166166

G00001

ANTIGENES OF THE ENVELOPE TRANSMEMBRANCE GLYCOPROTEIN OF A HUMAN RETROVIRUS OF THE HIV-2 TYPE, AND ANTIGENS PRESENTING WITH THEM AN IMMUNOLOGICAL RELATIONSHIP

The invention relates to antigens of the envelope transmembrane glycoprotein of an HIV-2 type human retrovirus, antigens which are immunologically related to them and more precisely antigens present exclusively during certain phases of the in vivo development of an infection due to a human retrovirus HIV-2. These antigens are characteristic of the process of formation and of maturation of the envelope transmembrane glycoprotein of this retrovirus, and are consequently involved in the development and the proliferation of the HIV-2 retrovirus and, consequently, in the propagation of the infection.

The invention also relates to antibodies which recognize these antigens, as well as to immunological compositions containing these antibodies.

The invention relates, in addition, to the preparation of the preceding antigens, as well as to their use for the diagnosis of an infection due to a human retrovirus HIV-2.

Etiological agents responsible for the development of lymphadenopathy syndromes (abbreviated LAS) or which contribute to the development of these syndromes or are responsible for the development of acquired immunodeficiency syndromes (AIDS) have been isolated, identified and characterized.

HIV (abbreviation for Human Immunodeficiency Virus) designates several human retroviruses capable, under certain conditions, of causing LAS possibly followed by AIDS in man.

Thus, a first virus called LAV-1 or HIV-1 has been isolated and described in Patent Application EP.84/401,834 (published under the No. EP-A-0 138 66) of 14/09/84. This virus has also been described by F. Barre Sinoussi et al. (1).

Variants of this HIV-1 virus, designated LAV ELI and LAV MAL, have also been isolated, characterized and described in Patent Application EP.84/401,834.

The isolation and characterization of retroviruses belonging to a separate class and having only a limited immunological relationship with the preceding

ones have been described in European Patent Application No. 87/400,151,4, published under the No. 239,425. These retroviruses which have been grouped under the name HIV-2, were isolated from several African patients having symptoms of a lymphadenopathy or AIDS.

The study of these HIV retroviruses, the isolation and the analysis of their nucleotide sequences, as well as the characterization of their antigenic proteins, has made it possible to carry out comparative studies between the different strains obtained, in relation to their relationship or on the contrary their specificity, both structural and functional.

These HIV-1 and HIV-2 retroviruses have also been studied in comparison with a retrovirus of simian origin (designated by the abbreviation SIV or STLV-III) and this study has made it possible to show some relationship between the proteins and glycoproteins of the HIV-2 retrovirus and its variants and those of the SIV retrovirus.

A relationship is especially noted at the level of the envelope protein of each of these viruses. Immunological cross-reactions have indeed been demonstrated between the envelope glycoprotein of the SIV retrovirus and antibodies directed against the envelope glycoprotein of the HIV-2 retrovirus, whereas this immunological cross-reaction does not occur between the SIV retrovirus and the HIV-1 retrovirus or between the HIV-1 and HIV-2 retroviruses.

Results relating to the envelope glycoprotein of the HIV-2 retrovirus encoded by the <u>env</u> gene of the genome of this retrovirus have been given in the above-mentioned European Patent Application No. 87/400,151,4 (published under the No. EP-A-0,239,425). In this EP patent application, this glycoprotein was designated by the abbreviation gp140 with reference to its molecular weight of about 140,000 d. It was specified, however, that the calculation of the molecular weight was evaluated at ±10%. In a previous French Patent Application (No. 86/03881 published under the

No. 2,596,063) a precursor of gp140 designated by gp160 (molecular weight of 160Kd ± 10%) had also been described.

More extensive investigations relating to the envelope glycoprotein of the HIV-2 retrovirus have been carried out and have allowed the inventors to perform other measurements of the molecular weights given so far and consequently to improve the results, from the molecular weight intervals described above. The envelope external glycoprotein has, in the HIV-2 viral particles - under the operating conditions of the present invention - a MW of about 125,000d (it will be designated by gp125). Under these same operating conditions, the precursor already detected and designated by gp160 in Patent Application FR.86/03881 has, according to the last results obtained, a molecular weight of about 140,000d. It will therefore be referred to as "gp140" in the present patent application.

Up until now, the existence of several phases of development leading to the production of envelope glycoproteins in a mature form, these mature envelope glycoproteins comprising an envelope external glycoprotein, gp125, and an envelope transmembrane glycoprotein gp36 has been demonstrated, different stages in the course of the viral replication cycle appearing when pathogenic disorders develop following an infection by a human retrovirus HIV-2 have been identified in this regard.

First results relating to the existence and the characterization of a precursor of the HIV-2 envelope external glycoprotein gp300 have been obtained. This glycoprotein has been described in a publication (13). For the purposes of the description, reference will be made in the following text to this glycoprotein which will be designated by gp300. The inventors have now identified and characterized a new glycoprotein which is involved in the HIV-2 viral cycle.

In parallel, they have carried out similar studies on the viral cycles of the HIV-1 and SIV retroviruses. These last studies have allowed the identifi-

cation of very specific new properties common to the HIV-2 and SIV retroviruses and, on the contrary, apparently inexistent during the viral replication cycle of HIV-1.

The invention therefore relates to new means for detecting an infection due to a human retrovirus HIV-2 or to a related virus, as well as for characterizing the affiliation of a retrovirus to the human retrovirus HIV-2 type.

In the light of the results obtained, the viral cycle specific to the HIV-2 and SIV retroviruses appears as a complex process of chain reactions of which only the combination leads to the formation of the envelope glycoproteins of the HIV-2 retrovirus in their final form. During this process, it was possible for new antigenic proteins or glycoproteins to be recognized, isolated and identified.

The invention therefore relates to antigenic glycoproteins produced following an infection by a human retrovirus HIV-2, having a peptide backbone in common with the transmembrane glycoprotein gp36 and which are capable of being dissociated during the course of the viral cycle, into two gp36 transmembrane glycoproteins.

Such a glycoprotein, in dimerized form, would correspond to the optimum form and conformation and would allow the fusion of the viral membrane with the cell membrane.

The invention therefore relates to a glycoprotein characterized by the following properties:

- it is formed from an HIV or SIV retrovirus envelope transmembrane glycoprotein dimer,
- it is capable of forming an antigen-antibody type immunological complex with antibodies directed against an antigenic glycoprotein gp80 having a molecular weight close to 80 kDa, recognized by polyclonal antibodies directed against the glycoprotein gp300 of a human retrovirus HIV-2, and comprising in association in the form of a dimer two components of the envelope transmembrane glycoprotein gp36 encoded by the genome of an

HIV-2 type human retrovirus capable of causing LAS or AIDS in man,
- it has a molecular weight of the same order as that of the abovementioned HIV-2 gp80.

According to a variant embodiment, the invention relates to an antigenic glycoprotein gp80, characterized by the following properties:
- it has a molecular weight (MW) close to 80 kDa,
- it is recognized by polyclonal antibodies directed against the glycoprotein gp300 of a human retrovirus HIV-2,
- it comprises in association in the form of a dimer two components of the envelope transmembrane glycoprotein gp36 encoded by the genome of an HIV-2 type human retrovirus capable of causing LAS or AIDS in man.

The above glycoprotein gp300 is the glycoprotein described in (13); it has a molecular weight of about 300 KDa and consists of the association of two units of a gp140 glycoprotein precursor especially of the envelope glycoprotein gp125 encoded by the env gene of the genome of a human retrovirus HIV-2 capable of causing LAS or AIDS.

After having isolated and characterized it, the inventors demonstrated that the serum of patients positive for the HIV-2 antigens recognize the glycoprotein gp80 in cell extracts, or in viral extracts. gp80 is a mature product of the transmembrane glycoprotein, constituting a stage necessary for the formation of the infectious or even pathogenic viral particles. The inventors therefore determined that by blocking the normal viral cycle for forming the particles of the HIV-2 type, especially at the level of the formation or of the dissociation of gp80, it is possible to act on the propagation of the infection. Such a blocking of the infection would be carried out using castanospermine, which is capable of inhibiting the maturation of the glycoprotein gp80.

The subject of the invention is therefore a glycoprotein characterized by the following properties:

- it is formed from an HIV or SIV retrovirus envelope transmembrane glycoprotein dimer,
- it has a molecular weight of about 80 kDa,
- it is capable of forming an antigen-antibody type immunological complex with antibodies directed against an antigenic glycoprotein gp80 having a molecular weight close to 80 kDa, recognized by polyclonal antibodies directed against the glycoprotein gp300 of a human retrovirus HIV-2, and comprising in association in the form of a dimer two components of the envelope transmembrane glycoprotein gp36 encoded by the genome of an HIV-2 type human retrovirus capable of causing LAS or AIDS in man.

A glycoprotein gp80 preferred according to the invention is further characterized by the following properties:
- it may be associated with cells infected by an HIV-2 type human retrovirus or/and with virions or/and with HIV-2 type viruses,
- it is not dissociated <u>in vitro</u> in the presence of a nonionic detergent such as 1% Triton® X-100 and it is dissociated <u>in vitro</u> in order to form the transmembrane glycoprotein gp36 of an HIV-2 type retrovirus, in the presence of an ionic detergent such as 1% SDS at a weakly acidic pH,
- it may be purified on an immunoaffinity column HIV-2 serum Sepharose® and it appears (during analysis by polyacrylamide gel electrophoresis), 3 to 4 hours after the beginning of the labelling of the infected cells.

Also entering within the framework of the invention are proteins derived from the glycoprotein gp80 defined above and which are characterized in that they correspond to the non-glycosylated peptide backbone of this glycoprotein gp80.

This protein, lacking the gp80 glycosylation groups is valuable within the framework of the invention for the properties which it has in common with gp80.

Parallel studies of the viral cycle of the simian retrovirus SIV and of the retrovirus HIV-1 have shown

that the SIV cycle comprises a stage of dimerization in the form of a glycoprotein gp65 of a molecular weight of about 65 kDa, which can lead to the formation of two envelope transmembrane glycoproteins gp32.

On the other hand, this process of dimerization of the envelope transmembrane glycoprotein was not observed in biological samples infected by HIV-1.

The production of a dimerized form of the envelope transmembrane glycoprotein could therefore be associated with a specific infection by a retrovirus of the HIV-2 type or a retrovirus which is immunologically related to the latter. This glycoprotein therefore constitutes a particularly suitable means of detecting a specific infection due to the HIV-2 retrovirus or a related retrovirus.

The invention also relates to polyclonal or monoclonal antibodies which recognize specifically, on the one hand, gp300, gp140, gp125 and gp80, which are present in cells infected by an HIV-2 type retrovirus, and on the other hand, gp125, gp80 and gp36 which are present in extracts of an HIV-2 type retrovirus and which do not recognize the proteins or glycoproteins of healthy cells or of cells infected by an HIV-1 type retrovirus.

Also entering within the framework of the invention are monoclonal antibodies, characterized in that they react specifically with the glycoprotein gp80 and in that they do not react with the proteins of cells infected by an HIV-1 type retrovirus or with viral aggregates of HIV-1.

Another monoclonal antibody according to the invention is characterized in that it recognizes, in addition, the HIV-2 glycoproteins gp300, gp140 and gp36.

In a particularly advantageous manner, a third monoclonal antibody conforming to the preceding definitions is characterized in that it recognizes an epitope common to the glycoprotein gp80, and to the following amino acid sequence of the peptide p39':

VTAIEKYLQDQARLNSWGCAFRQVCH

The invention also relates to antibodies directed

against the protein derived from gp80 and lacking glycosylated groups.

The above monoclonal antibodies may have immunogenic and neutralizing properties, and by acting on the process for the formation of gp80, they may interrupt the proliferation of the HIV-2 retrovirus.

Several methods for the production of the preceding antibodies may be suitable within the framework for carrying out the invention. For example, cultures in ascites in animals, especially in rodents such as murines, can be used. It will also be possible to produce the antibodies from cell cultures which are either immobilized or encapsulated or alternatively in suspension. The technique using the hybridomas obtained from the fusion of lymphocytes of an animal into which the HIV-2 retrovirus will previously have been inoculated will be employed in particular. Lymphocytes of the infected animal are then collected and fused with chosen myelomatous cells, in order to form hybrids, which are then selected for their capacity to produce specific antibodies directed against the antigenic proteins and glycoproteins described above.

A preferred process of preparation of the monoclonal antibodies of the invention comprises the following stages:

- immunization of BALB-C type mice if necessary several times by intraperitoneal injection with the purified glycoprotein gp80,
- fusion of cells of immunized mice, especially spleen cells with myelomatous cells, for example NS1 type cells, according to the Kohler and Milstein method (10),
- selection and recovery of the hybridomas producing the desired monoclonal antibodies.

The screening of the hybridomas in order to select those of them which lead to the production of the desired monoclonal antibodies may be carried out using RIPA tests or Western blot analyses.

Monoclonal antibodies specifically suitable for carrying out the invention will be obtained by one of the

methods described above and it will be possible to test their properties towards gp80 or an analogous protein corresponding to the above definitions in comparison with the results described with the monoclonal antibody 1H8 (Mab 1H8).

The invention also relates to hybridomas producing the monoclonal antibodies described above.

These monoclonal antibodies which are produced will, for example, be used to inactivate the proteins and/or glycoproteins with which they form an immunological complex or, alternatively, for their capacity to prevent the normal course of formation of the final glycoproteins of the HIV-2 envelope.

Another possible application of these antibodies would for example consist in the detection of viral antigens in biological preparations or alternatively in the purification of proteins and/or glycoproteins for example on an affinity column.

Having demonstrated the existence of gp80 and after having isolated and characterized it, the inventors developed means for carrying out the diagnosis of an infection due to a human retrovirus of the HIV-2 type.

To this end, the invention relates to an antigenic composition for the specific in vitro diagnosis of an infection due to an HIV-2 type human retrovirus, in a biological sample capable of containing antibodies formed after the said infection, characterized in that it comprises at least the glycoprotein gp80 defined in the preceding pages.

The biological sample may be a biological fluid, especially blood or serum or alternatively a biological tissue extract. The above compositions can be used for the detection of antibodies in an abovementioned biological sample.

The invention therefore also relates to a process for the in vitro diagnosis of an infection due specifically to an HIV-2 type retrovirus, characterized by the steps of:

- bringing a biological sample capable of containing

antibodies produced following an infection by an HIV-2 type retrovirus into contact with a glycoprotein gp80 corresponding to the above definitions or with an antigenic composition described above, under appropriate conditions allowing the formation of an antigen-antibody type immunological complex, and

- detecting the antigen-antibody complex which may be formed.

The invention relates, moreover, to an immunogenic composition characterized in that it comprises at least the glycoprotein gp80 in association with a pharmaceutically acceptable vehicle for the preparation of vaccines.

These compositions can be used for the manufacture of vaccines against an HIV-2 type retrovirus.

An immunogenic composition thus formed has a dose of antigenic proteins and/or glycoproteins so as to allow the administration of a dose of 10 to 100 μg per kilogram.

The invention also relates to a process for the preparation of gp80, characterized by the following steps:

- lysis of cells infected with a human retrovirus HIV-2 and separation of the supernatant and of the infected cells or lysis of the viral pellets prepared by centrifugation,
- incubation of the cellular extract and/or of the viral extract on an immunoaffinity column on which is deposited an immunoadsorbent containing purified antibodies as obtained from a serum from a patient infected with the human retrovirus HIV-2, this serum having the capacity to react strongly with the HIV-2 envelope proteins, the said antibodies being advantageously attached to a suitable support, in the presence of a buffer and for a sufficiently long period to obtain the formation of an antigen-antibody immunological complex,
- washing of the column with a buffer to remove the molecules not retained on the support, and
- recovery of the desired antigenic proteins.

According to a first embodiment of this preparation process, the recovery of the glycoproteins is made by electrophoresis and by electroelution of the proteins.

The infected cells would have been obtained in vitro from a culture of HIV-2-infected cells, in particular using HIV-2-infected T4 lymphocytes. Such cell cultures can be achieved on CEM lines.

According to another embodiment of this preparation process, the recovery of the glycoproteins is obtained by:

- elution of the proteins attached to the above immunoaffinity column, and
- purification of the products thus eluted on a chromatographic column containing, attached to the separation support, monoclonal antibodies which recognize gp80.

In a specific embodiment of the invention, the lysis of the infected cells is performed in a detergent solution, the antibodies included in the immunoadsorbent are attached to agarose beads and the procedure is carried out in the presence of an attachment buffer.

Also entering within the framework of the invention is a set or kit for the detection of antibodies in the serum or in another biological sample from a patient who may be infected with a human HIV-2 retrovirus, characterized in that it comprises:

- at least one antigenic glycoprotein gp80 or a composition,
- or alternatively a mixture of these different constituents and
- means permitting the reaction for the formation of the immunological complex between the antigens and the antibodies which may be present in the biological sample to be tested, especially, if required, one or more incubation buffers,
- a negative control sample, and
- means for visualizing the antigen-antibody complex formed.

The invention also relates to the glycoproteins

gp80 and gp65 labelled preferably with radioactive, enzymatic, fluorescent or chemiluminescent markers or choromophores.

The invention also provides a method for the detection of cells by the HIV-2 virus. This method comprises the treatment of a biological sample comprising cells capable of being infected by HIV-2, so as to expose the intracellular proteins, and the test for detecting the intracellular proteins exposed in order to investigate the presence of gp80 glycoproteins of HIV-2. The detection of the exposed intracellular proteins may be made for example by electrophoresis or by an immunological test with antibodies immunologically reactive with HIV-2 gp80. Such antibodies have for example been described in the preceding pages.

According to a specific embodiment of the diagnosis for the presence of gp80 antigen, the biological sample is a sample lacking cell debris.

The invention also provides the means for detecting a specific HIV-2 infection and for distinguishing an infection due to HIV-1, from dells capable of containing HIV-2 antigens. This method comprises bringing a biological sample containing intracellular proteins obtained from treated cells of a biological sample which it is desired to test, into contact with antibodies directed against gp80. A possible reaction of the antigen-antibody type is then investigated in order to detect the existence of an HIV-2 infection.

Other characteristics and advantages of the invention will emerge from the examples and the figures.

LEGEND TO THE FIGURES

Figure 1: Identification of a specific protein of 80 kDa in HIV-2-infected cells by Western blot analysis using a serum positive for HIV-1 and 3 sera positive for HIV-2 (A, B and C). Cell extracts of uninfected CEM cells (corresponding to the products of 10^6 cells) (lines 2), of cells infected with HIV-1 (lines 1) and of cells infected with HIV-2 (lines 3) were analysed by electrophoresis on a 7.5% polyacrylamide gel before the Western blot test. An autoradiograph is presented in the figure. On the left, the arrows indicate the position of the HIV-1 extracellular glycoprotein (gp120) and precursors of gag p55 and p40. On the right, the position of the glycoproteins specific for HIV-2, gp300, gp140 and gp80 is noted.

Figure 2: Synthesis of the glycoproteins linked to HIV-2. The cells infected with HIV-2 were labelled with [^{3}H]glucosamine (200 µCi/ml; 4×10^6 cells/ml) for 2, 3, 4, 6 and 8 hours. At different points, extracts (products of 10^7 cells) were prepared from infected cells and from viral aggregates (synonymous with pellets) (prepared by centrifugation of the culture medium at 100,000 g). Aliquots of these extracts (corresponding to 2×10^6 cells) were purified on HIV-2 serum-Sepharose® and the labelled proteins were analysed by polyacrylamide (12.5%) gel electrophoresis. A fluorograph is presented. On the left, the position of the molecular weight marker proteins is given: myosin: 200,000, phosphorylase B: 97,000, bovine serum albumin: 68,000, ovalbumin: 43,000, and carbonic anhydrase: 30,000.

Figure 3: Western blot analysis with polyclonal antibodies directed against gp300. On the left, extracts of CEM cells not infected (-) and infected with HIV-1 or HIV-2 are presented. On the right, extracts of CEM cells infected with HIV-2: cell extracts (line C) and viral aggregate (viral pellet) (line V). The samples were

analysed by polyacrylamide gel electrophoresis (7.5% gel on the left; 12.5% gel on the right) before the Western blot analysis using murine polyclonal antibodies directed against the purified protein gp300 (anti-gp300). The autoradiogram is presented, each sample corresponds to the product of 10^6 cells.

Figure 4: Western blot analysis using a monoclonal antibody mAb 1H8 is presented. The extracts of cells infected with HIV-1 or HIV-2 (lines C) and the extracts of viral aggregates (lines V) were analysed by electrophoresis on a 12.5% polyacrylamide gel before the Western blot test using the antibody mAb 1H8. The autoradiograph is presented, the monoclonal antibody was directed against the envelope transmembrane glycoprotein of $HIV\text{-}2_{ROD}$.

Figure 5: The peptide p39' blocks the binding between the antibody mAb 1H8 and gp80. Extracts of viral aggregates of HIV-2 were analysed by Western blotting using the antibody 1H8 (mAb section) or anti-gp300 polyclonal antibodies (section S). The incubation with each antibody was carried out in the absence (lines -) or in the presence (lines +) of 1 µg/ml of peptide p39'. The results of the autoradiography are presented.

Figure 6: Pulse chase experiments (labelling, monitoring of the marker) showing the production of gp80 in HIV-2-infected cells are presented. The HIV-2-infected CEM cells were labelled with [^{35}S]methionine (200 µCi/ml; 4×10^6 cells/ml) for 30 minutes (lines 0). The radioactive labelling was monitored in the culture medium containing 5 mM of cold methionine for 2 and 4 hours (lines 2 and 4). The culture medium was centrifuged at 100,000 g after 4 hours and the aggregates were extracted. All the samples were immunoprecipitated using anti-gp300 antibodies or mAb 1H8 antibodies. The labelled proteins of the preparations of the immune complex were eluted in electrophoresis buffer and analysed by electrophoresis on

a 7.5% polyacrylamide gel. A fluorograph is presented, in which C and V correspond to the cell and viral extracts respectively. Each sample represents the product of 10^6 cells.

Figure 7:

a) Incorporation of labelled glucosamine and of fucose into gp80. The HIV-2-infected CEM cells labelled with [^{3}H]glucosamine (200 µCi/ml) or with [^{3}H]fucose (200 µCi/ml) were tested by immunoprecipitation using anti-gp300 polyclonal antibodies (lines S) or monoclonal antibodies mAb 1H8 (lines M). All these samples were analysed by electrophoresis on a 12.5% polyacrylamide gel. The fluorograph is presented.

b) Effect of castanospermine on the production of gp125 and gp80. HIV-2-infected cells were labelled (16 hours) with [^{35}S]methionine (200 µCi/ml; 4×10^6 cells/ml) in the absence (lines -) or in the presence (lines +) of castanospermine (1 mM). The extracts of the culture medium containing the viral particles were purified on an immunoaffinity column using HIV-2 serum-Sepharose® and the purified proteins were tested by electrophoresis on a 12.5% polyacrylamide gel. The corresponding fluorograph is presented.

Figure 8: Dissociation of gp80. Section C: extracts of HIV-2-infected CEM cells labelled with [^{35}S]methionine, tested by immunoprecipitation using a monoclonal antibody mAb 1H8 (line 1). Another aliquot of the same preparation of cell extracts was first heated (95°C, 5 minutes) in the presence of 1% SDS. Next, it was diluted 10-fold in an RIPA buffer before the immunoprecipitation test (line 2). The preparations of immune complex were analysed by electrophoresis. Each sample corresponds to the extract of 10^6 cells. Section V: HIV virus aggregates obtained from cells labelled with [^{35}S]methionine (each corresponding to the product of 10^7 cells) were suspended in various buffers: (1) lysis buffer containing Triton (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% (V/V)

Triton® X-100 and 100 units/ml of aprotinin). (2) lysis buffer containing SDS (then heated as in (1) but containing 1% (v/v) SDS in place of Triton® X-100); (3) lysis buffer containing SDS and then heated at 95° for 5 minutes: (4) RIPA buffer (the same as in (1) also modified with 0.1% (v/v) SDS and 0.2% (v/v) deoxycholate). All the samples were then immunoprecipitated using an antibody mAb 1H8 and the labelled proteins were analysed by electrophoresis on a 12.5% polyacrylamide gel. The fluorograph is presented.

Figure 9: Dissociation of purified gp80 into gp36. HIV-2-infected CEM cells were labelled (17 hours) with [^{35}S]methionine and the viral aggregates were suspended in a lysis buffer containing Triton®. These viral extracts (products corresponding to 2×10^7 cells) were immunoprecipitated using antibodies mAb 1H8 and the gp80 was purified by preparative gel electrophoresis. Aliquots equal to the purified preparation of gp80 were freeze-dried and resuspended in 100 mM acetate at pH 6.8, 5.8 and 4.8 containing 1% (v/v) SDS, 100 units/ml of aprotinin and 5 mM EGTA (to inhibit calcium-dependent proteolysis). All the samples were incubated at 30°C for 60 minutes before dilution in a two-fold concentrated electrophoresis buffer. The samples were analysed by polyacrylamide gel electrophoresis (12.5%) and a fluorograph is presented.

Figure 10: The SIV transmembrane glycoprotein exists in dimer form. Cell section: SIV-mac-infected HUT-78 cells and HIV-2-infected CEM cells were labelled for 16 hours with [^{3}H]glucosamine (200 µCi/ml: 4×10^6 cells/ml). The extracts (prepared in a lysis buffer containing Triton®) of the infected cells (lines C) and of the viral aggregates (lines V) were purified by immunoprecipitation using mAb 1H8 and the labelled proteins were analysed by electrophoresis on a 12.5% polyacrylamide gel. The fluorograph is presented.

EXAMPLES

MATERIALS AND METHODS

MATERIALS

L-[^{35}S]Methionine (specific activity >1000 µCi/mmol), L-[6-^{3}H]fucose (specific activity: 45-70 µCi/mmol), D-[6-^{3}H]gluocsamine (specific activity: 20-40 µCi/mmol) were provided by Amersham (Amersham, UK). Castanospermine and tunicamycin were provided by Boehringer-Mannheim (Mannheim, Germany) poly(A).poly(U) is obtained from the Curie Institute in Paris and was prepared according to the method described by Hovanessian et al. -1982- (Journal Interferon Research vol.2 - p.209 to 216).

VIRUSES AND CELLS

The HIV-1_{BRU} isolate of the type I human immunodeficiency virus (12), the HIV-2_{ROD} isolate of the type 2 human immunodeficiency virus (4,5) and the Simian immunodeficiency virus, SIVmac$_{142}$ (6) were used for these examples.

The different cell lines and human lymphocytes were cultured in a suspension medium RPMI-1640 (GIBCO-BRL, Cergy-Pontoise France) containing 10% (v/v) of foetal calf serum; 2 µg/ml of polybrene (Sigma) were added for the culture of HIV-infected cells. The clone 13 CEM cells are derived from the human lymphoid line CEM (ATCC-CCL119) and express the T4 antigen at a high level. Five days after the infection with the HIV-1_{BRU} or HIV-2_{ROD} isolates, about 80-90% of the cells produce viral particles and can be identified by the cytopathogenic effect corresponding to the vacuolization of the cells and by the appearance of small syncytia. The cell line HUT-78 is another human lymphoid cell line positive for the T4 receptor (Gazdal et al. 1980) which is highly permissive for the replication of SIVmac$_{142}$ (Daniel et al. 1985). The peripheral blood lymphocytes of healthy donors were stimulated for 3 days with 0.2% (w/v) of fraction P of phytohaemagglutinin (Difco, Detroit, USA) in RPMI-1640

medium supplemented with 10% foetal calf. The cells were placed in culture in an RPMI-1640 medium containing 10% (v/v) T cell growth factor (TCFG, Biotest). After infection with HIV-2, the lymphocytes were placed in culture in the presence of 10% (v/v) TCGF and 2 µg/ml of Polybrene).

METABOLIC LABELLING OF THE CELLS

For the metabolic labelling of the proteins, the infected cells were incubated for 16 hours at 37°C in an MEM (abbreviation for "Minimal Essential Medium") minimum culture medium free of L-methionine and serum, the medium being however supplemented with 200 µCi/ml [^{35}S]methionine. For the metabolic labelling of the glycoproteins, the infected cells were incubated for 16 hours at 37°C in an MEM culture medium lacking serum and glucose but supplemented with 200 µCi/ml ^{3}H-fucose or 200 µCi/ml ^{3}H-glucosamine.

CELLULAR AND VIRAL EXTRACTS

The cellular extracts corresponding to 10^7 cells were resuspended in 100 µl of buffer: 10 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 100 units/ml of aprotinin (Iniprol®, Choay) before addition of 100 µl of the same buffer containing 2% (v/v) of Triton® X-100.

The cellular extracts were centrifuged at 12,000 g for 10 minutes, and the supernatant was stored at -80°C up to the time of use. For the preparation of the viral extracts, 100 µl of 10X lysis buffer (100 mM Tris-HCl pH 7.6, 1.5 M NaCl, 10 mM EDTA, 10% (v/v) Triton X-100, 100 units/ml of aprotinin) were added per ml of clarified supernatant of the CEM cells infected and treated as above. For the preparation of extracts from viral aggregates, a culture medium of infected cells was first centrifuged at 12,000 g for 10 minutes before a high-speed centrifugation at 100,000 g at 15 minutes (Beckman® TL100 centrifuge). The virus aggregates (product obtained from 10^7 cells) were then solubilized in 200 µl of lysis buffer.

PREPARATION OF AN IMMUNOADSORBENT WITH ANTIBODIES OBTAINED FROM A PATIENT SEROPOSITIVE FOR HIV-2

The serum immunoglobulins from a patient seropositive for HIV-2 were precipitated with 50% $(NH_4)_2SO_4$, dissolved in 20 mM sodium phosphate (pH 8.0) and then purified on a DEAE cellulose column (DE52, Whatman®) by elution with 20 mM sodium phosphate (pH 8.0). The immunoglobulins thus purified were judged to be pure at 90%. The antibodies were then coupled to a Sepharose® CL 4B column activated with CNBr in accordance with the technique described by Berg (2). Two milligrams of IgG were coupled per ml of Sepharose® CL 4B. In the remainder of the text, this immunoadsorbent will be designated "HIV-2 serum-Sepharose".

PREPARATION OF THE HIV-2 PROTEINS ON THE IMMUNOAFFINITY COLUMN

The cellular extracts of HIV-2-producing CEM cells were first diluted in two volumes of binding buffer (20 mM Tris-HCl pH 7.6, 50 mM KCl, 150 mM NaCl, 1 mM EDTA, 20% (v/v) of glycerol, 7 mM β-mercaptoethanol, 0.2 mM PMSF, 100 units/ml of aprotinin) before incubation with one volume of HIV-2 serum/sepharose. The extracellular virus was treated like the supernatants of the HIV-2 producing cells with 1/10th of the 10-fold concentrated binding buffer. The binding procedure was carried out overnight and then the column was sufficiently washed in the binding buffer. The proteins bound to the column were eluted by boiling in an electrophoresis buffer (125 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 2M urea, 20% of glycerol, 0.5% of β-mercaptoethanol). The eluted proteins were recovered by electrophoresis on 7.5% SDS-polyacrylamide gels containing 6M urea and 0.1% of bisacrylamide in place of 0.2% (w/v).

PREPARATIVE ELECTROPHORESIS

The recovery of the HIV-2 glycoproteins (gp300 or gp80) eluted from the affinity column was made by polyacrylamide gel electrophoresis as described above and the regions of the gel containing the viral glycoproteins were cut out with reference to the position of the pre-

established molecular weight marker proteins (BRL).

The glycoprotein gp300 was eluted by incubating for 16 hours at 4°C in an elution buffer (0.1M $NaHCO_3$, 0.5 mM EDTA, 0.05% (w/v) SDS, 0.2 mM PMSF). The glycoprotein fractions thus obtained were freeze-dried and stored in the refrigerator up to the time of use. gp80 was electroeluted in a buffer containing 4 mM Tris-HCl, pH 7.6, 2 mM sodium acetate and 2 mM EDTA

PREPARATION OF POLYCLONAL MURINE ANTIBODIES DIRECTED AGAINST GP300.

The HIV-2 envelope glycoprotein gp300 was purified from extracts of infected CEM cells (3×10^8 cells) by immunoaffinity chromatography on HIV-2 serum Sepharose® followed by preparative gel electrophoresis (13). The purified preparation of gp300 was dissolved in 10 ml of 150 mM NaCl containing 0.5M urea and 1 mg/ml of mouse serum proteins and then dialysed for 24 hours in contact with a solution containing 150 mM NaCl and 0.5M urea. The dialysis product was then centrifuged and 2 ml aliquots were stored at -80°C. Five mice (8 weeks old) received an intraperitoneal injection, 5 times at an interval of 12 days with 350 µl of preparation of gp300 (about 1 µg of gp300). A poly(A).poly(U) adjuvant (200 µg; 1 mg/ml in 150 mM NaCl) was administered intravenously during each immunization (17). Five days after the last injection, the mice received, by intraperitoneal injection, a suspension of 10^6 sarcoma cells 180/TG in order to prepare a hyperimmune ascitic fluid (8). Eight days after the injection, the mice were sacrificed and the ascitic fluids were collected. The cells of the ascites were removed by centrifugation (200 g, 5 minutes) and the peritoneal fluid was collected.

PREPARATION OF MONOCLONAL ANTIBODIES, mAb 1H8

The HIV-2 virions were cultured in CEM cells and purified from concentrated culture supernatants by forming bands, in sucrose gradients. The purified virus was dislodged solubilized) in 0.5% Triton®-100, 150 mM NaCl, 50 mM Tris, pH 8.0, 1% of aprotinin (Sigma®) and clarified by ultracentrifugation. The viral extract was

then passed over a Lentil-Lectin Sepharose® 4B affinity column (Pharmacia®), the column was washed and the attached glycoproteins were eluted with 0.5M methyl α-D-mannopyranoside (Sigma®) and dialysed overnight in contact with phosphate-buffered saline.

BALB/c mice were immunized by intraperitoneal injection with 0.3 ml of purified glycoproteins 2-5 µg, re-attached to Lentil-Lectin Sepharose® 4B (50-100 µl). The mice received a booster every 4-6 weeks for 24 hours with the same immunogen. These mice were monitored in order to detect the production of antibodies, qualitatively by an RIPA test on extracts of HIV-2 virions labelled with [^{35}S]methionine and quantitatively with the EIA test on the dislodged (solubilized) virions (Genetic System®).

Three days after the last injection, the spleen cells were fused with NS1 myeloma cells in accordance with the Kohler and Milstein method (10). 96-well fusion plates were screened in order to detect the presence of hybridomas secreting anti-HIV-2 antibodies using the EIA test on dislodged (solubilized) virions from Genetic System. Methods for the propagation and stabilization of the cloned hybridomas and for the production of ascites have already been described (7). The hybridoma culture supernatants were screened by RIPA and Western blot analyses. A monoclonal antibody (mAb) 1H8, which reacts with the glycoprotein, was then characterized with the amino acid sequence 579-604 of the corresponding HIV-2 transmembrane glycoprotein using an EIA test based on a synthetic peptide (16). The amino acid sequence 579-604 is highly conserved among all the HIV-2 and SIV isolates sequenced, the monoclonal antibody 1H8 produces a cross-reaction with all the HIV-2 and SIV isolates thus tested. The synthetic peptide p39' used was synthesized based on the amino acid sequence 579-604 deduced from the nucleotide sequence of the HIV-2_{ROD} envelope. The amino acid sequence of the synthetic peptide p39' is the following:

VTAIEKYLQDQARLNSWGCAFRQVCH

The cellular or viral extracts (20 µl) (product corresponding to 10^6 infected cells) were first diluted in two volumes of RIPA buffer [(10 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton® X-100 (v/v), 0.2% sodium deoxycholate (w/v), 0.1% SDS (w/v), 7 mM 2β-mercaptoethanol, 0.2 mM PMSF, 100 µ/ml of aprotinime (Iniprol®, Choay) and 20% of glycerol (v/v)]. The dilute extracts were then incubated (45 minutes, 4°C) with polyclonal or monoclonal antibodies (25 µl). Protein A-Sepharose® was then added and the samples were incubated for 3 hours at 4°C. These samples were then washed in an RIPA buffer. The proteins recovered by immunoprecipitation were eluted by heating (95°C, 5 minutes) in an electrophorèsis buffer [125 mM Tris-HCl, pH 6.8, 1% SDS (w/v), 20% glycerol (v/v), 0.5% 2 β-mercaptoethanol]. The eluted proteins were recovered by electrophoresis on 7.5 or 12.5% SDS-polyacrylamide gels containing 0.1% bis-acrylamide instead of 0.2% (w/v).

IMMUNOBLOT ANALYSIS AFTER ELECTROPHORETIC TRANSFER: WESTERN BLOT

The proteins were subjected to analysis by polyacrylamide gel electrophoresis before being electrophoretically transferred onto 0.45 µm nitrocellulose sheets (Schleicher and Schüll, Dassel, Germany) in an electrode buffer (20 mM Tris base, 150 mM glycine, 20% methanol, v/v) as described by (3). The electrophoresis blots were saturated with 5% (w/v) of skimmed powdered milk in PBS (9). They were then incubated in a sealed bag (overnight at 4°C) or with sera positive for HIV-1 or with sera positive for HIV-2 (at a dilution of 1:100) or with polyclonal or monoclonal mouse antibodies (at a dilution of 1:200) in PBS containing 10% FCS. The sheets were then washed in PBS, the PBS containing 5% of Nonidet P-40 and then resaturated with PBS containing skimmed milk (5%). The washed sheets were then incubated (2 hours at room temperature) in a sealed bag either with a preparation of protein A labelled with ^{125}I (Amersham®, >30 mCi/mg) in order to reveal the human polyclonal antibodies in the HIV-1 or HIV-2 sera or with a pre-

paration of anti-mouse goat immunoglobulins labelled with ^{125}I (Amersham®, 2-10 μCi/μg). The sheets were removed from the bags and again washed, dried and then autoradiographed (Kodak® RP Royal, X-Ray Films) for 24-48 hours.

RESULTS

DETECTION OF THE GLYCOPROTEIN OF 80 kDa IN CELLS INFECTED WITH HIV-2 AND IN THE VIRION

It has already been shown (13) that the precursor of the HIV-2 envelope glycoproteins is a 140-kDa protein (gp140) which requires the formation of a homologous dimer during its conversion into mature products, namely the extracellular (gp125) and transmembrane (gp36) glycoproteins. It has now been demonstrated that the transmembrane glycoprotein exists in the form of a homodimer having an electrophoretic mobility on polyacrylamide gels at a position corresponding to an 80-kDa protein (Figures 1 to 6). This protein is glycosylated and for convenience it will be designated by the expression gp80.

The crude extracts of CEM cells infected with HIV-1_{BRU} or HIV-2_{ROD} or alternatively of non-infected cells of this type were analysed by the immunoblot test after electrophoretic transfer (Western blot) using a serum positive for HIV-1 and 3 different sera positive for HIV-2 recovered from patients suffering from AIDS (Figure 1). The sera specific for HIV-2 identified the envelope precursors (gp140 and gp300) and, in addition, strongly recognized the 80 kDa glycoprotein (gp80). These sera were specific for the HIV-2 proteins since they did not recognize the following HIV-1 proteins which were detectable using a serum specific for HIV-1: the precursor of the envelope glycoprotein gp120 of HIV-1 and the precursors of the gag protein (p55 and p40 for HIV-1). The association of gp80 with the infection due to HIV-2 was demonstrated by several results in which gp80 was not identified in HIV-1-positive sera or in cells infected by HIV-1.

Western blot analysis of the virus pellets prepared by centrifugation (100,000 g for 30 minutes) of an infected culture medium showed that gp80 could also be detected in particles of HIV-2 at the same time as the extracellular glycoprotein gp125.

SYNTHESIS OF gp80 IN CELLS INFECTED WITH HIV-2

Preliminary experiments showed that all HIV-2-positive sera could immunoprecipitate gp80 as well as the envelope precursors, gp140 and gp300 as well as the extracellular glycoprotein gp125. To characterize the synthesis of gp80, an HIV-2-positive serum which reacts strongly with the envelope proteins was used. Antibodies purified from this serum were coupled to a sepharose® column activated with CNBr (HIV-2 serum-Sepharose), used as immunoaffinity column to purify the envelope glycoproteins. The cells infected with HIV-2 were labelled with [^{3}H]gluocosamine and at different times (2,3,4,6 and 8 hours) extracts were prepared from infected cells and from viral pellets. All the samples were purified on HIV-2 serum-Sepharose and the labelled proteins were analysed by polyacrylamide gel electrophoresis (Figure 2); after 2 hours, gp140 and gp300 are the only labelled proteins detectable in the infected cells. gp125 and gp80 became detectable 3 or 4 hours after the beginning of the labelling and they also became detectable in the virus aggregates (pellets) prepared from the culture medium. 6 or 8 hours after the beginning of the labelling, gp125 and gp80 could be clearly detected. These results indicate that gp80 is associated with the viral particles and suggest that this gp80 must be a mature product of a precursor which requires maturation.

In these experiments, one gp36 labelled with [^{3}H]glucosamine could also be detected but not intracellularly. A protein of 200 kDa also appears in Figure 2 and probably corresponds to a cellular protein since it was not immunoprecipitated by other HIV-2-positive sera.

The kinetic results relating to the synthesis of the HIV-2 envelope glycoproteins are in conformity with the results already obtained. In the cells infected with

HIV-2, gp140 is the first envelope product detectable 15 minutes after the labelling (pulse-labelling). During the period for monitoring the tracer (chase) the dimeric form of the envelope precursor (gp300) could be detected after 30 minutes whereas the mature extracellular glycoprotein (gp125) became detectable 1h 30 min to 3 hours after the beginning (13).

IDENTIFICATION OF gp80 BY POLYCLONAL ANTIBODIES DIRECTED AGAINST THE HIV-2 ENVELOPE PRECURSOR.

In order to characterize the HIV-2 envelope glycoproteins, polyclonal antibodies directed against the purified dimeric precursor gp300 were prepared. For that, gp300 was first partially purified by an immunoadsorbent with antibodies from a patient seropositive for HIV-2 before purification by preparative electrophoresis. Five mice were immunized with 5 µg of this purified preparation of gp300, administered intraperitoneally 5 times at 10-day intervals. Poly(A).poly(U) (200 µg) was used as adjuvant administered in the form of a mixture with the antigen (see Materials and Methods). All the mice developed antibodies against gp300. These antibodies are polyclonal antibodies against gp300 (anti-gp300 antibodies). Figure 3 shows the results of a Western blot analysis revealing the antibodies of one of the immunized mice. The anti-gp300 antibodies reacted specifically with gp300, but also with the gp140, gp125 and gp80 present in the cells infected with HIV-2. No specific signal was observed in the CEM cells infected with HIV-1 or in uninfected cells. The labelling sometimes observed for a 60 kDa protein with anti-gp300 antibodies was probably non-specific since it was observed in the cell extracts independently of viral infection (Figure 3, cells section) and in some experiments, it was not observed at all. These polyclonal antibodies were also used in a similar Western blot test using extracts of cells infected with HIV-2, as well as virus aggregates. In the cellular extracts, the antibodies recognized gp300, gp140 and gp80 (Figure 3, HIV-2 section, line C). In the viral extracts, they recognized gp125, gp80 and the 36 kDa

protein which is probably the transmembrane glycoprotein gp36 (Figure 3, HIV-2 section, line V). After prolonged exposure, it was possible to detect a signal corresponding to the position of gp36 in the cellular extracts. Advantageously, it is noted that the level of gp80 and of gp36 was much higher in the viral aggregate (pellet) compared with its level in the cellular extracts.

These results show that the polyclonal antibodies directed against the precursor of the envelope glycoprotein identified gp80, as well as all the components of the HIV-2 envelope. That is why gp80 can be linked with the HIV-2 envelope. The glycoprotein gp80 is associated with the virus; it is probably a mature product.

IDENTIFICATION OF gp80 SPECIFIC FOR THE HIV-2 TRANSMEMBRANE GLYCOPROTEIN BY MONOCLONAL ANTIBODIES 1H8.

The monoclonal antibody mAb 1H8 was used in a Western blot test to determine which viral proteins can be identified. In the cells infected with HIV-2, mAb 1H8 identified gp300, gp140 and gp80 whereas in the particles of HIV-2 it identified mainly gp80 and weakly gp36 and gp300 (Figure 4). The presence of a low level of gp300 in the viral aggregate was probably due to contaminations coming from lysed cells since it is a cellular protein (13). The weak signal with gp36 probably reflects the low level of this protein. mAb 1H8 did not recognize the extracellular glycoprotein gp125 (Figure 4). Furthermore, no protein of the extracts of cells infected with HIV-1 or of the virus aggregate was recognized. These results show that the antibody mAb 1H8 is specific for the HIV-2 envelope precursors (gp140 and gp300) and the transmembrane glycoprotein (gp36). The reactivity of mAb 1H8 was localized with the amino acid sequence 579-604 of the HIV-2 transmembrane glycoprotein, using a synthetic peptide p39'.

In order to show the specificity of the reactivity of mAb 1H8 with gp80, a Western blot test, using extracts of HIV-2 viral aggregate was carried out. After transferring the proteins, nitrocellulose sheets were incubated with antibodies mAb 1H8 or polyclonal anti-

bodies against gp300, in the absence or presence of peptides 39' (Figure 5). The mAb 1H8 gave a strong signal with gp80. Furthermore, a signal for gp36 was observed but only after prolonged exposure of the autoradiogram. Anti-gp300 antibodies reacted with gp125, gp80 and gp36; the signal obtained with the 60-kDa protein was not specific (like in Figure 3). The addition of peptide p39' completely abolished the signals obtained with mAb 1H8 but not those obtained with the anti-gp300 antibodies (Figure 5). These observations confirm that the reactivity of mAb 1H8 could be specific for the 26 amino acid residue corresponding to the sequence 579 to 604 of the HIV-2 transmembrane glycoprotein. Consequently, a sequence corresponding to that of the peptide p39' could be present in gp80. The reactivity of the anti-gp300 antibodies was not modified by the peptide P39'. That is why these polyclonal antibodies could interact with epitopes other than those corresponding to the peptide p39'.

IMMUNOPRECIPITATION OF gp80 BY ANTI-gp300 POLYCLONAL ANTIBODIES AND BY mAb 1H8

Polyclonal antibodies immunoprecipitated gp300, gp140 and gp125 as well as gp80, whereas mAb 1H8 immunoprecipitated gp300, gp140 and gp80 (Figure 6). Two cellular proteins (60 and 45-kDa) were also associated with the preparations of immune complexes using both the polyclonal and monoclonal antibodies (Figure 3, lines 0 and 2 hours). The presence of proteins of 60 and 45 kDa in the preparation of immune complexes was due to their attachment to the protein A Sepharose®; the latter was used so as to recover the immune complexes formed with the different antibodies.

Cells infected with HIV-2 were labelled for 30 minutes by the "pulse" technique and the marker was monitored (chase) for 2 to 4 hours. Extracts of labelled cells (times 0, 2 and 4 hours) and the viral aggregate recovered after 4 hours of monitoring (chase) were analysed by an immunoprecipitation test using polyclonal antibodies against gp300 and the mAb 1H8 antibody

(Figure 6). With the polyclonal and monoclonal antibodies, gp80 could not be detected during the labelling period (pulse). It became clearly apparent after 2 hours of monitoring (chase) in the infected cells. When the monitoring (chase) was prolonged up to 4 hours, then the gp80 labelled with [^{35}S]methionine became undetectable in the infected cells. Analysis of a viral aggregate produced after 4 hours showed that, like the extracellular glycoprotein gp125, gp80 was associated with the viral particles (Figure 6). It may be thought that gp80 is a product of maturation of the HIV-2 envelope. The fact that gp80 was identified by monoclonal antibodies specific for the HIV-2 transmembrane glycoprotein showed that it corresponds to the dimeric form of gp36 (confirmed by the results shown in Figures 8 and 9). The detection of gp80 was not restricted to the infected CEM cells since it was also detected in the T4 lymphocytes infected with HIV-2.

Comparison of the results obtained by Western blot analysis and by immunoprecipitation tests shows that the human sera positive for HIV-2, the murine polyclonal antibodies and mAb 1H8 recognize the denatured forms of gp80 and gp125 better than the native forms (Figure 1, 2, 3, 4 and 6). The native forms of these mature glycoproteins probably have conformations which mask the epitopes identified by the different antibodies.

INCORPORATION OF GLUCOSAMINE AND FUCOSE INTO gp80

Extracts of cells infected with the HIV-2 virus, metabolically labelled with [^{3}H]glucosamine and [^{3}H]fucose, were immunoprecipitated using antibodies mAb 1H8 and anti-gp300 polyclonal antibodies. In accordance with the preceding results (Figures 3-6), the anti-gp300 antibodies immunoprecipitated glycoproteins labelled with sugars, namely gp300, gp140, gp125 and gp80 whereas mAb 1H8 immunoprecipitated gp300, gp140 and gp80. All these proteins incorporated [^{3}H]glucosamine whereas the incorporation of [^{3}H]fucose appeared most of the time in gp125 and gp80 (Figure 7A). In these experiments, the labelling of gp36 with [^{3}H]fucose was weakly observed

after a prolonged exposure of the autoradiogram. gp80 can also incorporate [^{3}H]mannose in the same manner as gp300, gp140 and gp125. The incorporation of these labelled sugars into gp80 and into other glycoproteins was completely blocked with tunicamycin (15) an antibiotic which inhibits the glycosylation of the proteins linked to nitrogen.

The glycoprotein oligosaccharides linked to asparagine (containing N-acetylglucosamine, mannose and glucose) undergo greater maturation after their attachment to native proteins (11). The oligosaccharide chains are cut at the ends in the endoplasmic reticulum and in the Golgi apparatus before the transfer of the fucose residues and of sialic acid. That is why the incorporation of the fucose residues occurs long after the glycosylation steps. In the cells infected with HIV-2, the [^{3}H]fucose is incorporated predominantly into gp125 and gp80 (Figure 7), two proteins which correspond to mature products of HIV-2 envelope precursors (Figure 6). In order to confirm that gp120 was produced during the maturation of the envelope precursor, cells infected with HIV-2 were labelled with [^{35}S]methionine in the absence or in the presence of a glucosidase inhibitor, castanospermine (14). The culture supernatants were then tested by immunoprecipitation using a serum from a patient positive for HIV-2 which recognized several viral proteins. In the presence of castanospermine, the production of gp80 and of gp125 was considerably reduced whereas the production of the major nucleocapsid (core) protein (p26) was not significantly affected (Figure 7b).

DISSOCIATION OF gp80 INTO gp36

Experiments were carried out in order to optimize the conditions under which the dissociation of gp80 could take place, these conditions corresponding to a highly saline medium, an acidic pH, an ionic detergent EDTA or EGTA. We tested the method as described in (13) which makes it possible to dissociate gp300 by incubation in a weakly acidic buffer. Although this method may be suitable for the dissociation of gp80, with a buffer at a pH

below pH 6, most of the gp80 glycoproteins were degraded. In all the experiments, extracts of infected cells or of viral aggregates were prepared using a lysis buffer containing a nonionic detergent, Triton® X-100. Under these conditions, gp300 and gp80 are not dissociated, even after addition of ionic detergent SDS. The effect of SDS was investigated when it is substituted for Triton® for the preparation of the extracts. The cells infected with HIV were labelled with [^{35}S]methionine before preparation of the extracts by solubilization in a lysis buffer containing either 1% of Triton® X-100 or 1% of SDS. These extracts are then diluted 10-fold in a lysis buffer without detergent and immunoprecipitated using mAb 1H8. The preparations of immune complexes from extracts with Triton® showed the presence of bands labelled with [^{35}S]methionine corresponding to gp300, gp140 and gp80, and weak bands of gp36 (Figure 8, section C line 1). Moreover, when the extracts were prepared with SDS, then gp300 and gp80 were practically undetectable whereas the level of gp140 and gp36 was increased (Figure 8, section C, line 2). Therefore, in the presence of an ionic detergent, the dimeric forms gp300 and gp80 were dissociated, giving rise to the production of gp140 and gp36 respectively. The dissociation of purified gp300, labelled with [^{35}S]methionine gave rise only to the formation of gp140 (13). gp36 should therefore exist only after the dissociation of gp80. It is noted that the degradation of proteins may also occur in the presence of SDS so long as all the labelled gp300 and gp80 proteins have not been recovered among the dissociated proteins (Figure 8 section C). The presence of 200 units/ml of aprotinin and of 0.2 mM PMSF did not prevent such a degradation during incubation with SDS. It is possible that the dimeric forms of the proteins have a conformation such that they withstand proteolysis. The dissociation of gp300 and gp80 may then lead to conformational modifications which make the proteolytic sites accessible.

Dissociation experiments were also carried out

with viral aggregates of HIV-2. The viral proteins labelled with [^{35}S]methionine were solubilized in a lysis buffer containing 1% of Triton® or 1% of SDS in an RIPA buffer containing 0.1% of SDS and 1% of deoxycholate. The extracts in the lysis buffer containing 1% of SDS were also heated to 95°C. All the extracts were then immunoprecipitated with mAb 1H8 and analysed by polyacrylamide gel electrophoresis (Figure 8 Section V).

In the immune complex prepared from the Triton® extracts, gp80 and gp36 were the principal proteins immunoprecipitated by the antibody 1H8. Moreover, gp80 became undetectable whereas the level of gp36 was substantially increased in the SDS extracts. The degree of degradation of the SDS extracts was probably particularly high since most of the labelled constituents disappeared. A better result was obtained with the RIPA buffer in which most of the gp80 proteins were dissociated and about 30% of the labelling was found in the gp36 proteins (Figure 8 section V). In order to show that gp80 is solely composed of gp36, dissociation experiments were carried out using gp80 labelled with [^{35}S]methionine purified by immunoprecipitation with the antibodies mAb 1H8 and by preparative gel electrophoresis. The freeze-dried preparations of gp80 were then suspended directly in an acetate buffer containing SDS, at pH 6.8, 5.8 and 4.8. At pH 4.8, all the gp80 proteins were converted to gp36 (Figure 9).

CHARACTERIZATION OF A TRANSMEMBRANE GLYCOPROTEIN IN DIMERIC FORM IN SIV-mac

It was investigated whether the transmembrane glycoprotein was detectable in dimer form in the SIV samples.

Cells infected with SIV and HIV-2 were labelled with [^{3}H]glucosamine and extracts were prepared in a lysis buffer containing Triton®, then immunoprecipitated with the antibody mAb 1H8. As shown in Figure 7, the monoclonal antibody precipitated the gp300, gp140 and gp80 of the cells infected with HIV-2, whereas only gp80 was found in the viral particles (Figure 10). In the

cells infected with SIV, the monoclonal antibody precipitated 3 glycosylated proteins: the envelope precursor gp140, the dimeric precursor gp300 and a protein of 65 kDa (gp65) which probably corresponds to the counterpart of HIV-2 gp80. Like gp80, gp65 appeared to be associated with the viral particles of SIV (Figure 10).

It is noted that in these experiments, the monomeric forms of the transmembrane glycoprotein of HIV-2_{ROD} and SIV-mac were not detectable. The amino acid sequence 579-604 of HIV-2_{ROD} corresponds to the amino acid sequence 595-620 of SIV-mac (19,7). These two sequences exhibit strong homology and the antibody mAb 1H8 cross-reacts with the envelope proteins both of HIV-2_{ROD} and of SIV-mac.

The inventors have therefore now demonstrated that a protein with a molecular weight of about 65 kDa corresponds to a dimeric form of the SIV-mac virus gp32.

DISCUSSION

The dissociation of the dimeric forms (gp300 and gp80) can take place at a slightly acidic pH. In accordance with this result, it can be assumed that the dimerization of gp140 may be dependent on the pH and may take place in a compartment of the endoplasmic reticulum which promotes the fusion of 2 molecules of the precursor gp140.

The molecular weight of the dimeric form of the envelope precursor gp300 of HIV-2 was estimated by polyacrylamide gel electrophoresis under denaturing conditions (13). On a 5% polyacrylamide gel, containing 0.1% bisacrylamide instead of 0.2% (w/v), this dimeric precursor migrated to a position corresponding to a 280-kDa protein. To confirm the molecular weight of the dimer under the native conditions, gel filtration experiments were carried out using a Sephacryl® S-300 column, and extracts of cells infected with HIV-2 labelled with [^{35}S]methionine were produced which were prepared in a lysis buffer containing Triton. Under these experimental conditions, gp300 was eluted in the form of a second peak after the peak for the aggregated proteins. The dimer of

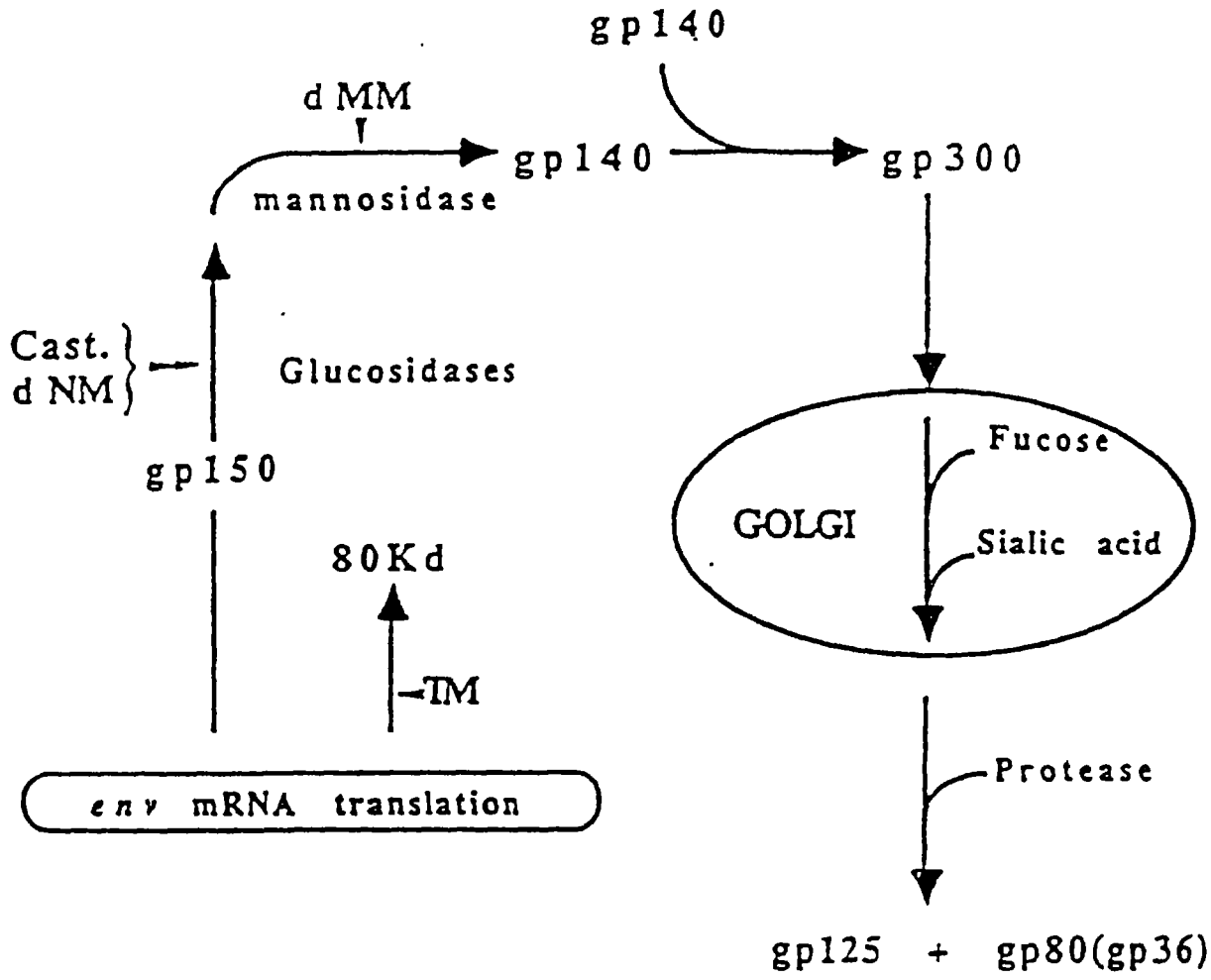
transmembrane glycoproteins corresponds to a protein, having a molecular weight of 75-80 kDa, eluted after the peak for gp125 and before the peak for bovine serum albumin (68 kDa) used as marker. These observations show that the molecular weight estimations for the native and denatured dimers gp300 and gp80 are comparable. In vitro the dissociation of these dimeric forms occurs at acidic pH and also in the presence of an ionic detergent SDS. When the extracts were prepared in lysis buffer containing Triton®, the dimeric forms gp300 and gp80 withstood dissociation by SDS. The nonionic detergent Triton® therefore preserves the native forms of the envelope dimers gp300 and gp80.

The dimeric forms of the envelope precursor gp300 and of the transmembrane glycoprotein were also observed in the cells infected with SIV but not with HIV-1. In particular, the transmembrane dimers of HIV-2 (gp80) and SIV (gp65) withstand dissociation by reducing agents. That is why the dimerization of the envelope transmembrane glycoprotein can be considered as a property specific for the expression of the envelope genes of HIV-1 and SIV. This property can be used for the characterization of new HIV isolates, as marker suitable for describing the relationship between the detected isolate and the HIV-1 type or the HIV-2 type. The dimerization of the envelope precursor could be essential for its maturation in order to produce mature envelope glycoproteins. Whereas the dimeric form of the transmembrane glycoprotein may be essential for the optimum formation of the structure of the virion and thus for its capacity to fuse with the cell membranes and to be infectious, the dissociation in vitro of the transmembrane dimer leads to a very substantial degradation. This may be due to the capacity of the dimeric form of this glycoprotein to withstand proteolysis by virtue of its conformation.

The table below and the diagram of the stages of production of the glycoproteins gp125 and gp80 of HIV-2 summarize some aspects of the invention set forth in the

examples.

HIV-1, HIV-2 and SIV envelope glycoproteins			
Envelope glycoprotein	$HIV\text{-}1_{Bru}$	$HIV\text{-}2_{ROD}$	SIV_{MAC}
Precursor	gp160	gp140	gp140
Dimer precursor	none	gp300	gp300
Extracellular	gp120	gp125	gp130
Transmembrane	gp41	gp36	gp32
Dimer transmembrane	none	gp80	gp75


gp140
d MM
gp140
gp300
mannosidase
Cast.
d NM
Glucosidases
gp150
Fucose
GOLGI
Sialic acid
80Kd
TM
Protease
env mRNA translation
gp125 + gp80(gp36)

CLAIMS

1. Glycoprotein characterized by the following properties:

- it is formed from an HIV or SIV retrovirus envelope transmembrane glycoprotein dimer,
- it is capable of forming an antigen-antibody type immunological complex with antibodies directed against an antigenic glycoprotein gp80 having a molecular weight close to 80 kDa, recognized by polyclonal antibodies directed against the glycoprotein gp300 of a human retrovirus HIV-2, and comprising in association in the form of a dimer two components of the envelope transmembrane glycoprotein gp36 encoded by the genome of an HIV-2 type human retrovirus capable of causing LAS or AIDS in man,
- it has a molecular weight of the same order as that of the abovementioned HIV-2 gp80.

2. Antigenic glycoprotein gp80 according to Claim 1, characterized by the following properties:

- it has a molecular weight (MW) close to 80 kDa,
- it is recognized by polyclonal antibodies directed against the glycoprotein gp300 of a human retrovirus HIV-2,
- it comprises in association in the form of a dimer two components of the envelope transmembrane glycoprotein gp36 encoded by the genome of an HIV-2 type human retrovirus capable of causing LAS or AIDS in man.

3. Glycoprotein gp80 according to Claim 2, characterized in that:

- it may be associated with cells infected by an HIV-2 type human retrovirus or/and with virions or/and with

HIV-2 type viruses,

- it is not dissociated in vitro in the presence of a nonionic detergent such as 1% Triton® X-100 and it is dissociated in vitro in order to form the transmembrane glycoprotein gp36 of an HIV-2 type retrovirus, in the presence of an ionic detergent such as 1% SDS at a weakly acidic pH.

4. Protein derived from a glycoprotein gp80 according to any one of Claims 1 to 3, characterized in that it lacks the glycosylated groups of gp80.

5. Polyclonal antibody characterized in that,

- it recognizes specifically, on the one hand, gp300, gp140, gp125 and gp80 according to any one of Claims 1 to 4, which are present in cells infected by an HIV-2 type retrovirus, and on the other hand, gp125, gp80 according to any one of Claims 1 to 4 and gp36 which are present in extracts of an HIV-2 type retrovirus, and
- it does not recognize the proteins or glycoproteins of healthy cells or of cells infected by an HIV-1 type retrovirus.

6. Monoclonal antibody characterized in that it reacts specifically with the glycoprotein gp80 according to any one of Claims 1 to 4, in that it does not react with the proteins and glycoproteins of cells infected by an HIV-1 type retrovirus or with viral aggregates of HIV-1 and in that it recognizes, in addition, the HIV-2 glycoproteins gp300, gp140 and gp36.

7. Hybridoma which produces monoclonal antibodies according to Claim 6.

8. Antigenic composition for the specific in vitro diagnosis of an infection due to an HIV-2 type human retrovirus, in a biological sample capable of containing antibodies formed after the said infection, characterized in that it comprises at least the glycoprotein gp80 according to any one of Claims 1 to 4.

9. Immunogenic composition characterized in that it comprises at least the glycoprotein gp80 according to any one of Claims 1 to 4, in association with a pharmaceutically acceptable vehicle for the preparation of vaccines.

10. Process for the in vitro diagnosis of an infection due specifically to an HIV-2 type retrovirus, characterized by the steps of:
- bringing a biological sample capable of containing antibodies produced following an infection by an HIV-2 type retrovirus into contact with a glycoprotein gp80 according to any one of Claims 1 to 4 or with an antigenic composition according to Claim 8, under appropriate conditions allowing the formation of an antigen-antibody type immunological complex, and
- detecting the antigen-antibody complex which may be formed.

11. Process for the preparation of an antigenic glycoprotein gp80 according to any one of Claims 1 to 4, characterized by the following steps:
- lysis of cells infected with a human retrovirus HIV-2 and separation of the supernatant and of the infected cells or lysis of the viral pellets prepared by centrifugation,
- incubation of the cellular extract and/or of the viral extract on an immunoaffinity column on which is deposited an immunoadsorbent containing purified antibodies as obtained from a serum from a patient infected with the human retrovirus HIV-2, this serum having the capacity to react strongly with the HIV-2 envelope proteins, the said antibodies being advantageously attached to a suitable support, in the presence of a buffer and for a sufficiently long period to obtain the formation of an antigen-antibody immunological complex,
- washing of the column with a buffer to remove the molecules not retained on the support, and
- recovery of the desired antigenic proteins.

12. Process according to Claim 11, for the preparation of the glycoprotein according to any one of Claims 1 to 4, characterized in that the recovery of the glycoproteins is made by electrophoresis and by electroelution.

13. Process according to Claim 11 for the preparation of the glycoprotein according to any one of Claims 1 to