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Detection of anti-Rev antibodies in human immunodeficiency virus type-1 patients using a recombinant 18kD Rev protein

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Summary

The proteins Rev, Tat and Tev encoded by the regulatory rev, tat and tev genes of HIV-1 were expressed using a pUC12-based expression vector in *Escherichia coli*. The production of an immunologically-reactive 18kD Rev protein was confirmed by Western blotting using specific antibodies against the authentic protein. Further Western blot analysis using the recombinant Rev protein against 47 HIV-1 patient sera indicated specific antibody reactions in 31.6% (6/19) of asymptomatic carriers, in 41.7% (5/12) of AIDS-related complex patients and in 18.7% (3/16) of AIDS patients. The relatively lower incidence of anti-Rev antibodies in the AIDS group may be associated with HIV-1 induced immunodeficiency. The absence of circulating antibodies to Rev in over half of the HIV-1 sera tested renders the use of Rev as a diagnostic index unreliable, although useful information may still be obtained in prognostic studies of the Rev-positive patients. No definite reactions were observed between the same patient sera and Tat or Tev proteins which be may explained by the low expression levels of the proteins we obtained, together with the reportedly low antigenicity of the regulatory proteins.

Key words: Rev protein, HIV-1 recombinant regulatory proteins, Western blot analysis, human and anti-Rev antibodies

Introduction

Diagnosis of HIV-1 infection is normally established by demonstrating antibodies to the structural proteins with over 90% of infected people having antibodies against the envelope proteins[1]. Although the regulatory proteins are under intensive investigation for the elucidation of the regulatory mechanisms which govern HIV replication, their role and effect in the immune response of the host has not been studied in detail. This is despite the fact that in the course of HIV infection, the first appearance of viral RNA in the cytoplasm of infected cells coincides with the synthesis of the regulatory proteins (Rev, Tat, Nef), structural proteins being produced later[2–4]. In the present report we describe the generation of a recombinant 18 kD Rev protein specifically designed to have the same immunological properties as the authentic protein, which we then applied in Western blots against HIV-1 patient and control sera to study the prevalence of anti-Rev antibodies in those patients. For comparison, Tat and Tev proteins were expressed also and used in the experiments.

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Materials and methods

Bacterial strains and plasmids

The *Escherichia coli* strain DH5a was used for plasmid propagation and standard cloning procedures[5]. The *E. coli* strain BU8049, a Δlon strain depleted of a protease, was used for expression of recombinant proteins[6]. Plasmid pUC12 modified to include a unique NcoI restriction site immediately 3' to a constitutive Lac promoter, at the polylinker region, served as the expression vector (pUC12N). The cDNA encoding for

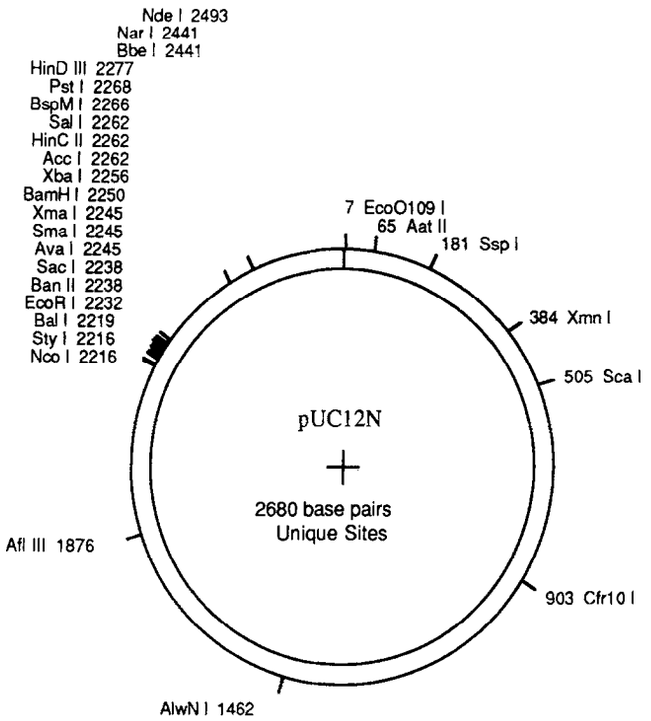

Figure 1. Schematic representation of the expression vector used for the generation of the recombinant regulatory proteins.

Rev and subcloned into the EcoRV of pBLUESCRIPT (Stratagen, US), was subject to PCR-mediated site specific mutagenesis[7], resulting in alternation of the codon usage of the gene according to *E. coli* translation machinery demands. An NcoI site was also created at the 5' end of the rev genespanning the rev AUG. The mutagenized gene was excised by digesting with NcoI and introduced to pUC12N linearized with NcoI to yield pUC12N-Rev (Figure 1). This assured the provision of an initiator AUG (from NcoI) located at the approximate position relative to the Shine–Dalgarno sequence on the plasmid. Similar subcloning was achieved with the tat and tev genes. Tat is another regulatory protein and Tev is a recently described tripartite Tat-Env-Rev fusion protein[8,9].

Bacterial cultivation and protein purification

The pUC12N-Rev transformed BU8049 *E. coli* cells were grown overnight at 37° C in Luria broth supplemented with 10 μg ml^{-1} ampicillin. Saturated cultures (5 ml) were diluted 50-fold into Super broth containing 10 μg ml^{-1} ampicillin and were further incubated at 37° C for another 6–7 hours. The cells were harvested, washed once with sterile water, resuspended at a density of 0.25 g ml^{-1} in 50 mM Tris-HCl, pH 8.0 containing 1 mM EDTA and 0.5 mM PMSF, disrupted by sonication and the lysate was clarified by centrifugation at 20 000 ***g*** for 20 min[10]. The supernatant containing the Rev protein was used directly for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blot analysis as described elsewhere[5].

Sera

SDS-PAGE purified Rev protein was used to immunize rabbits for polyclonal antibody production[6]. The serum against the authentic Rev was kindly provided by Dr G Pavlakis, National Cancer Institute, Frederic, Maryland, US. Human serum specimens used in this study, were provided by the National Retrovirus Reference Centre, Athens, Greece.

Western blot analysis

A small (30 μl) aliquot of the clarified bacterial lysate was subjected to SDS-PAGE according to standard procedures[4] and the gel electroblotted overnight onto nitrocellulose filter. Other gels were stained using Coomassie blue to visualize protein resolution. The transfer of proteins to filters was confirmed by staining filter strips with Amido black. The filters to be probed were initially blocked with bovine serum albumin to prevent non-specific binding of antibodies and subsequently allowed to immunoreact with anti-Rev antibody. Two methods were applied to detect specific reactions thereafter. When the proteins were present in small amounts,[125] I–protein A was used and the resultant binding of protein A to the previously applied antibody was visualized by autoradiography. A second approach involved the use of a goat anti-rabbit antibody conjugated to horseradish peroxidase diluted 1 : 2000 (NIDA Biometria, Heraklion, Greece) and immunoreactions were detected by enzymatic means[6]. In the case where human serum specimens were used, 1.5 ml of 1 : 5 diluted sample was applied to 1 cm strips, followed by a rabbit anti-human peroxidase conjugate at 1 : 750 dilution (NIDA Biometria, Heraklion, Greece) for antigen–antibody detection.

Results and discussion

Recombinant Rev and Tev protein products were analysed immunologically with antibodies raised against purified authentic Rev isolated from HIV-1 infected H9 lymphocytes. Using bacterial lysates containing expressed Rev and Tev in SDS-PAGE followed by Western blotting, the anti-Rev antibodies reacted with a 18 kD and a 28 kD protein band corresponding to the expected Rev and Tev respectively (Figure 2). The recombinant Rev protein showed similar immunological properties to the authentic Rev. Western blot experiments with an HIV-1 patient serum (stage CDC II) gave a reaction with the same 18 kD protein band as the rabbit anti-authentic Rev antibodies, whereas HIV-1 negative control human sera did not react (Figure 3). This was considered a strong indication that patient sera contained antibodies which recognized specifically the Rev protein. Further Western blot analysis of HIV-1 sera against *E. coli* lysates containing Tat or Tev proteins did not give any clear reactions with the corresponding recombinant

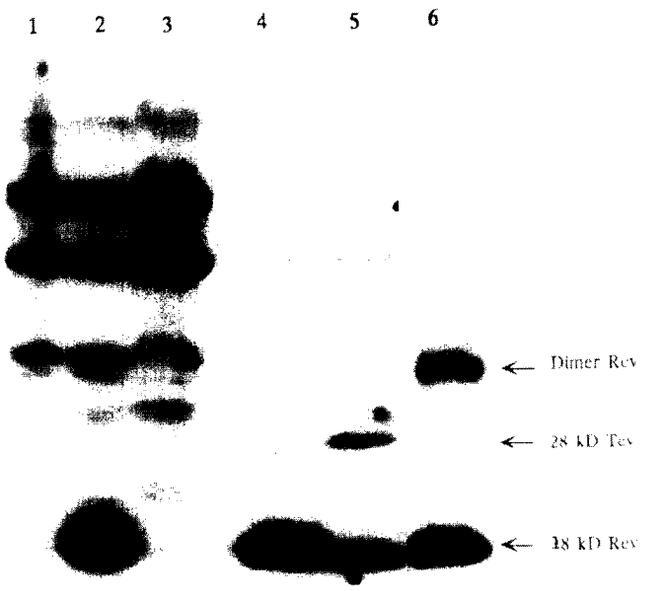

Figure 2. Immunological analysis of *E. coli* lysates containing recombinant Rev and Tev proteins. Autoradiograph of lysates after SDS-PAGE and Western blotting with anti-authentic REV antibodies using ^{125}I protein A for detection of immunoreactions. Lanes 1–3, crude lysates from pUC12N, pUC12N-REV and pUC12N-TEV transformed *E. coli*, respectively. The lanes show the expected common anti-*E. coli* reactions and a strong specific reaction in lane 2 with a 18 kD protein corresponding to Rev. Lane 4, partially-purified recombinant Rev protein. Lane 5, partially purified recombinant Tev showing reaction with a 28 kD protein (Tev) and with a 17 kD protein, termed 6DRev which is the product of an improper internal translational initiation event. Lane 6, purified Rev from HIV-infected H9 cells, showing also the presence of a dimer Rev protein.

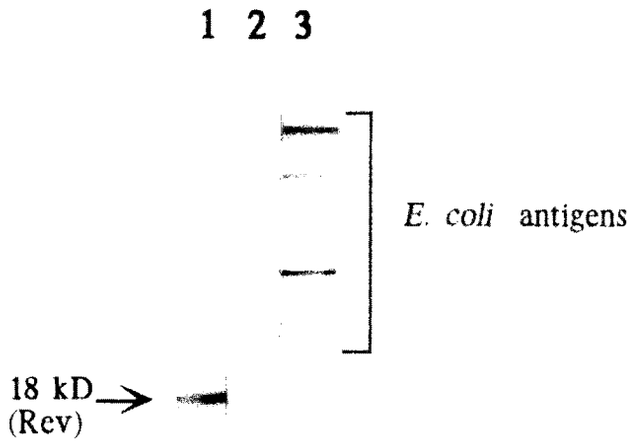

Figure 3. Immunoreaction of the recombinant Rev protein against a polyclonal anti-authentic Rev serum (donated by Dr G Pavlakis) and an HIV-1 patient serum after SDS-PAGE of *E. coli* lysates transformed with pUC12N-REV. Lane 1, reaction with rabbit anti-authentic Rev antibodies; lane 2, a weak reaction with the HIV-positive serum taken from a stage CDC-II patient; and lane 3, a negative control human serum (ELISA and PCR HIV-negative).

proteins (Figure 4). This may well be due to low levels of expression of these proteins. Attempts are currently being made to optimize the expression system. An additional reason may be the relatively lower antigenicity of the regulatory proteins[11].

To study the prevalence of anti-Rev antibodies and to assess the potential diagnostic or prognostic value of the recombinant Rev protein, we examined sera from 47 HIV-1 infected patients (all from the Athens area, Greece) using Western blot analysis with the partially purified protein[1]. The results (Table 1) showed that only a relatively small number of patients have circulating antibodies to Rev at detectable levels. The specific Rev-antibody reactions were weak and difficult to read, even at 1 : 5 serum dilutions, the most prominent being those from patients with AIDS-related conditions (ARC), in agreement with a previous report[1]. The distribution of anti-Rev antibodies was found to be higher in the ARC group (41.7%) and asymptomatic seropositive group (31.6%), as compared with the AIDS group (18.7%), in agreement with previous observations[11]. The lower incidence of Rev antibodies in the AIDS group is probably due to the overall immunodeficiency induced by the disease[11].

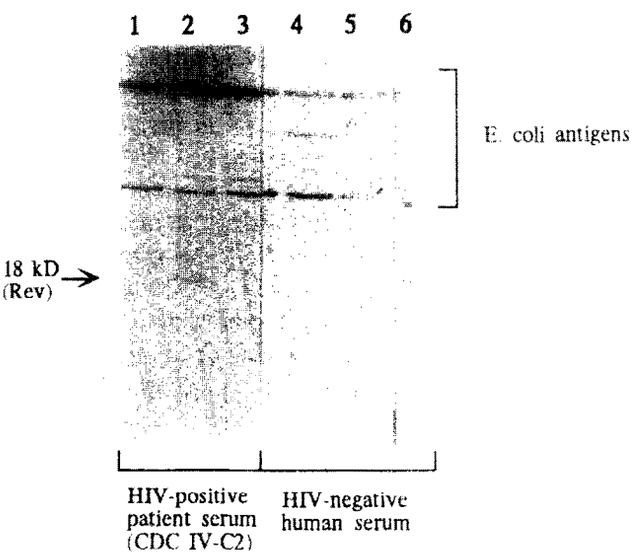

Figure 4. Comparison in immunoreactivity of an HIV-1 patient serum against recombinant regulatory proteins. Western blot analysis of *E. coli* lysates containing Tat protein (lanes 1, 4), Rev protein (lanes 2, 6) and Tev protein (lanes 3, 5). Lanes 1–3 show immunoreaction of an HIV-positive serum and lanes 4–6 of a negative control human serum (ELISA and PCR, HIV-negative).

Table 1. Prevalence of anti-Rev antibodies in asymptomatic seropositive patients, and in patients with AIDS-related conditions and AIDS

Patient group	*No. of sera tested*	*No. of sera reacted with Rev*
Asymptomatic, seropositive	19	6(31.6%)
AIDS-related conditions	12	5(41.7%)
AIDS	16	3(18.7%)
Healthy controls	30	0(0%)

The same sera tested by Western blotting against *E. coli* lysates containing Tat or Tev proteins failed to give any clear reactions with the corresponding recombinant proteins (Figure 4). This may well be due to the observed low levels of expression of these proteins. Attempts are currently being made to optimize the expression system. An additional reason may be the relatively lower antigenicity of the regulatory proteins[11].

The existence of anti-Rev antibodies in HIV-infected patients is not easily demonstrated because conventional ELISA and Western blots use either recombinant structural proteins or viral lysates which do not contain regulatory proteins whereas Rev protein is produced in the infected cell stage. The ability to apply recombinant regulatory proteins to detect circulating antibodies in HIV patients, as we demonstrated here, or using antibodies to detect these proteins in the patient serum, has potential applications in the early diagnosis and subsequent stages of the infection. It is well accepted that early stage expression of the virus is characterized by synthesis of short viral mRNA whereas protein synthesis is restricted to Rev which in turn regulates Tat and Nef protein production[12]. The suppressed expression of structural genes can cause a delay of a few weeks to several months in the appearance of an immune respone to the structural proteins[13] hence delaying also the diagnosis of the infection with conventional methods. Because of the production of Rev and Tat proteins during this early stage which is often associated with an acute syndrome[14], antibodies to these proteins may also appear at about the same time and be detected, thus achieving early diagnosis. Our results suggest that such antibodies exist in asymptomatic cases, although the poor negative predictive value of 47.5% (30/63) renders the use of Rev as a routine diagnostic tool unreliable. At the other end of the pathogenic spectrum, increased levels of Rev signal the final stage of replication with the initiation of virus particle synthesis[13]. This stage may be reflected with an increase in circulating anti-Rev antibodies, an event which could be associated with a progression to clinical disease[1]. Although this explanation has been disputed[11] as supported by evidence which showed no significant differences between AIDS attack and positive *versus* negative antibody profiles to Rev[15], our Western blot reactions with the ARC patient sera were relatively stronger than from sera of the other two groups.

The HIV regulatory proteins contain invariant and highly conserved regions which can serve as a target for an immune response[13]. The use of mutant forms of the recombinant proteins we described can facilitate the development of mechanisms to explore and induce an immune response which will neutralize most viral strains[13,16]. The methods developed here to detect antibodies to Rev and the recombinant proteins themselves could, additionally, serve as tools to address the physiological properties and temporal characteristics of the expression of the viral regulatory proteins[12]. Finally, having gained sufficient experience in expressing the regulatory proteins in *E. coli*, this allows us to express and investigate transdominant mutant forms of these proteins in infected cells which may lead to the development of an alternative mechanism of interference with viral replication.

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