

HIV-3 RETROVIRUS STRAINS AND THEIR USE

1

The present invention relates to the field of human immunodeficiency viruses.

More particularly, the present invention relates to new HIV-3 retrovirus strains, compositions comprising total extracts, lysates or antigens of said HIV-3 retrovirus strains, purified antigens of said HIV-3 retrovirus strains, more particularly p12, p16, p25 proteins and gp41 and gp120 glycoproteins to be used in the diagnosis of ARC or AIDS caused by said HIV-3 strains, as well as immunogenic composition containing the same to be used as vaccines.

Substantial progress has been made in our understanding of the acquired immunodeficiency syndrome or AIDS. The principal causative agent has been demonstrated to be a non-transforming retrovirus with a tropism for T4 helper/inducer lymphocytes (1,2) and it has been estimated that millions of people world-wide have already been infected. Infection with this virus leads, at least in a significant percentage of cases, to a progressive depletion of the T4 lymphocyte population with a concomitant increasing susceptibility to the opportunistic infections which are characteristic of the disease.

Epidemiological studies indicate that human immunodeficiency virus type 1 (HIV-1), the etiological agent responsible for the majority of AIDS cases and which is currently the most widely disseminated HIV, probably had its origins in Central Africa (3). The discovery of this virus did not necessarily imply the existence of other types of human immunodeficiency viruses. Nevertheless, a second group of human immunodeficiency-associated retroviruses, human immunodeficiency virus type 2 (HIV-2), was identified in West Africa (4,5). An HIV-2 virus is disclosed in EPO-85/00548. An HIV-1 virus is disclosed in EPO-0 239 425. Other similar, but not identical, retroviruses have also been isolated from simian sources (simian immunodeficiency virus, SIV) such as African green monkeys (6,7) and macaques (8,9). The simian isolates have been shown to be genetically more closely related to HIV-2 than HIV-1 but are nevertheless distinct (10).

One characteristic of human immunodeficiency viruses which complicates their comparison is their genetic variability; genetic variants arise spontaneously and with high frequency. A comparison of various HIV-1 isolates revealed that some regions of the genome are highly variable while others are reasonably well conserved (11-16). Similar polymorphisms have also been observed for HIV-2 (17). The regions with the greatest genetic stability are presumably those regions coding for the regions of viral proteins which are

This Sheet Has Been Replaced



REPLACED SHEETS	
☒ Description	: paged ... 1 ... to paged ... 51 ...
☒ Claim(s)	: paged ... 44 ... to paged ... 65 ...
☒ Drawing(s)	: paged ... 1/1 ... to paged ... 19/19 ...
☒ Abstract	: paged ... 65 ... to paged ... 7 ...
DATE OF RECEIPT	

structurally or enzymatically essential. The viral genes with the greatest overall genetic stability are the *gag* and *pol* genes, while some regions of the *env* gene and the genes coding for regulatory proteins such as *art*, *tat*, *src* and 3'orf exhibit a high degree of variability. Some of the major structural features of the *gag* and *pol* gene products are apparently shared not only by all of the variants of a particular HIV type, but have, at least to some extent, been conserved between virus types. Antiserum produced against HIV-1 crossreacts with the *gag* and *pol* gene products of HIV-2, albeit with a lower affinity than for the corresponding HIV-1 gene products. However, in spite of the demonstrable immunological crossreaction, at the nucleic acid level there is little sequence homology and no significant hybridization between these two viruses can be detected except under very low stringency conditions (17).

A higher degree of relatedness can be demonstrated between SIVagm (STLV-III agm, nearly or completely identical to Human Lymphotropic Virus type 4 (15)) and HIV-2. Immunological crossreaction is not limited only to the *gag* and *pol* gene products but extends to the *env* gene products as well. Nevertheless, genomic analysis of SIVagm and HIV-2 showed them to be genetically distinguishable (19). DNA probes specific for HIV-2, although able to hybridize to SIVagm sequences, hybridize preferentially to HIV-2 (18).

We now report the isolation and partial characterization of a novel human immunodeficiency virus from a Cameroonian woman and her partner. Geographically, this virus comes from a region in Africa located between West Africa where HIV-2 is endemic, and East-Central Africa where HIV-1 is endemic. This isolate is shown immunologically to be antigenically more closely related to HIV-1 than is HIV-2, yet an analysis of partial cleavage products obtained by chemical cleavage of the *gag* and *pol* gene products demonstrate that this isolate is neither HIV-1 nor HIV-2. This novel isolate could represent an evolutionary link between HIV-1 and HIV-2. This novel virus will be referred to as HIV-3 hereinafter.

Accordingly, the invention relates to an HIV-3 retrovirus or variants of this virus having the essential morphological and immunological properties of the retrovirus deposited in the European Collection of Animal Cell Cultures (ECACC) under n° V88060301.

A virus isolation was performed from blood from an asymptomatic Cameroonian woman who is the partner of an HIV-seropositive man with generalized lymphadenopathy. Serum from the woman was moderately positive (ratio O.D./cut-off of 4.5) in the enzyme-linked immunosorbent assay (EIA, Organon Teknika) and had a low titer (1/40) in the





immunofluorescent antibody assay for HIV-1 but gave ambiguous results in the HIV-1 Western blot assay with clear bands at p33, P53/55 and p64 but very weak bands at p24, gp41 and gp120. Virus was isolated by co-cultivation of the woman's lymphocytes with PHA-stimulated lymphocytes from healthy uninfected donors in a medium consisting of RPMI 1640 buffered with 20 mM HEPES (hydroxyethylpiperazine ethanesulfonate) and supplemented with 15 % fetal calf serum, 5 μ g/ml hydrocortisone, 75 u/ml interleukin-2 (IL-2) and 2 μ g/ml polybrene.

After 52 days in culture, virus was detected in the culture as judged by the presence of syncytia and on the basis of positive immunofluorescence observed when a laboratory reference anti-HIV antiserum was incubated with acetone-fixed cells from the culture. The presence of reverse transcriptase was also detected in the culture supernatant (10^4 cpm/ml, 27 X background). Cell-free culture supernatant was used to passage the virus on fresh lymphocytes. After 15 days, CPE was again observed and reverse transcriptase detected in the supernatant. The virus was further propagated in PHA-stimulated lymphocytes from healthy blood donors and was transferred to continuous cell lines of leukemic origin. Virus-containing supernatant was tested in parallel with culture supernatants known to contain HIV-1 in the differential antigen capturing test which is described in detail below. The results of this comparison indicated that the new isolate was not HIV-1.

The new virus was then characterized with respect to its protein antigens and nucleic acids. The cell lines used for propagating the virus can be, depending on the case, lines of the CEM, HUT, Molt-4, or MT4 type, or any other immortalized cell line which bears the T4 receptor on its cell surface.

A preferred cell line for the continuous propagation of HIV-3 is Molt-4. Molt-4 cells infected with HIV-3 were deposited with the ECACC on June 3, 1988 under number V88060301. Establishment of a chronically-infected cell line can, for example, be carried out as follows:

Molt-4 cells (10^6 /ml) and preferably Molt-4 clone 8 cells (obtained from N. Yamamoto, Yamaguchi, Japan) are cocultured with infected human lymphocytes (10^6 /ml) in RPMI 1640 culture medium buffered with 20 mM HEPES and containing 10 % fetal calf serum. Within one to two weeks, a cytopathic effect is observed in the culture which is followed by cell death. A fraction of the cells in the culture survive the infection and produce virus continuously. With continued culturing, these cells increase in number and can

be passaged. Supernatants from these cells can be used as a source of virus.

Furthermore, the invention relates to a purified retrovirus having the essential morphological and immunological properties described below. In many cases, the unique characteristics of HIV-3 can best be appreciated by comparison with the same type of characteristics relating to the other human immunodeficiency viruses, HIV-1 and HIV-2.

This Sheet Has Been Replaced



1. Morphology

Electron microscopy of HIV-3-infected MT4 cells revealed the presence of extracellular virus particles having a diameter of approximately 120 nm and consisting of an outer envelope which surrounds an inner elongated core which has a diameter of approximately 20 to 40 nm and which appears in some thin sections to be slightly cone-shaped in contrast to the more or less cylindrical appearance of the HIV-1 core. Nevertheless, HIV-3 is morphologically very similar to HIV-1 and HIV-2 but is readily distinguished from other human retroviruses such as HTLV-I and HTLV-II.

2. Protein and glycoprotein antigens.

The virus present in the culture supernatant of HIV-3-infected Molt 4 cells was concentrated by precipitation with polyethylene glycol (average molecular weight 6000) followed by centrifugation. The resulting pellet was resuspended in phosphate buffered saline, layered on top of a 20 % sucrose cushion and pelleted at 100,000 g for 1.5 hours. The pelleted virus was then dissociated in 62.5 mM Tris, pH 6.7, containing 2 % 2-mercaptoethanol, 1 % sodium dodecyl sulfate and 10 % glycerol and the principle viral antigens were separated by electrophoreses on a polyacrylamide gel (12.5 %) under denaturing conditions. Molecular weight markers were included on the same gel so as to provide a basis for estimating molecular weights. Once separated, the proteins were electrophoretically transferred to nitrocellulose paper (Western blot) which was then incubated with an antiserum derived from a person infected with an HIV. In the initial experiments, a high titer antiserum was used from an individual who was infected with HIV-1 and which had been previously shown to crossreact with HIV-2 gag- and pol-derived proteins. In this manner, the molecular weights of the HIV-3 gag and pol gene products could be compared with those of HIV-1 and HIV-2.

The apparent molecular weights observed for the HIV-3 proteins are close to those observed for both HIV-1 and HIV-2. Nevertheless, small yet reproducible molecular weight differences between HIV-3, and HIV-1 and HIV-2 proteins are also evident.

The protein blots revealed that HIV-3, like HIV-1 and HIV-2, possesses three core proteins. In the case of HIV-3, these proteins were found to have molecular weights of



approximately 12.000, 16.500 and 25.000 respectively. By convention, proteins are frequently referred to by a "p" for protein or "gp" for glycoprotein, followed by a number which, when multiplied by 1.000, gives the approximate molecular weight of the polypeptide. The three major core proteins of HIV-3 will be referred to hereafter as p12, p16, and p25 respectively.

The molecular weight values as determined are expected to be correct to within 10 % of the true values. Nevertheless, much confusion exists with regards to molecular weight values of proteins since the construction of the electrophoresis apparatus used and the source of the buffer components varies from laboratory to laboratory. It is therefore necessary when comparing the apparent molecular weights of the protein antigens of HIV-3 with respect to those of HIV-1 or HIV-2, to subject all samples to electrophoresis on the same gel. Such a gel can, for example, be seen in Fig. 5. In particular, it is evident that while, in the case of the major core protein, the molecular weight values of the homologous proteins of the three HIVs are very close, the protein derived from HIV-1 is the smallest. The major core protein of HIV-2 is somewhat larger than that of HIV-1, as has been previously reported. The homologous protein from HIV-3 is slightly larger than the major core protein of HIV-2. The calculated molecular weight of these proteins are given in Table 1.



Table 1 - Comparison of molecular weights of gag and pol gene products.

gag				pol		env	
				ENDO	REVERSE TRANSCRIP- TASE**	TRANS MEMBRANE PROTEIN	OUTER MEMBRANE PROTEIN
HIV-1	12 KD	17* KD,	24 KD	31 KD	49 KD/65 KD	gp41	gp120
ANT 70	12 KD	16.5 KD,	24.8 KD	31 KD	48.5 KD/62 KD	gp41	gp120
HIV-2	12 KD	16 KD,	24.3 KD	31 KD	53 KD/69 KD	gp41	gp120

* Some strain to strain variation in molecular weight has been observed for this protein.

** Molecular weights are given for both species of reverse transcriptase.



Similarly, molecular weight differences are also apparent between the three HIVs with respect to the second core protein which has, in most HIV-1 strains, a molecular weight of 18,000. Strain to strain differences in the molecular weight of this protein have, however, been documented in the case of HIV-1, and the molecular weight of this protein may be 17,000 in some isolates. The homologous protein from HIV-2 has a molecular weight of approximately 16,000 while the HIV-3 protein has an intermediate molecular weight of approximately 16,500.

By analogy with HIV-1 and HIV-2, HIV-3 also possesses two forms of the virally encoded enzyme reverse transcriptase. These two species also differ slightly in molecular weight from the corresponding species in HIV-1 and HIV-2 and are characteristic for HIV-3. These molecular weights are also summarized in Table 1.

HIV-3 possesses an additional *pol* gene-derived polypeptide which is an endonuclease with apparent molecular weight of 31,000 and which does not differ significantly in molecular weight from the homologous proteins from HIV-1 or HIV-2.

When protein blots containing HIV-3 proteins are incubated with serum obtained from an individual infected with this virus, two additional proteins can be seen. These proteins are derived from the *env* gene and are the viral envelope glycoproteins. The smallest protein, which is the transmembrane protein, migrates as a broad band with an apparent molecular weight of between 40,000 and 45,000. This protein will henceforth be referred to as gp41, with the understanding that the protein exhibits some intrinsic heterogeneity with respect to its apparent molecular weight and migration on polyacrylamide gels. The larger protein, which is the outer membrane protein, is similarly somewhat diffuse on polyacrylamide gels and has a molecular weight of approximately 120,000. This protein will henceforth be referred to as gp120. It should be noted that the apparent heterogeneous migration of these two species on polyacrylamide gel is not due to heterogeneity in the polypeptide chain but rather in posttranslational glycosylation. In particular, the gp120 is heavily glycosylated and the apparent molecular weight which one observes is to some degree influenced by the cell line used to produce the virus.

In addition to the Western blot, viral protein antigens can also be visualized by radioimmunoprecipitation assay (RIPA). For this purpose, viral proteins can be radioactively labeled metabolically *in vivo* by culturing HIV-3-infected cells in the presence of ³⁵S-cysteine and ³⁵S-methionine (200 μ Ci/ml) in RPMI 1640 medium devoid of these two amino acids

and supplemented with dialyzed fetal calf serum. After 16 hours, the labeled virus is harvested from the culture supernatant by centrifugation over a 20 % sucrose cushion at 100,000 g for 1.5 hours. The resulting pelleted virus is then resuspended in RIPA buffer (20 mM triethanolamine, pH 8.0, 0.5 M NaCl, 0.5 % Nonidet P40, 0.1 % sodium deoxycholate, and 1 mM phenylmethylsulfonylfluoride).

Alternatively, the virus may be radioactively labeled with ^{125}I using chloramine T by the technique familiar to persons versed in the art. In this case, virus is purified from the supernatant of infected cells by pelleting the virus through a cushion of 20 % sucrose, resuspending the virus in phosphate buffered saline and banding the virus by ultracentrifugation on a 20 to 50 % sucrose gradient at 60,000 g for 12 hours. The banded virus can be located in the fractionated gradient either by reverse transcriptase assay or by an antigen capturing assay. The fractions containing virus are pooled and Triton X-100 is added to a concentration of 0.5 %. The Triton X-100-lysed virus may then be iodinated.

For immunoprecipitations, 100,000 -200,000 cpm of labeled viral protein in RIPA buffer is reacted with 5 microliters of a test serum in a volume of 200 microliters for 16 hours at 4°C. The resulting immune complexes are then bound to Protein A-Sepharose (Pharmacia), washed extensively, and the bound proteins eluted with electrophoresis sample buffer containing 1 % SDS. The antigens are subsequently analyzed by electrophoresis followed by autoradiography.

The protein antigens of HIV-3 can be characterized with respect to those of HIV-1 and HIV-2 using two different but related approaches. On one hand, the antigens may be characterized on the basis of their ability to crossreact with antisera from persons infected with HIV-1 and HIV-2. On the other hand, antisera from persons infected with HIV-3, which contain antibodies produced in response to HIV-3 antigens, can be used to test crossreactivity to HIV-1 and HIV-2 proteins. The antigenic relationships between HIV-3, and HIV-1 and HIV-2 are substantially illustrated in the examples given below.

The results of these experiments indicate that HIV-3 is only distantly related to HIV-2 since crossreactivity is only observed with respect to the viral core proteins and *pol* gene products. No crossreactivity of the *env* gene products was observed when anti-HIV-2 antiserum was incubated with HIV-3 proteins or when anti-HIV-3 antiserum was incubated with HIV-2 proteins.

In contrast, HIV-3 is more closely related to HIV-1 since anti-HIV-3 antiserum



crossreacts not only with the *gag* and *pol* gene products of HIV-1 but also to some extent with the *gp41* and *gp120 env* gene products, albeit with a lower affinity. Anti- HIV-1 antiserum similarly crossreacts with all of the protein antigens of HIV-3, but with a lower affinity than for the proteins of HIV-1.

5 In the examples which follow, it is demonstrated that HIV-3 is antigenically substantially different from HIV-1 on the basis of 1.) a different pattern of reactivity with anti-HIV-1 antiserum than that observed for HIV-1, 2.) a drastically reduced ability to be recognized by mouse monoclonal antibodies raised against the HIV-1 p24 and p17 core proteins, and 3.) preferential recognition of HIV-3 proteins, including the envelope proteins,
10 over HIV-1 proteins by antisera from HIV-3- infected individuals.

In spite of the genetic variation characteristic of human immunodeficiency viruses, a test based, for example, on HIV-1 proteins derived from a particular strain will function satisfactorily for detecting antibodies raised in response to other HIV-1 variants. This can, in particular, also be seen in the example in which monoclonal antibodies were tested for
15 their ability to react with antigens derived from HIV-1, HIV-2 and HIV-3 isolates. In this case, the monoclonal antibodies were raised against the core proteins from the HIV-1 IIB strain, yet react very strongly to proteins derived from HIV-1 strain SF4. In contrast, these same monoclonal antibodies react only weakly or not at all with HIV-3 core proteins. This indicates that the antigenic differences between HIV-1 and HIV-3 are of such a magnitude
20 that immunological assays based on the use of HIV-1 proteins will not be suitable for testing sera from individuals infected with HIV-3.

Finally, in the examples given below, differences have been shown in the number and/or positions of methionine and tryptophan residues in the most highly conserved *gag* and *pol* gene products.

3. HIV-3 NUCLEIC ACIDS

A. HIV-3 viral RNA.

30 The RNA of HIV-3 when deposited on a Hybond-H (Amersham) filter according to the "dot blot" technique, did not hybridize to HIV-1 DNA under stringent hybridization



conditions.

By "stringent conditions" or "nonstringent conditions" are meant the conditions under which the actual hybridization and/or the subsequent wash steps are performed.

Dot blot hybridizations were performed by spotting dilutions of viral RNA from HIV-1 strain SF4, HIV-2 rod, HIV-3 strain ANT 70 and HIV-3 strain ANT 70 NA onto Hybond-H filters.

The dilution series for each virus corresponded to viral RNA pelleted from the equivalent of 5, 2.5, 1.25 and 0.62 milliliters of culture supernatant. The RNA was fixed onto the filter by U.V. irradiation for 2 min. and subjected to hybridization by bringing the filter into contact with a ^{32}P -labeled DNA probe. The probe chosen was derived from the HIV-1 sequence spanning nucleotides 487-4652 (Sac I - Hco R1) and includes a portion of the 5' long terminal repeat, the entire gag region and most of the pol gene, subcloned in the vector pUC 13. Hybridization of the ^{32}P -labeled probe with the filter was carried out under stringent conditions in 3 X SSC, 0.5 % milk powder, 1 % SDS, 10 % dextran sulfate, 50 % formamide (volume/volume) at 42°C for 18 hrs (1 X SSC corresponds to 0.15 M NaCl, 0.015 M sodium citrate). The subsequent wash steps were carried out under stringent conditions in 0.1 X SSC and 0.1 % SDS at 65°C (2 - 30 minute washes). The filter was then dried and autoradiographed with enhancing screens at -70°C. Following autoradiography, only spots were visible which corresponded to HIV-1 viral RNA. No hybridization was observed to HIV-2 or either of the two HIV-3 strains. HIV-3 therefore appears to be only distantly related to HIV-1.

B. cDNA and subclones of this cDNA derived from HIV-3.

The conditions under which cDNA corresponding to HIV-3 sequences was synthesized and cloned are described below. HIV-3 (strain ANT 70) from 1 liter of culture was precipitated with polyethylene glycol 6000, redissolved in phosphate buffered saline, and pelleted through a 20 % sucrose cushion. The resulting virus pellet was dissolved in 6 M guanidinium chloride in 20 mM dithiothreitol and 0.5 % Nonidet P-40. CsCl was added to a concentration of 2 molar and the solution containing disrupted virus was layered onto a 1.2 milliliter cushion of 5.7 M CsCl containing 0.1 EDTA. Viral RNA was pelleted by centrifuging for 20 hrs. at 25,000 rpm in a Beckman SW28 rotor at 15°C. The pelleted



RNA was redissolved, extracted with phenol and precipitated with ethanol and 2 M LiCl.

One-fifth of the viral RNA, prepared as described above, was used to direct the first step in the synthesis of cDNA which made use of an oligo (dT) primer which served to prime the synthesis of the first cDNA strand.

5 A commercially available kit supplied by Amersham was used for preparation of HIV-3 cDNA and made use of an exogenously added reverse transcriptase to synthesize the first strand. The synthesis of the second strand was performed using *E. coli* DNA polymerase I in the presence of RNase H to digest away the RNA strand of the RNA/DNA hybrid.

10 Second strand synthesis was performed in the presence of ^{32}p - dCTP to label the cDNA. The resulting cDNA was treated with T4 DNA polymerase to create blunt ends, the cDNA was methylated to protect possible internal EcoRI cleavage sites, and was then coupled to EcoRI linkers, also supplied by Amersham. The EcoRI restriction sites were then cleaved and the cDNA was sized on a 1.2 % agarose gel. The region in the gel corresponding to a
15 cDNA length of 500 to 2000 base pairs was excised and the cDNA was eluted and cloned in the vector pUC13 which had been cleaved with EcoRI and dephosphorylated. The DNA was then used to transform competent cells of *E. coli* MC1016 (lambda). The resulting colonies were transferred to Pall membranes (Pall Biodyne), lysed and denatured with 1.5 M NaCl, 0.5 M NaOH and neutralized with 3 M NaOAc, pH 5.5. Screening of colonies
20 harboring an insert of HIV-3 was performed under moderately stringent conditions in a buffer containing 5 X SSC, 5 X Denhardt's solution, 0.2 % SDS, 250 mg/ml denatured salmon sperm DNA, overnight at 65°C, using ^{32}P -labeled plasmid containing the SacI - EcoRI fragment of HIV-1 discussed above. Following hybridization, filters were washed as follows:

- 25
1. 1 hour in 2 X SSC, 0.1 % SDS at room temperature.
 2. 30 minutes in 0.1 X SSC, 0.1 % SDS at room temperature.
 3. 20 minutes in 2 X SSC, 0.1 SDS at 42°C.
 4. 20 minutes in 0.1 X SSC, 0.1 % SDS at 65°C.

30 Following autoradiography of the filter, several weakly positive colonies were identified which were then grown for analysis. It was expected that the positive signal would either be due to weak homology with the gag or pol regions of HIV-1, or due to some



sequence homology with the R region of the LTR.

C. Sequences contained in HIV-3 cDNA.

A clone carrying the largest insert, which was found to be approximately 1600 base pairs in length and is referred to as iso 70-11, was selected for sequence analysis. A number of subclones of the insert were prepared by digesting the insert with various restriction enzymes and subcloning the resulting fragments in the pUC 13 vector. Sequence determinations were performed according to the dideoxy-method, described by Sanger, (Proc. Natl. Acad. Sci. USA 74: 5463-5467, 1977), using a kit purchased from Boehringer which makes use of 17-mer M13 primers. Sequence analysis of cDNA clone iso 70-11 revealed that the insert corresponded to the 3' end of the viral genome which possessed a poly (A) chain at the 3' end.

The HIV-3 retrovirus contains a 3' LTR which is composed of a U3 region as well as an R region. Like the 3' LTR region of HIV-1, clone iso 70-11 contains an AATAAA polyadenylation signal located approximately 23 nucleotides from the 3' end of the R region. Analysis of the HIV-3 sequence revealed approximately 70 % homology with the corresponding 3' LTR sequence of HIV-1 and less than 55% homology with the corresponding sequence of HIV-2.

The retrovirus as deposited with the ECACC under number V88060301 contains in its env gene a sequence corresponding to the following nucleotide sequence:

10	20	30	40	50
GAATTC	CAAG	TGAAAT	GAAA	GAACCT
AACTC	CTTT	AGCAAT	TTTC	
60	70	80	90	100
ATTGGT	TTAG	CAATTACA	AC	CTTAATT
TAC		TTAATT	TATTG	CTATTT
CAAT				
110	120	130	140	150
GTCAC	TTAAT	GGTGGAT	CAT	TCTTTG
GCTT	CAAAG	ATTAC	ATGAG	TAGTT
160	170	180	190	200
TATTC	AAAAA	TGAAAA	AGCT	GGACGA
ATTG		TTTTT	GGTTT	AATTAAC
ATT				
210	220	230	240	
TGTATT	TTCAA	TTGGT	GTTCT	AGGTATT
TATT		AACGGT	TTTCT	CAATG

(SEQ ID NO 1)



The HIV-3 sequence exhibits less than 60% homology to the corresponding sequence in HIV-1 and HIV-2. The sequence is derived from a portion of the HIV-3 genome which encodes the transmembrane envelope protein.

It was found that the sequences contained in the RNA of the new virus hybridize neither with the *env* gene of HIV-1 nor with the sequences contained in the LTR region of the HIV-1 genome. The Sal I-Bgl II restriction fragment, which contains sequences located in the *env* gene of HIV-3, was excised from the DNA of clone iso 70-11, labeled with ³²P and hybridized under stringent conditions to a filter containing genomic DNA isolated from cells infected with HIV-1 (strain SF2) and HIV-3 (strain ANT 70). Hybridization was only observed with DNA corresponding to the cells infected with HIV-3. Similarly, when the entire iso 70-11 plasmid was labeled and used as a probe under stringent hybridization conditions, the only hybridization was detected with DNA from cells infected with HIV-3. This demonstrates that HIV-3 is only distantly related to HIV-1 and that the clone iso 70-11 is suitable for use as a hybridization probe to distinguish HIV-3 from the other two known human immunodeficiency virus.

Conversely, hybridizations using HIV-1 *gag* - *pol* sequences as the labeled probe to detect crosshybridization with HIV-3 RNA revealed no detectable hybridization when the hybridization was carried out under stringent conditions. This again indicates that the viruses are only distantly related and that a distinction can be made between HIV-1 and HIV-3 at the nucleic acid level in the region of the genome encompassing the *gag* and *pol* genes. This same labeled probe did, however, hybridize to RNA derived from HIV-1 strain SF4.

In addition, the invention relates to a composition comprising at least one antigen, in particular, a protein or glycoprotein of HIV-3 retrovirus. Such a composition can be used in methods for detecting antibodies and in kits for carrying out such methods.

The HIV-3 virus has proven to be usable as a source of antigen for detecting antibodies in people who have come into contact with HIV-3. As such, virus may be grown and concentrated by the methods already described and a lysate prepared by treating the virus with a suitable detergent. A preferred detergent for preparing a total viral lysate is Triton X-100, used at a concentration of 0.5 %. Another preferred detergent is Nonidet P-40 (NP-40), also used at a concentration of 0.5 %.

Alternatively, viral protein may be purified from lysate of the virus. A preferred method for purifying these proteins is affinity chromatography. For example, the viral



antigens may be separated on a preparative polyacrylamide gel and the individual antigens eluted in purified form. These may further be used to raise antisera in, for example, rabbits which is specific for the individual viral proteins. The IgG fraction derived from immune rabbit serum can be coupled to a solid phase such as CNBr-activated Sepharose 4B (Pharmacia) and used to selectively remove individual viral antigens from viral lysates. These proteins may then be eluted from the affinity support using a low pH buffer and further purified using standard chromatographic techniques of which an example is given by Montelaro et al., J. of Virology (1982) 42: 1029-1030.

The invention relates generally to any composition which can be used for the diagnosis of HIV-3 infection or for tests which have a prognostic value. These diagnostic procedures involve the detection of antibody in serum or other body fluid, which are directed against at least one of the antigens of HIV-3.

Preferred compositions are viral lysates or purified antigens which contain at least one of the viral core proteins, p12, p16, and p25 or envelope proteins gp41 or gp120. Especially preferred compositions are those which simultaneously contain, by way of example, the following proteins,

- p25 and gp120
- p25 and gp41
- p25, gp41 and gp120
- p12, p16 and p25
- p25, p31 and gp120

It should be understood however, that the above mentioned compositions are only meant to serve as examples and that the invention relates to all lysates or protein preparations containing one or more of the above mentioned proteins or glycoproteins.

The invention also relates to any composition in which either HIV-3 viral lysate in combination with similarly prepared proteins derived from HIV-1 and/or HIV-2 for the general diagnosis of infection or contact with human immunodeficiency virus without regard to the absolute identity of the virus being detected. For example, such compositions could consist of a mixture of lysates of HIV-1, HIV-2 and HIV-3 or could consist of the following:





- core proteins of HIV-1, HIV-2 and HIV-3, and in particular the major core protein of each virus type, homologous to the HIV-3 p25 protein.
- envelope glycoproteins of HIV-1, HIV-2 and HIV-3 and in particular the outer envelope glycoproteins of each virus type, homologous to the HIV-3 gp120.
- core proteins of HIV-1, HIV-2 and HIV-3 together with the envelope glycoproteins of HIV-1, HIV-2 and HIV-3, in particular the major core protein of each virus type, homologous to the HIV-3 p25 protein, together with the major outer envelope protein of each virus, homologous to the HIV-3 gp120.
- a combination of the core proteins and envelope proteins of HIV-1, HIV-2 and HIV-3 and in particular homologous to the HIV-3 proteins p25 and gp120 respectively and a protein derived from the pol gene of HIV-1, HIV-2 and HIV-3, in particular the proteins of each virus type homologous to the p31 endonuclease protein of HIV-3.

Furthermore, the invention relates to an antigen providing a single band in polyacrylamide gel electrophoresis, said antigen comprising, in common with one of the purified antigens of HIV-3 retrovirus, an epitope that is recognized by serum of persons carrying anti-HIV-3 antibodies. The amino acid sequences corresponding to these epitopes can readily be determined by isolating the individual proteins either by preparative electrophoresis or by affinity chromatography and determining the amino acid sequence of either the entire protein or the fragments produced enzymatically by trypsin or chymotrypsin digestion or chemically by the procedures described in detail below. The resulting peptide or polypeptides can subsequently be sequenced by Edman degradation. The invention relates therefore to any protein, glycoprotein or peptide, either derived directly from the virus or produced by cloning any cDNA fragments of the virus in bacterial expression vectors, or viral expression vectors for the expression of inserted DNA in mammalian or insect cells, and purifying the expressed protein by the methods described above. Furthermore, the invention also relates to synthetic peptides, produced either by Merrifield synthesis or Fmoc chemistry, which may be subsequently purified to homogeneity and which contain in their sequences



epitopes which are shared by the natural HIV-3 antigens.

Antigens which share epitopes with viral proteins may easily be recognized by their reaction with antibodies present in the serum of individual infected with HIV-3, either by Western blotting, or radioimmunoprecipitation. In the case of small peptides which are not able to bind to nitrocellulose, these peptides can be detected by binding to nylon membranes (Pall Biodyne or Amersham) and reacting the membrane with anti-HIV-3 antiserum. In particular, the invention relates to epitopes contained in any of the HIV-3 core proteins, p12, p16 and p25 or in a protein which may contain as part of its polypeptide chain epitopes derived from a combination of the core proteins. Furthermore, the invention relates to epitopes contained in either of the two HIV-3 envelope glycoproteins, gp41 and gp120 as well as any protein which contains, as part of its polypeptide chain, epitopes derived from a combination of the HIV-3 envelope glycoprotein or a combination of the HIV-3 envelope glycoproteins and HIV-3 core protein. The invention additionally relates to polypeptide whose synthesis is directed by expression vectors constructed by recombinant DNA methods which incorporate epitopes derived from HIV-3 proteins or glycoproteins together with epitopes derived from the protein or glycoproteins of either HIV-1 and/or HIV-2 into a single polypeptide chain. Preparing such a construction would involve excising the relevant coding regions from cDNA of HIV-3 as well as HIV-1 and HIV-2, and coupling the DNA in phase so as to form a coding sequence which, when inserted into an expression vector possessing the necessary signal sequences, directs the synthesis of a hybrid protein in which epitopes of the HIV-3, HIV-1 and HIV-2 are contained.

Furthermore, the invention relates to methods for the detection of antibodies against HIV-3 retrovirus in a biological fluid, in particular for the diagnosis of a potential or existing ARC or AIDS caused by HIV-3 retrovirus, characterized by contacting body fluid a person to be diagnosed with a composition containing one or more of the proteins or glycoproteins of HIV-3 or with a lysate of the virus, or with an antigen possessing epitopes common to HIV-3, and detecting the immunological conjugate formed between the anti-HIV-3 antibodies and the antigen(s) used.

Preferred methods include, for example, immunofluorescence assays or immunoenzymatic assays.

Immunofluorescence assay typically involve incubating, for example, serum from the person to be tested with cells infected with HIV-3 and which have been fixed and



permeabilized with cold acetone. Immune complexes formed are detected using either direct or indirect methods and involve the use of antibodies which specifically react to human immunoglobulins. Detection is achieved by using antibodies to which have been coupled fluorescent labels, such as fluorescein or rhodamine.

5 Immunoenzymatic assays may be performed, for example, as follows:

- a specific quantity of HIV-3 virus extract or of a composition referred to according to the invention is deposited in the wells of a microtitration plate.
- the excess unbound material is removed after a suitable incubation period by washing;
- 10 - a suitable dilution or dilutions of serum of other body fluid which is to be tested for the presence of antibodies directed against one or more of the protein or glycoprotein antigen of HIV-3 is introduced into the well.
- the microtitration plate is incubated for a period of time necessary for the binding reaction to occur.
- 15 - the plate is washed thoroughly.
- the presence of immune complexes is detected using antibodies which specifically bind to human immunoglobulins, and which have been labeled with an enzyme, preferably but not limited to either horseradish peroxidase, alkaline phosphatase, or beta-galactosidase, which is capable of converting a colorless or nearly colorless substrate into a highly colored product.
- 20 Alternatively, the detection system may employ an enzyme which, in the presence of the proper substrate(s), emits light.
- the amount of product formed is detected either visually,
- 25 spectrophotometrically, or luminometrically, and is compared to a similarly treated control.

Other detection systems which may also be used include those based on the use of protein A derived from *Staphylococcus aureus* Cowan strain I, protein G from group C *Streptococcus* sp. (strain 26RP66), or systems which employ the use of the biotin-avidin binding reaction.

30

Another method of immunoenzymatic detection of the presence of antibodies directed

against one or more of the HIV-3 antigens is the Western blot. The viral antigens are separated electrophoretically and transferred to a nitrocellulose membrane or other suitable support. The body fluid to be tested is then brought into contact with the membrane and the presence of the immune complexes formed are detected by the methods already described.

5 In a variation on this methods, purified viral antigen is applied in lines or spots on a membrane and allowed to bind. The membrane is subsequently brought into contact with the body fluid to be tested and the immune complexes formed are described using the previously described techniques.

10 The presence of antibodies in body fluid may also be detected by agglutination. HIV-3 lysates or a HIV-3 lysate, antigen or purified antigen composition referred to according to this invention, is used to coat, for example, latex particles which form an uniform suspension.

When mixed with serum containing antibodies to the antigen present, the latex particles are caused to agglutinate and the presence of large aggregates can be detected visually.

15 The present invention also relates to labeled extracts of HIV-3 or compositions as previously described. The labeling can be of any type, such as enzymatic, chemical, fluorescent or radioactive.

Furthermore, the invention relates to a method for detecting the presence of HIV-3 antigens in body fluids. This may, for example, be accomplished in the following manner:

- 20 - the IgG fraction of antiserum, derived either from humans infected with HIV-3 or animals injected with an HIV-3 lysate or composition already described, is placed in the wells of a microtitration plate.
- after a suitable period to allow adsorption, the excess unbound material is washed away.
- 25 - a body fluid containing the antigen to be detected is placed in the well.
- the microtitration plate is allowed to incubate for a suitable period of time to allow binding to occur.
- the plate is then thoroughly washed with a suitable buffer.
- the presence of bound antigen is detected either directly or indirectly, for
30 example, by using immunoglobulins which are similarly specific for the antigen(s) to be detected and which have been labeled, preferably with one of the aforementioned enzymes.





- an appropriate substrate is then added and the extent of reaction is compared to a control in order to measure the amount of antigen present.

Furthermore, the invention relates to a kit for the detection of anti-HIV-3 antibodies in biological fluids, comprising an HIV-3 lysate or a composition as referred to above and a means for detecting the immunological complexes formed.

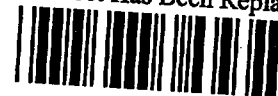
In the case of kits designed to detect specific antibodies by immunoenzymatic methods such, a kit would include:

- an HIV-3 lysate or composition of one of the types already described, preferably in a purified form, and preferably attached to a solid support such as a microtitration plate.
- a conjugate between an enzyme and an immunoglobulin fraction which is capable of binding to the antibodies to be detected, or a conjugate between an enzyme and bacterial protein A or protein G.
- a control antigen which possesses no epitopes which are shared by any human immunodeficiency virus.
- appropriate buffers for performing the assay.
- an appropriate substrate for the enzyme.

Kits for the detection of specific antibodies which make use of labeled antigen would include:

- an appropriately labeled antigen or combination of antigens of the types already described.
- protein A or anti-human immunoglobulins, preferably coupled to an insoluble support, such as Protein A-Sepharose 4B (Pharmacia) or an equivalent support.
- control antigen, which is not recognized by anti-HIV-3 antisera.
- appropriate buffers for performing the assay.
- if appropriate, substrates for the detection of enzymatically labeled antigen.

The invention further relates to kits, developed for the detection of HIV-3 antigens



in biological fluids, which comprise :

- anti-HIV-3 immunoglobulins, preferably coupled to a solid support such as a microtitration plate.
- anti-HIV-3 immunoglobulins conjugated to an enzyme.
- negative control antigen, which would not be recognized by anti-HIV-3 immunoglobulins.
- positive control antigen which consists of one of the HIV-3 antigens or compositions already described.
- appropriate buffers for conducting the test.
- an appropriate substrate for detection of bound enzyme.

Furthermore, the invention relates to an immunogenic composition containing an envelope glycoprotein of HIV-3 retrovirus, in particular, gp41 or gp 120, or a part of said glycoprotein, in combination with a pharmaceutically acceptable vehicle suitable for the constitution of vaccines effective against HIV-3. The invention additionally relates to any peptide or polypeptide which contains within its sequence all or part of the protein backbone of the HIV-3 retrovirus, as well as peptides which result from addition, substitution, or deletion of amino acids which do not affect the general immunological properties of said peptides.

The invention further relates to monoclonal antibodies characterized by their ability to specifically recognize epitopes contained in the HIV-3 antigens or compositions as previously defined, and in particular, monoclonal antibodies raised specifically against said antigens and produced by traditional techniques. The invention also relates to monoclonal antibodies of human origin produced by immortalizing B-cells derived from persons infected with HIV-3, for example, by transforming the B- cells with Epstein-Barr virus and subcloning the transformants.

The invention likewise relates to the production of polyclonal antisera in animals which recognize one or more HIV-3 antigens and which are produced by infecting animals with purified HIV-3, or an HIV-3 antigen, or a combination of antigens, and in particular the proteins or glycoproteins of HIV-3.

The antibodies, either polyclonal or monoclonal, can be used for a wide variety of



purposes which include neutralization of HIV-3 infectivity, the detection of HIV-3 antigens in biological fluids or in infected cells, and the purification of HIV-3 protein and glycoprotein antigens.

The invention further relates to nucleic acids, optionally labeled which are derived in part, at least, from RNA of the HIV-3 retrovirus or of variants of this virus.

The invention relates likewise to the use of cDNA or parts of the cDNA or the recombinants containing them, which are characterized by containing at least a portion of the cDNA corresponding to the entire genomic RNA of the HIV-3 retrovirus. Such cDNAs may be used as probes for the specific detection of HIV-3 sequences in biological fluids, tissues and cells. The probes are preferably also labeled, either radioactively or chemically, alternatively using enzymatic, fluorescent or chemiluminescent labels which enable the probes to be detected. Preferred probes for the specific detection of HIV-3 and diagnosis of HIV-3 infection are probes that contain all or a portion of the cDNA complementary to the HIV-3 genome. In this context, an especially advantageous probe can be characterized as one which contains, in particular, the nucleic acid sequence contained in clone iso 70-11 and which includes the viral LTR-R sequence which is located at both the 5' and 3' ends of viral genomic RNA and at both the 5' and 3' ends of integrated proviral DNA. In addition, sequences corresponding to the viral env gene and which are also included in clone iso 70-11 are also particularly advantageous.

It is nevertheless understood that the probes which can be used for the diagnosis of HIV-3 infection are in no way limited to the probes described above, and that the invention incorporates all sequences which originate from the HIV-3 genome or its naturally occurring variants and includes sequences encoding the viral core proteins (gag gene), the two forms of reverse transcriptase and the endonuclease (pol gene), as well as the two viral envelope glycoproteins (env gene).

The invention also relates to HIV-3 nucleic acid sequences which have been incorporated into a recombinant nucleic acid comprising a nucleic acid from a vector, and having said cDNA or part of said cDNA inserted therein. Such a construction could be used for replicating the viral cDNA or its fragments in an organism or cell other than the natural host so as to provide sufficient quantities of the probe to be used for diagnostic purposes.

A probe generated in such a manner can be employed in a diagnostic test specific detection of HIV-3 which incorporates the following essential steps :



- labeling of the probe generated as described above by the methods previously described.
- bringing the probe into contact under stringent hybridization conditions with DNA from infected cells or viral RNA from infected cells or biological fluids, once said DNA or RNA has been, preferably, applied to a membrane and has been rendered accessible to the probe.
- washing the membrane with a buffer under circumstances in which stringent conditions are maintained.
- detection of the labeled probe, preferably by autoradiography in cases in which the probe has been radioactively labeled, or by a suitable immunodetection technique in case the probe has been labeled chemically.

The invention further relates to a process for the production of HIV-3 retrovirus characterized by culturing human T4 lymphocytes or human lymphocytic cell lines of leukemic origin which carry the T4+ phenotype with lymphocytes or cell lines that have previously been infected with an isolate of HIV-3 retrovirus, as well as recovering and purifying the retrovirus from the culture medium.

The invention likewise relates to a process for the production of antigens of HIV-3 retrovirus, characterized by lysing the retrovirus, preferably with a detergent, and recovering the lysate containing said antigens.

The invention additionally relates to a process for the production of any of the proteins or glycoproteins p12, p16, p25, p31, gp41, gp120 or reverse transcriptase as previously defined, or a part thereof, characterized by inserting the nucleic acid encoding the proteins or glycoproteins in an expression vector, transforming a host with said vector, culturing the transformed host as well as recovering and purifying the expressed protein. The process includes vectors which may or may not direct the synthesis of fusion proteins and includes but is not limited to bacterial expression vectors, mammalian expression vectors such as vaccinia virus, and vectors based on baculovirus for the expression of cloned genes in insect cells.



BRIEF DESCRIPTION OF THE DRAWINGS

In figures 1 to 16, the designations HIV-3 (ANT 70) and HIV-3 (ANT 70 NA) refer to two strains of a new HIV-3 virus isolated from a Cameroonian woman and her partner from which HIV-3 (ANT 70) has been deposited under ECACC V88060301.

Figure 1 shows a procedure for preparing cleavage maps of viral proteins.

Figure 2 shows differential antigen capturing on virus-containing culture supernatants.

Differential antigen capturing was performed as described in the text. The solid line represents the results obtained using the broad-spectrum anti-HIV-1 IgG while the broken line depicts the results obtained using an IgG which was rather specific for HIV-1. The titrations shown in panels A-E are typical for HIV-1. Panel F shows the result obtained with HIV-3 (ANT 70) containing supernatant.

Figure 3 shows differential antigen capturing on HIV-1 and HIV-3 (ANT 70 NA) supernatants.

Differential antigen capturing was performed as described in the text. The solid line depicts the results obtained on plates coated with the broad spectrum anti-HIV IgG while the broken line represents the results obtained on plates coated with IgG which shows less crossreactivity with HIV types other than HIV-1.

Figure 4 shows the reactivity of anti-HIV sera on HIV-1 and HIV-2 Western Blot strips.

The reactivities of 3 different sera on HIV-1 and HIV-2 Western blot strips are shown. Sera: 1. anti-HIV-1, 2. anti-HIV-3 (ANT 70), 3. anti-HIV-2 (isolate 53). The molecular weights indicated are those given by the manufacturer (Dupont Biotech).

Figure 5 relates to the comparison of gag and pol proteins of several HIV-1 isolates, HIV-2rod and HIV-3 (ANT 70).

Proteins were separated electrophoretically and blotted as described. The blot was incubated with a broad-spectrum anti- HIV antiserum followed by (anti-human IgG) / alkaline phosphatase- labeled conjugate to visualize the proteins.



A. HIV-2rod, B. an HIV-1 laboratory isolate, C. HIV-3 (ANT 70), D. an HIV-1 laboratory isolate, E. HIV-1 (SF4).

Figure 6 shows a comparison of HIV-3 (ANT 70) and HIV-3 (ANT 70 NA) proteins.

Proteins were separated electrophoretically and blotted as described. The blot was incubated with the BSR antiserum followed by alkaline phosphatase: anti-human IgG conjugate to visualize the proteins. Lane 1: HIV-3 (ANT 70 NA), lane 2: HIV-3 (ANT 70), lane 3: HIV-1 (SF4). The apparent intensity difference between lanes 1 and 2 is caused by the difference in the amount of material loaded.

Figure 7 relates to the ability of various human anti-HIV-1 sera to capture viral antigens. A number of human sera were diluted 1:1000 and coated directly on microwell plates. Detergent-treated culture supernatants containing HIV-1 (SF4), HIV-3 (ANT 70), HIV-2rod or HIV-2 (isolate 53) were incubated and the bound antigen was detected using a broadspectrum (anti-HIV) / horseradish peroxidase conjugate. Sera 1-7 were of African origin while sera 8-11 were from Europeans. The greater ability of African sera to capture non-HIV-1 antigen can, in part, be explained by their higher anti-p24 titers (data not shown).

Figure 8 shows the effect of coating IgG dilution on the binding of HIV isolates.

Successive 2-fold dilutions were made of four different sera, beginning at a dilution of 1:1000 and were used to coat microwell plates. Detergent-treated supernatants of HIV-1 (SF4), HIV-3 (ANT 70), HIV-2rod and HIV-2 (isolate 53) were diluted to give approximately the same optical density on plates coated with the antiserum shown in panel B at a dilution of 1:1000. Bound antigen was detected using the broad-spectrum (anti-HIV IgG) / horseradish peroxidase conjugate.

Figure 9 shows antigen capturing of virus isolates using human polyclonal and mouse anti-HIV-1 monoclonal antibodies.

Wells were coated and incubated as described in the text. The IgGs used are as follows:

1. human polyclonal anti-HIV IgG , 2. MAb CLB 59, 3. MAb CLB 21, 4. MAb CLB 64, 5. MAb CLB 14, 6. MAb CLB 16, 7. MAb CLB 47, 8. MAb CLB 13.6 (anti-p18), 9. MAb



CLB 19.7, 10. Mab CLB 13.4 (anti-p18).

Figure 10 is a comparison of the reactivity of human anti-HIV antisera to different HIV types.

5 Lysates of HIV-1 (SF4), HIV-3 (ANT 70), HIV-2rod and HIV-2 (isolate 53) were separated electrophoretically on SDS-polyacrylamide gels, blotted onto nitrocellulose, and incubated with a high titer anti-HIV-1 antiserum (panel A), a lower titer anti-HIV-1 antiserum (panel B), serum from the woman from whom HIV-3 (ANT 70) was isolated (panel C), her partner from which HIV-3 (ANT 70 NA) was isolated (panel D) and anti-HIV-2 antiserum from the
10 person from whom HIV-2 (isolate 53) was isolated (panel E).

Figure 11 shows the titrations of anti-HIV sera by enzyme immunoassay.

Microwell plates were coated with lysates of HIV-1 (SF4), ANT 70 and HIV-2 (isolate 53). Serum from an HIV-1-infected European (left panel), antiserum to HIV-3 (ANT 70 NA)
15 (center panel) and antiserum to HIV-2 (isolate 53) (right panel) were titrated in 2-fold dilutions beginning at a dilution of 1:100 on all three coated plates.

Figure 12 shows the positions of methionine and tryptophan residues in viral gag and pol gene products.

20 Amino acid positions for the p17 gag proteins are given starting from the first methionine in the coding sequence. Positions for the p24 gag protein are given starting at the p17/p24 proteolytic cleavage site. Positions for the pol gene are shown after alignment with the highly conserved tryptophan doublet in the HIV-1 sequence at positions 556 and 557. The positions of a conserved protease sequence, the protease/reverse transcriptase cleavage site
25 and the reverse transcriptase/endonuclease cleavage site are indicated. In this case, the terms p24 and p17 are used in the genetic sense to refer to the largest and second largest viral core proteins respectively. The term "HIV-2 (LAV-2)" is a synonym for HIV-2 rod.

Figure 13 is a comparison of partial cleavage products of gag and pol gene products of HIV-1
30 (SF4) (HIV-1 in the figure), HIV-3 (ANT 70) (isolate 70 in the figure) (ANT 70), HIV-2rod (HIV-2 (LAV-2) in the figure) and HIV-2 (isolate 53) (isolate 53 in the figure). The terms p24 and p17 are used in the genetic sense to indicate the largest and second largest viral core



proteins, respectively.

Figure 14 shows hybridization of cDNA probes to viral RNA.

Viral RNA from HIV-1 (SF4), HIV-2rod, and HIV-3 (ANT 70) were spotted onto a membrane filter as described in Materials and Methods. The filters were hybridized under either nonstringent (A) or stringent conditions and autoradiographed.

Figure 15 shows hybridization of HIV-3 (ANT 70) cDNA (iso 70-11) to genomic DNA from uninfected and HIV-infected cells.

Genomic DNA from the cell lines listed below was subjected to restriction enzyme cleavage, separated electrophoretically and blotted. Hybridizations were carried out as described in Materials and Methods. The samples are as follows:

	Lane	DNA	Restriction enzyme digestion
15	A	lambda	Hind III
	B	iso 19 (HIV-1)	Pst I
	C	iso 19 (HIV-1)	Pst I, EcoRI
	D	lambda	Hind III
	E	HIV-3 (ANT 70)	Hind III
20	F	HIV-3 (ANT 70)	Pst I
	G	HIV-3 (ANT 70)	Pst I, EcoRI
	H	HIV-1 (SF4)	Hind III
	I	HIV-1 (SF4)	Pst I
	J	HIV-1 (SF4)	Pst I, EcoRI
25	K	MT4 (uninfected)	Pst I
	L	HUT 78 (uninfected)	Pst I

Panel A : Hybridization with iso 70-11

Panel B : Hybridization with iso 70-11 Bgl II/Sal I fragment.



Figure 16 relates to the location of a number of restriction enzyme cleavage sites in the HIV-3 insert contained in iso 70-11 DNA.

The fragment contains no internal cleavage sites for EcoRI, Bam HI, Hinc II, or Xba I.



EXAMPLES

MATERIALS AND METHODS

5 Virus and cell culture

a. Virus strains and cell lines.

HUT-78 cells chronically infected with ARV-4 (HIV-1 SI¹⁴), originally isolated by J. Levy, San Francisco, U.S.A. (20) and uninfected HUT-78 cells were kindly provided by S. Sprecher, Brussels, Belgium. LAV-2rod originally from L. Montagnier, Paris, and CRM cells were obtained from J. De Smeyter, Louven, Belgium. Isolate 53, an HIV-2 isolate, was isolated in this laboratory (21).

b. Virus isolations.

15 Virus isolations were performed in a manner similar to that described by Levy and Shimabukuro (22), with modifications. Lymphocytes from patients as well as healthy donors were isolated from heparinized whole blood on Lymphoprep (Nyegaard and Co., Oslo, Norway) and were cultured in RPMI 1640 containing 20 mM HEPES, 15 percent fetal calf serum (Gibco), 5 µg/ml hydrocortisone (Merck), 75 U/ml IL-2 and 2 µg/ml polybrene (Aldrich).

20 Lymphocytes from healthy donors were stimulated with 2 µg/ml phytohemagglutinin (PHA, Wellcome) for 3 days prior to use. Fresh PHA-stimulated lymphocytes were added to the virus isolation cultures every 3 to 4 days. Cultures were monitored for cytopathic effect, immunofluorescence, using a broad specificity, polyclonal reference antiserum (23), and the presence of antigen in the culture supernatants (Innotest VCA- HIV, Innogenetics). The broad specificity reference (BSR) anti-serum used was derived from an HIV-1-infected donor and was shown experimentally to have an exceptionally high titer $\geq 1,000,000$ in an enzyme immunoassay based on recombinant HIV-1 p24 protein) and to crossreact strongly with the gag and pol gene products of other HIV types, in particular, HIV-2. Reverse transcriptase was also assayed essentially as described (24).

30 In order to establish chronically infected, permanent cell lines, virus-infected primary



lymphocytes were co-cultured with Molt 4 clone 8 cells (25), kindly provided by N. Yamamoto, Yamaguchi, Japan, and monitored for cell growth. Virus production was monitored by the reverse transcriptase assay as well as antigen capturing.

5 Differential antigen capturing

A test system was developed whereby a distinction can be made between HIV-1 and other related human immunodeficiency viruses. The system is based on a comparison the ability of two different polyclonal IgG preparations, one with a broad anti-HIV specificity which is due to its exceptionally high titer, particularly against the major core protein, and
10 one with a lower titer which reacts preferentially with HIV-1, to capture detergent-treated virus in culture supernatants. Detection of captured antigen is achieved by using a (broad specificity IgG) / horseradish peroxidase conjugate.

The test detects primarily but not exclusively the p24 core protein.

15 Monoclonal antibodies to HIV-1

The panel of monoclonal antibodies used has been described (26). The antibodies were prepared against native viral proteins in Triton X-100-disrupted HIV-1 preparations.

Protein analysis

20 a. Electrophoresis.

Polyacrylamide gel electrophoresis of viral proteins was performed essentially as described by Maizel (27).

25 b. Protein blotting.

Blotting was performed either in a Bio-Rad transblot cell at 400 mA for 4 hours using the carbonate buffer described by Dunn (28) or using the LKB semi-dry blotting apparatus at 0.8 mA/cm² for 1 hour in 48 mM Tris, 39 mM glycine, 0.0375 % sodium dodecylsulfate
30 (SDS) and 20 % methanol.

c. Generation of partial cleavage products.



Viral proteins were analyzed by the technique shown in Figure 1. Advantage was taken of the fact that corresponding proteins from the various isolates have similar molecular weights. Proteins were separated on 12.5 percent SDS- polyacrylamide gels together with a marker lane of ARV-4 proteins which was excised following electrophoresis, blotted and incubated with an anti-HIV antiserum to reveal the positions of the viral proteins. The marker blot was in turn used to locate the approximate positions in the Coomassie blue stained portion of the gel of the viral proteins to be cleaved. Horizontal gel slices containing the proteins were excised, transferred to glass tubes and subjected to chemical cleavage.

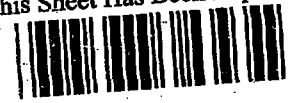
1. Cyanogen bromide cleavage

The gel slice was incubated with 10 ml of a freshly prepared 40 mg/ml solution of CNBr (Merck) in 0.3 N HCl for 3 hours at room temperature in a fume hood. Following the incubation, the gel slice was equilibrated with SDS-sample buffer for electrophoresis in the second dimension.

2. BNPS-Skatole cleavage.

The gel slice was incubated with 10 ml of a freshly prepared saturated solution of 2-(-2'-nitrophenylsulfenyl)- 3-methyl-3'-bromoindoline (BNPS-Skatole, Pierce) in 70 percent acetic acid; 30 % H₂O containing 0.1 % phenol, for 3 hours at room temperature, protected from light. Following the incubation, the gel slice was equilibrated by repeated washing in SDS-electrophoresis sample buffer.

Following cleavage, the individual lanes were excised from the gel slices, rotated 90° and placed on top of a 10 to 20 percent SDS-polyacrylamide gradient gel. On completion of electrophoresis, the gel was blotted onto nitrocellulose (Schleicher and Schuell) and blocked with PBS containing 1 mg/ml casein (Merck). Only cleavage products with molecular weights in excess of 10 kD are able to be visualized since peptides with lower molecular weights do not bind efficiently to nitrocellulose. Blots were incubated with a broad spectrum anti-HIV antiserum followed by goat anti-human IgG: alkaline phosphatase conjugate (Promega). Partial cleavage products were then visualized by reaction with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium (Sigma).



Viral Nucleic Acids

a. Hybridization to viral RNA.

Virus from culture supernatants was harvested by pelleting through cushions of 20 % sucrose by centrifugation at 26,500 rpm for 1.5 hrs. at 4°C and was disrupted in 10 mM Tris, pH 7.4, 10 mM NaCl, 10 mM EDTA containing 0.5 % sodium dodecylsulfate. Aliquots of the disrupted virus were spotted onto a membrane of Hybond II (Amersham) in amounts corresponding to 5, 2.5, 1.25 and 0.62 milliliters of original culture supernatants. The RNA deposited onto the filter was fixed to the membrane by irradiation with ultraviolet light for 2 min. The RNA bound to the filter was then subjected to hybridization with an HIV-1 cDNA probe which had been labeled by nick translation with ³²p-dCTP. The hybridization was carried out under stringent conditions in 3 X SSC, 0.5 % milk powder, 1 % SDS, 10 % dextran sulfate and 50 % formamide at 42°C for 18 hrs. Following hybridization, the filter was washed twice under stringent conditions in 0.1 X SSC and 0.1 % SDS for 30 minutes. Hybridization was detected by autoradiography at -70°C with enhancing screens. Hybridizations were similarly performed using a probe derived from the env region of HIV-2.

Hybridizations were also performed under nonstringent conditions in 5 X SSC, 25 % formamide, 5 X Denhardt's solution, 10 % dextran sulfate, and 100 µg/ml denatured salmon sperm DNA at 37°C overnight. The filter was subsequently washed 4 times for 15 minutes in 5 X SSC, 0.1 % SDS at room temperature and autoradiographed.

b. Preparation of ANT 70 cDNA

Virus was pelleted from 1 liter of culture supernatant using polyethylene glycol 6000, redissolved in PBS and pelleted through a 20 % sucrose cushion. The resulting pellet of virus was disrupted in 6 M guanidinium chloride in 20 mM sodium phosphate buffer, pH 6.5, containing 20 mM dithiothreitol and 0.5 % NP-40. Solid CsCl was added to a concentration of 2 molar. The solution containing disrupted virus was layered onto a cushion of 5.7 M CsCl containing 0.1 M EDTA and the viral RNA was pelleted by centrifugation at 25,000 in a Beckman SW 28 rotor at 15°C for 20 hrs. Following centrifugation, the RNA was redissolved, extracted with phenol and precipitated with ethanol and 2 M LiCl.



One-fifth of the viral RNA prepared was used to direct the first step in the synthesis of cDNA using a kit supplied by Amersham. cDNA synthesis was primed using oligo (dT).

The synthesis was carried out using the reverse transcriptase supplied with the kit. Second strand synthesis was performed using *E. coli* DNA polymerase I in the presence of RNase H to digest away the RNA strand of the RNA/DNA hybrid. The synthesis of the second strand was performed in the presence of ^{32}p -dCTP to label the cDNA. The resulting cDNA was treated with T4 DNA polymerase to create blunt ends, the cDNA was methylated to protect possible internal EcoRI cleavage sites, and was then coupled to EcoRI linkers (Amersham). The EcoRI sites in the linkers were then cleaved and the cDNA was sized on a 1.2 % agarose gel. The region of the gel corresponding to a cDNA length of 500 to 2000 base pairs was excised, and the cDNA was eluted and cloned in the vector pUC13 which had previously been cleaved with EcoRI and dephosphorylated. After ligation, the DNA was used to transform competent cells of *E. coli* MC1016 (λ). The resulting colonies were transferred to Pall membrane filters (Pall Biodyne), lysed and denatured with 1.5 M NaCl, 0.5 M NaOH and neutralized with 3 M NaOAc, pH 5.5. Screening of colonies harboring an insert of HIV-3 was carried out by hybridization under moderately stringent conditions in 5 X SSC, 5 X Denhardt's solution, 0.2 % SDS, 250 mg/ml denatured salmon sperm DNA overnight at 65°C. Hybridization was performed using the HIV-1 SacI-EcoRI fragment. Following hybridization, the filters were washed as follows:

1. 1 h. in 2 X SSC, 0.1 % SDS at room temperature.
2. 30 minutes in 0.1 X SSC, 0.1 % SDS at room temperature.
3. 20 minutes in 2 X SSC, 0.1 % SDS at 42°C.
4. 20 minutes in 0.1 X SSC, 0.1 % SDS at 65°C.

After washing, the filters were autoradiographed at -70°C using intensifying screens.

Hybridizations were also performed under the nonstringent conditions used for nonstringent hybridization of the HIV-1 and HIV-2 probe.

c. Analysis of cDNA clones.

Colonies giving a positive hybridization signal were grown for analysis. Plasmids

were isolated, cleaved with EcoRI and subjected to agarose gel electrophoresis to confirm the presence of an insert and to determine its size. Of 96 colonies analyzed 17 were found to contain inserts. Five were taken for further analysis and ranged in size from approximately 800 to 1600 base pairs in length.

d. Sequence determinations.

Nucleotide sequence determinations were performed according to the dideoxynucleotide method of Sanger (Proc. Natl. Acad. Sci. USA 74: 5463-6467, 1977), using a kit supplied by Boehringer. Sequencing was carried out using 17-mer M12 primers.

e. Hybridizations of ANT 70 cDNA to HIV-1 and HIV-2 viral RNA.

The ANT 70 cDNA clone containing the largest insert (iso 70-11) was used for hybridization to the filter onto which viral RNAs had been deposited.

Hybridization was performed under stringent conditions in 3 X SSC, 0.5 % milk powder, 1 % SDS, 10 % dextran sulfate, and 50 % formamide at 42°C for 18 hrs. Following hybridization, the filter was washed with 0.1 X SSC, 0.1 % SDS at 65°C (2-30 minute washes) after which the filter was autoradiographed at -70°C with an intensifying screen.

Genomic DNA was also prepared from cells infected with HIV-1 (SF4), isolate ANT 70, as well as uninfected control cells. Following restriction enzyme cleavage, the genomic DNA was separated on a 0.8 % agarose gel, transferred to a Hybond N membrane (Amersham) and was then hybridized with 32p-labeled DNA from clone iso 70-11 under stringent conditions in 50 % formamide, 10 % dextran sulfate, 3 X SSPE (1 X SSPE is 180 mM/NaCl, 10 mM NaH₂PO₄, pH 7.4, 1 mM EDTA, pH 7.4), 1 % SDS and 0.5 % milk powder. Following hybridization, the filter was washed as follows:

- 15 minutes with 2 X SSC, 0.1 % SDS at room temperature.
- 15 minutes with 0.5 X SSC, 0.1 % SDS at room temperature.
- 15 minutes with 0.1 X SSC, 0.1 % SDS at room temperature.
- 30 minutes with 0.1 X SSC, 1 % SDS at 52°C.





The filter was then autoradiographed at -70°C using an intensifying screen.

RESULTS

5

Virus isolation

10

15

20

25

As part of a continuing study on heterosexual transmission of HIV, a virus isolation was performed from blood from a Cameroonian woman and her partner. The woman is the partner of an HIV- seropositive man with generalized lymphadenopathy. Serum from the woman was moderately positive (ratio O.D./cut-off of 4.5) in the enzyme-linked immunosorbent assay (EIA, Organon Teknika) and had a low titer (1/40) in the immunofluorescent antibody assay for HIV-1 but gave ambiguous results in the HIV-1 Western blot assay with clear bands at p33, P53/55 and p64 but very weak bands at p24, gp41 and gp120. The woman had elevated serum IgG and IgM levels and a CD4/CD8 ratio of 0.46. Virus was isolated by co- cultivation of the woman's lymphocytes with PHA-stimulated lymphocytes from healthy uninfected donors. After 52 days in culture, virus was detected in the culture as judged by the presence of syncytia and on the basis of positive immunofluorescence observed when a laboratory reference anti-HIV antiserum was incubated with acetone-fixed cells from the culture. The presence of reverse transcriptase was also detected in the culture supernatant (10^4 cpm/ml, 27 X background). Cell-free culture supernatant was used to passage the virus on fresh lymphocytes. After 15 days, CPE was again observed and reverse transcriptase detected in the supernatant. A comparison of detergent-treated culture supernatant from this isolate (ANT 70) with other isolates by differential antigen capturing revealed, however, that this isolate was not HIV-1.

30

These results are illustrated in Figure 2. It is evident by the lower O.D. values that ANT 70 is, in contrast to the other isolates, poorly recognized by the HIV-1 specific IgG but, like the other isolates, was readily captured by the broad specificity IgG (panel F). The other isolates, which were subsequently all shown to be HIV-1 strains using an HIV-1 specific MAb (CLB MAb 14), all gave higher O.D. values on the plates coated with specific IgG than on plates coated with the broad specificity reference IgG.

An attempt was made to transfer the virus to a permanent cell line by co-cultivating



ANT 70-infected primary lymphocytes with Molt 4 clone 8 cells. In the initial phase of the infection, extensive cytopathic effect was observed with syncytium formation and cell death. Within several weeks, cell growth was detected. The cells gave a positive immunofluorescence when tested using a broad spectrum anti-HIV antiserum and the presence of antigen and reverse transcriptase was easily detectable in the culture supernatant.

Virus was similarly isolated from the partner of the woman from whom ANT 70 was isolated (strain ANT 70 NA). The man was suffering from lymphadenopathy and was classified as class 3 according to the CDC classification system. The man also had elevated serum IgM and IgG levels and a CD4/CD8 ratio of 0.4. Virus was detected in the supernatant of the culture on day 18. Detergent-treated supernatant containing this virus was also analyzed by differential antigen capturing and found to react in a manner similar to ANT 70 (Figure 3). The binding of antigen derived from this isolate was again less with HIV-1 specific IgG than with the broad specificity IgG.

Serum from the person from whom this isolate was derived was incubated with HIV-1 and HIV-2 Western blot strips (Biotech). Additional strips were also incubated with serum from a donor infected with HIV-1 as well as serum from the person from whom HIV-2 (isolate 53) was isolated. These results are shown in Figure 4. Serum from the person infected with ANT 70 NA crossreacted to a significant extent with virtually all HIV-1 proteins, including the envelope proteins. In contrast, serum from the HIV-2-infected individual crossreacted only with the gag p24 protein, p34 endonuclease and p68 reverse transcriptase. The anti-HIV-1 serum recognized only the p26 gag protein of HIV-2, while serum from the carrier of ANT 70 NA isolate recognizes this protein and the HIV-2 reverse transcriptase.

Characterization of viral proteins

Virus in the culture supernatant was precipitated using polyethylene glycol 6000 (Merck) and the resulting material was redissolved and pelleted through a 15 percent sucrose cushion. The pelleted virus was dissociated in SDS-sample buffer and analyzed by polyacrylamide gel electrophoresis followed by protein blotting. The blot, shown in figure 5, was incubated with a broad specificity anti-HIV serum to reveal the viral proteins.

In addition to reacting with all of the HIV-1 viral proteins, the BSR antiserum also

crossreacts with the gag and pol gene products of HIV-2. This antiserum clearly recognizes the gag and pol gene products of ANT 70 as well. It is evident that the molecular weights of the ANT 70 gene products differ from those of either HIV-1 or HIV-2. The molecular weights of the various viral proteins are summarized in table 1. The variability in the HIV-1 p17/p18 protein is due to a 6 amino acid insertion which is present in some strains between positions 120 and 121 in the HIV-1 HXB2 sequence. A comparison of the proteins from ANT 70 and ANT 70 NA are shown in figure 6. The molecular weights of all of the proteins of ANT 70 NA identical to those of ANT 70.

In order to investigate further the antigenic relationship between HIV-1, HIV-2 and ANT 70, a series of African and European anti- HIV-1 sera were diluted 1:1000 and used to coat microwell plates for antigen capturing.

Detergent-treated culture supernatant containing HIV-1, ANT-70, HIV-2 (LAV-2rod) and HIV-2 (isolate 53) were diluted and the ability of each antiserum to capture the four different isolates was analyzed. Representative results are shown in Figure 7. It can be seen from this experiment that the ability of the various sera to capture HIV-1 is in no way related to their ability to capture either HIV-2 or ANT 70. In contrast, the ability of these sera to capture LAV-2rod, the prototype HIV-2 strain, is strongly correlated with the ability of these sera to capture isolate 53, which is also an HIV-2 strain but an independent isolate. These data indicate that ANT 70 is neither HIV-1 nor HIV-2.

In a series of related antigen capturing experiments, four African anti-HIV-1 sera were chosen in order to assess their ability to bind HIV-1, ANT 70, HIV-2 (LAV-2rod) and HIV-2 (isolate 53) when the IgGs were coated at different dilutions. Culture supernatants were diluted so as to give approximately the same optical density when captured on plates coated with the IgG used in panel B of figure 8. Dilutions of the four sera were coated and virus- containing supernatant was added. The assumption was made that similar viruses should give rise to similar titration curves. Indeed, in Figure 5, LAV-2rod and isolate 53 both react similarly with the coated IgGs. On the other hand, ANT 70 gave more intense signals at higher IgG dilutions than did either of the HIV-2 isolates and the shapes of the curves obtained with ANT 70 resemble more closely the curves obtained for HIV-1, except that the optical densities are consistently lower.



Cross reactivity of mouse monoclonal antibodies directed against HIV-1 p24 core protein

A panel of mouse monoclonal antibodies (MAbs) prepared against the HIV-1 p24 core protein was tested for their ability to crossreact with ANT 70 and HIV-2 isolates. In principle, any panel of anti- HIV-1 p24 monoclonal antibodies can be used, as long as the series includes monoclonal antibodies which react with different epitopes on the HIV-1 p24 molecule. Ascites fluid containing the antibodies was diluted and used to coat microwell plates. Detergent-treated, virus-containing supernatants were then added to the coated wells. Bound antigen was detected using BSR-HIV IgGs conjugated to horseradish peroxidase. The results obtained are shown in Figure 9.

In control wells coated with polyclonal broad spectrum IgGs, all virus-containing supernatants gave optical densities which exceeded the limits of the microwell plate reader. However, when tested in wells coated with the various monoclonal antibodies, quite a different pattern emerged. Previous studies indicated that all of the MAbs tested react against different epitopes on the p24 molecule with the exception of MAbs CLB 59 and CLB 21 which have been shown to recognize the same epitope. Both of these two MAbs react strongly with HIV-1 as expected and also give a measurable signal with ANT 70 but fail to react with either of the HIV-2 strains. Two other MAbs, CLB 64 and CLB 14, bound HIV-1 well and showed a weak affinity for ANT 70 as well as the two HIV- 2 isolates. In particular, MAb CLB 14 has been shown to recognize all HIV-1 isolates well (> 150 tested). This MAb must therefore bind to a very highly conserved epitope, remnants of which can also be detected in other human immunodeficiency viruses. The other MAbs to p24 (CLB 16, 47 and 19.7) and two others which were raised against the HIV-1 p18 protein (CLB 13.4 and CLB 13.6), failed to recognize either ANT 70 or the two HIV-2 isolates but did capture the corresponding HIV-1 antigens.

Reaction of human anti-HIV antisera to viral proteins

Protein blots of viral proteins from HIV-1 (ARV 4), ANT 70 and HIV-2 (LAV2rod) were prepared after electrophoresis of detergent- solubilized extracts and incubated with various human sera (Figure 10). Panel A shows the reaction of the broad specificity laboratory reference serum with the three virus isolates. In panel B, an anti-HIV-1



antiserum was incubated with the blot and recognizes preferentially HIV-1 proteins. Serum from the woman from whom ANT 70 was isolated (panel C) and her partner (panel D) were tested for their ability to recognize other viral isolates. Both of these sera preferentially recognize ANT 70 including the gp120 envelope protein of this virus. Serum from the partner has a higher titer than serum from the woman from whom ANT 70 was isolated and recognizes the gp41 of HIV-1. Both of these sera have a higher affinity for ANT 70 than for HIV-1 or the HIV-2 isolates. In contrast, serum from the person from whom HIV-2 isolate 53 was isolated binds preferentially to HIV-2 proteins and recognizes the HIV-2 gp120 envelope protein of this virus as well as the gp41 transmembrane protein (panel E). It does not react with glycoproteins of HIV-1 or ANT 70. These results further indicate that ANT 70 is different from either HIV-1 or HIV-2.

Enzyme immunosorbent assays using coated viral proteins titrations of anti-HIV-1, anti-ANT 70 and anti-HIV-2 sera were performed in microwell plates coated with HIV-1 (ARV-4), ANT 70 and HIV-2 (isolate 53) viral lysates. Two-fold dilutions of each sera, beginning at an initial dilution of 1:100, were tested for their ability to bind to the coated antigen. Bound antibody was detected using a horseradish peroxidase-labeled goat anti-human IgG conjugate. These results are shown in Figure 11. The anti-HIV-1 serum recognized preferentially the HIV-1 proteins but shows a significant amount of crossreaction with ANT 70 proteins. The HIV-2 proteins were barely detected. In contrast, anti-ANT 70 serum preferentially recognized ANT 70 proteins, showed crossreactivity toward HIV-1 proteins, and reacted better with the HIV-2 coated wells than did the anti-HIV-1 serum as evidenced by the higher optical density values obtained. The anti-HIV-2 serum had a very low titer but nevertheless reacted best with HIV-2 proteins. No detectable signal was observed on HIV-1 or ANT 70 coated wells. The inability to detect crossreaction in this instance is undoubtedly related to the low anti-HIV titer of this serum.

Analysis of partial chemical cleavage products of viral proteins

The two reagents used for chemical cleavage, cyanogen bromide and BNPS-skatole, were chosen because of their high specificities for methionine (29) and tryptophan (30), respectively. These two amino acids are also rather hydrophobic and are therefore also less likely to be found located in epitopes on the outer surfaces of protein molecules (31).



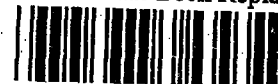
Examination of published amino sequences of the *gag* and *pol* gene products of HIV-1 (32-36), HIV-2 (19), SIVagm (10), SIVmac (9), equine infectious anemia virus (EIAV, 37) and Visna (38) reveals that while there is little amino acid homology between some of these diverse isolates, many of the positions of the methionine residues in these proteins and, to an even greater extent, the tryptophan residues, are strikingly conserved (figure 12). Furthermore, intraspecies variation in these residues is minimal or absent, at least in the case of HIV-1 (36) and probably holds true for all of the human and simian immunodeficiency retroviruses.

The partial digestion patterns of the *gag* and *pol* gene products of HIV-1, ANT 70, HIV-2 (LAVrod) and HIV-2 (isolate 53) are shown in Figure 13.

Inspection of the CNBr cleavage patterns of the p24 protein from the four isolates reveals that the patterns generated for HIV-2 (LAV-2rod) and HIV-2 (isolate 53) are identical.

Different patterns, however, are observed for HIV-1 and for ANT 70. Thus, significant differences exist in the locations of the methionine residues in the major core protein of HIV-1, ANT 70 and HIV-2. In the case of the p17 core protein, differences are observed between the two HIV-2 isolates. Inspection of the published sequence for HIV-2rod indicates that there is a methionine located 18 amino acids from the carboxyl terminus of this protein. We conclude that this methionine must be absent in the corresponding protein from isolate 53. From the cleavage pattern it is also possible to deduce the presence of a methionine near (10-15 amino acids) one of the termini of the p16 from ANT 70. CNBr cleavage of the retroviral reverse transcriptase reveals that again, the proteins from the two HIV-2 isolates are identical, while different patterns are observed for both HIV-1 and ANT 70 proteins. In the case of the p31 endonuclease derived from the 3'-portion of the *pol* gene, similarities can be deduced between all of the isolates although some minor differences are apparent.

BNPS-skatole cleavage of the p24 proteins from the four isolates results in strikingly similar patterns. It is evident from Figure 8 that this is to be expected since the tryptophan positions in this protein are very highly conserved, particularly for the retroviruses of human and simian origin. We conclude that the tryptophan positions in the ANT 70 p25 protein also conform to this pattern. Inspection of the patterns reveals, however, that minor differences can be observed, not in the overall appearance of the pattern but rather in the apparent



molecular weights of the species generalized by cleavage. In particular, differences are detected in the apparent molecular weights of the central spots in each pattern. As expected, the patterns for HIV-2 (LAV-2rod) and HIV-2 (isolate 53) are identical. The central spot in the pattern for ANT 70 has however, a larger apparent molecular weight while the central spot for HIV-1 (ARV-4) has a lower molecular weight. In regard to the p16, the positions of the tryptophans in the ANT 70 protein appear to resemble more closely the positions of the tryptophans found in the HIV-2 protein. The HIV-1 p17 has a tryptophan located 16 amino acids from the amino terminus of the protein and gives rise to an additional spot not seen in the ANT 70 and HIV-2 patterns following BNPS-skatole cleavage. The tryptophan corresponding to the one at position 36 in the HIV-1 p17 sequence is conserved in all isolates.

The patterns generated by cleavage of the reverse transcriptase from the four isolates are complex but it is once again apparent that the two HIV-2 isolates are identical. Patterns are obtained for ANT 70 which corresponds neither to the pattern obtained for HIV-1 nor to the HIV-2 pattern. Differences in apparent molecular weights of the cleavage products of the p31 endonuclease are also observed but the patterns generated from the corresponding proteins from HIV-1, ANT 70, and HIV-2 also show common features which suggests a conserved structure.

Viral Nucleic Acids

a. Hybridization of HIV-1 and HIV-2 cDNA to viral RNAs.

Nucleic acids crosshybridization between HIV-1 and RNA from the viruses HIV-2 and ANT 70 was evaluated by performing the hybridization with the SacI-BglII HIV-1 restriction fragment which had been inserted into the vector pUC13. This fragment contains a portion of the 5' LTR, including the R region, the entire gag gene and most of the pol gene of HIV-1. Under stringent hybridization conditions, hybridization was only observed between this probe and the RNA derived from HIV-1 (SF4). No hybridization was observed between the probe and either HIV-2 or ANT 70 (figure 14). This indicates that the gag and pol regions of HIV-2 and ANT 70 are significantly different from the corresponding region of HIV-1.



The HIV-2 probe used contains a sequence of approximately 100 base pairs derived from the env gene of HIV-2. This probe hybridized only to HIV-2 RNA under stringent hybridization conditions and no hybridization was observed with either HIV-1 or HIV-3.

5 b. Homology between ANT 70 cDNA and sequences of HIV-1 and HIV-2.

The cDNA clone iso 70-11 was used as a probe to assess the degree of nucleic acid homology between the various virus isolates. The filter onto which aliquots of HIV-1, HIV-2 and ANT 70 had been deposited was subjected to hybridization under stringent conditions. 10 The results are also shown in Figure 14. The experiment demonstrates that under stringent hybridization conditions, no crosshybridization can be detected between any of the virus isolates. The ANT 70 derived probe hybridizes only to ANT 70. DNA from ANT 70 cDNA clone iso 70-11 was used for hybridization to blots of restriction enzyme digests of cellular genomic DNA from cells infected with HIV-1 and ANT 70 as well as DNA from uninfected 15 cells. The results are shown in Figure 15. No hybridization was observed in any lane other than those lanes containing digests of genomic DNA from ANT 70 infected cells. This also indicates that the iso 70-11 insert is not derived from cellular sequences.

An additional hybridization was performed using the SacI-BglII fragment from the iso 70-11 insert which corresponds to the left end of the insert farthest from the sequences 20 presumably contained within the LTR and which, by analogy with HIV-1 and HIV-2, should correspond to env gene sequences. Hybridization with this fragment gave rise to the same bands as did hybridization with the entire fragment.

25 c. Sequence analysis of clone iso 70-11.

The locations of a number of restriction enzyme sites in the iso 70-11 ANT 70 insert are shown in Figure 16. Subclones of the insert were made in pUC13 and sequenced using the dideoxy-nucleotide-method.

The presence of a poly A tail confirmed that the iso 70-11 insert is derived from the 3' end 30 of the viral RNA. Adjacent to the poly A tail is the sequence corresponding to the R region of the viral 3' LTR. Sequence contained in the ANT 70 cDNA and the viral sequences to which they correspond are shown below:



1. HIV-3 LTR

```

      10      20      30      40      50
CCCCATGCATT TGAAGATACA CATAAAGAAA TACTGATGTG GAAGTTTGAAT
5      60      70      80      90     100
ACATCTCTAG GCAACACCCA TGTGCTATG ATAAC TCACC CAGAGCTCTT
      110     120     130     140     150
CCAGAAGGAC TAAAAACTGC TGACCTGAAG ATTGCTGACA CTGTGGAACAT
      160     170     180     190     200
10  TTCCAGCAAA GACTGCTGAC ACTGCGGGGA CTTTCCAGTG GGAGGGACAG
      210     220     230     240     250
GCCCCGCTTC GGGGAGTGGC TAACCCTCAG AAGCTGCATA TAAGCAGCCG
      260     270     280     290     300
CTTTCTGCTT GTACCGGGTC TCGGTTAGAG GACCAGGTCT GAGCCCGGGA
15      U3      R
      310     320     330     340     350
GCTCCCTGGC CTCTAGCTGA ACCCGCTCGT TAACGCTCAA TAAAGCTTGC
                                     Polyadenylation signal
      360
20  CTTGAGTGAG A-Poly A (SEQ ID NO 3)

```

This Sheet Has Been Replaced



2. Sequences from HIV-3 3'ORF

	10	20	30	40	50	60
	AACATGGGAAACGCATTGACAAAAGGTAAATTTGAGGGATCGGCAGCAGTAAGAGAAAGA					
5	AsnMetGLyAsnAlaLeuArgLysGlyLysPheGluGlyTrpAlaAlaValArgGluArg					
	70	80	90	100	110	120
	ATGAGAAGAACTAGAACTTTCCCTGAGTCTGAACCATGCGCACCTGGAGTAGGACAGATC					
	MetArgArgThrArgThrPheProGluSerGluProCysAlaProGlyValGlyGlnIle					
	130	140	150	160	170	180
10	TCCACGGAAATTAGCAGCTAGAGGAGGGATACCAAGTTCCTACTCCTCAAAACAATGCA					
	SerArgGluLeuAlaAlaArgGlyGlyIleProSerSerHisThrProGlnAsnAsnAla					
	190	200	210	220	230	240
	GCCCTTGCAATTCCTAGAAAGTCACCAAGAGGAACAAGTAGGTTTTCCAGTAGCACCTCAA					
	AlaLeuAlaPheLeuGluSerHisGlnGluGluGluValGlyPheProValAlaProGln					
15	250	260	270	280	290	300
	CTGCCTCTAAGGCCAATGACCTATAAAGGAGCATTTCACCTCAGCTTC'TTTTAAAGAA					
	ValProLeuArgProMetThrTyrLysGlyAlaPheAspLeuSerPhePheLeuLysGlu					
	310	320	330	340	350	360
	AAGCGAGGACTGCAAGGGTTAATTTACTCCATAAAAGAGCAGAAATCCTGGATCTTTGG					
20	LysGlyGlyLeuGluGlyLeuIleTyrSerHisLysArgAlaGluIleLeuAspLeuTrp					
	CTGTATAA					
	ValTyr					

25 (SEQ ID NO 4)

This Sheet Has Been Replaced



3. Coding sequences from the HIV-3 *env* gene

```

      10      20      30      40      50      60
ATTCAAAGTGAAATCAAAGAACCTAAAAAACTCCTTTAGCAATTTTTCATTGGTTTACGA
5  IleGlnSerGluMetLysGluProLysLysThrProLeuAlaIlePheIleGlyLeuGly
      70      80      90     100     110     120
ATTACAACCTTAATTTACTTAATTATTGCTATTTCAAATGTCACTTAATGGTGGATCATTC
IleThrThrLeuIleTyrLeuIleIleAlaIleSerMetSerLeuAsnGlyGlySerPhe
      130     140     150     160     170     180
10  TTTGGCTTCAAAGATTACATGAGTAGTTTATTCAAATAAGAAAGCTGGACGAATTGT
PheGlyPheLysAspTyrMetSerSerLeuPheLysAsnGluLysAlaGlyArgIleVal
      190     200     210     220     230     240
TTTCGTTTAATTAACATTTGTATTTCAATTGGTGTCTAGGTATTATTAACGCTTTCTCA
PheGlyLeuIleAsnIleCysIleSerIleGlyValLeuGlyIleIleAsnGlyPheSer
15
ATG
Met

```

(SEQ ID NO 6)

DISCUSSION

25 We have isolated a novel human immunodeficiency-associated retrovirus from a Cameroonian woman (ANT 70) and her partner (ANT 70 NA). At the time the original virus isolation was performed, the woman was only slightly seropositive, gave ambiguous results in the western blot test and was clinically asymptomatic. Since that time, the woman has begun to develop some of the symptoms of AIDS-related complex (ARC). In contrast, her

30 partner, from whom we were also able to isolate a virus with the same characteristics as the original isolate, was suffering from lymphadenopathy and has since developed other symptoms characteristic of AIDS. This novel isolate may therefore be considered to be a human immunodeficiency virus. The fact that this same virus could be isolated from sexual partners also suggests a mode of transmission which is similar to that of human retroviruses.





The virus was first recognized as being different from HIV-1 on the basis of its altered ability to be captured in a differential antigen capturing assay. This has proven to be a highly reliable test which is able to distinguish between HIV-1 and non-HIV-1 strains. That this isolate is not HIV-1 is borne out at the protein level by 1.) the differing molecular weights of the viral proteins, 2.) a different pattern of crossreactivity with anti- HIV-1 antiserum than HIV-1, 3.) a drastically reduced ability to be recognized by mouse monoclonal antibodies raised against HIV-1 p24 and p17 core proteins, 4.) preferential recognition of ANT 70 proteins over HIV-1 proteins by antisera from the virus carrier, and 5.) patterns of partial cleavage of four of the most highly conserved viral proteins which do not match the patterns obtained when HIV-1 proteins are subjected to the same treatment. Nevertheless, sera from the two individuals infected with this virus recognize the HIV-1 gp41 envelope protein. By the same criteria listed above, it is also clear that ANT 70 is not HIV-2. Indeed, the antigenic differences between ANT 70 and HIV-1 are smaller than those between HIV-2 and HIV-1. This is particularly evident from the results presented in Figures 8 and 10.

Additional compelling evidence that ANT 70 is a unique virus different from HIV-1 and HIV-2 comes from the partial peptide maps. We have shown that there are significant differences in the most highly conserved viral proteins. The two HIV-2 isolates which were used for comparison gave essentially identical cleavage patterns except in the case of CNBr cleavage of the p17 core protein. It should be noted, however, that the p17 core protein exhibits more variability than the p24 protein, at least in HIV-1 strains (34). Whether or not this also holds true for HIV-2 awaits sequence determination on more strains than have been analyzed to date.

In light of the fact that ANT 70 is antigenically more closely related to HIV-1 than is HIV-2, as evidenced by a higher degree of crossreactivity which extends even to the gp41 envelope protein, it was essential to establish that ANT 70 was more than simply a genetic variant of HIV-1. This was possible by investigating the locations of some of the most highly conserved amino acids in a number of viral proteins which are least subject to genetic variation. That major differences were noted in the cleavage patterns indicates that HIV-1, HIV-2 and ANT 70 are three genetically distinct viruses. On the other hand, the same series of experiments also revealed similarities between the viruses which may indicate that all three arose from a common progenitor.

The hybridization data also support the notion that ANT 70 is fundamentally different



from either HIV-1 and HIV-2. As long as the conditions under which the hybridization is performed are stringent, a distinction can easily be made between the three virus types.

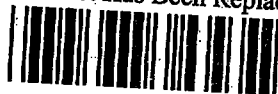
Analysis of the cDNA sequences revealed that the insert is derived from the 3' end of the viral genome. An analysis of the homology between these sequences and the sequence of HIV-1 and HIV-2 reveal that ANT 70 is somewhat more closely related to HIV-1, particularly in the LTR sequences (approximately 70% homology). The differences are nevertheless of such magnitude as to rule out the possibility that ANT 70 is simply a genetic variant of HIV-1. The ANT 70 3'LTR also contains the signal sequences which are typical of retroviral LTRs.

Sequences corresponding to the *env* gene of ANT 70 are found to be even less related to either HIV-1 or HIV-2 sequences. While it is true that retroviral *env* genes frequently exhibit genetic variability, the differences in sequence between variants is rarely more than 10 %. The degree of homology between ANT 70, and HIV-1 and HIV-2 was found, however, to be less than 60%. Such a difference in homology, together with the hybridizations results, indicates that ANT 70 belongs to a new class of human immunodeficiency retroviruses.

The existence of a third type of human immunodeficiency virus has immediate epidemiological implications and consequences for blood bank testing. As has been shown, antibodies from people infected with this virus react preferentially with this virus, although these antibodies also crossreact with HIV-1 proteins. While it was possible to detect a positive reaction in enzyme immunoassays, immunofluorescence assays and Western Blot assays based in HIV-1 proteins, the fact that the positive signal was due to a crossreaction inevitably implies that the sensitivity of such tests will be less for antibodies produced in response to this virus. This was amply demonstrated by the enzyme immunoassay results (Figure 11). Furthermore, one criterion for seropositivity in the Western blot assay is the presence of detectable antibodies to both a *gag* and/or *pol* protein and one of the envelope proteins. Since in the case of the two individuals infected with ANT 70 and ANT 70 NA, respectively, crossreaction was observed to both HIV-1 p24 and the envelope proteins, the conclusion which is invariably drawn is that these individuals are infected with HIV-1 but for some reason fail to develop high titers. It is possible therefore, that this virus is more widespread than is currently realized. From an epidemiological standpoint, it is essential to develop specific diagnostic tests for this virus in order to evaluate the limits of the



geographical area in Africa in which the virus can be found, and to evaluate the extent to which this virus has been disseminated.



REFERENCES

1. Dalglish, A.G., Beverly, P.C.I., Clapham, P.R., Crawford, D.H., Greaves, M.F. and Weiss, R.A. (1984).
5 The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.
Nature 312: 763-766.
2. Maddon, P.J., Dalglish, A.G., McDougal, J.S., Clapham, P.R., Weiss, T.A. and Axel, R. (1986).
10 The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain.
Cell 47: 333-348.
3. Wong-Staal, F. and Gallo, R.C. (1985).
15 Human T-lymphotropic retroviruses.
Nature 317: 395-403.
4. Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M.A., Santos-Ferreira, M.D., Laurent, A.G., Dauguet, C., Katlama, C., Rouzioux, C., Klatzmann, D.,
20 Champalimaud, J.L. and Montagnier, L. (1986).
Isolation of a new human retrovirus from West-African patients with AIDS.
Science 233: 343-346.
5. Albert, J., Bredberg, U., Chiddi, F., Bottinger, B., Penyo, E.M., Norrby, E. and Bibertfeld, G. (1987).
25 New pathogenic human retrovirus of West-African origin (SBL 6669) and its relationship to HTLV-IV, LAV-II and HTLV-IIIB.
AIDS Res.
6. Kanki, P.J., Kurth, R., Becker, W., Dreesman, G., McLane, M.F. and Essex, M.
30 Antibodies to simian T-lymphotropic virus type III in African green monkeys and recognition of STLV-III viral protein by AIDS and related sera.

Lancet 1985, i: 1330-1332.

7. Kanki, P.J., Alroy, J. and Essex, M. (1985):

Isolation of T-lymphotropic retrovirus related to HTLV- III/LAV from wild-caught African green monkeys.

Science 230: 951-954.

8. Daniel, M.D., Letvin, N.L., King, N.W., Kannagi, M., Sehgal, P.K., Hunt, R.D., Kanki, P.J., Essex, M. and Desrosiers, R.C. (1985).

Isolation of T-cell tropic HTLV-III-like retrovirus from macaques.

Science 228: 1201-1204.

9. Chakrabarti, L., Guyader, M., Alizon, M., Daniel, M.D., Desrosiers, R.C., Tiollais, P., and Sonigo, P. (1987).

Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses.

Nature 328: 543-547.

10. Franchini, G., Gurgo, C., Guo, H.-G., Gallo, R.C., Collati, E., Fargnoli, K.A., Hall, I.F., Wong-Staal, F. and Reitz, M.S. Jr. (1987).

Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses.

Nature 328: 539-543.

11. Benn, S., Rutledge, R., Folks, T., Gold, J., Baker, L.,

McCormick, J., Feorino, P., Piot, P., Quinn, T., and Martin, M. (1985)

Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire.

Science 230: 949-951.

12. Hahn, B.H., Shaw, G.M., Taylor, M.E., Redfield, R.R., Markham, P.D., Salahuddin, S.Z., Wong-Staal, F., Gallo, R.C., Parks, E.S. and Parks, W.P. (1986).

Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS.



Science 232: 1548-1553.

13. Magasiny, S., Spire, B., Barré-Sinoussi, F. and Chermann, J.-C. (1986).

Genomic variability of selected LAV-related AIDS retroviruses.

5 AIDS Res. 2: 19-30.

14. Alizon, M., Wain-Hobson, S., Montagnier, L. and Sonigo, P. (1985).

Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients.

10 Cell 46: 63-74.

15. Starcich, B.R., Hahn, B.H., Shaw, G.M., McNeely, P.D., Modrow, S., Wolf, H., Parks, E.S., Parks, W.P., Josephs, S.F., Gallo, R.C., Wong-Staal, F. (1986).

Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell 45: 637-648.

15

16. Willey, R.L., Rutledge, R.A., Dias, S., Folks, T., Theodore, T., Buckler, C.E. and Martin, M.A. (1986).

Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus.

20

Proc. Natl. Acad. Sci. USA 83: 5038-5042.

17. Clavel, F., Guyader, M., Guétard, D., Sallé, M., Montagnier, L. and Alizon, M. (1986).

25 Molecular cloning and polymorphism of the human immune deficiency virus type 2.

Nature 324: 691-695.

18. Hahn, B.H., Kong, L.I., Lee, S.-W., Kumar, P., Taylor, M.E., Arya, S.K. and Shaw, G.M. (1987).

30 Relationship of HTLV-4 to simian and human immunodeficiency-associated viruses.

Nature 300: 184-186.



19. Guyader, M., Emerman, M., Sonigo, P., Clavel, F., Montagnier, L. and Alizon, M. (1987).

Genome organization and transactivation of the human immunodeficiency virus type 2.
Nature 326: 662-669.

5

20. Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A., Shimabukuro, J.M. and Oshiro, L.S. (1984).

Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS.
Science 225: 840-842.

10

21. Vanderborght, B., De Leys, R.J., Van Heuverswyn, H., Merregaert, J., Prinsen, H., Nijs, P. Vercauteren, G., and van der Groen, G. (1988).
Submitted for publication.

15

22. Levy, J.A. and Shimabukuro, J. (1985).

Recovery of AIDS-associated retroviruses from patients with AIDS or AIDS-related conditions and from clinically healthy individuals.

J. Infect. Dis. 152: 734-738.

20

23. van der Groen, G., Vercauteren, G. and Piot, P. (1987).

Immunofluorescence tests for HIV antibody and their value as confirmatory tests.

J. Virol. Meth. 17: 35-43.

25

24. Hoffman, A.D., Banapour, B. and Levy, J.A. (1985).

Characterization of the AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions.

Virology 147: 326-335.

30

25. Kikukawa, R., Koyanagi, Y., Harada, S., Kobayashi, N., Hatanaka, M. and Yamamoto, N. (1986).

Differential susceptibility to the acquired immunodeficiency syndrome retrovirus in cloned cells of human leukemic T-cell line Molt-4.

This Sheet Has Been Replaced



J. Virol. 57: 1159-1162.

26. Winkel, I.N., Tersmetto, M., Miedema, P. and Huisman, J.G. (1987).

Identification of gag-epitopes by a panel of MAb in a series of HIV isolates.

Abstracts of the Third International Conference on AIDS, Washington D.C., USA, p. 116.

27. Maizel, J.V. (1971).

Polyacrylamide gel electrophoresis of viral proteins; in Methods in Virology, Vol. 5, pp: 180-246, K. Maramorush and H. Koprowski, Editor, Academic Press, New York, London.

28. Dunn, S.D. (1986)

Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies.

Anal. Biochem. 157: 144-153.

29. Gross, E. and Witkop, B. (1961).

Selective cleavage of methionyl peptide bonds in RNAase with CNBr.

J. Am. Chem. Soc. 83: 1510-1511.

30. Fontana, T.P. and woods, K.R. (1981).

Modification of tryptophanyl residues with BNPS-Skatole.

Meth. Enzymol. 25/ 419-423.

31. Hopp, T.P. and Woods, K.R. (1981).

Prediction of protein antigenic determinants from amino acid sequence.

Proc. Natl. Acad. Sci. USA 78: 3824-3828.

32. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985).

Nucleotide sequence of the AIDS virus, LAV.

Cell 40: 9-17.

33. Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran,



E.R., Rafalski, J.A., Whitcorn, B.A., Baumeister, K., Ivanoff, I., Petteway, S.R. Jr., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghayes, J., Chang, N.T., Gallo, R.C. and Wong-Staal, F. (1985).

Complete nucleotide sequence of the AIDS virus, HTLV-III.

5 Nature 313: 277-284.

34. Sanchez-Pescador, R., Power, M.D., Barr, P.J., Steimer, K.S., Stempion, M.M., Brown-Shimer, S.L., Gee, W.W., Renard, A., Randolph, A., Levy, J.A., Dina, D. and Luciw, P. (1985).

10 Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2)
Science 227: 484-492.

35. Muesing, M.A., Smith, D.H., Cabradillo, C.D., Benton, C.V., Laskey, L.A. and Capon, D.J. (1985).

15 Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus.
Nature 313: 450-458.

36. Human retroviruses and AIDS. (1987).

G. Moyers, A.B. Rabson, S.F. Josephs, T.H. Smith and F. Wong-Staal, Editors.

20 Los Alamos National Laboratory, Los Alamos, New Mexico, USA.

37. Kawakami, T., Sherman, L., Dahlberg, J., Gazit, A., Yaniv, A., Tronick, S. and Aaronson, S.A. (1987).

Nucleotide sequence analysis of Equine Infectious Anemia Virus proviral DNA.

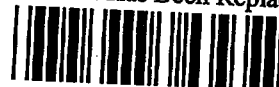
25 Virology 158: 300-312.

38. Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A. and Wain-Hobson, S. (1985).

Nucleotide sequence of the Visna lentivirus: relationship to the AIDS virus.

30 Cell 42: 369-382.

This Sheet Has Been Replaced



SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Innogenetics N.V.
 (B) STREET: Industriepark Zwijsnaarde 7 Bus 4
 (C) CITY: Gent
 (E) COUNTRY: Belgium
 (F) POSTAL CODE (ZIP): 9052
 (G) TELEPHONE: 00-32-09.241.07.11
 (H) TELEFAX: 00-32-09.241.07.99

(ii) TITLE OF INVENTION: HIV-3 retrovirus strains and their use

(iii) NUMBER OF SEQUENCES: 7

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: EP 88109200.1
 (B) FILING DATE: 09-JUN-1988

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 245 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 3..245

(ix) FEATURE:

(A) NAME/KEY: mat peptide
 (B) LOCATION: 3..245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

55	CA ATT CAA AGT GAA ATG AAA GAA CCT AAA AAA ACT CCT TTA GCA ATT	47
	Ile Cln Ser Glu Met Lys Glu Pro Lys Lys Thr Pro Leu Ala Ile	
	1 5 10 15	
60	TTC ATT GCT TTA GGA ATT ACA ACC TTA ATT TAC TTA ATT ATT GCT ATT	95
	Phe Ile Gly Leu Gly Ile Thr Thr Leu Ile Tyr Leu Ile Ala Ile	
	20 25 30	
65	TCA ATG TCA CTT AAT GGT GGA TCA TTC TTT GGC TTC AAA GAT TAC ATG	143
	Ser Met Ser Leu Asn Gly Gly Ser Phe Phe Gly Phe Lys Asp Tyr Met	
	35 40 45	





56

ACT ACT TTA TTC AAA AAT GAA AAA CCT GGA CGA ATT GTT TTT GGT TTA 191
 Ser Ser Leu Phe Lys Asn Glu Lys Ala Gly Arg Ile Val Phe Gly Leu
 50 55 60

5 ATT AAC ATT TGT ATT TCA ATT GGT CTT CTA GGT ATT ATT AAC GGT TTC 239
 Ile Asn Ile Cys Ile Ser Ile Gly Val Leu Gly Ile Ile Asn Gly Phe
 65 70 75

10 TCA ATG 245
 Ser Met
 80

(2) INFORMATION FOR SEQ ID NO: 2:

15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 81 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

25 Ile Gln Ser Glu Met Lys Glu Pro Lys Lys Thr Pro Leu Ala Ile Phe 15
 1 5 10 15

Ile Gly Leu Gly Ile Thr Thr Leu Ile Tyr Leu Ile Ile Ala Ile Ser 30
 20 25 30

30 Met Ser Leu Asn Gly Gly Ser Phe Phe Gly Phe Lys Asp Tyr Met Ser 45
 35 40 45

35 Ser Leu Phe Lys Asn Glu Lys Ala Gly Arg Ile Val Phe Gly Leu Ile 60
 50 55 60

Asn Ile Cys Ile Ser Ile Gly Val Leu Gly Ile Ile Asn Gly Phe Ser 80
 65 70 75 80

40 Met

(2) INFORMATION FOR SEQ ID NO: 3:

45 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 360 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

50 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

55 (iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

60 CCCATGGATT TCAACATACA CATAAAGAAA TACTGATCTC GAAGTTTGAT AGATCTCTAG 60

GCAACACCCA TGTTCCTATG ATAACACACC CAGAGCTCTT CCAGAAGGAC TAAAAACTCC 120

65 TGACCTGAAC ATTGCTGACA CTGTGGAAC TTCCACCAAA GACTGCTGAC ACTGCGGGGA 180

CTTCCAGTG GGAGGGACAC CCGGCGGTTT GGGGAGTGGC TAACCCTCAG AACCTGCATA 240

TAAGCAGCCG CTTTCTGCTT GTACCGGGTC TCGCTTAGAG GACCAGGTCT GAGCCCCGGA 300
 CCTCCCTGGC CTCTAGCTGA ACCCGCTCGT TAACGCTCAA TAAACCTTGC CTTGAGTTAG 360

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 368 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTISENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
 (B) LOCATION: 1..368

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
 (B) LOCATION: 1..366

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

1	AAC ATG GGA AAC CCA TTG AGA AAA GCT AAA TTT GAG GGA TGG GCA CCA	48
5	Asn Met Gly Asn Ala Leu Arg Lys Gly Lys Phe Glu Gly Trp Ala Ala	
10		
15	GTA ACA CAA AGA ATG AGA AGA ACT AGA ACT TTC CCT GAG TCT CAA CCA	96
20	Val Arg Glu Arg Met Arg Arg Thr Arg Thr Phe Pro Glu Ser Glu Pro	
25		
30	TGC GCA CCT GGA CTA GGA CAG ATC TCC AGG GAA TTA GCA GCT AGA CCA	144
35	Cys Ala Pro Gly Val Gly Gln Ile Ser Arg Glu Leu Ala Ala Arg Gly	
40		
45	CCC ATA CCA AGT TCC CAT ACT CCT CAA AAC AAT GCA GCC CTT GCA TTC	192
50	Gly Ile Pro Ser Ser His Thr Pro Gln Asn Asn Ala Ala Leu Ala Phe	
55		
60	CTA GAA AGT CAC CAA GAG GAA GAA GTA GGT TTT CCA CTA GCA CCT CAA	240
65	Leu Glu Ser His Gln Glu Glu Glu Val Gly Phe Pro Val Ala Pro Gln	
70		
75	CTG CCT CTA ACC CCA ATG ACC TAT AAA GGA GCA TTT GAC CTC ACC TTC	288
80	Val Pro Leu Arg Pro Met Thr Tyr Lys Gly Ala Phe Asp Leu Ser Phe	
85		
90	TTT TTA AAA GAA AAG GGA GGA CTC GAA GGG TTA ATT TAC TCC CAT AAA	336
95	Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly Leu Ile Tyr Ser His Lys	
100		
105	AGA GCA GAA ATC CTG GAT CTT TGG GTG TAT AA	368
110	Arg Ala Glu Ile Leu Asp Leu Trp Val Tyr	
115		
120		

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:

This Sheet Has Been Replaced



(A) LENGTH: 122 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

10 Asn Met Gly Asn Ala Leu Arg Lys Gly Lys Phe Glu Gly Trp Ala Ala
 1 5 10 15
 Val Arg Glu Arg Met Arg Arg Thr Arg Thr Phe Pro Glu Ser Glu Pro
 20 25 30
 15 Cys Ala Pro Gly Val Gly Gln Ile Ser Arg Glu Leu Ala Ala Arg Gly
 35 40 45
 Gly Ile Pro Ser Ser His Thr Pro Gln Asn Asn Ala Ala Leu Ala Phe
 50 55 60
 20 Leu Glu Ser His Gln Glu Glu Glu Val Gly Phe Pro Val Ala Pro Gln
 65 70 75 80
 25 Val Pro Leu Arg Pro Met Thr Tyr Lys Gly Ala Phe Asp Leu Ser Phe
 85 90 95
 Phe Leu Lys Glu Lys Gly Gly Leu Glu Gly Leu Ile Tyr Ser His Lys
 100 105 110
 30 Arg Ala Glu Ile Leu Asp Leu Trp Val Tyr
 115 120

(2) INFORMATION FOR SEQ ID NO: 6:

35 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 243 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

45 (iii) ANTI-SENSE: NO

(ix) FEATURE:

50 (A) NAME/KEY: CDS
 (B) LOCATION: 1..243

(ix) FEATURE:

55 (A) NAME/KEY: mat peptide
 (B) LOCATION: 1..243

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

60 ATT CAA AGT CAA ATG AAA GAA CCT AAA AAA ACT CCT TTA GCA ATT TTC 48
 1 5 10 15
 Ile Gln Ser Glu Met Lys Glu Pro Lys Lys Thr Pro Leu Ala Ile Phe
 65 ATT GGT TTA GGA ATT ACA ACC TTA ATT TAC TTA ATT ATT GCT ATT TCA 96
 20 25 30
 Ile Gly Leu Gly Ile Thr Thr Leu Ile Tyr Leu Ile Ile Ala Ile Ser
 ATG TCA CTT AAT GGT GGA TCA TTC TTT GGC TTC AAA GAT TAC ATG AGT 144



Met Ser Leu Asn Gly Gly Ser Phe Phe Gly Phe Lys Asp Tyr Met Ser
35 40 45

ACT TTA TTC AAA AAT GAA AAA GCT GGA CGA ATT GTT TTT GGT TTA ATT 192
Ser Leu Phe Lys Asn Glu Lys Ala Gly Arg Ile Val Phe Gly Leu Ile
50 55 60

AAC ATT TGT ATT TCA ATT CGT GTT CTA GGT ATT ATT AAC GGT TTC TCA 240
Asn Ile Cys Ile Ser Ile Gly Val Leu Gly Ile Ile Asn Gly Phe Ser
65 70 75 80

ATC 243
Met

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 81 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ile Gln Ser Glu Met Lys Glu Pro Lys Lys Thr Pro Leu Ala Ile Phe
1 5 10 15

Ile Gly Leu Gly Ile Thr Thr Leu Ile Tyr Leu Ile Ile Ala Ile Ser
20 25 30

Met Ser Leu Asn Gly Gly Ser Phe Phe Gly Phe Lys Asp Tyr Met Ser
35 40 45

Ser Leu Phe Lys Asn Glu Lys Ala Gly Arg Ile Val Phe Gly Leu Ile
50 55 60

Asn Ile Cys Ile Ser Ile Gly Val Leu Gly Ile Ile Asn Gly Phe Ser
65 70 75 80

Met





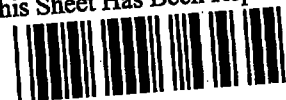
CLAIMS

1. HIV-3 retrovirus strain comprising in its nucleic acid sequence a contiguous R region
5 and an U3 region sequence showing a homology of more than 70% with the nucleic acid
sequence as represented in SEQ ID NO 3, and provided that said HIV-3 retrovirus strain has
at least one nucleotide difference in its sequence compared to the HIV-3 (ANT 70) retrovirus
strain deposited under ECACC N° V88060301.

10 2. HIV-3 retrovirus strain according to claim 1, further characterized in that said HIV-3
strain has the essential morphological and immunological properties of the HIV-3 (ANT 70)
retrovirus deposited under ECACC N° V88060301, with said properties being summarized
as follows :

- 15 - the virus exhibits a tropis for T4 lymphocytes;
- the virus is cytotoxic for the lymphocytes that is infects;
- the virus has a diameter of approximately 120 nm;
- the virus possesses a magnesium dependent reverse transcriptase activity;
- it can be cultivated in T4 receptor-bearing immortalized cell lines;
- 20 - lysates of the virus contain a p25 protein which is immunologically distinct
from the p19 protein of HTLV-1 and the p24 proteins of HIV-1 and HIV-2 as
determined by Western blot analysis and partial CNBr-cleavage, respectively;
- lysates of the virus contain a gp120 protein which is immunologically distinct
from the gp110 protein of HTLV-I, the gp120 of HIV-1 and the gp120 of
25 HIV-2 as determined by Western blot analysis;
- the lysate of the virus contains in addition a gp41 glycoprotein with a
molecular weight of 40,000 - 45,000;
- the genomic RNA of HIV-3 hybridizes neither with the sequences of HIV-1
nor with the sequences of HIV-2 under stringent hybridization conditions;
- 30 - lysates of the virus contain a p16 protein which differs from the p17 of HIV-1
and from HIV-2 as determined by partial CNBr-cleavage;
- lysates of the virus contain a p12 protein which differs from the p12 proteins





of HIV-1 and HIV-2 as determined by Western blot analysis;

- lysates of the virus contain a p31 endonuclease which differs from the p31 endonucleases from HIV-1 and HIV-2 as determined by partial CNBr-cleavage;
- 5 - lysates of the virus contain reverse transcriptase proteins which are lower in molecular weight than those from HIV-1 and HIV-2 as determined by Western blot analysis;

3. An HIV-3 retrovirus strain of any of claims 1 or 2, characterized in that its RNA
10 virtually hybridizes neither with the Env gene and the LTR close to it, in particular not with the nucleotide sequence 8352-9538 of HIV-1, nor with the sequences of the Pol region of the HIV-1 genome under stringent conditions.

4. An HIV-3 retrovirus strain of any of claims 1 to 3, characterized in that said strain
15 termed ANT 70 NA is isolated from the partner of the woman from whom the strain ANT 70 deposited under ECACC N° V88060301 was isolated.

5. A composition comprising a total extract or lysate of the HIV-3 retrovirus strain of
20 any of claims 1 to 4.

6. The composition of claim 5 further comprising a lysate of HIV-1, HIV-2, or a mixture
of both.

7. A composition comprising at least one antigen of an HIV-3 retrovirus strain as defined
25 in claim 2.

8. The composition of any of claims 5 to 7 further comprising proteins derived from
HIV-1 and/or HIV-2.

9. The composition of any of claims 7 or 8, characterized in that it contains at least one
30 of the internal core proteins of said HIV-3 retrovirus strain, in particular p12, p16 or p26 proteins having apparent molecular weights in the order of 12.000, 16.000 and 26.000





respectively.

10. The composition of any of claims 7 or 8, characterized in that it contains at least one of the envelope proteins of said HIV-3 retrovirus strain, in particular gp41 or gp120 proteins
5 having apparent molecular weights in the order of 40-45.000 and 120.000 respectively.

11. A purified antigen having the immunological characteristics of one of the HIV-3 strain retroviral proteins as defined in claim 2.

10 12. The antigen of claim 11 obtained by subjecting said HIV-3 strain total protein extract to preparative electrophoresis or to affinity chromatography and isolating the p12 protein in a manner known per se.

15 13. The antigen of claim 11 obtained by subjecting said HIV-3 strain total protein extract to preparative electrophoresis or to affinity chromatography and isolating the p16 protein in a manner known per se.

20 14. The antigen of claim 11 obtained by subjecting said HIV-3 strain total protein extract to preparative electrophoresis or to affinity chromatography and isolating the p26 protein in a manner known per se.

25 15. The antigen of claim 11 obtained by subjecting said HIV-3 strain total protein extract to preparative electrophoresis or to affinity chromatography and isolating the gp41 protein in a manner known per se.

16. The antigen of claim 11 obtained by subjecting said HIV-3 strain total protein extract to preparative electrophoresis or to affinity chromatography and isolating the gp120 protein in a manner known per se.

30 17. A polypeptide or peptide which is part of any of the antigens of claims 11 to 16, and which is characterized by the fact that it comprises an epitope which is preferentially recognized by serum of a patient carrying anti-HIV-3 antibodies as detected by means of



Western blotting or radioimmunoprecipitation.

18. A method for the detection of antibodies against an HIV-3 retrovirus strain of any of claims 1 to 4 in a biological liquid, such as serum or spinal fluid, in particular for the diagnosis of a potential or existing ARC or AIDS caused by said HIV-3 retrovirus strain, characterized by contacting body fluid of a person to be diagnosed with a composition of any of claims 5 to 10 or with an antigen of any claims 11 to 17, and detecting the immunological conjugate formed between said anti-HIV-3 strain antibodies and the antigen(s) used.

19. A kit for the detection of antibodies against an HIV-3 retrovirus strain of any of claims 1 to 4 in a biological fluid, comprising :

- a composition as defined in any of claims 5 to 10 or an antigen as defined in any of claims 11 to 17, and
- a means for detecting the immunological complex formed.

20. An immunogenic composition containing an envelope glycoprotein of an HIV-3 strain of any of claims 1 to 4, in particular an antigen according to any of claims 15 to 17, in combination with a pharmaceutically acceptable vehicle suitable for the constitution of vaccines effective against HIV-3.

21. Monoclonal antibodies characterized by their ability to specifically recognize one of the antigens as defined in any of claims 11 to 17, in particular monoclonal antibodies specifically raised against said antigens.

22. The hybridomas secreting the monoclonal antibodies of claim 21.

23. Nucleic acids, optionally labeled, comprising the sequence corresponding to the entire RNA genome derived from an HIV-3 retrovirus strains of any of claims 1 to 4.

24. Nucleic acids characterized by nucleotide sequences coding for any of the antigens or epitopes as defined in claims 11 to 17.





25. Recombinant nucleic acid comprising a nucleic acid from a vector having any of the nucleic acids of claim 24 inserted therein.
26. A process for the detection of an HIV-3 retrovirus strain of any of claims 1 to 4 or of its RNA in a biological liquid or tissue, characterized by contacting nucleic acids contained in said biological liquid or tissue with a probe containing a nucleic acid according to any of claims 23 or 24, or containing a part of any of said nucleic acids which does not hybridize to HIV-1 or HIV-2 strains under stringent hybridization conditions, washing the hybrid formed with a solution preserving said stringent conditions, and detecting the hybrid formed.
27. A process for the production of an HIV-3 retrovirus strain of any of claims 1 to 4 characterized by culturing human T4 lymphocytes, or permanent cell lines derived therefrom carrying the T4 phenotype, with lymphocytes or cell lines that have previously been infected with said HIV-3 retrovirus strain, as well as recovering and purifying the retrovirus from the culture medium.
28. A process for the production of the proteins or glycoproteins of any of claims 11 to 17 or of a part thereof, characterized by inserting the corresponding nucleic acid sequence according to claim 24 in an expression vector, transforming a host with said vector, culturing the transformed host as well as recovering and purifying the expressed recombinant protein.
29. A method for detecting antigen of an HIV-3 strain of any of claim 1 to 4, characterized by coating a surface with an immunoglobulin fraction raised against said HIV-3 strain, bringing a body or culture fluid to be analyzed into contact with the immunoglobulins, and detecting the complex formed between the immunoglobulins and the antigen.

