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BACTERIALLY EXPRESSED CORE AND ENVELOPE PROTEINS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1): COMPARATIVE EVALUATION IN DETECTION OF TYPE-SPECIFIC ANTIBODIES

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Summary. - Recombinant proteins derived from immunodominant conserved domains of HIV-1 *env* and *gag* genes were synthesized in *E. coli.* An immunoblot system using total cell lysates was employed for the analysis of recombinant bacterial clones. Together 427 serum samples obtained from asymptomatic anti-HIV seropositive individuals, AIDS patients, healthy donors and persons suffering from various conditions were comparatively evaluated for the presence of HIV-1 antibodies using recombinant peptides and commercially available western blot (WB) and ELISA assays. The recombinant antigen product of plasmid pEX41 was found to be superior, with respect to sensitivity and specificity, to the viral gp41 which represents a diagnostically important constituent of the WB.

Key words: HIV-1; recombinant antigens; western blot; expression; specificity; sensitivity

Introduction

Serological diagnosis of HIV-1 infection, etiologically linked with AIDS and related disorders (Barré-Sinoussi *et al.*, 1983; Popovič *et al.*, 1984; Levy *et al.*, 1984), is the subject of continuing methodical research. Well documented are interindividual differences in the prevalence of antibodies with specificity to particular viral antigens. The most consistently identified antibodies are directed against the *env* gene encoded glycoproteins (Sarngadharan *et al.*, 1985; Barin *et al.*, 1985; Allan *et al.*, 1985*a*). Reactivity with the major viral core antigen p24 correlates to a certain extent with the clinical status of individuals suffering from advanced stages of the infection and can serve as a serological marker of disease's progression (Kitchen *et al.*, 1984; Steimer *et al.*, 1986). Serum antibodies to the products of regulatory viral genes *nef, vif* and *tat* have

also been detected in infected individuals with varying frequency (Allan *et al.*, 1985*b*; Kan *et al.*, 1986; Barone *et al.*, 1986).

Early identification of persons infected with HIV is important in the prevention of virus spread. The first generation of enzyme-linked immunoassays (EIA) for detection of HIV antibodies, introduced in the United States early in 1985 (Petricciani, 1985) employed as antigenic material partially purified whole virus. They have proved to be highly sensitive and therefore effective in ensuring the safety of transfused blood. However, false-positive reactions, often due to antibodies directed against contaminating cellular antigens, require confirmative testing in order to obtain an accurate result. Western blotting (WB), indirect immunofluorescence and radioimmunoprecipitation are recommended methods for confirmation of the presence of HIV antibodies in specimens which scored positively in the screening EIA (WHO, 1986). However, they feature some drawbacks most relevant of which are the health risk resulting from propagation of virus infected cells, complicated nature of some of the test protocols and rather high costs linked with the bulk production of the virus. In recent years alternative assays were developed which rely on proteins expressed in bacteria (Kenealy *et al.*, 1987; Burke *et al.*, 1987; Hofbauer *et al.*, 1988). These recombinant antigens are encoded by conserved gene fragments of a particular cloned viral isolate. Yet, they show broad reactivity with sera specific for diverse HIV strains of the same type (Kenealy *et al.*, 1987).

An immunodominant epitope at the N-terminus of HIV-1 gp41 has been previously identified (Modrow *et al.*, 1987) and shown to exhibit a relatively high amino acid conservation. We have cloned 3 different *env* gene fragments containing this epitope and expressed them in *E. coli*. Nitrocellulose strips containing by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) fractionated total bacterial lysates were prepared and used for testing of previously characterized sera. Although it has been claimed that *gag*-derived antigens are not essential for the construction of sensitive assays for HIV antibodies (e. g. Burke *et al.*, 1987) we have prepared a recombinant peptide corresponding to nearly the entire viral core protein p24. Investigations on sera reactivity using *env/gag* immunoblots were carried out and the potential of those recombinant proteins for diagnostic purposes was assessed.

Materials and Methods

Serum samples. A panel of sera which were previously verified as positive in a screening EIA (Wellcozyme-Imula, Lachema, Brno) and WB (Biotech/Du Pont, Rockville, Md.) was obtained fom 90 patients at various stages of HIV-1 infection. They were acquired through The National Reference Laboratory for AIDS, Praque (M. B.), The National Institute of Hematology and Blood Transfusion, Budapest (E. U. and G. F.), and chosen further from the serum bank of The National Reference Laboratory for AIDS (Bratislava). As a control of the specificity of recombinant materials 261 anti-HIV-1 negative sera were obtained from blood donors. The majority of them scored

negatively upon screening testing (n=208) and in the rest the initial reactivity was not confirmed by WB. Further were examined 25 EIA positive samples which in HIV-1 WB scored as indeterminate. In addition, a panel was used consisting of 17 sera with high titers of antibodies to *E. coli* strains 026 and 055 (kindly supplied by G. Füst) and a panel of 34 serum specimens from patients with systemic lupus erythematosus.

Expression vectors, bacterial strains and HIV-1 proviral clone. E. coli strain JM105 was used as a recipient for recombinant expression plasmids pKK41, pKK24 and pUC1841 derived from pKK233-2 and pUC18 (both Pharmacia, Uppsala). The transformed bacteria were grown in LB medium containing 50 μg/ml of ampicillin (AMP) and 25 μg/ml of streptomycin at 37 °C. *E. coli* pop2136 was transformed with HIV *env* recombinant derivative of pEX1 (Boehringer, Mannheim) designated pEX41 and grown at 30 °C in LB containing 50 μg/ml AMP. HIV proviral clone pBT1 derived from LAV-1 isolate (Alizon *et al.*, 1984) was kindly provided by J. C. Chermann (U322 INSERM, Marseille).

Cloning procedures. Specific DNA fragments from the *gag* and *env* open reading frames of HIV-1 clone pBT1 (Wain-Hobson *et al.*, 1985) were engineered into *E. coli* expression vectors (Fig. 1) using standard procedures (Maniatis *et al.*, 1982).

pKK41 was constructed previously (Zachar *et al.*, 1990). Briefly, the 1429 bp *Bg*lII fragment of the *env* gene of HIV-1 was subcloned from pBT1 and amplified in pUC18 after insertion into the *Bam*HI site. Using *Pst*I and *Hind*III a 523 bp fragment was excised (nucleotides 7179 to 7702) and ligated into the homologous sites of pKK233-2. After transformation of *E. coli* recipient strain JM105, colonies were screened for the presence of recombinant plasmids by restriction analysis. Positive clones were induced and the synthesis of HIV-1 protein was evaluated by immunoblotting with anti-HIV-1 sera.

pKK24. The *Hind*III DNA fragment from the *gag* open reading frame of pBT1 was isolated. The expression plasmid pKK233-2 was linearised with *Hind*III and 3' recessed termini of the gene fragment, as well as of plasmid, were filled in. The segment 627 bp long (nucleotides 631 to 1258) was subsequently inserted into the dephosphorylated expression vector. Recombinant bacterial clones were analysed as described above.

pUC1841. The *Bg*lII fragment (1429 bp) of HIV-1 *env* gene was amplified in pUC18. Then in a two step-digestion a 544 bp *Hind*III fragment was isolated and in turn digested with *Sau*3AI. Resulting 157 bp long fragment (nucleotides 7546 to 7702) was ligated into *Bam*HI and *Hind*III sites of pU18. Transformation of *E. coli* JM105 was followed by immunoblot screening of the induced clones.

pEX41. The bacterial expression plasmid pEX1 was digested with *Sma*I and dephosphorylated. The 566 bp fragment of HIV-1 *env* gene containing the immunodominant epitope located in the N-terminal part of gp41 was isolated in two steps from the recombinant plasmid pUC18 containing the 1429 bp *Bg*lII fragment of the HIV-1 *env* gene. In the first step a 865 bp *Pst*I-*Bam*HI segment was excised and after isolation from low melting temperature agarose, it was further digested with *Bsp*RI. The resulting 566 bp segment (nucleotides 7403-7968) was inserted into the prepared vector. Transformed *E. coli* strain pop2136 was analysed for the HIV-specific recombinant protein by immunoblotting.

Immunoassay for recombinant proteins and HIV-1-specific antibodies. E. coli JM105 harbouring recombinant plasmids pKK41, pKK24 and pUC1841 were grown at 37 °C until the cultures reached optical density A_{600}=0.7. IPTG was then added to 1 mmol/l final concentration and the induced cultures were further incubated for 2 hr. Cells were spun down by low speed centrifugation, and pellets were resuspended in 10 mmol/l Tris.HCl (pH 8.0) containing 1 mmol/l EDTA, 1 % SDS and 5 % mercaptoethanol to 1/10 of the original volume and boiled for 10 min. Recombinant clones of *E. coli* pop2136 transformed with pEX41 were grown at 30 °C. When the optical density A_{600}=0.7 was reached, the culture temperature was raised to 42 °C and protein induction continued for 2 hrs. Bacteria were then pelleted, resuspended to 1/10 of the original volume in the same buffer and boiled for 10 min. The proteins were fractionated by SDS-PAGE in 12.6 % gel and electrotransferred onto nitrocellulose membrane. For immunodetection, the membranes were soaked for 30 min in 10 mmol/l Tris.HCl (pH 7.6) with 150 mmol/l NaCl and 0.05 % Tween20

(washing buffer) and blocked for 10 min by incubating in 5 % nonfat dry milk in 10 mmol/l Tris.HCl (pH 7.6) with 0.1 % sodium azid and 0.2 % Nonidet NP40 (blocking solution). The procedure was proceeded by incubating the membranes overnight with a serum sample diluted 1:100 in blocking solution. Unbound antibodies were washed off and membranes were reacted with swine anti-human immunoglobulin conjugated with peroxidase (ÚSOL, Prague) for 2 hrs. After repeated washing the immune complexes were visualised using 4-chloro-1-naphthol as a substrate.

Results

Expression of HIV-1 gag and env gene fragments in E. coli

We have constructed four recombinant plasmids pKK24, pKK41, pUC1841 and pEX41 (Fig. 1) which coded for amino acids 99-302 of the HIV-1 *gag* gene (pKK24) and amino acids 472-645, 594-645 and 547-743 of the HIV-1 *env* gene, respectively. The constructs pKK24, pKK41 and pUC1841 produced recombinant proteins which with the exception of several amino acids at C- or N--terminus, derived from the vectors, contained no *E. coli* sequences. The recombinant p24 with a molecular weight of 25 kD consisted of 176 amino acids of p24 and 33 amino acids of the C-terminal part of core protein p17. The product of pKK41 was a 21 kD protein which contained 45 amino acids of the C-terminal part of the external envelope glycoprotein gp120 and 128 amino acids of the N-terminal end of the transmembrane glycoprotein gp41. A smaller N-terminal fragment of the gp41 gene comprising an immunodominant epitope has been inserted into the expression vector pUC18. This fragment was made shorter of a hydrophobic stretch of amino acids that is present in the N-terminal sequences of gp41 because it is known that hydrophobic sequences may have a deleterious effect on recombinant protein synthesis in bacteria (Windheuser *et al.*, 1988). This was indeed exemplified by the observation that the level of recombinant protein production directed by the pUC1841 was slightly higher than that of pKK41 in spite of the *trc* promoter of pKK233-2 which is claimed to be 10 times stronger than the simple *lac* promoter of pUC18 (Amann *et al.*, 1983) (data not shown). The size of the pUC1841 recombinant protein corresponded to that of the predicted product of the insert, suggesting that the expected synthesis of a fusion product of HIV-1 DNA sequences and *lac*Z gene did not occur. This may be explained e. g. by instabilty of respective mRNA or metabolic degradation of the hybrid gp41/*lac*Z protein (Zabeau *et al.*, 1982; Bachmair *et al.*, 1986). In order to optimize the level of expression we have employed the pEX1 expression plasmid and created a hybrid gene consisting of a short gp41 gene sequence fused with the *cro-lac*Z gene. After heat induction of the p_R promoter of pEX1 an expected 138 kD protein was synthesized. This protein could be detected with Coomasie Brilliant Blue staining of SDS-PAGE gels. To evaluate the immunoreactivity of our recombinant peptides we have prepared a set of nitrocellulose strips using total cell lysates of the clones pKK24 and of the host cell strains.

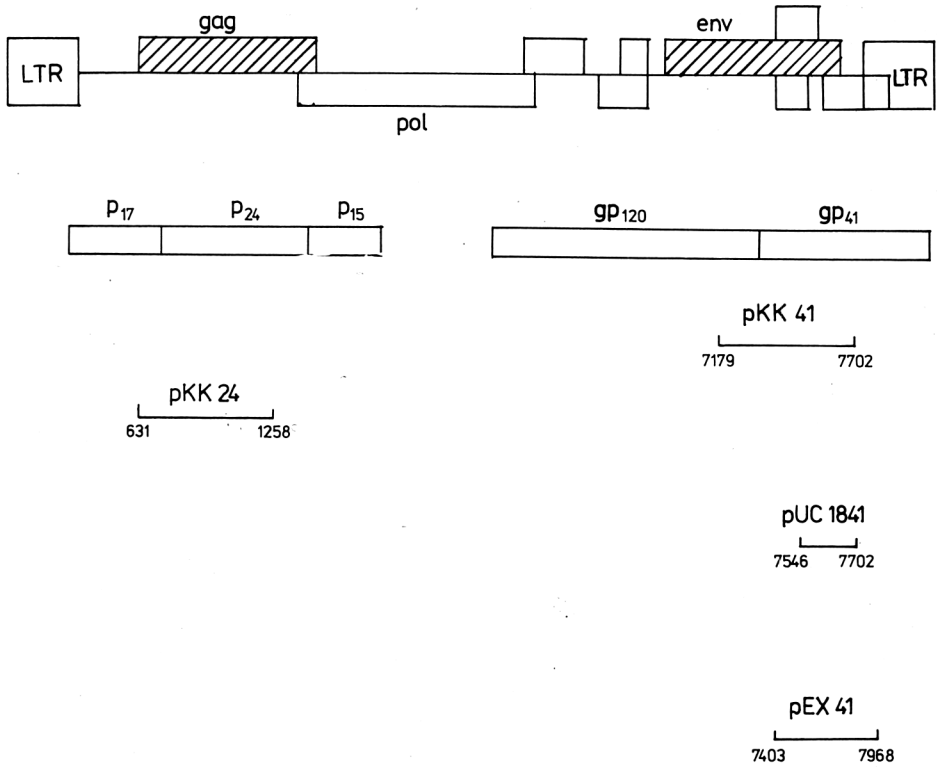

Fig. 1
Schematic representation of the genome structure of HIV-1 and assignment of DNA fragments used for construction of recombinant plasmids pKK41, pKK24, pUC1841, and pEX41 Numbers below the gene fragments denote nucleotides (Wain-Hobson *et al.*, 1985).

Immunoreactivity of HIV recombinant polypeptides

Results obtained with the specimens from seropositive asymptomatic donors and AIDS patients in comparison with commercial WB (Biotech/Du Pont) and screening competitive EIA (Wellcozyme-Imula, Lachema, Brno) are shown in Table 1. All 90 sera scored positively in each of the three assays. According to the criteria defined by the producer of the WB test, a serum sample was considered to be positive when antibodies to at least one product of each to the structural genes were detected. We have compared the anti-gp41 and -p24 reactivity by the WB and the recombinant immunoblot. The recombinant p41 proved to be more sensitive than the viral gp41 of the WB test. The WB failed to detect antibodies to gp41 in 14 specimens and in 6 it has produced only weak reactions. When analysing the p24 reactivity discrepant results were

obtained in 10 serum samples. However, we assume that the majority of these cases could be resolved after purification of the recombinant p24 and its application in a higher concentration on the nitrocellulose membrane. Table 2 documents the reactivity of HIV-1 seronegative donor sera. In the group were comprised 53 sera showing false-positive reactions in the screening EIA. None of them reacted unequivocally positively in WB or recombinant immunoblot. In this group no p41 or gp41 bands have been observed and only faint reaction was apparent with recombinant p24 (2 specimens) and viral p24 (1 specimen), respectively. 25 specimens that yielded indeterminate results in WB were also examined with recombinant antigens (Table 3). Apart from one serum (weak reaction with both antigens) the other 24 did not give any reaction with the recombinant p41. 12 specimens of these 24 were additionally confirmed as

Table 1. Reactivity with gp41/env and p24/gag antigens in WB+and recombinant immunoblot of 90 HIV-1 seropositive sera++

No. of sera+	p41 (rec.)	gp41 (WB)
70	+	+
6	+	+/-
14	+	-
	p24 (rec.)	**p24 (WB)**
79	+	+
2	+/-	+
1	+/-	-
1	+	-
1	-	-
2	-	+/-
4	-	+

\+ Commercial western blot kit (Biotech/Du Pont).
++ All sera scored positively in screening EIA (Wellcozyme-Imula).

Table 2. Reactivity with gp41/env and p24/gag antigens in WB and recombinant immunoblot of 62 donor sera HIV-1 seronegative according to WB criteria (Biotech/Du Pont)

No. of sera+	p41 (rec.)	gp41 (WB)
62	-	-
	p24 (rec.)	**p24 (WB)**
59	-	-
2	+/-	-
1	-	+/-

\+ Further 199 sera negative in screening EIA scored negatively in recombinant immunoblot.

Table 3. Reactivity with gp41/env and p24/gag antigens in WB and recombinant immunoblot of 25 sera HIV-1 indeterminate according to WB criteria (Biotech/Du Pont)

No. of sera	p41 (rec.)	gp41 (WB)
22	-	-
1	+/-	-
2	-	+/-
	p24 (rec.)	**p24 (WB)**
1	+	+
2	+/-	+
17	-	+
5	-	-

negative when a second sample withdrawn after 6 months was tested by WB. 17 samples of *E. coli* immune sera as well as 34 sera from patients with lupus erythematosus did not give positive reactions with the recombinant antigens.

Discussion

At present, the diagnostic assessment of HIV-1 antibody status requires further confirmation of the screening EIA results (reactive sera) by further testing. In practice, proteins of complete virions separated by SDS-PAGE are most frequently used for this purpose (Western blotting). The WB is a technique that permits the identification of antibodies directed to individual structural polypeptides of HIV-1. In general, for positivity two criteria have been accepted, each describing a distinct pattern of reactivity. The first criterion requires the presence of antibodies to products of the *env* gene and at least one other structural gene of HIV (Hausler, 1988; CDC, 1989; The Consortium for Retrovirus Serology, 1988). The second criterion requires the reactivity against the products of each of the three structural genes (Sandler *et al.*, 1988). A WB test cannot be definitely interpreted when the bands which appear do not comply with the established criteria. Testing of specimens repeatedly reactive in EIA demonstrated that the frequency of WB indeterminate results can be as high as 20 % when dealing with a population with low prevalence of HIV-1 infection (Genesca *et al.*, 1989). Even among persons clearly not infected with HIV-1, whose screening tests give negative results for HIV-1 antibodies, up to 15 % may produce some bands on a blot (product insert, Du Pont Western blot kit for antibodies to HIV-1). Supposedly, the reasons of such HIV-1 indeterminate Western blot results can fall in two categories:

First, reactivity may be caused by the particular antigens used. Confusing reactions may rely on the components of the cells used to grow the virus (nonviral peptides, particulary actin or an antigen of the HLA complex) (Dodd

et al., 1990), lot-to-lot variations in test kits, or transient phenomena in the donor's sera.

Second, some findings are due to reactivity against HIV-1-encoded proteins. Testing systems employing recombinant HIV-1 proteins have shown that the majority of samples producing a HIV-1 indeterminate WB really contains antibodies which react with epitopes encoded by HIV-1 genes (Kleinman *et al.*, 1988). It has been hypothesised that donors of such samples may be actually infected wih a related retrovirus exhibiting a certain serological cross-reactivity mostly with polypeptides encoded by the *gag* gene of HIV (evolutionary the most ancient gene of retrovirus family). This hypothesis has not yet been experimentally confirmed.

The major difficulty in interpreting indeterminate blot patterns (particularly those characterized by the presence of reactivity to the *gag* peptide p24), is the possibility that the pattern may be a sign of developing specific seroconversion. In fact, this appears to be infrequent in population at low risk for infection (Dodd *et al.*, 1990; Kleinman *et al.*, 1988). Further studies have documented that a stable indeterminate blot pattern is almost certainly not due to infection with HIV-1 (Dock *et al.*, 1988; van de Poel *et al.*, 1989; Josephson *et al.*, 1989). At present, withdrawal of a second sample after 3 to 6 months is recommended to determine whether an indeterminate pattern is associated with specific seroconversion. Although p24 reactivity may actually be the first sign of HIV-1 seroconversion when the WB method is used, it has been demonstrated that antibodies to envelope antigens appear concurrently and can be detected utilizing methods that optimize their detection (Kenealy *et al.*, 1987).

Consequently it would be possible to resolve an indeterminate blot pattern by supplementary testing of the initial sample using an assay with suitable recombinant HIV-1 *env* antigens. In this respect, especially the transmembrane envelope glycoprotein gp41 seems to be an optimal substrate in the sense of sensitivity and specificity. The bacterially expressed pEX41 gave rise to an antigen which even unpurified performed superior to the gp41 of the commercial WB. Therefore we consider this plasmid as a source of HIV-1-specific protein that has the potential, to be practically useful when used as a supplementary or together with other HIV-1 gene encoded products (*gag, pol*) even as an alternative assay.

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