

1

The invention relates to precursor antigens of the envelope glycoproteins of a HIV-2 human retrovirus and to antigens cross-reacting immunologically with specific monoclonal antibodies recognizing the above antigens, precursors of the envelope glycoproteins of the HIV-2 retrovirus

More precisely, the invention relates to antigens present exclusively during certain phases of the in vivo development of an infection due to a HIV-2 human retrovirus. These antigens are characteristic of the formation and maturation processes of the envelope glycoprotein of this retrovirus and, consequently, are implicated in the development and proliferation of the HIV-2 retrovirus and, consequently, in the propagation of the infection.

The invention also relates to antibodies recognizing these antigens as well as to immunogenic compositions containing these antibodies.

The invention also relates to the preparation of the forementioned antigens as well as to their use for the diagnosis of an infection by a HIV-2 human retrovirus.

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

Several human retroviruses capable, under certain conditions, of causing an LAS possibly superseded by an AIDS in man have been designated by HIV (an abbreviation for Human Immunodeficiency Virus).

Thus a first virus designated LAV-1 or HIV-1 was isolated and described in the patent application GB.83/24.800 corresponding to the application EP. 84/401.834 of 14/09/84 published under the number 0138667. This virus has also been described by F. Barre-Sinoussi et al. in Science, 220 No. 45-99, 20, pages 868-871.

Variants of this HIV-1 virus designated as LAV ELI and LAV MAL have also been isolated, characterized and described in the patent application EP.84/401.834.

The isolation and the characterization of retroviruses belonging to a distinct class and having only a limited immunological relationship to the foregoing have been described in

the European patent application No. 87/400.151.4, published under the No. 239.425. These retroviruses, which have been classed under the designation HIV-2, were isolated from several African patients exhibiting symptoms of a lymphadenopathy or an AIDS.

The study of these HIV retroviruses, the isolation and 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 with regard to their relatedness or, conversely, their structural and functional specificity.

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 made it possible to show a certain relatedness between the proteins and glycoproteins of the HIV-2 retrovirus and its variants and those of the SIV retrovirus. A relationship is observed particularly at the level of the envelope protein of each of these viruses. Immunological cross-reactions have in fact 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 nor between the retroviruses HIV-1 and HIV-2.

Results concerning the envelope glycoprotein of the HIV-2 glycoprotein were given in the previously mentioned European patent application No. 87/400.151.4 (published under the number 0239425). In this EP patent application this glycoprotein was designated by the abbreviation gp140 in reference to its molecular weight of about 140,000 D. It was specified however that the calculation of the molecular weight was estimated at $\pm 10\%$. In a previous French patent application (No. 86/03881 published under No. 2.596.063) a precursor of the gp140 designated as gp160 (molecular weight of 160 kD $\pm 10\%$) had also been described.

More detailed investigations relating to the envelope glycoprotein of the HIV-2 retrovirus have been conducted and have enabled the inventors to carry out other measurements of the molecular weights given hitherto and, consequently, to make the results relating to the molecular weight values described above more precise. The external envelope glycoprotein exhibits a MW of

about 125,000 D (it will be designated as gp125) in the HIV-2 viral partides - under the operating conditions of the present invention. Under these same operating conditions the precursor already identified and designated as gp160 in the patent application FR 86/03881 has a molecular weight of about 140,000 D, according to the latest results obtained. It will thus be referred to as "gp140" in the present patent application.

In the context of the invention the inventors have demonstrated the existence of several phases of development leading to the production of envelope glycoproteins in a mature form, these mature envelope glycoproteins including a glycoprotein pg125 and a glycoprotein gp36. The inventors have thus identified different stages in the progression of the viral replication cycle which appear when pathogenic disorders develop as the result of an infection by a HIV-2 human retrovirus. The inventors have emphasized in this connection the importance of several steps occurring in the cells infected by the HIV-2 retrovirus, in particular in human T4 lymphocytes.

In parallel, they have conducted similar studies on the viral cycles of the HIV-1 and SIV retroviruses. These latter studies have revealed very specific properties common to the HIV-2 and SIV retroviruses and, conversely, apparently non-existent during the viral replication cycle of HIV-1.

The invention thus provides novel agents to detect specifically an infection attributable to a HIV-2 human retrovirus.

In view of the latest results obtained the viral cycle intrinsic to the HIV-2 and SIV retroviruses appears as a complex process of chain reactions, only the entire sequence of which is likely to lead to the formation of the external glycoprotein present in the envelope of the HIV-2 retrovirus in its definitive form. During this process, it has been possible to recognize, isolate and identify different antigenic precursors. All possess in particular a structural relatedness based on the peptide skeleton of the envelope glycoprotein encoded in the env gene of the HIV-2 retrovirus described in the EP patent application previously mentioned.

The invention thus relates to proteins or glycoproteins produced following an infection by a HIV-2 human retrovirus

during the various stages of the formation and maturation cycle of the external envelope glycoprotein gp125 of HIV-2, which has in common the peptide skeleton of the external envelope glycoprotein of the HIV-2 human retrovirus or of a glycoprotein exhibiting an immunological cross-reaction with the above-mentioned gp125 and differing by the presence and/or the composition of possible oligosaccharide chains.

The specific properties of the proteins and glycoproteins of the invention are acquired during each step essential to the formation and maturation of the mature external envelope glycoprotein of the HIV-2 human retrovirus.

As a result of the study of the viral cycle, the inventors have suggested the possibility of intervening during the particular stages of the formation of the mature envelope glycoproteins of the HIV-2 human retrovirus, thus providing agents to interrupt the normal development of the viral cycle and to prevent the propagation of the retrovirus which is infectious for man and pathogenic under certain conditions.

In agreement with the foregoing the invention relates to an antigenic retroviral glycoprotein characterized in that it is :

- either a glycoprotein called gp 300, having a molecular weight (MW) of about 300 kD and is constituted by the association of two units of gp140, a glycoprotein precursor of the envelope glycoprotein gp125 encoded by the env gene of the genome of a human retrovirus of the HIV-2 type capable of causing a LAS or an AIDS,
- or a dimer of the precursor of the envelope glycoprotein of a retrovirus which cross-reacts immunologically with antibodies directed against said glycoprotein gp300.

It is observed that the precursor gp140 is also the precursor of gp 36 of a HIV-2 human retrovirus. The inventors have observed that the previously mentioned association of the two units of gp140 is an intrinsic property of their peptide skeleton.

The antigenic protein gp300 is also characterized by the following properties :

- it is present in the cells of a biological sample of a patient infected by a retrovirus of the HIV-2 family, in particular in human T4

lymphocytes or in permanent lines derived from these T4 lymphocytes,

- its isoelectric point (pI) lies in the range pH 6.8 - 7.8,
- it is glycosylated by oligosaccharide chains linked to nitrogen,
- it is stable to ionic detergents, in particular to 1% SDS, to non-ionic detergents, in particular 2% Triton^R X-100, 4M urea, to high salt concentrations and reducing agents,
- it dissociates spontaneously in vitro into two glycoproteins of molecular weight of about 140 kD between pH 4-5.
- after isolation and purification on gel electrophoresis, it dissociates in acetate buffer in vitro into two glycoproteins of molecular weight of about 140 kD at pH values equal to or less than 6,
- it exhibits one band on electrophoresis on a 7.5% polyacrylamide - SDS gel in the presence of 6M urea, corresponding to a molecular weight of 300 kD after purification on an affinity column of the HIV-2 type serum-Sepharose^R containing specific antibodies obtained from the serum of a patient infected by a HIV-2 type human retrovirus.

The inventors have observed that the formation of the dimer consisting of gp300 is a transient stage in the viral cycle and probably necessary for the maturation of the envelope glycoproteins of the HIV-2 type human retrovirus. In fact, the above antigenic protein gp300 is characterized in that it is not revealed by electrophoretic analysis of a biological medium constituted of viral particles present in the culture medium specific for the cells infected by a HIV-2 type human retrovirus after labelling with ³⁵S-methionine (200 µCi /ml ; 4 x 10⁶ cells/ml) for 18 hours and purification of the extracts obtained containing the infected cells and their corresponding culture medium whereas it is revealed under the same conditions in the above-mentioned infected cells.

This has led them to consider gp300 as a specific transient precursor of the final envelope glycoproteins. This property of the viral cycle of a HIV-2 type human retrovirus resulting in the production of a gp300 protein is original and unusual compared with what is known of the general mechanisms of glycoprotein formation containing oligosaccharide glycosylation chains linked to nitrogen.

Furthermore, the inventors have made the really interesting observation that the formation and maturation of the envelope glycoproteins of the SIV_{mac} retrovirus also involves a dimerization step leading to the production of a glycoprotein of the gp300 type characterized in fact for a HIV-2 type human retrovirus. On the other hand, the parallel observation of the viral cycle of the HIV-1 human retrovirus has not led to the observation of any dimerization step resulting in the formation of a glycoprotein related to gp300.

These results indicate quite surprisingly that the formation of a dimer from the gp140 precursor of the envelope glycoprotein of a HIV-2 type human retrovirus is a specific property of the expression of the env gene of the HIV-2 type human and SIV retroviruses.

Advantage may be taken of this observation in the research into and development of agents for the selective diagnosis of an infection due to a HIV-2 type human retrovirus capable of causing a LAS or an AIDS under certain conditions. We will come back to this later when discussing these suitable diagnostic agents.

The invention also relates to a protein exhibiting properties identical with those of gp300 with respect to the peptide skeleton, characterized in that it lacks glycosylation groups and in that its molecular weight is about 200 kD.

This p200 protein to which we will return in the examples which follow was detected during digestion with endo-beta-N-acetylglucosaminidase H (also designated "endo-H"). This protein which represents the non-glycosylated form of gp300 might be of particular interest owing to the fact of the possible existence of an epitope common to gp300 and to p200 which proved to be only weakly antigenic in gp300 because of the presence of oligosaccharide chains important for the final conformation of gp300 but nonetheless a hindrance for accessibility to the interesting epitope.

During the study of the viral cycle another glycoprotein was detected: it is a purified glycoprotein gp150, characterized in that it is detected in vitro (uniquely in cells of a patient infected by a HIV-2 type human retrovirus) after pulse-labelling of these cells with a radioactive tracer added in the presence of an inhibitor of glucosidases, in particular castanospermine, then elimination of this radioactive tracer by washing and replacement by non-radioactive

molecules prior to a step involving the determination of the radioactivity in samples taken at different time intervals, a step which makes it possible to trace this tracer in the proteins and glycoproteins formed after said labelling. The observation of gp150 is made after a time equal or similar to that necessary for the detection of gp300.

The glycoprotein gp150 is also characterized by :

- a molecular weight of about 150 kD,
- the peptide skeleton of the precursor of the envelope glycopeptide gp125 encoded in the env gene of the genome of a HIV-2 type human retrovirus or of a retrovirus whose external envelope glycoprotein is recognized by the antibodies of a serum of a patient infected by said HIV-2 type human retrovirus, in particular the antibodies directed against gp125,
- terminal glucose residues on an oligosaccharide chain.

The proteins and glycoproteins previously described and preferably gp300 have proved to be advantageous for use in antigenic compositions for the diagnosis of the presence of antibodies in the cells of a biological sample of a patient infected by a HIV-2 type human retrovirus, these compositions being characterized in that they contain an antigenic protein or glycoprotein defined above.

These compositions are particularly interesting in that they permit the formation of immunological complexes of the antigen-antibody type with antibodies of the serum of a patient infected by a HIV-2 type human retrovirus. Consequently, these compositions and/or the antigens they contain would advantageously be expected to be used to carry out the specific diagnosis of an infection due to a HIV-2 type human retrovirus.

Furthermore, the invention relates to monoclonal antibodies characterized in that they recognize the gp300 defined in the preceding pages and in that they do not recognize the mature envelope glycoprotein gp 125 and the mature gp36.

Other monoclonal antibodies are characterized in that they recognize an epitope which in the above gp300 is exposed and accessible to the antibody, this epitope being however either not exposed or masked in the natural conformation of the mature gp125 and gp36.

Such monoclonal antibodies may also be neutralizing, in particular by preventing the binding of a HIV-2 type human retrovirus to its receptor, in particular to the T4 receptor of the human lymphocytes which bear it.

Other useful monoclonal antibodies in the framework of the invention recognize the p200 defined above and do not recognize the mature envelope glycoproteins gp125 and gp36.

Other monoclonal antibodies are characterized in that they recognize an epitope on the p200, this epitope being however either not exposed or masked on the gp125 and gp36 and/or on gp300 such as obtain in their natural conformations.

The above monoclonal antibodies may have immunogenic or neutralizing properties, and interrupt the proliferation of a HIV-2 type human retrovirus.

Several methods for the production of the preceding antibodies may be suitable in the framework of the embodiment of the invention. For example, recourse should be had to ascites cultures in animals, in particular in rodents like the mouse. The antibodies can also be produced from cell cultures either immobilized or encapsulated or even in suspension. Use should be made in particular of the hybridoma procedure obtained by fusion of the lymphocytes of an animal which has previously been injected with a HIV-2 type human retrovirus. Lymphocytes of the infected animal are then taken and fused with myeloma cells selected to form hybrids subsequently selected for their capacity to produce specific antibodies directed against the antigenic proteins and glycoproteins described above.

These monoclonal antibodies produced will for example be used to inactivate the proteins and/or glycoproteins with which they form an immunological complex or also for their capacity to prevent the normal course of formation of the final envelope glycoproteins of a HIV-2 type human retrovirus. Furthermore, given the relatedness of the peptide skeleton of the proteins and glycoproteins of the invention, the monoclonal antibodies formed against these glycoproteins, in particular against the gp300, the p200 might prove to be efficacious against the gp125.

Another possible use of these antibodies would consist for example in the detection of viral antigens in biological preparations

or also in the purification of proteins and/or glycoproteins for example on an affinity column.

Generally, the invention is not limited to glycoproteins and proteins such as previously described when they are obtained only from a HIV-2 type human retrovirus. On the contrary, they encompass proteins and glycoproteins exhibiting antigenic, immunological, even immunogenic properties in common with the proteins and glycoproteins described above, these proteins and glycoproteins being then defined for example by their capacity to be recognized by antibodies specifically directed against the corresponding proteins characteristic of a HIV-2 type human retrovirus.

This remark applies for example to the gp300 glycoproteins and other precursors of the external envelope glycoprotein of a SIV_{mac} retrovirus whose viral cycle studied by the inventors has shown common properties with the proteins and glycoproteins of a HIV-2 type human retrovirus previously mentioned.

The invention also relates to an immunogenic composition containing a gp300 glycoprotein such as defined above in combination with a pharmaceutically acceptable vehicle suitable for the preparation of vaccines.

An immunogenic composition thus formed is titrated in antigenic proteins and/or glycoproteins so as to make possible the administration of a dose of 10 to 100 µg per kilogram.

The invention also relates to a procedure for the preparation of the gp300 antigenic glycoprotein characterized by the following steps :

- lysis of cells infected by a HIV-2 type human retrovirus and separation of the supernatant and the cell extract,
- incubation of the cell extract and/or supernatant on an immunoaffinity column on which is loaded an immunoabsorbent containing purified antibodies such as those obtained from the serum of a patient infected by a HIV-2 typ human retrovirus, bound to a suitable support in the presence of a buffer and for a time sufficiently long to lead to the formation of an antigen-antibody immunological complex,
- washing of the column with buffer to remove the molecules not retained on the support,

- resolution of the proteins retained after elution by electrophoresis and electroelution,
- recovery of the desired antigenic proteins.

The infected cells will have been taken from a patient infected by a HIV-2 type human retrovirus. They will also have been obtained in vitro from a cell culture infected by HIV-2. Such cell cultures may be generated using CEM lines.

Another procedure for the preparation of this glycoprotein is analogous to the previous one, the electroelution step being replaced, however, by the following steps :

- elution of the proteins bound to the above immunoaffinity column,
- purification of the products thus eluted on a chromatography column containing monoclonal antibodies directed against the gp300 bound to the separation support.

In a particular embodiment of the invention the lysis of the infected cells is carried out in a detergent solution, the antibodies contained in the immunoadsorbent are bound to agarose beads and the experiment is performed in a coupling buffer.

The invention also relates to a procedure for the in vitro diagnosis of the presence of antibodies in the cells of a biological sample of a patient for the purpose of verifying a possible infection by a HIV-2 type human retrovirus, characterized by :

- the placing of this biological sample in contact with an antigen described above, or an antigenic composition of the invention under conditions permitting the formation of an immunological complex of the antigen-antibody type.
- the detection of the antigen-antibody complex formed.

The biological sample on which the diagnosis is performed may be a biological fluid, in particular a serum, an extract of biological tissue provided that it is likely to contain cells infected by a HIV-2 type human retrovirus.

Such a procedure may be particularly interesting in that it might make possible the early detection of an infection due to a HIV-2 type human retrovirus or to a related retrovirus provided that the inventors have detected the appearance of the gp300 at an early stage of the viral replication cycle of the retrovirus responsible for the infection.

For the implementation of the preceding diagnostic procedure the inventors have designed a kit for the detection of antibodies in the cells of a biological sample of a patient likely to be infected by a HIV-2 type human retrovirus, characterized in that it contains :

- at least one antigenic protein and/or glycoprotein previously described or
- a composition made up from these components or also a mixture of these different constituents and
- agents enabling reaction to occur leading to the formation of the immunological complex between the above antigens and the antibodies possibly present in the biological sample to be tested, in particular one or more incubation buffers if necessary,
- a negative control sample,
- agents for the revelation of the antigen-antibody complex formed.

Having now defined the different stages of the formation of the infectious HIV-2 retrovirus as well as the importance of certain of them in the development of the infection, the inventors have suggested the development of a pharmaceutical composition characterized in that it contains at least one inhibitor of the glucosidases I and/or II, in particular castanospermine or deoxyjirimycin, or a mixture of these inhibitors in a dose sufficient to block the removal of the terminal glucose residues of the oligosaccharide chains of the gp150 glycoprotein during the viral cycle, in combination with a physiologically acceptable excipient.

Other characteristics and advantages of the invention will become apparent on reading the examples which follow as well as from the Figures whose legends are given hereafter :

Figure 1 composed of the Figures 1A and 1B show the envelope glycoproteins of HIV-2 :

- Figure 1A : Comparison of the high molecular weight proteins of HIV-1 and HIV-2.

CEM cells infected with HIV-1 or HIV-2 were labelled with ^{35}S -methionine (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) for 18 hours. Extracts of infected cells (CELL) and their corresponding culture medium (SN) were purified on specific immunoaffinity columns : HIV-1 serum-Sepharose column, specific for HIV-1 proteins (Krust et al., 1988) and a HIV-2 serum-Sepharose column, specific for HIV-2 proteins (see "experimental procedures"). The purified proteins

were analyzed by electrophoresis on a 7.5% polyacrylamide-SDS gel containing 6M urea. A fluorograph of the gel is shown in Figure 1A. The sizes of the HIV-1 and HIV-2 proteins are indicated on the left and right of the lines. The p68 and p55 are the reverse transcriptase and the gag precursor, respectively. gp160 and gp120 are the precursor HIV-1 envelope glycoprotein and its cleavage product. gp300, gp140 and gp125 are the precursors of the glycoprotein and the cleavage product of the HIV-2 envelope protein.

- Figure 1B : Identification of the HIV-2 glycoproteins :

CEM cells infected by HIV-2 and T lymphocytes were labelled with ^3H -glucosamine (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) for 18 hours. Extracts of infected cells (line C) and the culture medium containing the virus (line V) were purified on a HIV-2 serum-Sepharose column and the labelled proteins were analyzed by electrophoresis of the HIV-2 glycoproteins synthesized in the CEM cells on a 7.5% gel. In the line at the extreme left the electrophoresis on a 12.5% acrylamide gel shows the presence of the gp36. This part of the Figure needed to be overexposed to reveal the gp36, that is why the gp140/125 is resolved in the form of a thick band.

- Figure 2 : Gel electrophoretic analysis in two dimensions of the HIV-2 glycoproteins.

The glycoproteins gp300, gp140 and gp125 purified from CEM cells infected by HIV-2 (CELL) were labelled with ^{35}S -methionine. The glycoproteins as well as the culture medium containing the virus were analyzed (SN represents the result obtained with the culture medium; the conditions of preparation are similar to those of Figure 1A for two-dimensional gel electrophoresis) (see "experimental procedures"). The pH gradient for isoelectric focussing (first dimension) corresponds to that given. In the second dimension, the proteins were resolved on a 7.5% polyacrylamide-SDS gel containing 6M urea. Fluorographs of the gels are shown in Figure 2.

- Figure 3 : Dissociation of the native (a) and denatured (b and c) gp300 protein.

a/ - Extracts of CEM cells infected by HIV-2 and labelled with ^{35}S -methionine were purified on a HIV-2 serum-Sepharose column. This sample was then divided into two equal aliquots : one was incubated in coupling buffer (line 1) whereas the other was

incubated in a buffer containing 30 mM sodium acetate at pH 4.0, 0.2 mM PMSF, 100 units/ml of aprotinin and 5 mM of beta-mercaptoethanol (line 2). After 1 hour at 37°C, the acidic medium was neutralized and the two samples were analyzed by electrophoresis.

b/ - The gp300 labelled with ^{35}S -methionine, purified and lyophilized, was suspended in a sodium acetate buffer (100 μl) at pH 4 as indicated above (line 2). Incubations were conducted for 30 minutes at 37°C before two additions of electrophoresis buffer containing 2M urea were made. The results of line 1 correspond to lyophilized gp300 directly suspended in the electrophoresis buffer.

c/ - The gp300 labelled with ^{35}S -methionine, purified and lyophilized, was suspended in a solution containing 30 mM Tris-HCl, 0.2 mM PMSF and 100 units/ml of aprotinin and buffered with HCl at pH 7.5; 7.0; 6.5; 6.0 (as indicated on the Figure). After 60 minutes at 37°C electrophoresis buffer was added twice and the samples were analyzed by electrophoresis.

Fluorographs of these gels are shown in a, b and c. In section c, the band characteristic of the gp300 is dissociated into gp140, it being possible to quantify this dissociation by carrying out a densitometric scan of this fluorograph.

- Figure 4 : Digestion of the HIV-2 glycoproteins with endo-H

The glycoproteins gp300, gp140/gp125 and gp125, purified and lyophilized (see "experimental procedures") were suspended in a buffer containing 150mM sodium citrate at pH 5.5; 0.1% SDS (w/v), 0.5 mM PMSF before being heated for 2 minutes at 90°C. Aliquots of these samples were incubated (2 hours 30°C) without endo-H (line 1) or with 0.4 (line 2), 2 (line 3) and 10 (line 4) milli-units of endo-H.

All these reactions were stopped by two additions of electrophoresis buffer. Electrophoresis was performed as described in Figure 1. Fluorographs of the different gels are shown. The arrows p90 and p80 on the right indicate the position of the digestion product. The conditions for endo-H digestion have been described by Tarentino et al. 1974.

- Figure 5 : Isotopic labelling of the HIV-2 glycoproteins with ^{14}C -mannose or ^3H -fucose.

Cells infected by HIV-2 were labelled for 18 hours with ^{14}C -mannose (25 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) or ^3H -fucose (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml). Extracts of the infected cells (line C) and of the culture medium containing the virus (line V) were purified on a HIV-2 serum-Sepharose column. The labelled glycoproteins were then analyzed by electrophoresis on polyacrylamide gel. The fluorograph is shown in Figure 5.

- Figure 6 : Inhibition of nitrogen-linked glycosylation by tunicamycin.

Cells infected by HIV-2 in the absence (line C) or presence (line TM) of tunicamycin (2 $\mu\text{g/ml}$) were labelled with ^{35}S -methionine (box entitled ^{35}S -met ; 200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) or with ^3H -glucosamine (box entitled H-GlcNAc; (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) for 16 hours. The cells treated with tunicamycin were first incubated (37°C) with the antibiotic (2 $\mu\text{g/ml}$) for 2 hours before being labelled with ^{35}S -methionine or ^3H -glucosamine. Extracts of infected cells (CELL) and of the culture medium containing the virus (SN) were purified on a HIV-2 serum-Sepharose column and analyzed by electrophoresis on a 7.5% polyacrylamide gel. Fluorographs of these gels are shown. The positions of the non-glycosylated precursor of the envelope (p90/p80) and of the non-glycosylated dimer (200 kD) are indicated by small arrows on the right. The proteins 90/80 kD and 200 kD do not incorporate ^3H -glucosamine (box ^3H -GlcNAc, CELL line TM).

- Figure 7 : Effect of the inhibitors of oligosaccharide cleavage on the process of formation of the HIV-2 envelope glycoprotein.

CEM cells infected by HIV-2 were labelled (16 hours, 37°C) with ^{35}S -methionine (200 $\mu\text{Ci/ml}$; 4×10^6 cells/ml) in the absence (lines T) or in the presence of an oligosaccharide cleavage inhibitor : 1 mM bromoconduritol (lines Bro); 1 mM castanospermine (lines Cast.); 10 $\mu\text{g/ml}$ swainsonine (lines Sw); 3 mM deoxynojirimycin (lines dNM) and 1 mM deoxymannojirimycin (lines dMM). Extracts of infected cells (box entitled CELL) and of the culture medium containing the viral particles (box SN) were purified on a HIV-2 serum-Sepharose column in order to identify the viral glycoproteins gp125, gp140, gp300 in the infected cells and gp125 in the culture media. All of the samples were analyzed by electrophoresis on a 7.5% polyacrylamide gel. In order

to show that the inhibition of the production of the gp125 by the cells treated with different inhibitors is specific for the viral glycoprotein, the culture medium was tested leading to the detection of the presence of the core protein, p26, by an immunoprecipitation test making use of a serum seropositive for HIV-2 (Clavel et al., 1986a, 1987). The p26 was analyzed by electrophoresis on a polyacrylamide gel (12.5%). The Figures present the fluorograph showing only one part of each gel.

- Figure 8 : Effect of castanospermine and monensin on the process of formation of the HIV-2 glycoproteins

a/ - Pulse-labelling experiments using a radioactive tracer followed by the removal of this tracer and its replacement by non-radioactive molecules ("pulse-chase" experiment) were conducted on CEM cells infected by HIV-2 in the absence of castanospermine (control) or in the presence of 1 mM castanospermine (Cast.).

Control : The infected cells were incubated for 1 hour at 37°C in a medium lacking methionine before pulse-labelling for 15 minutes with ^{35}S -methionine (200 $\mu\text{Ci}/\text{ml}$; 4×10^5 cells/ml line 1). The radioactive tracer was removed and replaced by culture medium containing 5 mM cold methionine for 0.5; 1.5 and 3 hours (result reported in lines 2, 3 and 4, respectively). Cast. : The CEM cells infected by HIV-2 were incubated (one hour at 37°C) in a medium lacking methionine containing castanospermine before pulse-labelling for 30 minutes with ^{35}S -methionine. After removal of the radioactive tracer and replacement by non-radioactive molecules as described above these cells were then incubated in the presence of castanospermine for 0.5; 1.5 and 3 hours (lines 2, 3 and 4, respectively).

b/ - Labelling experiments by a radioactive tracer and a chase of this tracer under the conditions described above in the cells infected by HIV-2 in the absence of monensin (control) and in the presence of 1 μM monensin were carried out. Infected cells were incubated (1 hour, 37°C) with and without monensin in a medium lacking methionine before being pulse labelled (30 minutes) with ^{35}S -methionine (line 1). The labelled cells were then chased in the culture medium containing 5 mM cold methionine for 0.5, 1.5 and 3 hours (lines 2, 3 and 4, respectively).

Extracts were purified on a Sepharose-HIV-2 serum column and the labelled proteins were analyzed by electrophoresis on a 7.5% polyacrylamide gel. The results are shown on the fluorographs. The p55 represents the gag precursor in section A, line 1.

- Figure 9 : Precursors of the SIV glycoproteins

HUT-78 cells infected by SIV were labelled (16 hours, 37°C) with ^{35}S -methionine (200 $\mu\text{Ci}/\mu\text{l}$; 4×10^6 cells/ml), ^3H -fucose (200 $\mu\text{Ci}/\mu\text{l}$; 4×10^6 cells/ml) and ^{14}C -mannose (25 $\mu\text{Ci}/\mu\text{l}$; 4×10^6 cells/ml). Extracts of infected cells (lines C) and of the culture medium containing SIV (lines V) were purified on a HIV-2 serum-Sepharose column. A HIV-2- positive serum was used to carry out the immunoprecipitation reaction on the SIV proteins, since the HIV-2 and SIV proteins exhibit immunological cross-reactions. All of the samples were analyzed by polyacrylamide gel (7.5%) electrophoresis (see "Experimental procedures"). A fluorograph of the different gels is shown. The electrophoretic mobility of the gp300_{SIV} and the gp140_{SIV} corresponds to that of the gp300 and gp140 glycoproteins of HIV-2. The gp130_{SIV} has a slightly greater mobility than that of the gp125 of HIV-2. The p55 labelled with ^{35}S -methionine is probably the gag precursor of SIV.

- Figure 10 : Schematic representation of the steps in the formation of the HIV-2 envelope glycoprotein.

The 80 kD polypeptide synthesized and glycosylated gives rise to the formation of an "immature glycoprotein precursor", gp150, which is rapidly deglycosylated by the glucosidases of the rough endoplasmic reticulum (RER). A transformation of the environment (particularly of the pH) then leads to dimerization giving rise to the formation of the gp300, "intermediate precursor in dimeric form". The gp300 is cleaved by the mannosidases of the RER before being transported to the Golgi apparatus. Other cleavages of the gp300 are made by the mannosidases of the Golgi before transfer of fucose and sialic acid residues in the medial-Golgi and the trans-Golgi. Finally, the dimer is dissociated into a "mature precursor" gp135 as a result of the acidic pH prevailing in the trans-Golgi network (TGN) compartment. The gp135 is transported into the plasma membrane and cleaved by the cell protease to lead to mature envelope glycoproteins of HIV-2, gp125 and gp36.

Tunicamycin (TM) inhibits the assembly of the dolichol-P-P-glycan residues; castanospermine (Cast.) and deoxynojirimycin (dNM) inhibit the glucosidases of the RER; deoxymannojirimycin (dMM) inhibits the mannosidases in the RER and in the cis-Golgi and in the medial-Golgi; monensin inhibits the transport of the membrane glycoproteins and secretory proteins from the Golgi apparatus.

EXAMPLES

EXPERIMENTAL PROCEDURES

I - MATERIALS

L-(³⁵S) methionine (specific activity : 1000 Ci/mmol), L-(6-³H) fucose (specific activity : 45-70 Ci/mmol), D-(6-³H) glucosamine (specific activity : 20-40 Ci/mmol) and D-(U-¹⁴C) mannose (specific activity : 200-300 mCi/mmol) were obtained from Amersham (Amersham, UK).

Bromoconduritol, castanospermine, deoxymannojirimycin (dMM), 1-deoxynojirimycin (dNM), swainsonine and tunicamycin were obtained from Boehringer-Mannheim (Mannheim, FRG).

Endo-beta-N-acetylglucosaminidase H was obtained from Calbiochem (San Diego, USA).

The ampholines were supplied by Pharmacia (Uppsala, Sweden).

II - VIRUSES AND CELLS

A HIV-1BRU isolate of type 1 human immunodeficiency virus (described by Montagnier et al. 1984), a HIV-2ROD isolate of type 2 human immunodeficiency virus (described by Clavel et al. 1986a) and a simian immunodeficiency virus SIVmac142 (described by Daniel et al. 1985) were used in this study.

The different cell lines and the human lymphocytes were cultured in a RPMI-1640 suspension medium (GIBCO-BRL, Cergy-Pontoise France) containing 10% (v/v) of fetal calf serum; 2 µg/ml of polybrene (SIGMA) were added to the cell cultures infected by HIV. The cells of the clone CEM13 are derived from the CEM line of human lymphoid cells (deposited with the ATCC under No CCL119) and express the T4 antigen at a high level. Five days after

infection with the HIV-1BRU isolate or with the HIV-2ROD isolate, about 80-90% of the cells produce viral particles and can be identified by cytopathogenic effects corresponding to vacuolization of the cells and the appearance of small syncytia.

The HUT-78 cell line is another cell line of positive human T4 lymphocytes (Gadzdar et al. 1980), highly permissive for the replication of SIVmac142 (Daniel et al. 1985). Peripheral blood lymphocytes of healthy blood donors were stimulated for 3 days with 0.02% (w/v) of fraction P of phytohemagglutinin (DIFCO, Detroit, USA) in a RPMI-1640 medium, supplemented with 10% of fetal calf serum. The cells were then cultured on a RPMI-1640 medium containing 10% (v/v) of T cell growth factor (TCGF, Biotest). After infection with HIV-2, lymphocytes were cultured in the presence of 10% (v/v) TCGF and 2 µg/ml of polybrene.

III - METABOLIC LABELLING OF CELLS

For the metabolic labelling of the proteins infected cells were incubated for 16 hours at 37°C in a MEM (abbreviation for Minimal Essential Medium) culture medium without L-methionine or serum, but supplemented with 200 µCi/ml of L-(³⁵S) methionine. For the metabolic labelling of the glycoproteins infected cells were incubated for 16 hours at 37°C in a MEM culture medium without serum or glucose, but supplemented with 200 µCi/ml of ³H-fucose and 200 µCi/ml of D-(6-H)-glucosamine or 25 µCi/ml of ¹⁴C-mannose.

IV - CELLS AND VIRAL EXTRACTS

Cell pellets corresponding to 10⁷ cells were resuspended in 100 µl of the following buffer : 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.2 mM PMSE, 100 units per ml of aprotinin (Iniprol^R, CHOAY) before the addition of 100 µl of the same buffer containing 2% (v/v) of Triton^R X-100. Cell extracts were centrifuged at 12,000 g for 10 minutes and the supernatant was stored at -80°C until used. For the preparation of viral extracts, 100 µl of 10 x lysis buffer (100 mM Tris-HCl, pH 7.6, 1.5 M NaCl, 10 mM EDTA, 10% (v/v) Triton^R X-100, 100 units/ml aprotinin) were added per ml of clarified supernatant of infected CEM cells. These preparations were used as follows.

V - PREPARATION OF AN IMMUNOABSORBENT WITH ANTIBODIES PRESENT IN THE SERUM OF A PATIENT SEROPOSITIVE FOR THE HIV-2 VIRUS.

Immunoglobulins of a serum of a patient seropositive for HIV-2 were precipitated with 50% $(\text{NH}_4)_2\text{SO}_4$ dissolved in 20 mM sodium phosphate (pH 8.0) then purified on a DEAE cellulose column (DE 52, Whatman) by elution with 20 mM sodium phosphate (pH 8.0). These immunoglobulins thus purified were judged to be 90% pure. The antibodies were then coupled to SEPHAROSE^R CL 4B activated by CNBr following the procedure described by Berg (1977). Two milligrams of IgG were coupled per ml of SEPHAROSE^R CL 4B. This immunoabsorbent is designated by the reference "HIV-2 serum Sepharose".

VI - BINDING OF HIV-2 PROTEINS TO AN IMMUNOAFFINITY COLUMN.

Cell extracts of CEM cells producing HIV-2 were first diluted with two volumes of coupling buffer (20 mM Tris-HCl pH 7.6, 50 mM KCl, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton^R X-100, 20% (v/v) glycerol, 7 mM mercaptoethanol, 0.2 mM PMSF, 100 units/ml of aprotinin) before incubation with one volume of "HIV-2 serum-Sepharose". Supernatants of cells producing the HIV-2 virus were treated in the same manner as the cell extracts except that 1/10th of the 10x concentrated coupling buffer was added per volume of supernatant. The coupling step was conducted overnight, then the column was washed adequately with coupling buffer. The proteins bound to the column were eluted by boiling them in an electrophoresis buffer (125 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 2M urea, 20% glycerol, 1% beta-mercaptoethanol). The eluted proteins were analyzed on 7.5% polyacrylamide-SDS electrophoresis gels containing 6 M urea and 0.1% instead of 0.2% bis-acrylamide (w/v).

VII - PREPARATIVE ELECTROPHORESIS

The HIV-2 glycoproteins eluted from the affinity column were analyzed by electrophoresis on a polyacrylamide gel as previously described and the regions of the gel containing the viral glycoproteins were excised by reference to the position of protein markers of known molecular weight (BRL).

The glycoproteins were eluted by incubation for 16 hours at 4°C in an elution buffer (0.1 M NaHCO_3 , 0.5 mM EDTA, 0.05%

(w/v) SDS, 0.2 mM PMSF). The glycoprotein fractions thus obtained were lyophilized and stored in the refrigerator until used.

VIII - TWO-DIMENSIONAL GEL ELECTROPHORESIS

Two-dimensional gel electrophoresis was carried out as described by O' Farrel (1975) with the following modifications : proteins labelled with L-(³⁵S) methionine bound to a column of "HIV-2 serum-Sepharose" were eluted by boiling them in an electrophoresis buffer such as previously described, before dilution in one volume of buffer containing 9.5 M urea, 8% (v/v)-mercaptoethanol, 1.6% (w/v) of ampholines at a pH in the range 6.5-9, 0.4% (w/v) of ampholines at a pH in the range 3-10 and 100 units/ml of aprotinin.

EXAMPLE 1 - CHARACTERIZATION OF THE GLYCOPROTEINS gp300 AND gp140.

The resolution pattern obtained on 2-dimensional gel electrophoretic analysis of the glycoproteins gp300, gp140 and gp125 labelled with ³⁵S- methionine shows that gp300 and gp140 are related. These two proteins exhibit distinct locations on the gel but they possess an identical isoelectric point (pI) for a pH varying from 6.8 to 7.8. These results are reported in Figure 2. This similarity between the proteins gp140 and gp300 suggests that gp300 is a dimeric form of gp140.

The gp125 exhibits less heterogeneity both in the infected cells and in the viral particles and migrates to its isoelectric point at pH values between 6.2 and 6.5.

In the infected cells the presence of minor sub-entities of the gp125 protein having an isoelectric point for pH values located between 7.2 and 7.3 were observed. This latter category of proteins is not present in the HIV-2 virion, that is why it probably does not represent an authentic glycoprotein constituent.

The acidic nature of mature gp125 may be due to the sialic acid attached to certain carbohydrate chains added during the maturation of the envelope glycoprotein.

The envelope glycoprotein gp300 is very stable because it is resistant to ionic (1% SDS) and non-ionic (2% Triton^R X-100) detergents, to urea (2-6 M), to high salt (1M NaCl) and to reducing agents (1% beta-mercaptoethanol). However, it has been possible to

establish that gp300 can be dissociated in a medium at acidic pH to form two glycoproteins gp140. In these experiments proteins bound to an immunoaffinity column were incubated in an acetate buffer at pH values varying between 4 and 7. These samples were then analyzed by electrophoresis on polyacrylamide gel. Figure 3A in which the results are reported shows that a band of gp300 is converted into a band corresponding to the position of gp140 when the sample is incubated at pH 4. Other experiments were performed by using purified preparations of gp300 obtained from preparative electrophoresis gels as previously described. Such denatured samples of gp300 were completely dissociated in an acetate buffer at pH 6.0. The results of these assays are reported in Figure 3B. The efficacy of the dissociation of the purified glycoprotein gp300 is probably due to the lowering of the pH and to the presence of SDS residues in the lyophilized sample, given that the native glycoprotein gp300 bound to the column could not be dissociated in the same buffer at pH values higher than 5.

In a Tris-HCl buffer the dissociation is less effective. At pH 7.5, only a weak dissociation of the gp300 into the gp140 form is observed; however this increases as the pH value is lowered. In a Tris buffer at pH 6.0, the dissociation was about 80% as is shown in Figure 3C. On dissociation of the pure gp300 in another acetate or Tris-HCl buffer no other protein than gp140 could be detected (the experiments were conducted on a 15% polyacrylamide gel). These results indicate that gp300 is a dimeric form of gp140, itself precursor of the HIV-2 envelope glycoprotein. Consequently, it seems probable that for the constitution of the envelope glycoprotein two molecules of gp140 participate in a pH-dependent fusion mechanism.

EXAMPLE 2 - CHARACTERIZATION OF THE OLIGO-SACCHARIDE SIDE CHAINS OF THE HIV-2 GLYCOPROTEINS

Digestion with endo beta-N-acetylglucosaminidase H (endo H) has made it possible to demonstrate the presence in the HIV-2 glycoproteins of oligosaccharides linked to nitrogen (Tarentino et al. 1974). The gp300, the mixture of gp140 and gp125, and gp125 alone were purified by affinity chromatography and preparative electrophoresis (see the experimental procedures). The lyophilized

samples were suspended in an endo H digestion buffer which does not favour the dissociation of gp300 into gp140.

After digestion with endo H, the electrophoretic mobility of the gp300 is lower and corresponds to that of a protein of 200-250 kD. A small fraction of gp300 dissociated into gp140 was digested to give rise to a protein of 80 kD. The results are reported in Figure 4, section gp300. The sample containing the gp140 + gp125 mixture was digested by endo H to give proteins of 90 and 80 kD whereas the gp125 alone was converted into a protein of 90 kD after a similar digestion as the sections gp140/125 and gp125 of the Figure 4 show. Digestion of gp140 and gp125 with endo H leads to products of molecular weights of 80 and 90 kD, respectively.

In fact, by metabolically labelling the cells with ^{14}C -mannose and ^3H -fucose it was shown that mannose is incorporated into gp300, gp140 and gp125 whereas only gp300 and gp125 were capable of incorporating fucose (Figure 5). The fucose residues are usually transferred to oligosaccharide chains during an advanced stage of the glycosylation cycle following the action of processing enzymes of the endoplasmic reticulum and the Golgi apparatus (Kornfeld and Kornfeld, 1985; Fuhrmann et al. 1985).

For these reasons it may be thought that in all probability the low resistance of gp125 to digestion with endo H compared with gp140 is due to the conversion of certain oligosaccharide side chains of the mannose type into complex oligosaccharides during the transformation of the envelope glycopeptide (Kornfeld and Kornfeld, 1985). The fact that gp140 does not contain a fucose residue is in agreement with the fact that it constitutes a precursor of gp300 and gp125.

EXAMPLE 3 - EFFECT OF THE GLYCOSYLATION INHIBITOR TUNICAMYCIN ON THE FORMATION OF THE HIV-2 GLYCOPROTEINS.

All of the glycoproteins bearing oligosaccharide linked to nitrogen receive their oligosaccharide chain $\text{Glc}_3\text{Man}_9\text{-GlcNAc}_2\text{-pp-dolichol}$ as a result of a reaction carried out by a protein-oligosaccharide transferase which catalyses the block transfer of the oligosaccharide chains to asparagine residues (see references in Kornfeld and Kornfeld 1985). Tunicamycin blocks such nitrogen-

bound glycosylation by inhibiting the production of N-acetylglucosamine pyrophosphoryldolichol, the first step in the assembly of lipid-linked oligosaccharides (Li et al. 1978; Heifetz et al. 1979). In the presence of 2 µg/ml of tunicamycin, all of the nitrogen-bound glycosylation of the HIV-2 envelope glycoproteins in the infected cells is blocked. This result is demonstrated by the absence of the incorporation of ^3H -glucosamine into the viral glycoproteins gp300, gp140 and gp125 (Figure 6). Under these experimental conditions, protein synthesis has however not been affected in the infected cells treated with tunicamycin. Such cultures labelled with isotopic ^{35}S -methionine exhibit two major proteins of apparent molecular weight of 200 and 80-90 kD migrating as wide bands (figure 6). The molecular weight of these proteins coincides with the products of digestion of gp300, gp140 and gp125 with endo H (Figure 4), suggesting that the proteins of molecular weights 200 kD, 80-90 kD correspond to non-glycosylated forms of the HIV-2 envelope glycoproteins.

The molecular weight of the protein of 80-90 kD corresponds to the expected molecular weight of the non-glycosylated precursor of the HIV-2 envelope, estimated on the basis of its nucleic acid sequence (Guyader et al. 1987). The protein of 200 kD is probably the dimeric form of the non-glycosylated precursor of the envelope protein. These results confirm that the HIV-2 envelope proteins contain polysaccharide chains linked to nitrogen. In addition to the inhibition of glycosylation, treatment with tunicamycin inhibits the formation and exportation of the envelope glycoprotein since the 80-90 kD protein has not been detected in the extracellular medium, as is shown in Figure 6, line SN.

The results of these experiments suggest that the oligosaccharide chains of the HIV-2 envelope protein are probably implicated in cell transport through the Golgi apparatus. The absence of non-glycosylated forms of the envelope protein in the extracellular medium of cells treated with tunicamycin might possibly be due to its rapid degradation. Many reports have suggested that in general the non-glycosylated form of a protein is more sensitive to the action of proteases than its glycosylated form (Olden et al. 1978. Schwartz et al. 1976). In agreement with these results the low molecular weight proteins present in the cells

labelled with ^{35}S methionine cultured in the presence of tunicamycin probably represent partial degradation products of the non-glycosylated envelope protein (Figure 6).

EXAMPLE 4 - EFFECTS OF THE INHIBITORS OF OLIGOSACCHARIDE PROCESSING ON THE SYNTHESIS AND TRANSFORMATION OF HIV-2 GLYCOPROTEINS

Oligosaccharides ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) bound to the asparagine of glycoproteins undergo extensive modifications or transformation following their attachment to proteins (see Kornfeld and Kornfeld, 1985). The reactions entailing the removal of glucose and mannose from the extremities by enzymes take place in the lumen of the rough endoplasmic reticulum (RER) and in the Golgi apparatus by the action of specific mannosidases and glucosidases.

It is possible to interfere with the transformation of the oligosaccharide chains of the glycoproteins by using specific inhibitors of the glucosidases and mannosidases (reported by Schwarz and Datema, 1984; Fuhrmann et al. 1985). In these experiments different processing inhibitors were used to investigate the localization of the precursors of the HIV-2 glycoprotein and also to study the role of glycosylation in the transformation of the envelope precursor. The inhibitors used are the following : castanospermine, a plant alkaloid which inhibits glucosidase I (Saul et al. 1983); deoxynojirimycin (dNM), a glucose analogue which inhibits glucosidases I and II responsible for the removal of terminal glucose residues (Lemansky et al. 1984); a deoxymannojirimycin (dMM), a mannose analogue which inhibits the reactions catalyzed by mannosidase (Fuhrmann et al., 1984); bromoconduritol (6-bromo-3, 4, 5-trihydroxycyclohex-1-ene) which inhibits glucosidase II (Datema et al., 1982); swainsonine, an indolizidine alkaloid which inhibits mannosidase II of the Golgi apparatus (Tulsiani et al. 1982).

The cells infected by HIV-2 were labelled with the isotope ^{35}S -methionine in the absence or presence of different processing inhibitors and intracellular and extracellular proteins were purified by affinity chromatography (Figure 7). It was checked that the infected cells contain gp300, gp140 and gp125, whereas only gp125 is observed in the extracellular medium (the results are reported in Figure 7, sections "cells" and SN, lines T). In the cells treated with

castanospermine or dNM, a normal level of gp300, the absence of gp125 and a small quantity of a protein of 150 kD which probably corresponds to the glycosylated form of gp140 were observed. Consequently, in such cells the transformation of the envelope glycoprotein was blocked since no gp125 is detected in the extracellular medium despite the production of p25, a core protein of HIV-2 (Figure 7, lines Cast. and dNM).

These results show that the removal of the terminal glucose residues from the oligosaccharide chains of the envelope glycoprotein precursor is necessary for its transformation and cleavage by the cellular protease. Bromoconduritol which acts on glycosidase II also inhibits the normal production of gp125 to 70-90%, but the levels of gp140 and gp300 remained normal (Figure 7, lines Bro.). Unlike castanospermine and dNM (which inhibit the removal of the terminal glucose residue), the treatment with bromoconduritol (which inhibits the removal of the two internal glucose residues) does not completely block the transformation of the HIV-2 envelope glycoprotein. In fact, a low level of gp125 may be detected in the intracellular and extracellular media. This latter result suggests that a low level of removal of mannose can occur without removal of the two internal glucose residues.

Mannosidase inhibitors swainsonine and dMM did not lead to apparent modifications in the intracellular level of gp300, gp140 or gp125 but the extracellular level of gp125 was 50% lower than the corresponding form in the control cells (Figure 7, lines Sw and dMM). Thus although the oligosaccharide has only undergone a deglycosylation the precursor of the glycoprotein is cleaved by proteolytic enzymes producing a protein similar to gp125 but containing more mannose, which probably affects the cellular transport of gp125. The molecular weight of the extracellular glycoprotein produced in the presence of dMM is slightly higher than that of the protein produced in the absence of the inhibitor. This is probably due to a higher concentration of mannose residues in the extracellular protein synthesized by the cells treated with dMM (Figure 7, section SN).

It can be deduced that the effects of the inhibitors of processing enzymes on the transformation of the HIV-2 envelope

glycoprotein are specific since the synthesis and production of p26 of HIV-2 are not affected at all (Figure 7, section SN).

EXAMPLE 5 - EFFECT OF CASTANOSPERMINE AND MONENSIN ON THE TRANSFORMATION OF THE HIV-2 GLYCOPROTEINS.

In order to study the intracellular transformation of the HIV-2 glycoproteins, experiments making use of (pulse-chase) tracers were carried out. The cells infected by HIV-2 were pulse-labelled with radioactive ^{35}S methionine for 15 minutes, then the incorporation of the tracer was chased for 0.5, 1.5 and 3 hours (Figure 8, control). The gp140 is the first protein detected 15 minutes after labelling. During the chase of the incorporation of the radioactive tracer gp300 can be detected after half an hour, whereas gp125 is only detected after 1.5 to 3 hours. The fact that gp300 is observed after the synthesis of gp140, and that gp125 is only detected after formation of gp300 (Figure 8A, lines 1-4) suggests that the dimerization is a intermediate stage necessary for the transformation of the oligosaccharide into a glycoprotein which has reached maturation, the gp125. This suggestion is confirmed by the use of castanospermine which inhibits the removal of the terminal glucose residue of the polysaccharide chains. After 30 minutes of pulse labelling in the presence of castanospermine a protein of 150 kD is detected with the gp300 (Figure 8, Cas., line 1). The protein of 150 kD could correspond to the gp140; the slight increase in the molecular weight of the first precursor is attributed to the presence of glucose residues on the oligosaccharide chains. Thus, the gp140 synthesized in cells infected by HIV-2 represents the precursor of the glycoprotein without the glucose residues. In agreement with this result, the 150 kD protein (gp150) represents the first glycoprotein of the HIV-2 envelope which has still not attained maturation. The removal of the glucose residues in the control cells corresponds to a rapid transformation taking place during or immediately after the cotranslational translocation of the glycoprotein precursors into the endoplasmic reticulum (Lemansky et al., 1984). After 30 minutes of pulse-labelling and a 3 hours chase of the incorporation of the tracer in the presence of castano-spermine, the level of gp150 is gradually reduced whereas the quantity of gp300 increases (Figure 8, Cast.

lines 1-4). Under these experimental conditions the precursor was not cleaved to give gp125.

Another characterization of the HIV-2 envelope glycoprotein was performed during experiments relating to the incorporation of radioactive tracer (pulse-chase) using monensin, a cationic ionophore which inhibits the transport of proteins from the Golgi apparatus towards the plasma membrane or in certain cases blocks the transport of proteins at the cisternae of the median Golgi apparatus (Tartakoff and Vassali, 1977; Johnson and Schlesinger, 1980; Strous and Lodish, 1980; Griffiths et al. 1983). Cells infected by HIV-2 on the one hand in the absence, on the other hand in the presence of monensin were labelled with a radioactive tracer (pulsed) for 30 minutes then the incorporation of the tracer was chased for 0.5, 1.5 and 3 hours (Figures 8B). In the presence of monensin, the cells infected by HIV-2 synthesized normal levels of the gp140 as well as its dimeric form. On the other hand no trace of gp125 could be detected in cells treated with monensin. After 1.5-3 hours of chasing the incorporation of the tracer, the cells treated with monensin have accumulated a protein of 135 kD (gp135) which is probably the dissociation product of the precursor of the dimer. The slightly lower molecular weight of the gp135 compared with the gp140 is due to the removal of certain mannose residues by the action of the mannosidases of the RER and the Golgi apparatus. This gp135 can then be transported into the plasma membrane and cleaved by the cellular protease. The inhibition of protein transport by monensin blocks the glycoprotein gp135 in the "trans" part of the Golgi apparatus. No mature envelope glycoprotein is detected in the cells treated with monensin, whether extracellularly or intracellularly, whereas the core protein p26 is synthesized and excreted.

EXAMPLE 6 - DIMERIZATION OF THE GLYCOPROTEIN PRECURSOR IN THE CELLS INFECTED BY SIV_{mac}

The nucleotide sequence of the HIV-2 envelope glycoprotein shows considerable homology (75% of amino acids identical) to that of SIV (Guyader et al. 1987; Chakrabarti et al. 1987; Franchini et al., 1987). For these reasons investigations were made in order to determine whether the dimerization of the envelope glycoproteins

can also be detected in cells infected by SIV. SIV proteins were purified on an immunoaffinity column containing specific antibodies directed against the HIV-2 proteins as a result of the fact that the gag, pol and env proteins of HIV-2 and SIV exhibit antigenic cross-reactions. Figure 9 shows that the cells infected by SIV synthesize three proteins of high molecular weight, similar to those synthesized in the cells infected by HIV-2 : gp300, gp140 and gp130. The proof that the glycoproteins present in the cells infected by SIV are glycoproteins was given by isotopic labelling with ^{14}C -mannose and ^3H -fucose. These three proteins have incorporated mannose but only the gp300/SIV and the gp130/SIV have incorporated fucose. The gp300/SIV and the gp140/SIV are intracellular proteins whereas the gp130/SIV is an extracellular glycoprotein. the fact that the gp300/SIV and the gp130/SIV are capable of incorporating fucose shows that these proteins are transformation products of gp140/SIV.

These results indicate that formation of the precursor of the envelope glycoprotein as a dimer is a specific property of the expression of the gene of the envelope of HIV-2 and SIV. On the other hand it is possible to maintain that the HIV-1 envelope glycoprotein does not undergo dimerization during its transformation. The cells infected by HIV-1 in the presence of castanospermine or dNM do not accumulate envelope dimer, as is the case for HIV-2 or SIV.

DISCUSSION :

As the examples show, the inventors have succeeded in defining the process of formation of the HIV-2 envelope glycoprotein and they have demonstrated an original mechanism in the synthesis of glycoproteins comprising oligosaccharide chains linked to nitrogen. The HIV-2 envelope glycoproteins, namely the extracellular glycoprotein gp125 and the transmembrane glycoprotein gp36 are derived from a common precursor glycoprotein (Guyader et al., 1987). An unexpected element reported in the invention is that, in order to be transported and transformed in the Golgi apparatus, the glycoprotein precursor implicates the formation of a homologous dimer. The mechanism of

dimerization of the envelope glycoprotein has not been completely clarified. The fact that the dimer purified in the framework of the invention can be dissociated at acidic pH (pH 6.0) suggests that the dimerization might be pH-dependent.

The inventors have demonstrated the fact that the oligosaccharide chains present in the natural precursor of the glycoprotein are not essential for the formation of the dimer. This was demonstrated by two experiments : (1) the digestion with endo H leads to a modification of the electrophoretic mobility of the dimer without its dissociation; (2) in the presence of tunicamycin the cells infected by HIV-2 synthesize a non-glycosylated envelope precursor (80-90 kD capable of forming a dimer (200 kD). These results testify to the fact that the formation of a dimer is an intrinsic property of the polypeptide skeleton of the envelope precursor.

Pulse-labelling experiments with a radioactive tracer, followed by a step involving the removal of this tracer by washing and its replacement by non-radioactive molecules and the chase of the radioactivity in samples taken at different time intervals , with or without castanospermine (Figure 8) show that the dimerization of the precursor of the glycoprotein normally occurs immediately after the removal of the glucose residues. Since the glucosidases are associated with the membranes of the endoplasmic reticulum it may be supposed that the dimerization occurs in the RER. In the presence of castanospermine the dimer is accumulated in the RER and is not transformed. However, when the glucose residues are removed the inhibition of the mannosidases of the RER does not prevent the transformation of the dimeric glycoproteins through the Golgi apparatus (Figure 7). This suggests the hypothesis that the glucose residues of the oligosaccharide chains of the dimeric precursor prevent its expulsion from the RER. In agreement with this, the hypothesis can be put forward that the cleavage of the glucose residues is necessary for efficient transport from the RER to the Golgi, this being possibly due to the fact that the deglycosylated oligosaccharides form a part of the recognition site for a receptor for transport (Lodish and Kong, 1984, Lemansky et al., 1984). It is also possible that the removal of glucose is important so that the dimeric precursor can assume the correct functional configuration

(Schlesinger et al., 1984). This functional configuration would facilitate the processing action of the mannosidases.

In the light of these results the inventors have put forward a scheme showing the transformation of the HIV-2 envelope glycoproteins (Figure 10). The presumed size of the polypeptide skeleton of the precursor of the envelope glycopeptide is about 80 kD (Figures 4 and 6). The oligosaccharide chain is transferred from dolichol-P-P to an envelope precursor (80 kD) probably to amino acids residues of the asparagine type which function as acceptors (Kornfeld and Kornfeld, 1985). Tunicamycin inhibits the assembly of the dolichol-P-P and the glycan and for this reason the 80 kD protein is not glycosylated. The addition of oligosaccharide chains to the 80 kD protein leads to the formation of the first precursor of the envelope glycoprotein, the gp150. This precursor might exist as such or, on the other hand, does not exist in infected cells since addition of polysaccharide chains and cleavage of the glucose residues probably takes place during the translation of the precursor. However that may be, the gp150 is rapidly deglycosylated to lead to the gp140. At this stage an environmental difference, perhaps of pH, would direct the formation of the dimer by fusion of 2 molecules of the gp140. The resulting gp300 can then be cleaved by a mannosidase of the RER and transported to the Golgi apparatus. In the presence of castanospermine or dNM the gp150 is dimerized and accumulates in the RER. This dimer is not transformed because it is glucosylated. However, for as long as the dimer is present in a deglycosylated form it can be transported into the Golgi; the inhibition of the mannosidase of the RER by dMM does not block the production of the dimeric precursor.

In the Golgi, the gp300 passes through the different compartments probably by vesicular transport (Griffiths and Simons, 1986). During this transport the oligosaccharide chain is cleaved by the mannosidases of the Golgi before the addition of other sugars such as fucose and sialic acid. The demonstration of the incorporation of fucose was obtained by isotopic labelling of the gp300 with ^3H -fucose. The demonstration of the incorporation of sialic acid was obtained indirectly by digestion of the gp300 with neuraminidase, an enzyme capable of hydrolyzing the terminal N-acetylneuraminic acids of various glycoproteins (Peyrieras et al.

1983). The gp300 of HIV-2 is capable of being digested with neuramidase as is shown by the significant diminution of the electrophoretic mobility of the dimer. The results are in agreement with the observation that the precursor retains its dimeric form throughout its transport and transformation in the cis, medial and trans cisternae of the Golgi before being transported into the trans-Golgi network (TGN; Griffiths and Simons, 1986) where it is dissociated as a result of the fall in pH in this compartment. The dissociated dimer leads to glycoproteins (gp135) of slightly lower molecular weight than that of the first detected precursor of the glycoprotein (gp150-140). The gp135 might be transported towards the plasma membrane and thus be cleaved by the cellular protease before leading to mature glycoproteins of the HIV-2 envelope, gp125 and gp36. Monensin probably inhibits transport from the Golgi towards the plasma membrane; for this reason the gp135 accumulates in the Golgi.

It is known that the Golgi apparatus is implicated in the sorting mechanism of secretory proteins and proteins of the plasma membrane. This mechanism seems to occur in the terminal Golgi compartment designated as TGN (Griffiths and Simons, 1986). This compartment corresponds to the "trans" side of the Golgi stacks sometimes designated as lysosomes of the Golgi endoplasmic reticulum (GERL) and designated recently as post vacuoles of the Golgi or the innermost cisternae of the Golgi complex (Novikoff, 1976; Saraste and Kuismanen 1984; Orci et al. 1987). In a quite interesting manner the pH of the TGN was studied and it was observed that it was moderately acidic, i.e. about 6 (Anderson and Pathak, 1985; Griffiths and Simons, 1986)-. The acidic pH in the TGN might take part in the dissociation of the dimer formed.

The results previously discussed illustrate the fact that the formation and transformation of the HIV-2 envelope glycoproteins enter into a process comprising several stages implicating in particular the synthesis of an immature precursor gp150-140, the synthesis of an intermediate dimeric precursor or gp300 and, finally, of a mature precursor, gp135. In spite of their relatedness, the retroviruses HIV-1 and HIV-2 have different mechanisms for the formation of their envelope glycoproteins, which may be a quite

advantageous characteristic for distinguishing between an infection due to HIV-1 and an infection due to HIV-2.

1



CLAIMS

1/. Antigenic retroviral glycoprotein characterized in that it is :

- either a glycoprotein called gp300, having a molecular weight of about 300 kD and constituted by the association of two units of a glycoprotein gp140, precursor of the envelope glycoprotein gp125 encoded by the env gene of a human HIV-2 type retrovirus capable of causing a LAS or AIDS,
- or the dimer of the precursor of the envelope glycoprotein of a retrovirus which cross-reacts immunologically with antibodies directed against the said glycoprotein gp300.

2/. Antigenic glycoprotein gp300 according to Claim 1, characterized by the following properties :

- it is present in the cells of a biological sample taken from a patient infected by a human HIV-2 type retrovirus, in particular in human T4 lymphocytes or in permanent lines derived from these T4 lymphocytes,
- its isoelectric point (pI) lies in the pH range 6.8 - 7.8,
- it is glycosylated by oligosaccharide chains linked to nitrogen,
- it is stable to ionic detergents, in particular 1% SDS, non-ionic detergents, in particular 2% Triton^R X-100, 4M urea, strong salts and reducing agents,
- it dissociates spontaneously in vitro into two glycoproteins of a molecular weight of about 140 kD between pH 4 - 5,
- after isolation and purification on gel electrophoresis, it dissociates in acetate buffer in vitro into two glycoproteins of molecular weight of about 140 kD at pH values equal to or less than 6,
- it exhibits one band on electrophoresis in a 7.5% polyacrylamide-SDS gel in the presence of 6M urea, corresponding to a molecular weight of 300 kD after purification on an immunoaffinity column of the Sepharose^R HIV-2 type serum containing specific antibodies obtained from the serum of a patient infected by a human HIV-2 type retrovirus.

3/. Antigenic glycoprotein according to Claim 1 or Claim 2, characterized in that it is not revealed by electrophoretic analysis of a biological medium constituted by viral particles present in the culture medium specific for cells infected by a human HIV-2 type



retrovirus after labelling with ^{35}S -methionine (200 $\mu\text{Ci/ml}$ 4×10^6 cells/ml) for 18 hours and purification of the extracts obtained containing the infected cells and their corresponding culture medium whereas it is revealed under the same conditions in the above-mentioned infected cells.

4/. Protein p200 derived from the glycoprotein according to any one of the Claims 1 to 3, characterized by a non-glycosylated skeleton identical with that of the said glycoprotein having a molecular weight of about 200 kD and differing from the said glycoprotein by the absence of glycosyl groups.

5/. Purified glycoprotein gp150 capable of being detected in vitro in the cells of a patient infected by a human HIV-2 type retrovirus, characterized by :

- a molecular weight of about 150 kD,
- the peptide skeleton of the precursor of the envelope glycoprotein gp125 encoded in the env gene of the genome of a human HIV-2 type retrovirus or of a retrovirus whose external envelope protein is recognized by the antibodies of the serum of a patient infected by the said human HIV-2 type retrovirus, in particular the antibodies directed against gp125,
- terminal glucose residues on an oligosaccharide chain.

6/. Antigenic composition for the diagnosis of the presence of antibodies in the cells of a biological sample taken from a patient infected by a human HIV-2 type retrovirus, characterized in that it comprises a protein or antigenic glycoprotein according to any one of the Claims 1 to 5.

7/. Monoclonal antibody characterized in that it recognizes the glycoprotein according to any one of the Claims 1 to 3, and in that it does not recognize the mature envelope glycoproteins gp125 and gp36.

8/. Monoclonal antibody characterized in that it recognizes an epitope which in the glycoprotein according to any one of the Claims 1 to 3 is exposed and accessible to the antibody, this epitope being however either not exposed or masked in the mature gp125 and gp36 in their natural conformation.

9/. Antibody according to Claim 8, characterized in that it is in addition neutralizing in particular in that it prevents the binding of



a human HIV-2 type retrovirus to its receptor, in particular to the T4 receptor of the human lymphocytes which bear it.

10/. Monoclonal antibody characterized in that it recognizes p200 according to Claim 4, and in that it does not recognize the mature glycoproteins gp125 and gp36.

11/. Monoclonal antibody characterized in that it recognizes an epitope of the p200 according to Claim 4, this epitope being however either not exposed or masked on the gp125 and the gp36 and/or on the gp300 in their natural conformations.

12/. Monoclonal antibody according to Claim 11, characterized in that it is neutralizing, in particular in that it prevents the binding of a human HIV-2 type retrovirus to its receptor, in particular to the T4 receptor of human lymphocytes.

13/. Immunogenic composition containing a glycopeptide according to any one of the Claims 1 to 3 or the p200 according to Claim 4, in combination with a pharmaceutically acceptable vehicle, suitable for the preparation of vaccines.

14/. Immunogenic composition according to Claim 11, characterized in that it is dosed in protein and antigenic glycoprotein according to any one of the Claims 1 to 4 so as to permit the administration of a dose of 10 to 100 µg per kilogram.

15/. Procedure for the preparation of the antigenic glycoprotein according to any one of the Claims 1 to 3 characterized by the following steps :

- lysis of cells infected by a human HIV-2 type retrovirus and separation of the supernatant from the cell extract,
- incubation of the cell extract and/or the supernatant on an immunoaffinity column on which is deposited an immunoabsorbent containing purified antibodies such as those obtained from the serum of a patient infected by a human HIV-2 type retrovirus, these antibodies being advantageously bound to a suitable support in the presence of a buffer and for a sufficiently long to lead to the formation of an antigen-antibody immunological complex,
- washing of the column with a buffer in order to remove the molecules not retained by the support,
- resolution of the glycoproteins eluted,
- recovery of the desired antigenic proteins.



16/. Procedure according Claim 15 for the preparation of the glycoprotein according to any one of the Claims 1 to 3, characterized in that the resolution of the glycoproteins is performed by electrophoresis and by electroelution of the proteins retained on the column.

17/. Procedure according to the Claim 15 for the preparation of the glycoprotein according to any one of the Claims 1 to 3, characterized in that the resolution of the glycoproteins is obtained by

- elution of the proteins bound to the above immunoaffinity column,
- purification of the products thus eluted on a chromatographic column containing as separation support monoclonal antibodies recognizing gp 300 bound.

18/. Procedure for the in vitro diagnosis of the presence of antibodies in the cells of a biological sample taken from a patient for the purpose of verifying a possible infection by a human HIV-2 type retrovirus, characterized by :

- the placing of this biological sample in contact with an antigen according to any one of the Claims 1 to 5 or an antigenic composition according to Claim 6 under conditions permitting the formation of an antigen-antibody type immunological complex,
- the detection of the antigen-antibody complex formed.

19/. Kit for the detection of antibodies in the cells of a biological sample of a patient likely to be infected by a human HIV-2 type retrovirus, characterized in that it contains :

- at least one protein and/or antigenic glycoprotein according to any one of the Claims 1 to 5,
- or a composition according to Claim 6,
- or even a mixture of these different constituents and
- agents permitting the formation of the immunological complex between the above antigens and the antibodies possibly present in the biological sample to be tested, in particular if necessary one or more incubation buffers,
- a negative control sample,
- agents for the revelation of the antigen-antibody complex formed.