporation, San Diego, CA).

The immunoglobulinic promoter of the plasmid 1 is an integral part of V_H62, while the viral promoter of the plasmid 2 was derived from the plasmid phMGFP (Promega, WI, USA). The gene for resistance to neomycin also confers a resistance to kanamycin for selective growth in prokaryotic cells.

The polyadenylation sequence is in position 5178:5183 of the plasmid and follows the human constant region. The sequence is as follows: AATAAA. The origin of bacterial replication is of *Escherichia coli* and is situated in position 8324:9264.

The particular feature of the plasmids of the invention is that the regions determining the complementarity (CDR) of the encoded protein may be mutagenized for the purpose of introducing therein

epitopes (from 5 to 25 amino acid residues) with antigenic properties (Gerloni et al *Nature Biotechnology 1997*). Such properties make it possible to use the plasmid in question for inducing a specific immune response either by means of a transfection *ex vivo* with the inoculum of cells transfected spontaneously or by means of direct inoculation *in vivo* (genic immunization).

Accordingly, an object of the present invention is the use of the plasmids of the invention for the preparation of a pharmaceutical formulation for DNA vaccination, in particular for inducing an immunoresponse in a human or animal organism.

Furthermore, another object of the invention is a formulation containing at least one plasmid according to any one of the preceding claims together with pharmaceutically acceptable excipients and/or coadjuvants.

The formulation of the invention may be used for prophylactic or therapeutic purposes.

The formulation of the invention may be used in particular in a human or animal organism that is or was affected by:

-tumours belonging to the family of carcinomas and/or adenomas and/or sarcomas and/or lipomas and/or solid and/or ascitic tumours, by prostate or pancreatic, renal or pulmonary carcinoma. Preferably, in this case said organism is or was affected by the presence of tumour cells having on the surface at least one antigenic epitope, the encoding sequence of which is contained in the plasmid.

-bacterial, viral, fungal and/or parasitic infections.

A further object of the present invention is a method for inducing an immunoresponse in a human or animal

organism. Said method comprised transfection of prokaryotic and/or eukaryotic cells *ex vivo* and the subsequent inoculation of said prokaryotic and/or eukaryotic cells into said human or animal organism. Preferably, said transfected cells belong to the family of limphocytes and are preferably taken from the peripheral vessels of said human or animal organism. Alternatively, said method comprises inoculation of the plasmid *in vivo* into said human human or animal organism.

Inoculation of the plasmid or of the prokaryotic and/or eukaryotic cells into said human or animal organism is preferably carried out by means of injective or transmucosal administration.

The protein encoded by the plasmids of the invention, like all the immunoglobulins, possesses 3 CDRs, CDR1 with a restriction site usable for the insertion of peptide sequences AfiIII, CDR2 with a Ncol site and CDR3 with an Acc65I site.

It is therefore possible to insert into them various peptide sequences capable of evoking various immune responses both of the humoral type (mediated by B cells) and of the cellular mediated type (mediated by T-cells CD4 and CD8); for example, it is possible to insert at least a single sequence on each CDR, for a total of three sequences capable of evoking various immune responses; it is further possible to insert one or more sequences, optionally fused with one another, on each CDR.

An example of insertion of antigenic epitopes into the CDRs is shown in Figure 3.

The antigenic epitopes preferred for the purposes of the present invention are:

-tumoral antigens, such as for example that of telomerasis and, even more preferably, p540 (ILAKFLHWL), p572 (RLFFYRKSV), pY572 (YLFFYRKSV) and p865 (RLVDDFLLV);

5 -antigens deriving from infective microorganisms, such as, for example, that of influenza and, preferably, the epitope pNP (ASNENMETM).

The transgenic product encoded plasmids 1 and 2 is a protein with a molecular weight of around 156.000
10 daltons (Figure 4). The heavy region encoded is chimeric in nature: part human (the constant region) and part murine (the variable region). Nevertheless, the murine part contains sequences 80% homologous with the human variable regions.

15 Important characteristics of the aforesaid plasmids are the absence of the gene for resistance to ampicillin and the presence of the gene for resistance to kanamycin which provides for the safe use thereof in subjects with potential allergies to the betalactamic
20 antibiotics. Another advantageous factor is that kanamycin is an antibiotic stable at 37°C (conditions of culture of the plasmid) for 24-48 hours, while ampicillin is stable only for 3-4 hours, consequently allowing a culture yield of the plasmid which is
25 greater (less expensive production) and more stable.

The aforesaid plasmids are also devoid of "useless" sequences (for example the sequence SV40 or pieces of genomic material) which would represent greater risks of homology with the genome of the host
30 cell and therefore greater risks of integration in the cell itself.

Analysis of the restriction map of the plasmids

The restriction map obtained by digestion with restriction enzymes is the first criterion to be

considered in order to define the identity of a plasmid. In particular, the map shown in Figure 5 identifies in an exclusive manner the plasmids 1 and 2. There is also shown in succession an image of the fragments of the plasmid 1 after digestion, run on agarose gel in parallel with a standard of known dimension (1Kb ladder) in order to determine the exact dimension thereof. The sequence of the plasmid 1 is shown in SEQ ID NO. 1, while the sequence of the plasmid 2 is shown in SEQ ID NO. 2.

Biological characterization of the plasmids

It has already been indicated previously that a plasmid encoding immunogenic epitopes in a method of transfection is one of the systems that can be used for inducing a specific immune response in some pathologies characterized by the appearance of infecting agents, or by spontaneous or induced cellular mutation (carcinogenesis), with modification of the apoptic course of a selected cell or cellular line.

A further use for such a plasmid is the direct injection *in vivo* into immuno-competent organisms in order to induce an immune response against proteins of a foreign nature and pathogenic microorganisms (Tang et al., *Nature* 1992, Ulmer et al., *Science* 1993, Gerloni et al., *Nature Biotechnology* 1997). In this field with the inoculation of functional genes the induction of humoral responses (mediated by antibodies) and cellular mediated responses (mediated by T-lymphocytes of type CD4 and CD8) effective in the treatment or prevention of pathologies of infective and cancerous origin was demonstrated.

Consequently, the concept of genic immunization is now adopted by vaccinologists all over the world, who use plasmids encoding antigens deriving from bacteria,

viruses and parasites and also from various types of tumour in order to evoke specific and protective immune responses. Clinical trials are currently under way for the therapy or prophylaxis of HIV, herpes, influenza, avian influenza, SARS, hepatitis B and C and carcinomas of various kinds.

The essential components of a plasmid to be used *in vivo* are the gene encoding the antigen (or pieces thereof) of interest, a promoter sequence (normally derived from cytomegalovirus, CMV) which guides the transcription of the antigen, a region of polyadenylation which ensures the translation thereof.

Furthermore, together with the origin of replication for the amplification of the plasmid in bacterial cells there is also a gene which encodes antibiotic resistance in order to ensure the selection of the bacterial population and to eliminate contamination during culture.

Another intrinsic property of the DNA vaccines is that plasmids of bacterial origin contain sequences of non-methylated cytosine together with residues of guanosine (CpG). These CpG units have the capacity of increasing the immunogenic capacity of the plasmids themselves and therefore function as adjuvants.

The direct inoculation of nucleic acids into somatic cells appears to mimic the immunity induced by natural infections and offers various advantages, including the possibility of producing and testing such plasmids in an inexpensive, easy and rapid manner. Moreover, the plasmids are much more stable than conventional vaccines and may be preserved as lyophilisates.

The plasmid is usually inoculated *in vivo* by the intramuscular or intradermal route, although other

routes such as the oral, vaginal, endovenous, intraperitoneal and subcutaneous routes are applicable. The plasmids are administered in a variety of diluents which include distilled water, saline or sugar solutions, physiological buffers, isotonicising compounds, preservative or cryoprotective substances in case the processes of lyophilisation are necessary.

The dose of plasmid used in the immunization protocols varies from case to case but, as a rule, amounts of from 25 to 200 μg per dose are used with 3 doses/injections at intervals of three weeks.

A further object of the present invention is therefore constituted by two recombinant plasmids characterized by a sequence corresponding to SEQ ID NO. 1 and SEQ ID NO.2, respectively or a sequence at least 90% homologous, preferably 95% homologous to SEQ ID NO. 1 and SEQ ID NO.2. These plasmids have dimensions that are reduced but suitable for the purpose and a transfection method that is suitable, reproducible, safe and effective.

The use of such plasmids has specific characteristics:

-optimum yield in the production process, inasmuch as a plasmid of measured content is simpler to produce and gives rise to better production yields if reduced in amplitude;

-optimum stability of the cellular culture, since the plasmids contains the gene for resistance to antibiotic kanamycin stable at 37°C for 24-48 hours (as opposed to ampicillin stable only for 4-6 hours at the same temperature) the stability of the bacterial culture is consequently significantly increased, with undoubted advantages in terms of yield;

-high efficiency of spontaneous transfection (capacity of penetration into the cell) because of contained molecular dimensions and lesser steric bulk, a not negligible detail in the case of protocols in which the use of spontaneous transfection is envisaged;

-the absence of the possibility of anaphylactic reactions that can be induced in the subjects treated, if predisposed to an allergic reaction to ampicillin, inasmuch as the plasmid, not possessing the gene for resistance to ampicillin, is not grown in the presence of that antibiotic;

-low, if not zero, possibility of integration in the genome of the host cell inasmuch as the minimum quantity of plasmid used for transfection renders practically negligible the risk of integration (consequently reduced possibility of induction of oncogenic mutations in the host cell);

-low, if not zero, possibility of direct integration in the genome of the host cell inasmuch as the plasmid does not possess extraneous sequences (such as SV40) which could have homologies with the genome of the cell itself and represent greater risks of integration;

-high flexibility of use of the plasmid also for direction injection *in vivo* in genic immunization protocols. In fact, by substituting in the nucleotidic sequence the specific promoter for B cells with a viral promoter (CMV) with wider expression, the use of the plasmid is extended by simple transfection *ex vivo* to the use of direct inoculation in higher organisms.

A further object of the present invention is constituted by the use of a method of transfection *ex vivo* without the use of any physical or chemical means which might facilitate the process. Such a method does not induce disturbances in the functionality of the

transfected cells and does not induce genetic transformation thereof. The method is characterized by separation of the specific cellular material to be transfected from the remainder of the corpuscular and fluid part of the peripheral blood, obtainable with processes with less handling than the normal centrifuging methods: the use of apheresis makes it possible to separate a large number of lymphocytic cells on which to carry out transfection in a manner which is painless for the patient and very much more useful for experimental purposes. In fact:

-it does not disturb the functionality of the cells with gravitational or mechanical shocks;

-it allows the harvesting of a large number of cells, to be used for the process and to keep stored as reference for the subsequent phase of therapeutic monitoring;

-it does not impoverish the functional and structural resources of the patient or his coagulative or reparative processes;

-it does not add any risk of contamination of the biological material and/or of the patient.

An object of the present invention is constituted by the use of a plasmid as described for effecting the transfection *ex vivo* of selected cells by means of a spontaneous process with the adoption of the following modalities:

1. transfer of the peripheral blood originating from a patient into an instrument capable of directly separating the family of lymphocytes from the rest of the corpuscular fraction and from the serum (apheresis process);
2. isolation of a quantity of lymphocytic cells suitable for the application of the treatment

described hereinafter; then transfer of the isolated lymphocytes and washing with PBS (without Ca^{++} and Mg^{++}) and further centrifuging;

3. dilution 1:1 with tryptan blue and counting of the lymphocytes via haemocytometer and microscope in order to verify that they have preserved at least 90% of vitality;
4. re-suspension of the lymphocytes at a concentration of around 20×10^6 cells/ml in PBS (without Ca^{++} and Mg^{++}) and re-division of an aliquot into plates with U-shaped wells, where 25 µg of a plasmid having the sequence shown in SEQ ID NO. 1 are added;
5. incubation of the plates with the transfection wells in an incubator for 30-90 minutes at 37°C and 5% of CO_2;
6. transfer of a suitable aliquot of cells treated with plasmid into a suitable culture medium and leaving to incubate for one night;
7. verifying that the cellular vitality is maintained above a threshold judged to be appropriate (typically 70%) and calculating the dose to be transferred to the patient;
8. transfer into a phleboclysis bag of a suitable volume of transfected cells containing the dose to be re-administered to the patient (typically variable from a few thousands to a few tens of millions of cells);
9. within the scope of the treatment intended to arouse an immune response against the cells which express at the surface the proteins of which the epitopes are contained in the plasmid, inoculation into the patient of the blood contained in the

bag, transgenized by means of the use of the plasmid having the sequence shown in SEQ ID NO. 1.

As an alternative to the method described above, the isolation of the lymphocytes to be transfected may also be carried out by a classic method based on centrifuging; in that case the treatment of apheresis described above in item 1 and item 2 may be substituted by the following:

1. transfer of the peripheral blood originating from a patient into test tubes, addition of buffer with Ficoll and centrifuging until the lymphocytes stratify into an unmistakable band;
2. transfer of the isolated lymphocytes and washing with PBS (without Ca^{++} and Mg^{++}) and further centrifuging.

The use of the plasmid of reduced dimensions and a selected population of cells as described above makes it possible to obtain advantages from the process, such as a reduced time for handling of the blood and of its lymphocytic fraction, a reduced incubation time, a substantially reproducible process yield, the possibility of applying a high degree of automation to the various steps of the process and the possibility of performing the transfection process directly inside an isolated device which does not require the adoption of sterile conditions of the working ambience outside the device itself, which are required by the handling of biological fluids intended for human administration.

A object of the present invention is constituted by the use of a plasmid of contained dimension and characterized by the sequence described, with a method of isolation of the material to be transfected with reduced handling, with transfection conditions

described for inducing immune responses in the treated patients against the agent responsible for the infection or the mutation, of which the specific imprint at the sequential and conformational level is
5 contained in the genetic material represented in the plasmid.

A further object of the present invention is constituted by the use of a device in which to carry out the incubation of the plasmid with the cells,
10 characterized by the presence of a space in which to accommodate the desired volume of cells, a port through which to insert a needle in order to deposit a solution of the plasmid and an optional other port through which to effect the sampling of the transfected cells.
15 In a typical application of the present invention, the plasmid with the sequence shown in SEQ ID NO. 1 is used on a population of lymphocytic cells separated from the blood by the method of apheresis; these transfected cells, after incubation under the normal
20 conditions used for biological fluids, are injected back into a patient in order to induce an immune type of response.

In a further application of the present invention, the plasmid constructed as described is used under the
25 conditions described for transfecting a population of lymphocytic cells harvested by means of apheresis or centrifuging from a patient affected by a tumour of the pancreas, or prostate, lung, kidney, or skin, or affected by some other type of adenomatous or
30 carcinomatous or other tumoral form, either solid or acytic in form, in order to induce in the patient himself a selective immune response against only the cancerous cells, expressing superficially the proteic fraction contained in the plasmid and by these caused

to express the lymphocytes, used as APC (cells presenting the antigen).

In a further application of the present invention, the plasmid shown in SEQ ID NO. 1 is modified by the
5 substitution of the promoter for lymphocytic cells with a promoter of viral origin (CMV). Such substitution gives rise to a plasmid of slightly larger dimensions (300 bp), shown in SEQ ID NO. 2, and used for inducing an immune response in an organism by means of direct
10 parenteral (intramuscular, intradermal, subcutaneous or endovenous) administration or transmucosal administration (via the nasal, oral, intestinal or vaginal mucosa).

The plasmids described were evaluated for:

15
1. the capacity for spontaneously transfecting human lymphocytes;
2. the capacity for inducing an immune response in laboratory mice when injected with transfected cells *ex vivo*;
20
3. the capacity for inducing an immune response in laboratory mice when injected directly with the plasmid 2 in a genic immunization protocol.

The following examples are intended purely as non-
25 limiting illustrations of the invention.

The plasmid 1 was constructed according to the following protocol:

1. Modification of the plasmid PSV2 neo
   - The plasmid PSV2 neo was digested with the
30 restriction enzymes AhdI and XmnI in order to remove the segment for resistance to ampicillin (amp^r)

- PSV2 neo (amp^{r} minus) was digested with BsmBI and HindIII in order to remove the origin of replication SV40 (SV40)
- PSV2 neo ($amp^{r\ and\ SV40}$ minus) was digested with AatII and BstBI. At the end of this process a fragment of 2737 pairs of bases containing the origin of replication of *E. Coli* and the gene for resistance to neomycin/kanamycin was obtained. This fragment was isolated from agarose gel, purified on a column and maintained at 4°C until subsequent use.

2. Modification of the plasmid γ1Vh62

- The plasmid $\gamma 1V_{H}62$ was digested with the restriction enzymes Fsel and BamHI in order to remove the segment of 4787 pairs of bases encoding human genomic DNA
- The plasmid thus derived ($DNA^{genomic}$minus) was circularised with the new dimension of 10721 pairs of bases. It was digested with AatII and BstBI and the fragment containing the variable and constant region was purified for the subsequent ligation reaction

3. Construction of the final plasmid 1

- The plasmid PSV2 neo ($amp^{r\ and\ SV40}$minus) deriving from the passage 1 was digested with AatII and BstBI.
- The modified plasmid $\gamma 1V_{H}62$($DNA^{genomic}$minus) deriving from the passage 2 was digested with AatII and BstBI.
- The two modified plasmids thus digested were bonded together and used for transfecting competent cells of *E. coli*.

- The plasmidic DNA extracted from the cells of *E. coli* was then used for the tests of identification by means of PCR, restriction map and sequencing.

4. Construction of the final plasmid 2
   - The plasmid 1 was digested with DraII and BclI in order to remove the immunoglobulinic promoter contained therein
   - From the plasmid phMGFP, by the same digestion, the viral promoter CMV was removed
   - To the plasmid 1, without promoter, by means of PCR, the promoter CMV was added so as to obtain the plasmid 2.

The following examples are intended purely as a non-limiting illustration of the invention.

Example 1.

An aliquot of around 50 ml of peripheral blood originating from a patient, with the addition of a suitable aliquot of anticoagulant, is transferred into 50 ml tubes, diluted 1:1 with buffer, 20 ml of Ficoll-Paque™ are added and centrifuging is carried out for 20 minutes at 2000 rpm.

After centrifuging, the lymphocytes, contained in the interface band, are harvested and transferred into a new tube and washed 3 times in PBS (without Ca^{++} and Mg^{++}) with recovery by centrifuging.

An aliquot of lymphocytes is diluted 1:1 in tryptan blue and counted in a haemocytometer with the microscope in order to determine the vitality thereof (at least 90%).

After the count, the lymphocytes are re-suspended at a concentration of $20X10^6$ cells per ml in PBS and aliquoted into plates provided with wells with U-shaped bases. There are then added 25 µg of a plasmid, having

the sequence shown in SEQ ID NO. 1, and the plate is placed in an incubator for 60-90 minutes at 37°C and 5% of CO_2.

5 The cells are then diluted to a concentration of $1X10^6$ cells per ml in suitable culture medium, placed in flasks inside an incubator and left for one night at 37°C and 5% of CO_2. After having verified that the cellular vitality is above 70%, the cells are washed twice in saline solution, the number of total cells 10 containing the dose to be inoculated is then calculated, corresponding to a number of cells variable from 10,000 to 100 million and the latter are then re-suspended in an endovenous administration bag, which is then used for inoculation into the patient.

15 An aliquot of the transfected lymphocytes is tested before use by means of the extraction of DNA and mRNA in a nested PCR test in such a way as to evaluate the transfection of lymphocytes that has occurred and give a quantification thereof, even approximate.

20 Figure 6 shows the amplification with a PCR test of the DNA from human lymphocytes transfected with plasmidic DNA. Ladder is the reference for the determination of the dimension of the amplified fragments. The numbers refer to the number of cells 25 amplified, and Naïve means lymphocytes not transfected with DNA (negative control). The figure demonstrates the transfection of the patient's lymphocytes which has taken place, and above all denotes its specificity, inasmuch as no fragment is amplified from lymphocytes 30 not subjected to transfection.

Example 2.

Proceeding from the assumption that the lymphocytes transfected with a DNA encoding antigenic sequences are capable of evoking an immune response

after inoculation into laboratory animals and that the plasmid of the present invention, shown in SEQ ID NO. 1, encodes an epitope capable of evoking a cellular response on the part of the T CD4 lymphocytes, in order to verify the biological activity of the lymphocytes transfected by DNA, an experiment was planned for the verification of the immunogenicity of such lymphocytes *in vivo*, devised as described hereinafter.

- 4 C57/B16 mice were inoculated with 5,000 murine lymphocytes transfected with the plasmid as described in the protocol contained in the present invention. Inoculation was carried out endovenously in the tail vein;
- 14 days after inoculation, the mice were sacrificed and the cells of the spleen were used in a test of proliferation in the presence of the epitope encoded by the plasmid used for transfection;
- the splenocytes were cultured for 72 hours with the epitope of reference, and then tritiated timidine (a radioisotope capable of showing the cellular proliferation) was added thereto;
- after 18 hours, the cells were harvested and the radioactivity measured with a beta counter instrument.

The proliferation was expressed as stimulation index (SI), which is calculated by dividing the radioactive counts in the presence of the specific epitope by the radioactive counts in the presence of an uncorrelated epitope (non-specific proliferation). Conventionally, an SI of more than 3 is considered positive.

Table I : example of immune response induced by the inoculation into laboratory animals of lymphocytes transfected with the plasmid in question. The specific peptide refers to the epitope encoded by the DNA

incorporated in the plasmid of the invention, the control peptide has an uncorrelated sequence.

Mouse	SI Control peptide	SI Specific peptide
#1	0.8	11.8
#2	1.3	14.3
#3	1	13.3
#4	1.1	16.3

The experiment described above shows a specific immune response induced in mice when immunized with lymphocytes transfected with the plasmidic DNA, proving a clear biological property thereof when used in a test *in vivo*.

Example 3.

- Using a plasmid as having the sequence shown in SEQ ID NO. 2, 10 C57/B16 mice were inoculated intramuscularly, in the quadriceps, with 100 µg of DNA diluted in 100 µl of saline solution (group A). The immunization protocol consisted of a total of 3 injections separated by three weeks' interval. Another group of 10 C57/B16 were immunized in the same way, using the same plasmid with the CDRs empty (without any epitope) (group B) as control of specificity of the vaccination;
- 14 days after the last inoculation, the mice were sacrificed and the cells of the spleen were used in a

test of cytotoxicity in comparison with cells pulsed with epitope (NP) encoded by the plasmid used for transfection. The specific epitope is a cytotoxic sequence of the nucleoprotein of Influenza Virus A/PR8;

- the splenocytes were cultured for 5 days with the epitope of reference and then used as effectric cells in a cytotoxicity test;
- the cytotoxicity test consists in measuring the radioactivity (^{51}Cr) released by cells (EL-4) pulsed with epitope NP which are killed by the effectric cells from the immunized mice; the cytotoxicity is expressed as a percentage of lysis at a certain ratio of Effectrics:Target (E:T), which is calculated by dividing the amount of radioactivity released by the cells pulsed with the peptide by the amount of radioactivity released by the non-pulsed cells (specific cytotoxicity).

Table II: Example of cytotoxicity mediated by specific T CD8 cells in animals immunized with the plasmidic DNA #2.

The experiment described above shows the specific immune response induced in mice when immunized with

Group	% of specific lysis
A	50%±12
B (control)	3%±2

plasmidic DNA, proving a clear biological property thereof when used in a test *in vivo*.

C L A I M S

1. A recombinant plasmid having a length of between 7 and 12 kbases and comprising a sequence encoding the heavy chain of immunoglobulin.
2. A plasmid according to claim 1, characterized in that it has a length of between 8 and 12 kbases.
3. A plasmid according to claim 1, characterized in that it has a length of between 9 and 11 kbases, preferably of between 9 and 10 kbases.
4. A plasmid according to claim 1, characterized in that said heavy chain of the immunoglobulin is chimeric.
5. A plasmid according to claim 4, characterized in that said heavy chain of the immunoglobulin comprises a murine variable region and a human constant region.
6. A plasmid according to claim 5, characterized in that said murine variable region is V_H62.
7. A plasmid according to claim 6, characterized in that said murine variable region V_H62 is cloned from a mouse hybridoma secreting a monoclonal antibody with anti-tyroglobulin activity.
8. A plasmid according to claim 5, characterized in that said human constant region is Igγ1.
9. A plasmid according to claim 8, characterized in that said human constant region Igγ1 is cloned from the vector pNγ1.
10. A plasmid according to claim 1, characterized in that it comprises a promoter for lymphocytic cells, preferably of around 50 bp, or a viral promoter, preferably of around 742 bp.

11. A plasmid according to claim 1, characterized in that it comprises a polyadenylation sequence AATAAA.
12. A plasmid according to claim 1, characterized in that it comprises a bacterial replication origin of *Escherichia coli*.
13. A plasmid according to claim 1, characterized in that it expresses resistance to neomycin and/or that it comprises the replication origin PBR322.
14. A plasmid according to claim 1, characterized in that it does not express resistance to betalactamic antibiotics and/or it does not comprise a replication origin of SV40.
15. A recombinant plasmid characterized in that it has the sequence SEQ ID NO. 1 or a sequence at least 90% homologous, preferably at least 95%, to SEQ ID NO. 1.
16. A recombinant plasmid having the sequence SEQ ID NO. 2 or a sequence at least 90% homologous, preferably at least 95%, to SEQ ID NO. 2.
17. A plasmid according to any one of the preceding claims, characterized in that it contains at least one sequence encoding an antigenic epitope.
18. A plasmid according to any one of the preceding claims, characterized in that said antigenic epitope is selected from among p540 (ILAKFLHWL), p572 (RLFFYRKSV), pY572 (YLFFYRKSV), p865 (RLVDDFLLV) and pNP (ASNENMETM).
19. A formulation containing at least one plasmid according to any one of the preceding claims together with pharmaceutically acceptable excipients and/or coadjuvants.
20. The use of a plasmid according to any one of the preceding claims for the preparation of a

pharmaceutical formulation for inducing an immune response in a human or animal organism.

21. The use according to claim 20, characterized in that said human or animal organism is or was affected by tumours belonging to the family of carcinomas and/or adenomas and/or sarcomas and/or lipomas and/or solid and/or ascitic tumours, by prostate or pancreatic, renal or pulmonary carcinoma.

22. The use according to claim 20, characterized in that said human or animal organism is or was affected by bacterial, viral, fungal and/or parasitic infections.

23. The use according to claim 20, characterized in that such a formulation is usable for prophylactic purposes.

24. The use according to claim 20, characterized in that said organism is or was affected by the presence of tumour cells having on the surface at least one antigenic epitope, the encoding sequence of which is contained in said plasmid.

25. Method for inducing an immunoresponse in a human or animal organism comprising the transfection of prokaryotic and/or eukaryotic cells *ex vivo* by means of a plasmid according to claims 1-18 and/or a formulation according to claim 19 and the subsequent inoculation of said prokaryotic and/or eukaryotic cells into said human or animal organism.

26. The method according to claim 25, characterized in that the cells to be transfected belong to the family of lymphocytes.

27. The method according to claim 26, characterized in that said cells are taken from the peripheral vessels of said human or animal organism.

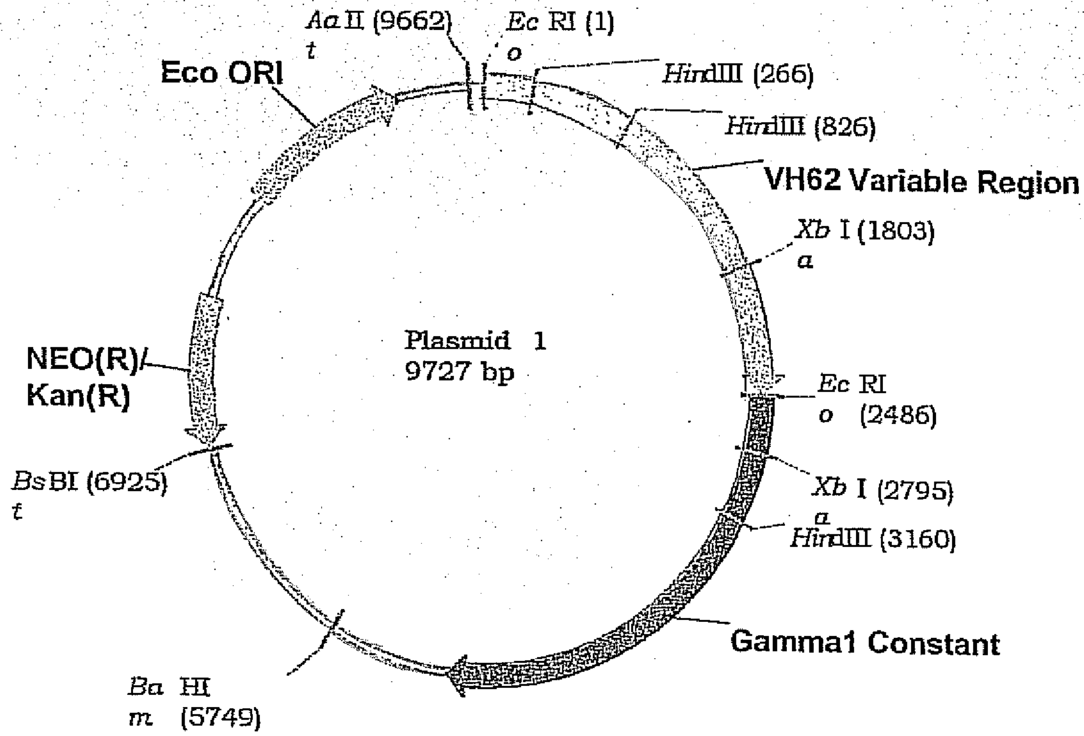
28. The method according to claim 25, characterized in that said plasmid has the sequence SEQ ID NO. 1 or a sequence at least 90% homologous, preferably at least 95%, with respect to SEQ ID NO. 1.

29. Method for inducing an immunoresponse in a human or animal organism comprising the inoculation *in vivo* into said human or animal organism of a plasmid according to claims 1-18 and/or of a formulation according to claim 19.

30. The method according to claim 29, characterized in that said plasmid has the sequence SEQ ID NO. 2 or a sequence at least 90% homologous, preferably at least 95%, to SEQ ID NO.2.

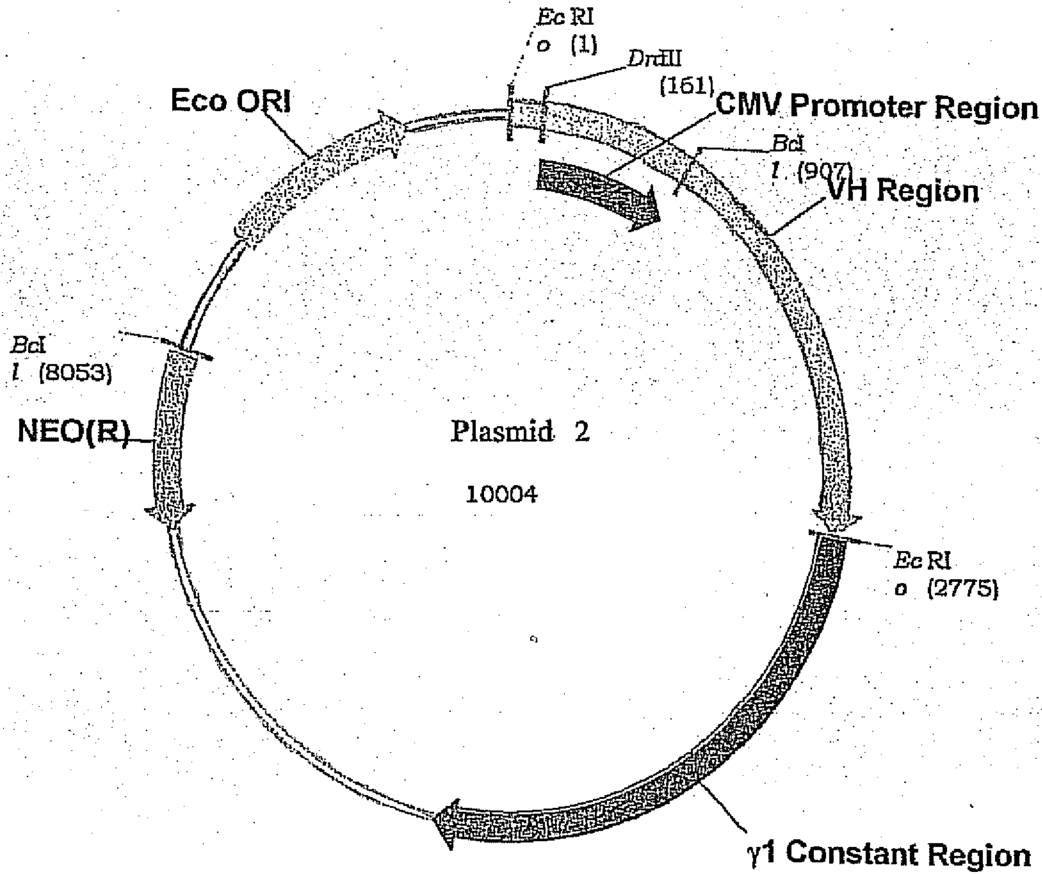
31. The method according to any one of the preceding claims, wherein the inoculation of the plasmid or of the prokaryotic and/or eukaryotic cells into said human or animal organism is carried out by means of injective or transmucosal administration.

Figure 1. Map of plasmid 1



1-2483bp	Segment encoding the variable region Vh62
2484-5077 bp	Segment encoding the constant region γ1
6943-7737bp	Segment encoding the gene for resistance to neomycin
9324-9267bp	*E. coli* origin of replication

Figure 2. Map of plasmid 2



1 to 2775	Segment encoding the variable region Vh62
161 to 907	Viral promoter CMV
2776 to 5366bp	Segment encoding the constant region γ1
7232 to 8026bp	Segment encoding the gene for resistance to neomycin
8613bp to 9553bp	*E. coli* origin of replication

Figure 3. Diagrammatic representation of a model of insertion of epitopes into the CDRs of the plasmid

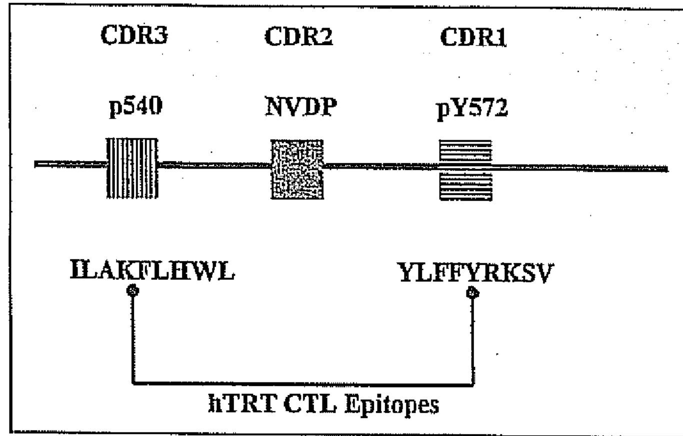


Figure 4. Hypothethised structure of a protein with antigenic epitopes that is encoded by the plasmid described

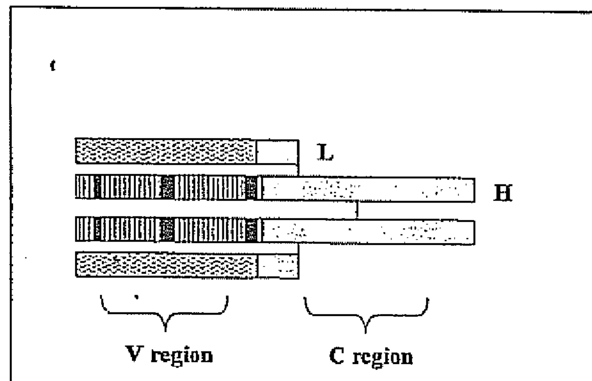


Figure 5. Restriction map of the plasmids

Restriction enzyme	Originated fragments Plasmid 1	Originated fragments Plasmid 2
EcoRI	2.5kb + 7.2kb	2.6kb + 7.2kb
XbaI	1kb + 8.7kb	1kb + 8.8kb
BamHI	9.7kb	9.8kb
HindIII	0.5kb + 2.4kb + 4.9kb + 1.9kb	0.5kb + 2.3kb + 7.0kb + 1.9kb

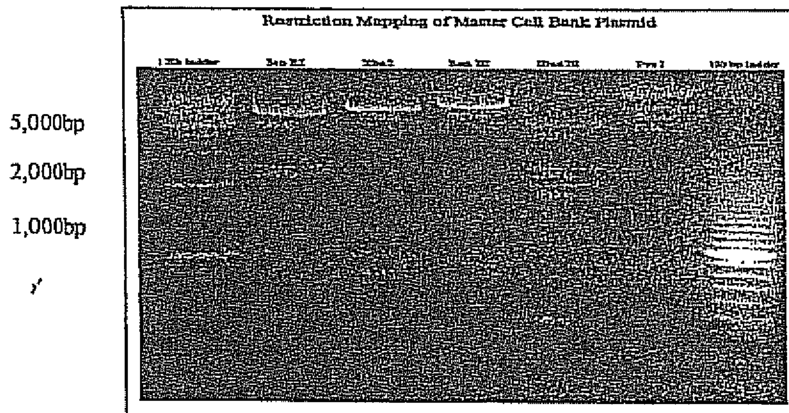
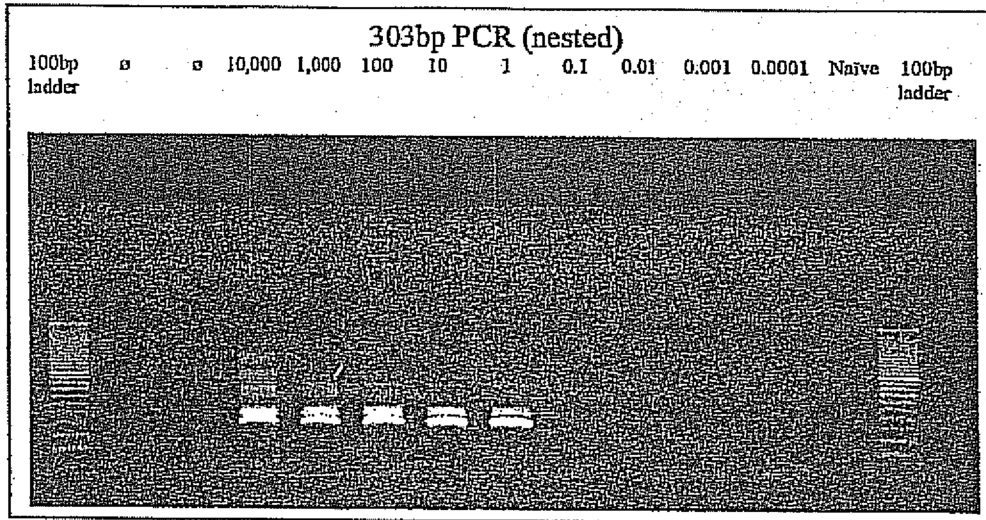


Figure 6. Amplification by means of PCR test of the plasmid from lymphocytes transfected *ex vivo*



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/057224

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/145 C12N15/79 A61K48/00 A61P35/00 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)
C12N

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, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00/64488 A2 (ZANETTI MAURIZIO [US] EUROGEN HOLDING S A [LU]; ZANETTI MAURIZIO [US]) 2 November 2000 (2000-11-02) cited in the application claims 1-50; examples 1-10	1-17, 19-31
Y		18
X	WO 02/088306 A2 (LILLY CO ELI [US]; TSURUSHITA NAOYA [US]; VASQUEZ MAXIMILANO [US]) 7 November 2002 (2002-11-07) pages 15-16; claims 1-17; sequence 16	1-15
X	US 5 583 202 A (ZANETTI MAURIZIO [US]) 10 December 1996 (1996-12-10) the whole document	1-17,19, 20,22,23
Y		18,21

-/--

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

[X] See patent family 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
29 October 2007	14/11/2007
Name and mailing address of the ISA/ 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	Authorized officer Bernhardt, Wiebke

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GERLONI M ET AL: "IMMUNITY TO PLASMODIUM FALCIPARUM MALARIA SPOROZOITES BY SOMATIC TRANSGENE IMMUNIZATION" NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 15, no. 9, September 1997 (1997-09), pages 876-881, XP008032322 ISSN: 1087-0156 abstract page 880, left-hand column	1-14,17, 19,20, 23,29
Y		18
X	XIONG S ET AL: "ENGINEERING VACCINES WITH HETEROLOGOUS B AND T CELL EPITOPES USING IMMUNOGLOBULIN GENES" NATURE BIOTECHNOLOGY, NATURE PUB. CO, NEW YORK, NY, US, vol. 15, no. 9, September 1997 (1997-09), pages 882-886, XP000918882 ISSN: 1087-0156 abstract; figures 1-4	1-17,19, 20,22, 23,29,31
Y	WO 00/02581 A (NORSK HYDRO AS [NO]; GAUDERNACK GUSTAV [NO]; ERIKSEN JON AMUND [NO]; M) 20 January 2000 (2000-01-20) claim 12; sequence 9	18,21
Y	WO 97/30721 A (BOEHRINGER INGELHEIM INT [DE]; SCHMIDT WALTER [AT]; BIRNSTIEL MAX [AT]) 28 August 1997 (1997-08-28) page 40; example 8	18
A		22
Y	US 2004/072240 A1 (KOSMATOPOULOS KOSTAS [FR] ET AL) 15 April 2004 (2004-04-15) tables 7,8; sequences 10, 60, 61	18
X	XIONG S ET AL: "IN VIVO ROLE OF B LYMPHOCYTES IN SOMATIC TRANSGENE IMMUNIZATION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 94, June 1997 (1997-06), pages 6352-6357, XP000918878 ISSN: 0027-8424 page 6352	1-14

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEN S S ET AL: "Cytotoxic T-cells specific for natural IgE peptides downregulate IgE production" CELLULAR IMMUNOLOGY, ACADEMIC PRESS, SAN DIEGO, CA, US, vol. 233, no. 1, January 2005 (2005-01), pages 11-22, XP004953600 ISSN: 0008-8749 pages 11-12	1-14,17
X	BILLETTA R ET AL: "MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I-RESTRICTED PRESENTATION OF INFLUENZA VIRUS NUCLEOPROTEIN PEPTIDE BY B LYMPHOMA CELLS HARBORING AN ANTIBODY GENE ANTIGENIZED WITH THE VIRUS PEPTIDE" EUROPEAN JOURNAL OF IMMUNOLOGY, WEINHEIM, DE, vol. 25, no. 3, March 1995 (1995-03), pages 776-783, XP009058917 ISSN: 0014-2980 pages 776-778; figure 1	1-4,17, 18
X	SOLLAZZO M ET AL: "MOLECULAR CHARACTERIZATION OF THE V-H REGION OF MURINE AUTOANTIBODIES FROM NEONATAL AND ADULT BALB-C MICE" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 19, no. 3, 1989, pages 453-457, XP009077021 ISSN: 0014-2980 abstract; figure 1	1-8
A	WO 2005/021595 A (EURO CELTIQUE SA [LU]; KYLE DONALD JAMES [US]; BROGLE KEVIN CHRISTOPHE) 10 March 2005 (2005-03-10) claims 1-117	1-31

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 25-31 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/composition.

Continuation of Box II.1

Claims Nos.: -

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/057224

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 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:

see FURTHER INFORMATION sheet PCT/ISA/210

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 No. III Observations where unity of invention is lacking (Continuation of item 3 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 allsearchable claims.

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

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search reportcovers 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 and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/057224

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 0064488	A2	02-11-2000	CA	2369616	A1	02-11-2000
			EP	1181320	A2	27-02-2002
			JP	2002542305	T	10-12-2002
WO 02088306	A2	07-11-2002	EP	1385545	A2	04-02-2004
US 5583202	A	10-12-1996	NONE			
WO 0002581	A	20-01-2000	AT	247484	T	15-09-2003
			AU	756094	B2	02-01-2003
			AU	4534099	A	01-02-2000
			CA	2336743	A1	20-01-2000
			CN	1313773	A	19-09-2001
			DE	69910580	D1	25-09-2003
			DE	69910580	T2	24-06-2004
			DK	1093381	T3	08-09-2003
			EP	1093381	A1	25-04-2001
			ES	2200526	T3	01-03-2004
			HU	0104889	A2	29-04-2002
			JP	2002520293	T	09-07-2002
			NO	983141	A	10-01-2000
			PL	345541	A1	17-12-2001
			PT	1093381	T	30-01-2004
			TW	261617	B	11-09-2006
			US	2006106196	A1	18-05-2006
			US	7030211	B1	18-04-2006
WO 9730721	A	28-08-1997	AT	274350	T	15-09-2004
			AU	722264	B2	27-07-2000
			AU	1875997	A	10-09-1997
			BG	63682	B1	30-09-2002
			BG	102714	A	30-06-1999
			BR	9707694	A	27-07-1999
			CN	1211926	A	24-03-1999
			CZ	9802689	A3	14-07-1999
			EE	9800255	A	15-02-1999
			EP	0881906	A1	09-12-1998
			ES	2225951	T3	16-03-2005
			HK	1017257	A1	01-04-2005
			HR	970100	A2	30-04-1998
			HU	9901186	A2	28-07-1999
			ID	16038	A	28-08-1997
			IL	125361	A	14-08-2002
			JP	2000506125	T	23-05-2000
			NO	983850	A	21-10-1998
			NZ	332020	A	28-02-2000
			PL	189413	B1	31-08-2005
			PT	881906	T	29-10-2004
			RO	119344	B1	30-08-2004
			SK	114598	A3	11-06-1999
			TR	9801649	T2	21-12-1998
			TW	585774	B	01-05-2004
			US	7105162	B1	12-09-2006
US 2004072240	A1	15-04-2004	AU	7855501	A	05-02-2002
			CA	2416761	A1	31-01-2002
			EP	1309860	A2	14-05-2003
			FR	2812087	A1	25-01-2002

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/057224

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004072240 A1		WO 0208716 A2	31-01-2002
WO 2005021595 A	10-03-2005	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005)