Description

The present invention relates to novel vaccine formulations, methods for preparing them and to their use in therapy. In particular, the present invention relates to novel formulations for treating Herpes Simplex Virus infections, more particularly Herpes Simplex virus 2 (HSV-2) infections.

HSV-2 is the primary etiological agent of herpes genitalis and together with HSV-1 (the causative agent of herpes labialis) are characterised by their ability to induce both acute diseases and to establish a latent infection, primarily in neuronal ganglia cells.

Genital herpes is estimated to occur in about 5 million people in the U.S.A. alone with 500,000 clinical cases recorded every year (primary and recurrent infection),. Primary infection typically occurs after puberty and is characterised by the localised appearance of painful skin lesions, which persist for a period of between 2 to 3 weeks. Within the following six months after primary infection 50% of patients will experience a recurrence of the disease. About 25% of patients may experience between 10-15 recurrent episodes of the disease each year. In immunocompromised patients the incidence of high frequence recurrence is statistically higher than in the normal patient population.

Both HSV-1 and HSV-2 virus have a number of glycoprotein components located on the surface of the virus. These are known as gA, gB, gC, gD and gE etc.

Glycoprotein D is located on the viral membrane, and is also found in the cytoplasm of infected cells (Eisenberg R.J. et al; J of Virol 1980 35 428-435). It comprises 393 amino acids including a signal peptide and has a molecular weight of approximately 60 kD. Of all the HSV envelope glycoproteins this is probably the best characterised (Cohen et al J. Virology 60 157-166). In vivo it is known to play a central role in viral attachment to cell membranes. Moreover, glycoprotein D has been shown to be able to elicit neutralising antibodies in vivo (Eing et al J. Med. Virology 127: 59-65). However, latent HSV-2 virus can still be reactivated and induce recurrence of the disease despite the presence of high neutralising antibodies titre in the patients sera.

The ability to induce neutralising antibody alone is insufficient to adequately control the disease. In order to prevent recurrence of the disease, any vaccine will need to stimulate not only neutralising antibody, but also cellular immunity mediated through T-cells. The present invention achieves these aims.

The present invention provides a vaccine comprising HSV glycoprotein D or an immunological fragment thereof in conjunction with 3-o-deacylated monophosphoryl lipid A (3D-MPL) a deacylated derivative of monophosphoryl lipid A, and a suitable carrier. Typically the glycoprotein D will be from HSV-2. The carrier may be an oil in water emulsion, or alum, 3D-MPL will be present in the range of 10μg - 100μg preferably 25-50μg per dose wherein the antigen will typically be present in a range 2-50μg per dose.

3D-MPL may be obtained according to the methods described in British patent No. 2220211 (RIBI).

An embodiment of the invention is a truncated HSV-2 glycoprotein D of 308 amino acids which comprises amino acids 1 through 306 naturally occuring glycoprotein with the addition Asparagine and Glutamine at the C terminal end of the truncated protein devoid of its membrane anchor region. This form of the protein includes the signal peptide which is cleaved to yield a mature 283 amino acid protein. The production of such a protein in Chinese Hamster ovary cells has been described in Genentech's European patent EP-B-139 417.

The mature truncate preferably is used in the vaccine formulations of the present invention as is designated rgD_2t.

The HSV antigen may be chemically or otherwise conjugated to a particulate carrier. A particularly preferred approach is to chemically conjugate to particulate Hepatitis B surface antigen through free sulfhydryl groups located on the surface of the Hepatitis B surface antigen. See copending U.K Patent application No. 9027623.9.

The formulations of the present invention are very effective in inducing protective immunity, even with very low doses of antigen (e.g. as low as 5 μg rgD_2t).

They provide excellent protection against primary infection and stimulate, advantageously both specific humoral (neutralising antibodies) and also effector cell mediated (DTH) immune responses.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, e.g. squalane or squalene, a emulsifier, e.g. Tween® 80, in an aqueous carrier. The aqueous carrier may be for example, phosphate buffered saline.

The present invention in a further aspect provides a vaccine formulation as herein described for use in medical therapy, particularly for use in the treatment or prophylaxis of Herpes Simplex viral infections.

The vaccine of the present invention will contain an immunoprotective quantity of HSV gD or immunological fragment thereof and this maybe prepared by conventional techniques.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described,

for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 µg of protein, preferably 2-100 µg, most preferably 4-40 µg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

In addition to vaccination of persons susceptible to HSV infections, the pharmaceutical compositions of the present invention may be used to treat, immunotherapeutically, patients suffering from HSV infections.

In a further aspect of the present invention there is provided a method of manufacture as herein described, wherein the method comprises mixing HSV-2 glycoprotein D or an immunological fragment with a carrier, e.g. an oil in water emulsion or alum, and 3D-MPL.

Comparison of adjuvant efficacy of a recombinant Herpes Simplex Virus Glycoprotein D Subunit Vaccine

In this study, the ability of several adjuvants to improve the protective immunity of a recombinant glycoprotein D from Herpes Simplex Virus (HSV) type 2 (rgD_2t) was evaluated in a guinea pig model. Adjuvants tested were aluminium hydroxide, aluminium hydroxide in combination with 3 Deacyl-Monophosphoryl Lipid A, and 3 Deacyl-Monophosphoryl Lipid A delivered in an oil in water emulsion.

1. Description of the antigen

HSV rgD_2t is a genetically engineered recombinant truncated glycoprotein produced in transfected Chinese hamster ovary (CHO) cells (European Patent No. 0 139 417).

2. Antigen-Adjuvant preparations and immunization schedules

Two separate experiments were performed to evaluate the protective immunity of several rgD_2t formulations in the guinea pig model. In the first experiment, groups of guinea pigs were immunized three times with a low antigen dose (5 µg of rgD_2t) in 4 adjuvant formulations prepared as described below. Two weeks after the last immunization, they were challenged intravaginally with HSV type 2 and were monitored daily for the development of primary and recurrent HSV2 disease. In the second experiment, these formulations were further evaluated on larger animal groups. Factors influencing efficacy of these formulations were also tested such as antigen dose and adjuvant composition.

2.1. Antigen-Adjuvant preparations

In the first experiment, guinea pigs were immunized with the following adjuvant preparations. Each dose (5 µg) was administered in a 0.25 ml volume.

2.1.1. rgD2t /Alum (Aluminium Hydroxide)

Alum was obtained from Superfos (Alhydrogel, (Boehimte) Superfos, Denmark). Five µg of purified rgD_2t was adsorbed overnight at 4°C on aluminium hydroxide (alum) corresponding to 0.25 mg equivalents Al^{3+} in 0.25 ml of 150 mM NaCl 10 mM phosphate buffer pH 6.8.

2.1.2. rgD2t / Aluminium Hydroxide plus 3D-MPL

3 D-MPL was obtained from Ribi Immunochem Research, Inc. After an overnight adsorption of 5 µg gD_2t on alum as described in 2.1.1., the adjuvant preparation was centrifuged and its supernatant removed. An equal volume of adsorption buffer containing 100 µg 3D-MPL was then added to the alum-bound rgD_2t.

For both rgD_2t/Alum preparations, more than 98% of the rgD_2t was found to be incorporated in aluminium hydroxide adjuvant.

2.1.3. rgD2t/3D-MPL in an oil in water emulsion (R)

The oil in water emulsion was prepared using 12% w/v lecithin added to Squalene oil and 0.08% Tween® 80. 3D-MPL was added at a concentration 100 fold higher than the final desired concentration. 1% of this preparation was then mixed in a 0.25 ml volume to 5 µg rgD_2t in aqueous phase, yielding a 1% oil in water emulsion containing 100 µg 3D-MPL.

Similar adjuvant formulations prepared as above but containing different amounts of rgD_2t and/or immunostimulator were used in the second experiment. They were administered in a total volume of 0.5 ml. These formulations are described below.

rgD2t/Alum: Five or 20 µg rgD_2t; 0.5 mg equivalents Al^{3+} per 0.5 ml dose.

rgD2t/Alum plus 3D-MPL: Five or 20 µg rgD_2t; 0.5 mg equivalents Al^{3+}; 50 µg 3D-MPL per 0.5 ml dose.

rgD2t/3D-MPL in o/w emulsion (R): Five or 20 µg rgD_2t were formulated in an 1% o/w emulsion as described above (2.1.3). A 0.5 ml dose contained 5 µg or 20 µg rgD_2t, 50 µg 3D-MPL in a 1% o/w emulsion.

rgD2t/3D-MPL in o/w emulsion (S): The vehicle was prepared as follows: To phosphate buffered saline (PBS) containing 0.4% (v/v) Tween® 80 are added 5% (v/v) Pluronic® L121 and 10% squalane and the resulting mixture microfluidized ten times through a microfluidizer (Model M/110 Microfluidics Corp.,) such that the resulting emulsion comprises only submicron particles. 50µg of 3D-MPL was then added to the emulsion. One volume of this emulsion, containing 3D-MPL was mixed with an equal volume of twice concentrated antigen and vortexed briefly to ensure complete mixing of the components. The final preparation consisted of 0.2% Tween® 80, 2.5% Pluronic® L121, 5% Squalane, 50µg 3D-Mpl and 5 µg or 20 µg rgD_2t in a 0.5 ml dose.

2.2. Immunization schedule

Groups of female Hartley guinea pigs (200-250 gr) were immunized three times at day 0, 28 and 95 with 5 µg rgD_2t formulated in 4 different adjuvant formulations.

Immunizations were done subcutaneously with injection volume of 0.25 ml. Control animals were injected according to the same protocol with adjuvant alone or were untreated.

The different groups were immunized as follows:

Group 1 (n = 4) : 5 µg rgD_2t/3D-MPL (100 µg) in o/w emulsion (R)
Group 2 (n = 4) : 5 µg rgD_2t/Alum plus 3D-MPL (100 µg)
Group 3 (n = 4) : 5 µg rgD_2t/Alum
Group 4 (n = 5) : Alum alone
Group 5 (n = 5) : 3D-MPL (100 µg) alone
Group 6 (n = 8) : untreated

Animals were bled every 2 weeks for antibody determinations by ELISA and neutralization assays as described below.

The different formulations were also tested for their ability to induce T cell mediated immunity, as measured by the induction of delayed-type hypersensitivity responses. The read-outs applied for evaluation of the humoral and cellular immune responses induced by the different rgD_2t formulations are described below.

In order to compare the protective immunity induced by the rgD_2t formulations, all the guinea pigs were challenged intravaginally with 10^5 plaque-forming units (pfu) of HSV2, strain MS, 2 weeks after the last immunization. They were monitored daily for clinical signs of acute infection as well as for evidence of recurrent herpetic diseases. Vaginal swab samples were collected on day 5 after viral challenge and titered for infectious virus.

A detailed description of the guinea pig intravaginal model is given below.

In the second experiment, the immunogenicity of the following rgD_2t formulations was evaluated in larger animal groups. Two antigen doses were compared (5 and 20µg) and different adjuvant composition were tested. A dose of 50µg 3 DMPL was used and its effects compared to the 100µg dose previously used.

Groups of female Hartley guinea pigs were immunized three times at days 1, 28 and 84, as follows:

Group I (n = 8) : 20µg rgD_2t/3DMPL (50µg) o/w emulsion (R)
Group II (n = 8) : 5µg rgD_2t/3DMPL (50µg) o/w emulsion (R)
Group III (n = 10) : 20µg rgD_2t/3DMPL (50µg) o/w emulsion (S)
Group IV (n = 10) : 5µg rgD_2t/3DMPL (50µg) o/w emulsion (S)
Group V (n = 10) : 20µg rgD_2t/Alum + 3DMPL (50µg)

Group VI (n = 10) : 5µg rgD$_2$t/Alum + 3DMPL (50µg)
Group VII (n = 4) : Alum + 3DMPL (50µg) alone
Group VIII (n = 4) : 3DMPL (50µg) o/w emulsion (R) alone
Group IX (n = 8) : untreated

Immunizations were given in a 0.5 ml dose. Control groups were immunized according to the same protocol with adjuvant alone (Groups VII and VIII) or were intreated (Group IX).

A last group (Group X) was immunized with a gD$_2$t Alum + 3D-MPL formulation containing 100µg 3D-MPL in a 0.25 ml dose, according to the protocol described in the first prophylactic experiment:

Group X (n = 10) : 5µg rgD$_2$t/Alum plus 3DMPL (100mg).

Animals were bled every two weeks for individual antibody determinations by ELISA and neutralization assays, as described below. Vaginal washings were collected after the second immunization and were assayed for the presence of systemic antibodies specific for gD$_2$t (anti-gD$_2$t antibodies of IgG class). Guinea pigs were challenged intravaginally with 105 pfu HSV2 (strain MS) 2 weeks after the last immunization. After challenge, they were monitored daily for clinical signs of acute infection (days 4 to 12 post challenge) as well as for evidence of recurrent herpetic disease (days 13 to 39 post challenge).

3. Read-outs

Several read-outs were set up to evaluate the specific antibody and cell mediated responses induced by vaccination with rgD$_2$t formulations. The protective value of these formulations was assessed in the guinea pig intravaginal model.

3.1. ELISA

An ELISA was designed to detect and quantify gD-specific antibodies in guinea pig sera and vaginal washings, using rgD$_2$t as the coating antigen.

3.1.1. Detection of IgG antibodies specific for rgD2t in sera

Antigen and antibody solutions were used at 50 µl per well. Antigen was diluted to a final concentration of 1 µg/ml in PBS and was adsorbed overnight at 4°C to the wells of 96 wells microtitre plate (Maxisorp Immuno-plate, Nunc, Denmark). The wells were then washed 5 times with PBS Tween® 0.1% (wash buffer) and incubated for 1 hour at 37°C with PBS containing 1% bovine serum albumin, 4% newborn calf serum and 0.1% Tween® (saturation buffer). Threefold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the rgD$_2$t-coated wells and incubated for 2 hrs at room temperature. The plates were washed as above and biotin-conjugated sheep anti-guinea pig IgG (IgG1 and IgG2 specific, Serotec, Sopar Biochem., Belgium) diluted 1/3000 in saturation buffer was added to each well and incubated for 1 h.30 min. at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex (Amersham, UK) diluted 1/1000 in saturation buffer was added and incubated for 30 min. at 37°C. Plates were washed as above and incubated with a solution of o-phenylenediamine

(Sigma) 0.04% H_2O_2 0.03% in 0.1 M citrate buffer at pH 4.5.

Color reaction was stopped after 15 min by the addition of H_2SO_4 2 M and the absorbance was readed at 492 nm.

ELISA titer was defined as the reciprocal of serum dilution which produced an absorbance (optical density measured at 492 nm equal to 50% of the maximal absorbance value (midpoint titer).

ELISA titers were calculated by a 4 parameter linear regression analysis using a computer program.

3.1.2. Detection of IgG antibodies specific for rgD2t in vaginal washings

Vaginal washings were first calibrated for their total IgG content by ELISA as follows. Maxisorp Immuno-plates were coated overnight at 4°C with 1 µg/ml (50 µl per well) of purified goat anti-guinea pig IgG (Sigma, Belgium) diluted in PBS. The plates were washed and incubated with saturation buffer as above. Vaginal washings were diluted serially with two-fold dilutions (starting at a 1/100 dilution) in the saturation buffer and added to the plates. A standard curve of purified guinea pig IgG (Sigma, Belgium) was included (two fold dilution starting at a 100 ng/ml concentration) in each plate.

After a 2 hrs incubation at room temperature, the plates were washed as above and biotin-conjugated sheep antibodies specific for guinea pig IgG1 and IgG2 (Serotec, Sopar Biochem, Belgium) diluted 1/1000 in saturation buffer was added to each well and incubated for 1 h 30 min at 37°C. Next steps (addition of

streptavidin-biotinylated peroxidase complex and color revelation) were as described above (3.1.1.).

The concentration of total IgG present in the vaginal washings was determined from the IgG standard curve, by a 4 parameters mon-linear regression analysis using a computer program.

After calibration of their total IgG content, vaginal washings were tested for the presence of IgG antibodies specific for rgD_2t using the same ELISA as described for anti-gD antibody sera quantifications. Results were expressed as optical densities measured at 492 nm per 0.5 µg/ml total IgG.

3.2. Neutralization assay

A 96 well format neutralization assay was set up as follows:

Serial two-fold dilutions of the samples to be tested were prepared directly in the 96 W plates (25 ul/well of each serum dilutions, duplicates). Fifty microliters of a mixture containing 4000 pfu of virus HG52 and complement (1/100 final dilution in the well) were added to each well. The plates were incubated for 1 hour at 37°C. One hundred microliters of BHK 21 cell suspension at 4.10^5 cells/ml were then added to each well (4.10^4 cells/well). The plates were centrifuged for 5 minutes at 1000 rpm and incubated for five days at 37°C in the presence of 7% CO_2.

After this period, the culture medium was gently removed and 100 µl of a solution of cristal violet (10% methanol, 90% H_2O, 0.3% cristal violet) were added to each well and incubated for 20 min. at room temperature. The plates were then abundantly washed with tapwater. The presence of plaques can easily be monitored by microscopic examination.

The neutralizing titer was defined as the reciprocal of the highest serum dilution at which no viral plaque was observed (100% protection of cytopathogen effect). It is important to note that at this time point, a complete cytopathogen effect (100% lysis of the cell monolayer) was observed in the control wells.

3.3. Delayed-Type Hypersensitivity (DTH)

The different rgD_2t formulations were also tested for their ability to induce a T cell specific immune response as measured by the induction of delayed-type hypersensitivity responses.

The adjuvant formulations prepared for the first experiment were used in this study. These preparations contained 5 µg of rgD_2t per 0.25 ml dose. The immunization schedule was as follows: primary immunization: 0.25 ml of vaccine formulation given intramuscularly; booster immunization: 0.25 ml of vaccine formulation given intramuscularly 21 days later; skin test: 5 µg rgD_2t given intradermally (in saline) 8 days later. All guinea pigs were skin tested with saline as control.

In addition, control guinea pigs (non immunized animals) were skin tested with rgD_2t. Erythema and induration at site of intradermal injection were monitored 24 and 48 hrs later.

3.4. Guinea-pig intravaginal model

The guinea pig model for HSV genital infection has been described by LR Stanberry et al (J. of Infectious Diseases 1982, 146:397-403; Intervirology 1985, 24:226-231).

Briefly, 2 weeks after the last immunization, the guinea pigs were challenged with 10^5 pfu of HSV2 strain MS by intravaginal instillation. The clinical course of the primary infection was monitored by daily observation of the incidence and severity of external genital skin lesions during the 12-day post-challenge period.

Vaginal swabs were collected on day 5 after viral challenge and titered for infectious HSV2 by plaque assay, as described below. Animals were then examined daily for evidence of recurrent herpetic lesions from days 13 to 60. The herpetic lesions on the external genital skin were quantitated by using a lesion score scale ranging from 0 to 4 (0 = no lesion or redness; 0.5 = redness; 1 = vesicle; 1.5 = ≥ 4 small vesicles; 2 = larger vesicles; 2.5 = several large vesicles resulting from the fusion of vesicles as in score 2; 3 = size and number of vesicles increase; 3.5 = lesions covering all the surface of the genital skin; 4 = ulcerated lesions with maceration).

The degree of protection provided by the different rgD_2t vaccines was evaluated according to the criteria defined below.

Protection against primary disease (days 0 - 12)

The animal was considered to be not protected if the following lesions were recorded:

- more than one red area at any time,

- one red area persisting in the same area for at least 3 successive days (0.5 lesion score),
- one or several vesicles (≥ 1 lesion score).

Protection against recurrent disease (days 13 - 60)

The animal was scored positive for recurrent disease either if a 0.5 lesion score was recorded for 2 successive days at least or if a lesion score ≥ 1 was observed at any day. An episode of recurrent disease was preceded and followed by a day without any lesions or redness.

The lesion severity for an animal is calculated as the sum of the scores measured during the primary infection (days 1 - 12). The lesion incidence represents the number of animals showing a lesion of ≥ 1 during the observation period (days 1 - 12 [primary disease] or days 13 - 60 [recurrent diseases]).

3.5. Virus titration in vaginal swabs

Vaginal swabs were collected at day 5 after viral challenge. The vaginal vault was swabbed with a calcium alginate tipped swab premoistered in Basal Eagle's medium supplemented with 2% fetal calf serum, 2 mM L glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml gentamycin and 1 µg/ml amphotericin B (swab medium).

Each swab was broken and put into a sterile 12 x 75 mm 5 ml polyallomer tube containing 1 ml of swab medium. The tubes were then vortexed in order to take the virus out and frozen until use. For the titration itself, 6 wells culture plates containing 5.10^5 cells /well were incubated overnight at 37°C. The tubes were thawed and serial dilutions of the samples in swab medium were prepared. After removal of the culture medium in the 6 wells, 200 µl of each samples dilution were transferred in duplicate on the cell monolayers and kept for one hour at 37°C. Four ml of a culture medium containing 1.5% carboxymethylcellulose were added to each well. The plates were then incubated for 2 days at 37°C. After this incubation period, the medium was gently removed and 1 ml of a solution of cristal violet (10% methanol, 90% H_2O, 0.3% cristal violet) was added to each well for 15 min. The plates were then thoroughly rinsed and the plaques were counted. HSV2 titer was expressed in pfu/ml.

4. Results

In a first set of experiments, groups of guinea pigs were immunized with a low antigen dose (5 µg rgD_2t) formulated in 4 different formulations. This suboptimal antigen dose was chosen in order to select the more potent rgD_2t adjuvant combination that could provide protection against primary and recurrent HSV disease when administered to guinea pigs prior to intravaginal HSV2 inoculation (prophylactic trials).

4.1. Induction of humoral immunity

As shown in Table 1, groups vaccinated with rgD_2t formulations containing 3D-MPL as immunostimulant showed higher ELISA and neutralizing titers in their sera than the group immunized with the rgD_2t/Alum vaccine. Good mean neutralizing titers were induced after 3 immunizations with rgD_2t 3D-MPL o/w (R) or rgD_2t Alum 3D-MPL.

4.2. Induction of effector T cell response (DTH)

Skin test results (Table 2) showed that rgD_2t formulated in 3D-MPL o/w emulsion induced the strongest DTH response. A specific DTH response was also induced by rgD_2t Alum 3D-MPL. Similar experiments conducted in mice also revealed that rgD_2t combined with Alum plus 3D-MPL was very potent in inducing an in vivo effector T cell response, in contrast to rgD_2t Alum formulation.

4.3. Effect of vaccination on HSV primary disease

Two weeks after the third immunization, guinea pigs were challenged intravaginally with HSV2. The effect of vaccination on the clinical and virological course of primary HSV2 infection is illustrated in Figure 1 and summarized in Table 3. As compared to the control groups (Groups 4 to 6) that became infected and experienced acute primary disease, 100% of the animals vaccinated with the rgD_2t 3D-MPL o/w formulation showed no evidence of herpetic disease, as monitored by skin lesion incidence and severity. Moreover, these animals did not show any viral replication in the vaginal tract as determined by vaginal virus titration

at day 5 post challenge. Very similar results were obtained in the group vaccinated with rgD$_2$t/Alum 3D-MPL. This group never developed herpetic vesicles during the observation period (lesion score < 1). Moreover, very low viral replication could be detected in the vaginal swabs collected. In contrast animals rgD$_2$t adsorbed on alum were poorly protected (75% skin lesion incident).

4.4. Effect of vaccination on HSV recurrent disease

Results are illustrated in Figure 1 and summarized in Table 4.

Vaccination with rgD$_2$t formulations containing 3D-MPL (Groups 1 and 2) significantly altered the development of recurrent herpetic diseases. Two groups had significantly fewer recurrent episodes and recurrent day numbers than control or rgD$_2$t Alum treated groups.

In order to further evaluate the factors influencing the efficacy of prophylactic rgD$_2$t vaccines containing 3DMPL, a second set of experiments was initiated on larger guinea pig numbers.

Two antigen doses were compared (5 and 20µg) and different adjuvant compositions were tested. Three immunizations were administered at days 0, 28 and 84. Animals were bled every two weeks for individual antibody determination by ELISA and neutralization assays. Vaginal washings were collected after the second immunization and were tested for the presence of systemic antibodies specific for rgD$_2$t.

Induction of humoral immunity

Results (Table 5) indicated that all the rgD$_2$t formulations containing 3D-MPL were able to stimulate high ELISA and neutralizing titers in the guinea pig sera.

The mean ELISA and neutralizing titers induced after three immunizations were very similar in the sera of groups vaccinated with a rgD$_2$t formulation containing either 5µg or 20µg gD$_2$t. There was no significant difference in the humoral response measured in the groups immunized with a rgD$_2$t Alum vaccine containing either 50µg 3D-MPL (Group VI) or 100mg 3D-MPL (Group X).

It is interesting to note that systemic anti-rgD$_2$t antibodies (IgG class) could be detected in the vaginal washings of all vaccinated groups. This mucosally located anti-rgD$_2$t antibody response may play an important protective role by decreasing the load of infectious virus in the genital tract during primary infection.

Effect of vaccination on HSV primary disease

Two weeks after the third immunization, guinea pigs were challenged intravaginally with HSV2. The effect of vaccination on the clinical and virological course of primary HSV2 infection is summarized in Table 6. As compared to the controls, animals vaccinated with a 5µg rgD$_2$t Alum 3D-MPL formulation containing either 50µg or 100µg 3D-MPL (Groups VI and X) showed significantly ($p<0.05$) reduced skin lesion severity as well as reduction of skin lesions incidence.

Very similar results were observed in the group vaccinated with 5µg rgD$_2$t in a 3D-MPL o/w emulsion (Group III). In the three vaccinated groups, very low viral replication could be detected in the vaginal swabs collected 5 days after the challenge.

Effect of vaccination on HSV recurrent disease

Results are given in Table 6. As compared to the control groups, the incidence of skin lesions and the recurrence day number were significantly ($p>0.05$) reduced in the three vaccinated groups. These groups had also fewer recurrent episodes than control groups.

5. Conclusions

Results obtained in guinea pigs clearly show that vaccination with a rgD$_2$t formulation containing 3D-MPL delivered in an oil in water emulsion or combined with aluminium hydroxyde is very effective in providing protection against primary and recurrent HSV2 disease when administered to guinea pigs prior to HSV2 inoculation. Such rgD$_2$t 3D-MPL formulations are able to improve specific humoral (neutralizing antibodies) and effector cell mediated (DTH) immune responses. These results are obtained using a low dose of rgD$_2$t (5µg).

6. Immunogenicity of gD2t formulations in primates

6.1 Comparative immunogenicity of rgD_2t/Alum and rgD_2t/Alum 3D-MPL form

The immunogenicity of rgD_2t/Alum and rgD_2t/Alum 3D-MPL vaccines were evaluated in cercopithecus aethiops (African Green Monkeys, AGM). Three immunizations were given at 0, 1 and 3 months. Specific humoral (ELISA and neutralizing titors) and effector cell mediated (DTH) immune responses were measured.

6.1.1. Experimental procedure

Each formulation contained 20mg rgD_2t and 0.5mg equivalents AL^{3+}/dose. A dose of 50µg 3D-MPL was used. Groups of cercopithecus aethiops (AGM) were immunized 3 times at days 0, 28 and 84. Immunizations were given intramuscularly in a 0.5ml dose (20 rgD_2t). Animals were bled every ± 2 weeks for antibody determination by ELISA and neutralization assays. The two formulations were also tested for their ability to induce T cell mediated immunity, as measured by the induction of delayed-type hypersensitivity (DTH) responses. Monkeys were given intradermally on the belly different rgD_2t doses (20, 5 and 1µg) in saline 13 days after the second immunization. They were also skin tested with saline alone as control. Erythema and induration at site of intradermal injection were monitored 24 hrs and 48 hrs later.

6.1.2. Results

a) Induction of humoral immunity

Before vaccination, none of the monkey sera showed any anti-HSV2 antibody activity (data not shown). As shown in table 7, both vaccines induced good ELISA and neutralizing titers after the second immunization. This antibody response was not boosted with a third immunization in the rgD_2t/Alum vaccinated monkeys. In contrast, monkeys receiving a third immunization with rgD_2t/Alum 3D-MPL produced increased ELISA and neutralizing antibody responses (mean ELISA titer: 10056; mean neutralizing titer: 950).

b) Induction of effector T cell response (DTH)

Skin test results (table 8) showed that rgD_2t combined with Alum plus 3D-MPL was very potent in inducing an *in vivo* effector T cell response, in contrast to the rgD_2t Alum formulation. A strong DTH response was observed in all rgD_2t Alum 3D-MPL vaccinated animals skin tested with 20mg rgD_2t. Specific DTH responses were also measured with the lower gD_2t concentrations (5 and 1µg) in the majority of the monkeys (3/4 for the 5µg dose and 2/4 for the 1µg dose). These rgD_2t doses induced weaker skin test responses than the 20mg rgD_2t concentration.

6.2. Immunogenicity of rgD_2t/Alum 3D-MPL formulations in rhesus monkeys

The immunogenicity of rgD_2t/Alum 3D-MPL vaccines containing different rgD_2t doses (100µg, 10µg, or 5µg) was compared in rhesus monkeys.

6.2.1. Experimental procedure

Each formulation contained 0.5µg equivalents Al^{3+} and 50µg 3D-MPL per dose. Three groups of rhesus monkeys (4 monkeys/group) were immunized three times at days 0, 28 and 77, as follows:

Group 1 : 100µg rgD_2t Alum plus 3D-MPL (50µg)

Group 2 : 20µg rgD_2t Alum plus 3D-MPL (50µg)

Group 3 : 5µg rgD_2t Alum plus 3D-MPL (50µg)

Immunizations were given intramuscularly in a 1 ml dose. Animals were bled every ± 2 weeks for antibody determination by ELISA and neutralization assays.

6.2.2. Induction of humoral immunity

Before vaccination, none of the monkey sera showed any anti-HSV2 antibody activity. Good ELISA and neutralizing titers were observed in the three vaccinated groups receiving either 100, 20 and 5mg gD_2t in

Alum + 3D-MPL. (Data not shown).

6.3. Conclusions

Results obtained in cercopithecus aethiops clearly indicate that a rgD$_2$t vaccine containing a combination of Alum with 3D-MPL significantly improve humoral (neutralizing antibodies) and effector cell mediated (DTH) specific immune responses. As compared to this vaccine, a rgD$_2$t Alum formulation is less potent in inducing neutralizing antibodies and is unable to induce an *in vivo* DTH response.

Results obtained in rhesus monkeys also show that a rgD$_2$t Alum + 3D-MPL formulation is very effective in inducing a specific humoral response, even with low doses of antigen (5µg or 20µg rgD$_2$t).

7. General Conclusions

Results obtained in guinea pigs clearly indicate that adjuvant formulations containing either 3D-MPL delivered in an oil in water emulsion or combined with aluminium hydroxide are very effective in inducing a protective immune response with a recombinant HSV glycoprotein vaccine in the intravaginal guinea pig challenge animal model, even with very low doses of antigen (5 µg rgD$_2$t). Protection data also show that these rgD$_2$t 3D-MPL formulations are more potent in providing protection. Such 3D-MPL formulations are able to improve specific humoral (neutralizing antibodies) and effector cell mediated (DTH) immune responses.

Furthermore, the rgD$_2$t Alum 3D-MPL formulation was shown to also improve immunogenicity at the antibody level and to induce an effector T cell response in primates, suggesting that this adjuvant effect is not restricted to small animal species.

TABLE 1 : Anti-HSV antibody response in sera of guinea pigs immunized with rgD_2t formulations before and after viral challenge.

Group	Vaccine (1)		Pre-challenge (2)		Post-challenge (3)	
	Antigen	Adjuvant	ELISA titer	Neutralizing titer	ELISA titer	Neutralizing titer
1	rgD_2t	3D-MPL o/w (R)	81291 ± 20822	1600	68720 ± 24648	2200 ± 765
2	rgD_2t	Alum 3D-MPL	39897 ± 30165	2000 ± 800	27224 ± 13093	1800 ± 765
3	rgD_2t	Alum	20346 ± 23704	600 ± 400	28622 ± 24024	1333 ± 461
4	-	Alum	< 100	< 50	737 ± 878	142 ± 85
5	-	3D-MPL	< 100	< 50	259 ± 244	1275 ± 1304
6	untreated	-	< 100	< 50	225 ± 194	119 ± 141

(1) rgD_2t dose = 5 µg. Animals were immunized three times at days 0, 28 and 95. They were challenged 2 weeks later with 10^5 pfu HSV2.

(2) Sera collected the day before challenge (= 14 days after the third immunization)

(3) Sera collected 2 weeks after challenge.

Values are given as arithmetic mean titers ± SD.

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TABLE 2 : Skin Test Results (DTH) in guinea pigs vaccinated with rgD$_2$t formulations.

Formulation	Guinea Pig #	24 hr reading		48 hr reading	
		E (mm)	E (mm)	E (mm)	I (mm)
rgD2^t 3D-MPL o/w (R)	1	20	15	14	10 (N)
	2	15	10	10	3
	3	20	17 (N)	15	12 (N)
rgD2^t Alum 3D-MPL	1	10	8	10	4
	2	15	12	12	3
	3	11	9	12	0
- Alum 3D-MPL	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
untreated	1	0	0	0	0
	2	0	0	0	0

Guinea pigs were immunized at days 0 and 21 with 5 µg rgD$_2$t formulation (given intramuscularly). They were given intradermally 5 µg rgD$_2$t in saline at day 29. Skin test was read at 24 h and 48 h.
E = erythema at site of ID injection in millimeters.
I = induration at site of ID injection in millimeters.
N = necrosis at skin test site.

TABLE 3 : Effect of immunization with rgD$_2$t formulations on the clinical and virological course of primary HSV2 infection in guinea pigs.

Group	Vaccine (1)		Incidence of Skin Lesions (2)	Skin Lesion Severity (3)	Vaginal Virus Titers (4)
	Antigen	Adjuvant			
1	rgD$_2$t	3D-MPL o/w (R)	0/4	0.1 ± 0.3	0
2	rgD$_2$t	Alum 3D-MPL	0/4	1 ± 0.4	6.25 ±12.4
3	rgD$_2$t	Alum	3/4	4.4 ± 2.7	3575 ± 6010
4	-	Alum	5/5	6.2 ± 2.6	5216 ± 6295
5	-	3D-MPL	4/5	5.1 ± 3.6	3298 ± 4475
6	untreated	-	7/8	7.3 ± 4.7	2214 ± 4519

(1) rgD$_2$t dose = 5 µg. Animals were immunized three times at days 0, 28 and 95. They were challenged 2 weeks later with 10^5 pfu HSV2.

(2) Number animals showing a lesion score ≥ 1 during the 12 days observation period

(3) Sum of the lesion scores (days 1 - 12), arithmetic mean ± SD

(4) Peak HSV titer (pfu/ml) in vaginal swabs collected 5 days post challenge.

TABLE 4 : Effect of immunization with rgD$_2$t formulations on the recurrent genital HSV2 disease in guinea pigs.

Group	Vaccine (1)		Incidence of Skin Lesions (2)	Episodes of recurrent disease (3)	Recurrence (4) days Numbers
	Antigen	Adjuvant			
1	rgD$_2$t	3D-MPL o/w (R)	1/4	1 ± 2	0.7 ± 1.5
2	rgD$_2$t	Alum 3D-MPL	2/4	1 ± 0.8	1.7 ± 3.5
3	rgD$_2$t	Alum	3/3	4.3 ± 1.5	8.3 ± 5
4	-	Alum	4/5	3.8 ± 3.3	7.6 ± 6.5
5	-	3D-MPL	5/5	2.6 ± 1.1	6 ± 4.4
6	untreated		6/8	3.5 ± 2.2	9.9 ± 6

(1) rgD$_2$t dose = 5 µg. Animals were immunized three times at days 0, 28 and 95. They were challenged 2 weeks later with 10^5 pfu HSV2.

(2) Number animals showing a lesion score ≥ 1 during the observation period (days 13 - 60)

(3) One recurrent episode is preceded and followed by a day without lesion and characterised by at least two days with erythema (score = 0.5) or one day with vesicle(s) (lesion score≥1). Results expressed as arithmetic mean ± SD (observation period: days 13-60).

(4) Total days animals experienced a recurrent herpetic episode, arithmetic mean ± SD (observation period: days 13-39).

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TABLE 5 : COMPARISON OF THE EFFECT OF DIFFERENT ADJUVANT FORMULATIONS ON THE IMMUNOGENICITY OF rgD2t IN GUINEA PIGS

GROUP	VACCINE (1)		Anti-HSV antibody response after two immunisations			Anti HSV antibody response after three immunizations - prechallenge titer (3)	
			IN SERA		IN VAGINAL (5)	IN SERA (4)	
	rgD2t dose	Adjuvant	Elisa titer	Neutral titer	WASHINGS	Elisa titer	Neutral titer
I	20 µg	3DMPL (50 µg) o/w (R)	31462 ± 9087	850 ± 396	0.780 ± 0.376	19958 ± 10171	3400 ± 1994
II	5 µg	3DMPL (50 µg) o/w (R)	35015 ± 14395	412 ± 264	1.000 ± 0.177	51688 ± 40120	4342 ± 2879
III	20 µg	3DMPL (50 µg) o/w (S)	16720 ± 12641	1380 ± 756	0.700 ± 0.232	36647 ± 24126	4080 ± 2883
IV	5 µg	3DMPL (50 µg) o/w (S)	14992 ± 9885	840 ± 571	0.570 ± 0.200	45082 ± 24221	4560 ± 2502
V	20 µg	Alum 3DMPL (50 µg)	14452 ± 7476	740 ± 499	0.620 ± 0.175	16015 ± 7846	3280 ± 2276
VI	5 µg	Alum 3DMPL (50 µg)	10174 ± 4219	420 ± 301	0.520 ± 0.175	20488 ± 9562	2640 ± 1510
VII	-	Alum 3DMPL (50 µg)	<100	<50	<0.020	<100	<50
VIII	-	3DMPL (50 µg) o/w (R)	<100	<50	<0.020	<100	<50
IX	-	untreated	<100	<50	<0.020	<100	<50
X	5 µg	Alum 3DMPL (100 µg)	4602 ± 3953	163 ± 151	0.671 ± 1.187	16588 ± 6945	2560 ± 1678

(1) Animals were immunized three times at days 0, 28 and 84. They were challenged 2 weeks later with 10^5 pfu HSV2.
(2) Sera and vaginal washings collected 14 days after the second immunization.
(3) Sera collected the day before challenge (= 14 days after the third immunization).
(4) Values are given as arithmetic mean titers ± SD
(5) Corresponds to the optical density (at 492 nm) per 0.5 µg/ml total IgG measured in the standard anti-gD2t ELISA assay, arithmetic mean ± SD.

TABLE 6 : EFFECT OF IMMUNIZATION WITH rgD2t FORMULATIONS ON THE CLINICAL AND VIROLOGICAL COURSE OF HSV2 INFECTION IN GUINEA PIGS

	VACCINE			
	5µg rgD2t 3DMPL o/w (s) Group III	5µg rgD2t Alum 3DMPL(50µg) Group VI	5µg rgD2t Alum 3DMPL(100µg Group X	CONTROLS Groups VII - VIII - IX
PRIMARY HSV2 INFECTION				
Incidence of skin lesions (%)	1/9 11%	1/10 10%	0/10 0%	12/14 86%
Skin lesion severity	1,2 ± 1	0.7 ± 0.7	0.9 ± 1	8.6 ± 5.1
Vaginal virus titers (pfu/ml)	0	0	1.5 ± 4.7	1077 ± 1682
RECURRENT HSV2 INFECTION				
Incidence of skin lesions (%)	2/9 22%	2/10 20%	3/10 30%	11/14 79%
Recurrence day number	1 ± 2.3	1.6 ± 2.1	1.6 ± 2.7	7.3 ± 6
Recurrence episode number	0.2 ± 0.4	0.6 ± 0.7	0.5 ± 0.8	1.9 ± 1.2

Experimental schedule : 3 immunizations at days 0, 28 and 84.

Challenge 2 weeks after the last immunization with 10^5 pfu HSV2.

Primary HSV2 infection : (observation period days 4 to 12 post challenge)
Incidence of skin lesions (%) : number of animals with vesicle(s) (lesion score ≥ 1)
Skin lesion severity : sum of the lesion scores (for the days 4 to 12), arithmetic mean ± SD
Vaginal virus titers : virus titers (pfu/ml) in vaginal swabs collected 5 days after the challenge

Recurrent HSV2 infection : (observation period days 13 to 39 post challenge)
Incidence of skin lesions (%) : number (%) of animals with vesicle(s) (lesion score ≥ 1)
Recurrence day number : total days animals experienced a recurrent herpetic disease, arithmetic mean ± SD. Animals were scored positive for recurrent disease either if a 0.5 lesion score (erythema) was recorded for 2 successive days at least or if a lesion score ≥ 1 (vesicle(s)) was observed at any day.
Recurrence episode number : arithmetic mean, ± SD

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TABLE 8

COMPARATIVE IMMUNOGENICITY OF GD2T ALUM AND GD2T ALUM 3D MPL FORMULATIONS IN AFRICAN GREEN MONKEYS: SEROLOGICAL RESPONSES

		Post II					Post III	
		14 days		28 days	56 days		14 days	
VACCINE*	MONKEY NB	ELISA TITER	NEUT TITER	ELISA TITER	ELISA TITER	NEUT TITER	ELISA TITER	NEUT TITER
gD2t ALUM	JO 358	1388	400	6572	1135	200	2050	400
	JO 359	4731	400	3232	1866	100	2110	200
	JO 363	1376	200	2316	1300	50	1205	50
	JO 364	5914	1600	5275	3740	400	6323	800
	JO 366	21104	400	3696	2550	200	2302	200
	Arit mean	***6902***	***600***	***4218***	***2118***	***190***	***2798***	***330***
	± SD	***± 8190***	***± 565***	***± 1697***	***± 1062***	***± 134***	***± 2015***	***± 290***
gD2t ALUM/ 3D MPL	JO 348	7120	200	10175	4490	200	11082	400
	JO 349	14437	1600	15409	7361	800	15848	1600
	JO 375	7990	800	5170	2953	800	6797	1600
	JO 515	6515	200	7246	3660	100	6497	200
	Arit mean	***9015***	***700***	***9500***	***4616***	***475***	***10056***	***950***
	± SD	***± 3664***	***± 663***	***± 4442***	***± 1934***	***± 377***	***± 4392***	***± 754***

* Each vaccine dose contains 20 µg gD2t
ELISA titer = midpoint titer
NEUT titer = reciprocal of the highest serum dilution giving 100% protection against the cytopathogen effect

Claims

1. A vaccine formulation comprising an HSV glycoprotein D or an immunological fragment thereof in conjunction with 3Deacylated monophosphoryl lipid A and a suitable carrier.

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Claims

1. A vaccine formulation comprising an HSV glycoprotein D or an immunological fragment thereof in conjunction with 3Deacylated monophosphoryl lipid A and a suitable carrier.

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2. A vaccine formulation as claimed in claim 1 wherein the carrier is alum.

3. A vaccine formulation as claimed in claim 2 wherein the carrier is an oil in water emulsion.

4. A vaccine formulation as claimed in any of claims 1 to 3 wherein the glycoprotein D is an HSV-2 glycoprotein D or immunological framgment thereof.

5. A vaccine formulation as claimed in claims 1 to 4 wherein the glycoprotein D is a truncated protein.

6. A vaccine formulation as claimed in claim 5 wherein the truncated protein is $HSVgD_2$ and is devoid of the C terminal anchor region.

7. A vaccine formulation as claimed in claims 1-6, 8 or 9 wherein the glycoprotein D is conjugated to a particulate carrier.

8. A vaccine formulation as claimed in claims 1-7 or 9 wherein 3Deacylated monophosphoryl lipid A is present is the range of 10μg - 100μg per dose.

9. A vaccine formulation as claimed in claims 1-8 for use in medicine.

10. Use of HSV glycoprotein gD or an immunological fragment thereof in conjunction with 3Deacylated monophosphoryl lipid A in the manufacture of a medicament for the prophylaxis or treatment of HSV infections.

11. A method of producing a vaccine according to any of claims 1 to 8 wherein the method comprises mixing HSV glycoprotein D or an immunological fragment thereof with a carrier and 3Deacylated monophosphoryl lipid A.

Patentansprüche

1. Impfstoff-Formulierung, umfassend ein HSV-Glycoprotein D oder ein immunologisches Fragment davon in Verbindung mit 3-deacyliertem Monophosphoryl-Lipid A und einem geeigneten Träger.

2. Impfstoff-Formulierung nach Anspruch 1, wobei der Träger Alaun ist.

3. Impfstoff-Formulierung nach Anspruch 2, wobei der Träger eine Öl-in-Wasser-Emulsion ist.

4. Impfstoff-Formulierung nach einem der Ansprüche 1 bis 3, wobei das Glycoprotein D ein Glycoprotein D von HSV-2 oder ein immunologisches Fragment davon ist.

5. Impfstoff-Formulierung nach den Ansprüchen 1 bis 4, wobei das Glycoprotein D ein verkürztes Protein ist.

6. Impfstoff-Formulierung nach Anspruch 5, wobei das verkürzte Protein $HSVgD_2$ ist und der C-terminale Ankerbereich fehlt.

7. Impfstoff-Formulierung nach den Ansprüchen 1 bis 6, 8 oder 9, wobei das Glycoprotein D an einen teilchenförmigen Träger konjugiert ist.

8. Impfstoff-Formulierung nach den Ansprüchen 1 bis 7 oder 9, wobei 3-deacyliertes Monophosphoryl-Lipid A im Bereich von 10 μg bis 100 μg pro Dosis vorhanden ist.

9. Impfstoff-Formulierung nach den Ansprüchen 1 bis 8 zur Verwendung als Medikament.

10. Verwendung von HSV-Glycoprotein gD oder einem immunologischen Fragment davon in Verbindung mit 3-deacyliertem Monophosphoryl-Lipid A für die Herstellung eines Medikamentes für die Vorbeugung gegen oder die Behandlung von HSV-Infektionen.

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11. Verfahren zur Herstellung eines Impfstoffes nach einem der Ansprüche 1 bis 8, wobei das Verfahren das Mischen von HSV-Glycoprotein D oder einem immunologischen Fragment davon mit einem Träger und 3-deacyliertem Monophosphoryl-Lipid A umfaßt.

Revendications

1. Formulation de vaccin comprenant une glycoprotéine D de HSV ou un fragment immunologique de celle-ci, en conjonction avec un monophosphoryl lipide A 3-déacylé, et un véhicule convenable.

2. Formulation de vaccin selon la revendication 1, dans laquelle le véhicule est l'alun.

3. Formulation de vaccin selon la revendication 2, dans laquelle le véhicule est une huile en émulsion aqueuse.

4. Formulation de vaccin selon l'une quelconque des revendications 1 à 3, dans laquelle la glycoprotéine D est une glycoprotéine D de HSV-2, ou un fragment immunologique de celle-ci.

5. Formulation de vaccin selon les revendications 1 à 4, dans laquelle la glycoprotéine D est une protéine tronquée.

6. Formulation de vaccin selon la revendication 5, dans laquelle la protéine tronquée est la gD_2 du HSV, et est dépourvue de la région d'ancrage du C terminal.

7. Formulation de vaccin selon les revendications 1 à 6, ou 8 ou 9, dans laquelle la glycoprotéine D est conjuguée à un véhicule particulier.

8. Formulation de vaccin selon les revendications 1 à 7, ou 9, dans laquelle le monophosphoryl lipide A 3-déacylé, est présent en quantité comprise entre 10 µg et 100 µg par dose.

9. Formulation de vaccin selon les revendications 1 à 8, pour l'utilisation en médecine.

10. Utilisation de la glycoprotéine gD du HSV, ou d'un fragment immunologique de celle-ci, en conjonction avec le monophosphoryl lipide A 3-déacylé, dans la fabrication d'un médicament pour la prophylaxie ou le traitement des infections par le HSV.

11. Méthode de production d'un vaccin selon l'une quelconque des revendications 1 à 8, laquelle méthode comprend le mélange de la glycoprotéine D du HSV ou d'un fragment immunologique de celle-ci, avec un véhicule, et avec le monophosphoryl lipide A 3-déacylé.

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