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**HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTIONS;
STRAIN AND TYPE VARIATIONS;
DIAGNOSIS AND PREVENTION**

MIDTERM REPORT

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The results show that:						
a) the replicative capacity of HIV-2, like HIV-1, correlates with the severity of immunodeficiency;						
b) molecular clones of a rapid/high HIV-1 isolate may have restricted replicative capacity compared to the genetically heterogenous isolate. Transfection of the same molecular clones into different cell types may yield progeny viruses with different phenotypes;						
c) monocyte tropism is a general property of HIV isolates. In some cases silent infection is established. Silently infected monocyte/macrophages harbor virus that can be recovered by cocultivation with peripheral blood mononuclear cells. Since the						
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virus recovered differs from the original isolate in replicative and cytopathic characteristics we suggest that monocytes may contribute to virus variability;

d) neutralizing antibody response in HIV-1 infection is concomitant with seroconversion and isolate-specific. Variant viruses resistant to neutralization by autologous antibody emerge months or a year later. The lack of neutralizing antibodies to autologous virus in HIV-1 infected individuals may frequently be encountered;

e) virus neutralizing epitopes in the HIV-1 and HIV-2 envelope roughly coincide as demonstrated by use of synthetic peptides. In addition, at least one ADCC epitope has been identified for HIV-2 that is coincident with a neutralizing epitope.

Methodologically, the establishment of a simple, sensitive and specific polymerase chain reaction (PCR) protocol for the detection of HIV-1 in PBMC of infected individuals has been important. By using appropriate primers, amplification followed by direct sequencing of the amplified products, will now be possible.

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INTRODUCTION

The studies described herein have focused on HIV variability, in particular on biologic and antigenic variation and their relevance for pathogenesis. Biologic characterization involved tests for replicative capacity, cytopathic effect and monocyte tropism of virus isolates and molecular clones, whereas antigenic characterization involved virus neutralization in autologous system. By use of synthetic peptides, epitopes for virus neutralization and antibody dependent cellular cytotoxicity (ADCC) have been mapped in the envelope of HIV-1 and HIV-2.

Previous studies in our laboratory (1, 2) have shown that naturally occurring HIV-1 variants show distinct biologic features that correspond to the severity of HIV-1 infection. Virus from asymptomatic carriers or individuals with mild disease replicates slowly and inefficiently in the patients peripheral blood mononuclear cell (PBMC) cultures. Attempts to passage these viruses in CD4 positive cell lines usually fail or result in transient replication only. In contrast, viruses from patients with severe immunodeficiency replicate rapidly and efficiently in PBMC as well as in cell lines. Hence the designation slow/low and rapid/high, respectively. These two groups of viruses can also be distinguished by the type of cytopathogenicity exerted in PBMC (2). Rapid/high viruses are characterized by extensive syncytia formation, whereas syncytia are rarely seen with slow/low viruses. Instead, cultures infected with slow/low viruses show signs of cell death or no cytopathic changes at all. These results have been confirmed and extended by several laboratories world wide (3) and indicate that the biologic characteristics of the virus seem to play a key role in pathogenesis.

Primary HIV infection is accompanied by viremia which is rapidly cleared concomittant to seroconversion and development of isolate specific neutralizing antibodies (4). It seems therefore that neutralizing antibodies play a crucial role in clearing the initial viremia. The question arises why then continued virus replication occurs in the course of HIV infection? Is the virus replicating at a later stage different from the virus replicating early during HIV infection? If so, can the immune system cope with the new variants? In our studies we have focused on the role of neutralizing antibodies and antibodies mediating cellular cytotoxicity reactions (ADCC).

These studies provide the basis for our attempts to identify critical target immunogens for vaccine development.

In collaboration with Dr G. Biberfeld's group we have evaluated different forms of immune interventions in HIV-2 (5) and SIV_{sm} infected Cynomolgus macaque monkeys (6). It was demonstrated that infection with the non-pathogenic HIV-2 protects against the pathogenic consequences of a subsequent SIV_{sm} infection (7) and that immunization with inactivated HIV-2 material prevents infection with homologous virus in these animals (8). More recently we have demonstrated that passive transfer of antibodies in the form of serum from healthy SIV_{sm} infected animals or from animals hyperimmunized with HIV-2 can prevent infection with homologous virus (9). In the same system we are currently evaluating the possibilities for establishment of a protective immunity by immunization with synthetic peptides representing selected regions of the glycoproteins of HIV-2 and SIV_{sm}. In the selection of peptides for use in these studies advantage was taken of the knowledge of important immunoprotective domains in the HIV-1 glycoproteins including the principle neutralizing domain (10).

METHODOLOGY

Biological characterization of virus isolates: 1) replication in T-lymphoid and monocytoïd cell lines. First, peripheral blood mononuclear cells (PBMC) are infected with 5-20.000 cpm reverse transcriptase (RT) activity and 7-10 days later, when cultures are RT positive, 1×10^6 PBMC are cocultivated with 3×10^6 cells of each of the cell lines, Jurkat, Jurkat-tat, U937 clone 2, CEM and/or HUT-78 (2). 2) Cytopathogenicity is scored in PBMC cultures 7-10 days postinfection (2). 3) Replication in fresh monocyte/macrophage cultures. Monocyte cultures are prepared by seeding 2.5×10^7 PBMC into 25 cm^2 plastic culture flasks in RPMI medium supplemented with 10% heat-inactivated pooled HIV-1 negative human serum and 20% fetal calf serum (FCS). After 5 days at 37°C the cultures are extensively washed with PBS to remove nonadherent cells and maintained thereafter in RPMI medium with 20% FCS (11). Virus replication is followed by measuring HIV-Ag (p24) (12) and RT activity (13) in culture supernatants.

Virus neutralisation assay: Virus titrations are done in PBMC according to the method described by McDougal et al. (14) and modified by Albert et al. (4). In short, virus aliquots are diluted in medium, six fivefold dilution steps starting with a 1:5 dilution. First, 75µl of each virus dilution was added to five parallel wells of a round-bottom 96-well culture plate (Nunc, Roskilde, Denmark) and, second, 1×10^5 PHA-P stimulated blood donor PBMC in 150 µl medium was added to each well. The culture medium is changed on each of the following 3 days and on day 7 a 100 µl sample from each well is analyzed in an in-house HIV-Ag ELISA assay (12). The ID-50 was defined as the reciprocal of the virus dilution resulting in 50% positive wells (Reed-Muench calculation).

Neutralization assays were run simultaneously with each virus titration using three virus dilutions, 1:5, 1:25 and 1:125, for each serum dilution. Five twofold dilution steps of sera, starting with a 1:10 dilution, were added in triplicate (75µl each) to 96-well culture plates. Virus (or medium) was then added in an equal volume and the plate incubated for 1 h at 37°C. Subsequently, 10^5 PHA-P stimulated PBMC in 75µl were added and the plate further incubated overnight. Subsequent washing and testing as above. The negative virus controls consist of two wells with virus but no cells to check the washing procedure and five wells with cells but no virus. Sera from two asymptomatic homosexual men with high-titer neutralizing antibodies against HIV-1_{IIIB} are used as positive serum controls. All sera and virus isolates obtained from one patient are assayed simultaneously. The neutralizing titer of a serum is estimated at virus dilution(s) containing 10-50 ID-50 and defined as the reciprocal of the highest serum dilution giving a complete (or 80%) reduction in absorbance value in the HIV-Ag assay as compared to the mean -2SD of five negative wells containing cells only.

ADCC: the test was performed as described previously (15). In short, U937 clone 2 cells chronically infected with HIV-2SBL6669 were used as target cells. PBMC were derived from normal healthy donors and collected by density centrifugation on Ficoll-Isopaque, and adherent cells removed by the scrubbed nylon wool technique (16). ^{51}Cr -labeled target cells (1×10^4) and isolated lymphocytes as effector cells (2×10^5) were mixed with serum dilutions. Supernatant was harvested after 3 hours and released radioactivity was calculated.

Peptide synthesis. The peptides were synthesized in 30-70 mg quantities by the method of simultaneous multiple-peptide synthesis (17) and were cleaved with liquid hydrogen fluoride in a 24 vessel apparatus (18). Their composition was controlled by amino acid analysis and their purity by high performance liquid chromatography characterization using a reverse phase Vydac C4 column.

Peptide-ELISA. Peptides dissolved in 0.1 M carbonate-bicarbonate buffer pH 9.3 were added to microtiter plates in a final amount of 1µg/well. After incubation at room temperature over night the plates were washed with PBS and then blocked for one hour at room temperature with 0.5% bovine serum albumin in PBS. After removal of blocking buffer the test was run as previously described (19).

Western blotting was performed as previously described (20).

RESULTS

Biological properties of HIV-2 isolates.

Whether the biological properties of HIV-2 isolates are similar to those of HIV-1 is a particularly important question, since HIV-2 has been suggested to be less pathogenic than HIV-1 (21, 22). We have collected 13 HIV-2 isolates in order to compare their in vitro biological properties to those of HIV-1 isolates. Similarly to HIV-1, virus isolated from HIV-2 infected immunodeficient individuals fulfills the rapid/high criteria (23, 24). Accordingly, these viruses infect cell lines and give rise to continuous virus replication. On the other hand, HIV-2 infected asymptomatic individuals carry slow/low type of HIV-2. These viruses replicate in cell lines transiently or not at all. Still another similarity to HIV-1 is that rapid/high type of HIV-2 causes extensive syncytia formation in PBMC cultures, whereas slow/low type of HIV-2 isolates often lack this capacity. These similarities suggest that in cases where HIV-2 causes disease, albeit rarely or with longer incubation period than HIV-1, the pathogenic mechanisms operating are similar.

Biological characterization of progeny viruses obtained from molecular clones of a HIV-1 isolate with rapid/high replicative capacity.

In order to study the molecular determinants underlying the biologic characteristics, molecular clones were obtained from a HIV-1 isolate with high replicative capacity (25). The virus isolate, 4803, has only been passaged in PBMC prior to cloning. High molecular weight DNA of 4803 infected PBMC was restricted with Xba-1 and appropriate size fragments cloned in the bacteriophage λ -dash. Seven recombinant phages were identified. The clones were shown to be highly related to each other and differed only at 1 or 2 restriction sites of 26 tested. Following transfection by electroporation into various kinds of cells the phenotype of progeny viruses depended on the cell type. Viruses recovered from PBMC cultures differed from the parental isolate in that they did not form syncytia and lacked the capacity to replicate in cell lines. Since transfection of PBMC yielded progeny virus within one week, this phenotype is considered to be the true phenotype of the clones. Transfection into the T-lymphoid HUT-78 cell line and the monocytoid U937-2 cell line yielded virus after considerable delay (more than one month). Progeny viruses from HUT-78 cells were similar to the parental isolate, they formed syncytia in PBMC and replicated in all cell lines tested. Progeny viruses from U937-2 cells showed an intermediate phenotype in that they replicated in U937-2 cells but not in T-lymphoid cell lines. The results suggest that molecular clones of a rapid/high HIV-1 isolate may have restricted replicative capacity compared to the genetically heterogenous isolate. Our hypothesis is that the phenotype of naturally occurring HIV-1 may be dependent on the interaction of variant viruses that are always present in the genetically heterogenous isolate. Diversification and selection during replication following transfection may be different in different cell types resulting in alteration of the viral phenotype in a cell type dependent manner.

Infection of monocyte/macrophage cultures by HIV-1, HIV-2 and SIV.

Studies on more than 70 HIV-1, 12 HIV-2 and two SIV isolates have shown that all viruses can infect fresh monocyte/macrophage cultures derived from the peripheral blood of normal donors. Replication and cytopathogenicity by SIV is more pronounced (26) than by HIV. While replication of HIV is influenced by the maturation stage of monocytes, replication of SIV

is not, suggesting different replicative mechanism for these viruses in human macrophages. Accordingly, HIV replication in macrophages can no longer be detected one month after infection. Virus can, however, be recovered by cocultivation with PBMC (11). The virus thus recovered differs from the original isolate in replicative and cytopathic characteristics. Our results demonstrate that HIV can establish a persistent, nonproductive infection in monocyte/macrophages and, consequently, these cells may serve as infectious reservoir in the infected individual. Moreover, passage of HIV through macrophages may contribute to the biological diversity of virus isolates.

Establishment of a simple, sensitive and specific polymerase chain reaction (PCR) protocol for the detection of HIV-1 in PBMC of infected individuals.

In this test protocol (27) we amplify crude cell lysates in a two-step PCR, first with outer primers then with inner primers, nested within the first and the PCR product is visualized by agarose gel electrophoresis and ethidium bromide staining. The samples are analyzed with three (or four) sets of nested primers designed to amplify HIV-1 gag, pol and env gp41 (and env gp120) sequences, respectively. We were able to amplify HIV-1 sequences in all samples from 90 HIV-1 seropositive individuals with mostly mild symptoms. Samples from 26 healthy blood donors as well as cells infected in vitro with HIV-2 and HTLV-I were negative in PCR, thus demonstrating the specificity of the amplification. Since this technique avoids conventional DNA extraction as well as hybridization for the detection of the PCR product it may be widely used in clinical virology practice. Moreover, the use of a primer set spanning the V3 region of the envelope gp120 protein (variable region 3, also called the immunodominant loop) allowed us to specifically study this region in neutralization resistant HIV-1 variants (described in next chapter and in 28).

Within the frame of studies on pediatric HIV infection we have examined the presence of HIV-1 sequences in altogether 165 blood samples from 47 HIV-1 antibody positive mothers and 87 children born to HIV-1 antibody positive mothers. Early and reliable diagnosis of HIV infection in children is a particularly important issue. We were able to amplify HIV-1 sequences in samples from all mothers and all children found to be HIV-1

seropositive or showing clinical signs of immunodeficiency (29).

Seronegative children or those without clinical signs of HIV-1 infection (91 samples of 76 children) were also PCR negative

Studies on the kinetics of appearance and specificity of HIV-1 neutralizing antibodies.

We have used a unique autologous system. HIV-1 has been isolated during symptomatic primary HIV-1 infection (30) and repeatedly thereafter and tested against autologous sera collected in parallel (4). Isolate-specific low-titer neutralizing antibodies developed within 2 - 4 weeks after onset of symptoms and the titers to the first isolate increased with time. In three patients we could document the emergence of virus variants with reduced sensitivity to neutralization by autologous, but not heterologous, sera. These virus variants were, however, not resistant to neutralization per se, since they were readily neutralized by the positive control serum. Our patients did not develop antibodies capable of neutralizing the new virus variants during the observation period. This seems to suggest either a progressive impairment of immunoglobulin production or a mechanism by which the new virus variants evade detection by the immune system.

As a continuation, we have studied the V3 region of the envelope gp120 protein (also called the immunodominant loop) of these neutralization sensitive and resistant HIV-1 variants, using amplification by the polymerase chain reaction (PCR) followed by nucleic acid sequencing. The use of a primer set spanning the V3 region of the gp120 portion of the envelope (27) allowed us to specifically study this region (nt 7137-7572) by use of a direct solid phase DNA sequencing technique (28). The results show that in three of four individuals tested, nucleic acid sequence of the envelope V3 region of variant viruses is identical to the V3 region of the first, neutralization sensitive isolate. In the fourth individual three point mutations were present in the V3 region 118 weeks after the onset of primary HIV-1 infection. The individual was asymptomatic at the time of the collection of this last isolate. The results indicate that resistance to neutralization by autologous antibody may arise from changes outside the V3 region. For a more detailed mapping of neutralizing epitopes within the

envelope, we proceed to determine the entire nucleotide sequence of the envelope.

Mapping of virus neutralizing and ADCC epitopes in the HIV-1 and HIV-2 envelope with synthetic peptides.

HIV-1

Four major sites involved in neutralization were identified by use of a panel of 80 HIV-1 antibody positive human sera (31). A correlation between high neutralization of a certain virus strain and strong reactivity with a selected homologous peptide suggested that the corresponding region might be involved in virus neutralization. In order to substantiate the role of a certain region in neutralization peptides were used to block the neutralizing activity of selected sera. Four sites were identified, two sites on each of the envelope glycoproteins. In gp120 one was the V3 domain and the other was the carboxyl terminal end, amino acids 489-508. The latter site is conserved and was previously reported to be highly antigenic. Our results show that this site reacts with neutralizing antibodies. The gp41 sites encompass the previously identified, presumably intracellular, region of this molecule (amino acids 732-746) and in addition a previously unrecognized, conserved site with an extra-membrane position, amino acids 647-671. The results indicate that this conserved epitope of the HIV-1 envelope elicits a virus neutralizing antibody response during natural infection in humans and may therefore be considered for inclusion in a vaccine against HIV-1.

HIV-2

Twenty five peptides representing different regions in the HIV-2SBL6669 (clone Isy in Meyr's et al. Human retroviruses data base) envelope proteins were synthesized. The peptides were selected to represent regions which in HIV-1 had been documented to be of antigenic or immunogenic importance.

Evaluation of the antigenic activity of the HIV-2 env peptides demonstrated a dominating activity in four different regions (32). These were 1) the V3 region and an adjacent (carboxy terminal) amino acid stretch located in the carboxy terminal half of the external glycoprotein. 2) the extreme carboxy terminal end of gp120. 3) a uniquely antigenic site in

the amino terminal part of the transmembrane glycoprotein and 4) a preferentially HIV-2 antigenic site located further about 50 amino acids towards the carboxy terminal of the latter site.

In order to characterize the immunogenic activity of peptides guinea pigs were immunized with KLH-coupled material. Antisera showed high ELISA titers in tests with homologous peptide antigen and reacted in about 65% of cases with intact protein as determined in whole antigen ELISA and Western blot studies. All sera were tested for their capacity to neutralize and to mediate ADCC with the homologous virus. Seven peptides representing five different regions elicited virus neutralizing antibody activity. The highest titer was seen with sera against two overlapping peptides representing the carboxy terminal part of the V3 loop. High neutralizing activity was also found in sera against peptide Ala¹¹⁹-Cys¹³⁷, Thr⁴⁸⁹-Gly⁵⁰⁹ (with weaker activity in sera against an overlapping peptide) and His⁷¹⁴-Glu⁷²⁹. A weak activity was seen in sera against peptide Cys⁵⁹⁵-Thr⁶¹⁴. Several anti-peptide sera showed ADCC activity but in only one case did this activity occur concomitantly with a neutralizing activity (in one of the V3-specific sera. Significant ADCC activity was also found in sera against peptides Glu²⁹¹-Ser³¹¹ and Arg⁴⁴⁶-Phe⁴⁶¹.

CONCLUSIONS

The biologic properties of HIV-1 and HIV-2 are similar and correspond to the severity of HIV infection. Both show a slow/low replicative capacity when isolated from individuals with normal levels of CD4 cells in blood and, conversely, a rapid/high replicative capacity when obtained from severely immunodeficient patients. All HIV-2 isolates tested, like those of HIV-1, infect and replicate in fresh monocyte/macrophage cultures. Monocyte tropism seems to be a general property of HIV isolates. The results indicate that the pathogenic mechanisms operating in HIV-1 and HIV-2 infection are similar.

Monocyte/macrophages may have an impact on virus variability, since viruses rescued from silently infected monocyte/macrophages have different biologic properties than the infecting virus. The notion that the cell type is important in determining the biological properties of HIV has

been substantiated and further extended in experiments using molecular clones of an HIV-1 isolate. The results show that transfection into different cell types alters the viral phenotype in a cell type dependent manner. Our present conception of pathogenic mechanisms operating in HIV infection has been put forward in a recent review (33). Work with the molecular clones will be extended in order to pinpoint the genetic differences underlying the phenotypic changes.

Neutralizing antibody response in HIV-1 infection is concomitant with seroconversion and isolate-specific. Neutralizing antibodies seem to have a role in clearing the initial viremia since as the antibodies appear viremia disappears. However, virus isolated a few months later cannot be neutralized by the antibodies present in the patients serum indicating the emergence of neutralization resistant variants. In fact, our extended work as well as results from another group (34) emphasize that the lack of neutralizing antibodies to autologous virus in HIV-1 infected individuals is a general phenomenon. Efforts to localize the change leading to neutralization resistance in the viral envelope have shown that the amino acid sequence of V3 region may (one of four tested) or may not change in variant viruses. Peptides corresponding to each patient-specific V3 sequence will be used to dissect whether the early, isolate-specific neutralizing antibody response is directed to the V3 region. In addition, the nucleotide sequence of the entire envelope will be determined. The same approach, autologous neutralization in combination with nucleotide sequencing and peptide synthesis, will be used to test HIV-1 antibody positive mothers that do or do not transmit infection to their child.

For the first time, immunogenic regions in the HIV-2 envelope could be mapped using synthetic peptides and guinea pig antisera. Based on these results attempts will be made to immunize cynomolgus macaque monkeys with single HIV-2 or SIV_{sm} peptides or pools of them. The development of neutralizing antibodies and the susceptibility to challenge of animals with homologous virus will be determined.

We have not succeeded to find a test system for autologous ADCC. Similarly to autologous neutralization, autologous ADCC should make use of the patient's own isolates and antibodies. The problem has been the target cell: in ADCC this is a virus-infected cell line. Since most of HIV-1 and HIV-2

isolates have a slow replicative capacity, they will not replicate in cell lines. Jurkat-tat cells support replication of slow/low viruses (35) but at most 20% of cells express viral antigens. The fraction of cells with viral antigen is thus too low for a satisfactory in vitro ADCC test system. We are now in the process of cloning virus-infected Jurkat-tat cultures, in order to enrich for virus expressing cells. Another approach is to infect the CD4+ fraction of PBMC with slow/low virus and use these cells as targets for ADCC.

Based on the present results, it is our working hypothesis that the biologic and antigenic characteristics of HIV are closely linked. It seems that neutralizing antibodies, alone or together with other immune mechanisms, efficiently control virus replication following the initial viremia. Variants with slow replicative capacity, however, seem to evade detection by the immune system. The continuous slow replication of these viruses may in turn brake down the immune defence of the HIV infected individual and lead to AIDS.

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