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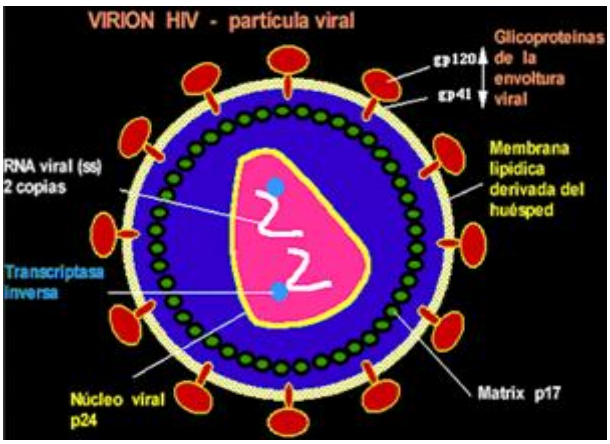
EXPRESSION OF HIV TYPE 1 GLYCOPROTEIN 120 FROM A MEXICAN AIDS PATIENT IN A BACULOVIRUS SYSTEM



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Introduction

Acquired Immunodeficiency Syndrome (AIDS) is the result of the pandemic human immuno-deficiency virus (HIV) infection, for which effective surveillance, diagnostic and treatment are required. AIDS/HIV has become a serious public health problem worldwide, with psychological, social, ethical, political, and economical involvement. It was estimated that 14,000 individuals became daily infected with HIV during 2003 (1). Because we do not have an effective vaccine against HIV yet, is important to evaluate different approaches to generate vaccines capable to stimulate a strong immune response, and thus serve as prophylactic and therapeutic measures against the AIDS disease.

In order to be approved as a vaccine, the immunogenic compound must be demonstrated to be safe and effective, in addition to induce a long-term immune response and/or prevent the infection process. HIV proposed vaccines can be divided into five categories, based on the production methodology: i) synthetic peptides or peptidic sub-units, ii) live recombinant vectors, iii) alive attenuated vaccines, iv) inactivated complete particles, and v) DNA vaccines (2). Synthetic peptides or peptidic sub-unit protein-based vaccines have small viral peptides or pieces of viral peptides. These antigenic subunits can be produced using genetic engineering in bacteria, yeasts, mammal cells or baculovirus (3). The HIV envelope protein might become the most important target, because it attaches to the human cell and allows the viral DNA entrance. HIV envelope glycoproteins, particularly the gp120, have been reported as good candidates for vaccine production (4).

The insect culture cell-baculovirus expression vector system (IC-BEVS) has been used as a recombinant protein production system. It has advantages over other systems including low-cost production, safety for mammals, and simple to scale up. In addition, it is possible to use it for complex post-traductional processes, such as the O-glycosilation and N-glycosilation, among others (5). To date, 16 recombinant protein productions using IC-BEVS have been recognized, and represent almost half of the products obtained with recombinant DNA technology approved for application as commercial products (6).

In Mexico, new HIV-1 subtype B sequences, isolated from Mexican AIDS patients, have been reported (7). In order to produce a peptide with potential as a vaccine, we decided to use the gp120 isolated from a Mexican AIDS patient, and expressed in IC-BEVS.

Materials and Methods

Glycoprotein gp120 isolation

The pro-viral DNA from two HIV sero-positive patients (confirmed by ELISA and western blot), was isolated using a QIAGEN system (Qiamp blood, Qiagen Chatsworth, CA., USA), according to supplier´s instructions. Isolated gp120 DNA from Mexican patients (referred as patients # 18 and # 69), and the reference plasmid HXB2 were amplified using a nested PCR. For this, we selected the primers ED3 and ED14 reported by Delwart *et al.* (8), to obtain a 2,000 bp, a second reaction using the first reaction product as template, with the specific gp120 designed primers gpF 5' GTC GAC GCC ACC ATG AGA GTG AAG GAG AAA TAT CAG C 3' and gpR 5' CTA TCT TTT TTC TCT CTG CAC CAC TCT TCT 3'. The 1,532bp products were cloned using the TOPO TA Cloning system (Invitrogen, CA), following the manual instructions (9). The cloning resulting plasmids were characterized using a restriction analysis with the *EcoR*I (1U/mgr, Promega, WI, USA) and *Kpn*I (1U/mgr, Promega) endonucleases by 12 h. Then, the gp120 sequences were extracted from the plasmids using the *Sal*I (1U/mgr, Promega) and *EcoR*I restriction enzymes by 12 h. The extracted gp120 sequences were then used to be subcloned in a pFastBac1 plasmid, following the Bac-to-Bac baculovirus expression system (Invitrogen, CA) (10).

Subcloning of glycoprotein gp120 sequence in pFastBac1

The pFastBac1 donor plasmid has the advantage of that the protein sequence to be expressed is linked to a multiple cloning site (MCS), which is regulated by the polihedrin promoter pPolh. The promoter pPolh has two specific and interchangeable small elements (mini-Tn7), to each side of MCS, which have the role to integrate the cassette of foreign gene (gp120) to the baculovirus genome, by site-directed transposition (11). In order to linearise the pFastBac1 plasmid, we used *EcoR*I and *Sal*I (1U/mgr, Promega) to allow linking of the gp120 plasmid sequences with the use of T4 DNA-ligase enzyme (12). To multiply the plasmid numbers we used alpha transformation; resulting plasmids were characterized using restriction analysis with *EcoR*I and *Sal*I endonucleases.

Recombinant vector construction

The thermal shock ultra-competent transformation bacteria, DH10Bac, have a bMON14272 bacmid (AcMNPV baculovirus genome) (13). This bacmid contains a mini-F replicon with small number of copies, a kanamycine resistance gene, and a DNA segment which codifies the *lac*Z cloning vector based on pUC (14). The N-end of *lac*Z gene is a short segment that contains the union sites to Tn7 transposon; these sites do not alter the peptide lecture frame. They also have the pMON7124 plasmid, which acts as co-operator plasmid. This plasmid has tetracycline resistance and trasposase genes (15), and induces the recombination by site-directed transposition to the mini-attTn7 pFastBac1 sites, to the bacmid miniTn7, from the gene group found among them (16). For this, E. coli DH10Bac was transformed with the pFactBac1 containing either the Mexican patient or the HXB2 gp120 sequence. Plasmid transposition to bacmid was stimulated by the addition of tetracycline and gentamicine to the media culture. After this, bacmids were isolated by minipreps for characterization (17).

Recombinant vector analysis

The chromogenic substrate (X-gal) and its IPTG inductor in the bacterial media culture, facilitate the phenotypic identification of the bacterial colony growth if a transposition event expression occurred (18). Based on this, white-coloured colonies were selected for the recombinant vector isolation, due that those were incapable to produce β-galactosidase, which unfold the X-gal, generates a blue-coloured colonies. For the nested PCR analysis, ED3 and ED14 internal HIV-1 gp120 primers were used, whereas ED5 and ED12 were utilized in a second reaction to amplify the C2-V5 region (1200 bp) (19). PCR-antibiotic positive clone isolates were phenotypically analyzed by using both kanamicine$^{(+)}$, and ampicilline$^{(-)}$. Clones were then confirmed with positive transposition using a specific PCR with the universal primer M13F bond to the bacmid and the internal gp120 ED12, as reported previously (20).

In vitro Sf9 insect cells transfection

The recombinant viral genome transfection of the two described gp120 bacmids and controls, was performed in triplicate, as previously described by Invitrogen (21). Transfection samples were prepared using non-encapsulated viral DNA, liposome encapsulated viral DNA, empty liposomes, and entrapped DNA-suspension buffer (TE buffer, 10mM Tris-Cl, 1mM EDTA, pH 7.4) in liposomes. Transfection was done by using 2 ml SF900II culture media containing 9 x 10^5 *Sf9* insect cells, showing 97% viability, in 6-well sterile ELISA microplates (22). One mililiter transfection mixture was then added to all wells, and incubated 5 h at 27ºC. After that, mixture was removed, and SF900II fresh medium was the added to all wells. Plates were incubated 72 h at 27ºC and supernatants were collected in 15-ml centrifuge tubes. Tubes were then centrifuged 10 min at 4,000 rpm, and supernatant and pellet were independently kept at 4°C in dark until use inI immunodetection analysis.

Detection of gp120 in Sf9 cells

The cellular pellets obtained from the transfection process described above, were used to detect the recombinant gp120. For this, cellular pellets were subjected to sodium dodecyl sulphate (SDS) 8% polyacrilamide-gel electrophoresis (PAGE), and were electrophoretically transferred to nitro-cellulose membranes, for Western blot immunodetection analysis. To identify the recombinant gp120, a murine monoclonal antibody gp120 anti-HIV-1 IgG_1 isotype, from P4D1-A7-F3 hybridome clone (Chemicon International, CA, USA), and a murine anti-IgG conjugated to peroxidase were used in Western blot analysis, and were detected with luminol by chemiluminescence (ECL Western Blotting Analysis System, Amersham Biosciences, London, England), and hydrogen peroxide hydrolysis by chemical reaction with carbazole (23).

Results

Glycoprotein gp120 isolation

DNA samples from two Mexican AIDS patients (# 18 and # 69), and HXB2 plasmid DNA were amplified to obtain the gp120 sequences. For analysis, we selected one of the patients´s (# 69) amplified Gp120 DNA, for its obtained amount and integrity (Figure 1). PCR products were cloned in the pCR2.1 TOPO plasmid and then they were characterized by restriction analysis using *Eco*RI and *KpnI*. Ten *E. coli* colonies were tested with each DNA product (Mexican patient and HXB2).

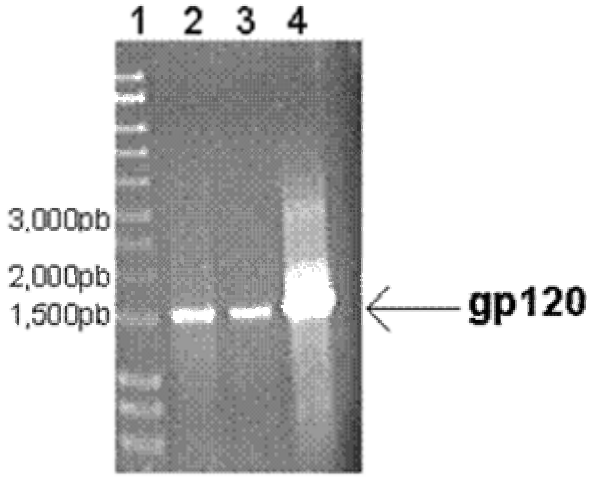


Figure 1. Nested PCR reaction with the amplification of the HIV-1 gp120. One percent agarose electrophoresis gel stained with ethidium bromide. Lane 1, 100bp molecular weight markers, lanes 2, 3 and 4, nested PCR HIV-1 gp120 products from two Mexican patient samples (18 and 69) and the positive control HXB2, respectively.

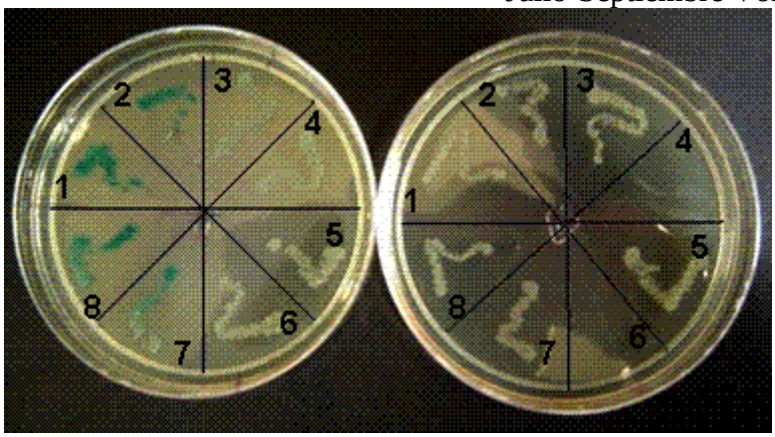
Colonies showing the presence of a 1,532 pb insert, corresponding to gp120 carrying plasmids, were selected and plasmids were identified as pTTA-120-69-3 and pTTA-120-HX-9 for Mexican patient and HXB2, respectively.

Gp120 subcloning in pFastBac1and vector construction

pTTA-120-69-3 and pTTA-120-HX-9 plasmids were used to be subcloned in pFastBac1 vector. After subcloning and transforming in *E. coli*, ten colonies were selected for each plasmid which might contain the gp120 insert. *Eco*RI and *Sal*I restriction enzyme analysis revealed that pFB1-HX-5 and pFB1-HX-10 plasmids obtained from reference HXB2 actually have the gp120 insert, as well as pFB1-69-5, from the Mexican patient (data not shown).

Recombinant vector analysis

Detection of the recombinant vector after the DH10Bac bacterial transformation with pFB1-HX-5 and pFB1-HX-10 plasmids and transposition induction were performed using phenotypic and genotypic analysis. Nested PCR to detect the C2-V5 region from the gp120 was used to the first recombinant vector detection. PCR results detected eight *E. coli* strains with the presence of presumptive positive bacmids, Bac-HX-1, Bac-HX-2, Bac-HX-3, Bac-HX-4, Bac-HX-6, Bac-69-1, Bac-69-5, and Bac-69-6, which may contain the gp120 sequence, because they were capable to amplify a 1,200 bp product. Strains with a positive gp120 bacmid were selected to perform two phenotypic analyses to detect the integrity of the *lacZ* reporter gene, which confers a blue strain to the *E. coli* colony, and the kanamycine resistance conferred by the bacmid (Figure 2a). Results showed that strains Bac-HX-3, Bac-HX-4, Bac-HX-6 and Bac-69-1, had the lecture frame for β-galactosidase gene interrupted by the gp120 gene, thus indicated by white-stained *E. coli* colonies, and suggesting that it was highly possible to have the gp120 insert by transposition. In addition, all the strains were shown to grow in a culture medium supplemented with kanamycine, suggesting the presence of the antibiotic resistance gene. Based on these results, a second phenotypic analysis was used to guarantee the absence of the donor plasmid, which confers resistance to ampicilline. Selected strains were inoculated in a culture media containing ampicilline. Ampicilline-susceptible strains Bac-HX-4 and Bac-69-1 (Figure 2b) were then selected for the transfection process.



(a) **(b)**

Figure 2. Phenotypic analysis of pFastBac-1 transformed *Escherichia coli* DH10Bac, using the HIV-1 gp120 DNA sequences from the AIDS patient # 69, and the HXB2 control. *E. coli* strains were grown in (*a*) LB agar supplemented with kanamycine (100µg/ml), 40µL of IPTG (100mM) and 40µL of X-Gal (40mg/mL), and (*b*) LB agar supplemented with ampicilline (100µg/ml), as explained in the text. 1, Bac-HX-1; 2, Bac-HX-2; 3, Bac-HX-3; 4, Bac-HX-4; 5, Bac-HX-6; 6, Bac-69-1; 7, Bac-69-5; 8, Bac-69-6.

In vitro Sf9 insect cells transfection

Baculovirus transfection using the Bac-HX-4 and Bac-69-1 recombinant strain bacmids was performed as described above. The first transfection mixture had the recombinant baculovirus genome (Bac-HX-4 or Bac-69-1) encapsulated in liposomes, and was capable to generate a cytopathic effect on *Sf9* insect cell culture. None cytopathogenic effect was observed with insect cell culture exposed to other transfection mixture treatments.

Detection of gp120 in Sf9 cells

Bac-HX-4 and Bac-69-1 cellular pellets were subjected to Western blot analysis for gp120 detection. Western blot analysis using chemiluminescence (luminol) or an enzymatic reaction (peroxidase) revealed the gp120 production by the transfected insect cells (Fig. 3). Supernatants collected from the transfected cells were also shown to contain the gp120-recombinant baculovirus.

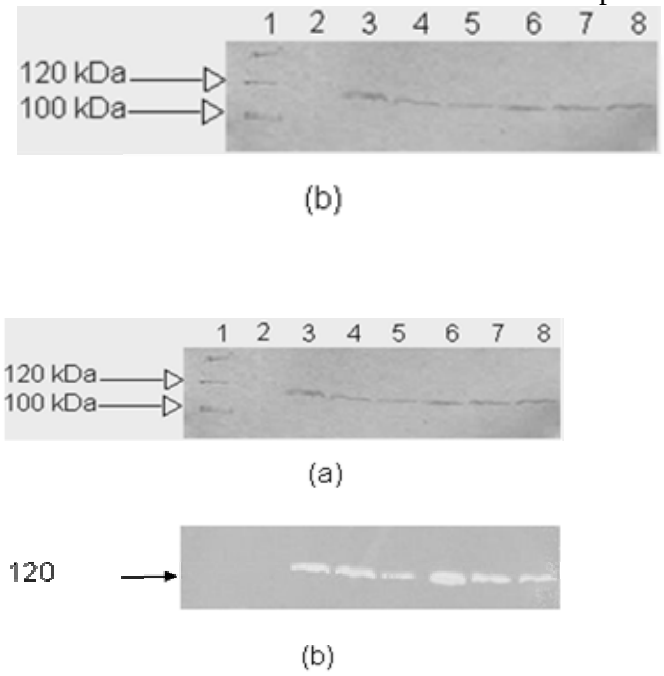


Figure. 3. Detection of the HIV-1 gp120 (patient # 69) in Sf9 insect cells infected with recombinant AcMNPV, carrying the Bac-69-1 and Bac-HX-4 bacmids, using Western blot analysis. (*a*) Immunodetection by a chemiluminescence reaction with luminal and anti-HIV-1 gp120 mouse IgG_1, and (*b*) immuno-detection by enzymatic reaction based on hydrogen peroxide hydrolysis using an anti IgG mouse-peroxidase conjugate

Discussion

The recombinant protein production using the IC-BEVS system has the advantage over *E. coli*, *Staphylococcus aureus*, yeast or mammal cells, to produce a high-expression level product, as well as being the only approved system to test in clinical trials by the food and drug administration (FDA) agency (The Recombinant Protein Handbook, Amersham Biosciences, UK). It is possible to develop post-traductional process for the protein maturation, as N- and O-glycosilation, phosphorilation, fatty acid acylation, α-amidation, N-end amidation, carboxymethylation, isoprenylation, trucked signed peptide, proteolytic cut site, by using the IC-BEVS system (24). In the present study, it was possible to express the gp120 HIV-1 glycoprotein using the IC-BEVS system. To achieve this, two recombinant baculovirus were constructed, both showing the HIV-1 gp120 recombinant protein, with differences in the DNA source (Mexican AIDS patient and HXB2 reference plasmid). In recent years, IC-BEVS system has been selected to express and produce recombinant proteins from several animal viruses including canine and swine parvovirus, classic swine pest virus, or the African equine pest virus (25). Also, it has been possible to express virus-like particles, such as the blue tongue, which is very immunogenic. Using the IC-BEVS, it was only possible to produce a classic swine pest virus sub-unit vaccine, with the gp55 glycoprotein. Gp55 induces neutralizing antibodies capable to confer protection against the wild-type virus infection (26).

Other examples of medical-clinical impact of the recombinant protein production using IC-BEVS, are the immunogenic hepatitis-B virus surface antigen (27), the hepatitis-C virus NS3 protease (28), and the antigen associated to HCA587 hepatocellular carcinoma (29). The bovine diarrheic virus gp48 and gp53 glycoproteins, which showed similar immune response to the native virus (30), and the growth human hormone (31) and the interferon-β (32), are other examples of the versatility of the IC-BEVS system for vaccine or medical drugs production. We selected the IC-BEVS system for its potential for recombinant protein production to be used as

a vaccine (33). We used Mexican AIDS patient DNA samples to produce the recombinant gp120 because they have shown some differences compared with the HIV-1 subtype B found in America (34). We believe that a the Mexican DNA sequence will produce a immunogenic glycoprotein useful for the Mexican population. Recently, different strategies for the production of a safe HIV-1 immunogenic compound have been reported, many of which are based on the structural viral subunits, with either the gp160 (which is the virus *env* gene translation product), or with the gp120 (excision gp160 product), which directly binds I to CD4 cell receptor (35). Furthermore, clinical trials have been conducted by VaxGen, Corp. (AIDSVAX MN and AIDSVAX B/B), using HIV-1 subtype B immunogens, and currently in phase II; Chiron Co. in Thailand who uses CM235/SF-2, a gp120 obtained from HIV-1 subtypes B and E, which is phases I and II; and Aventis-Pasteur (USA) who uses THO23/LAI-DID, a gp160 obtained from HIV-1 subtypes B and E, which is phases I and II (36).

An important goal of the present study was the selection of the glycoprotein subunit from a Mexican patient which was able to be expressed in IC-BEVS system. Considering AIDS as one of the most important social health problems in the world, each country must attempt to produce an effective and safe vaccine against its ethnical HIV virus strain (37). It has been suggested that an optimum immune response against HIV-1 can be achieved after treatment with the gp120 which still carries the carbohydrates resulting from the post-traductional processes. One reported study revealed a limited immune response after the treatment with the wild-type gp160 modified at 4^{th}, 5^{th} and 6^{th} N-glycosilation sites (38). It has also been reported that removal of the gp120 glycosilation conserved sites, did not improve the humoral immune response, whereas the loss of the carboxyl end significantly reduced the cytotoxic T lymphocyte efficacy against the virus (39).

Other results have suggested that the effectiveness of the immune response not only depends in the post-traductional process, but also in the transfer process, because the immune response has been enhanced after some gp120 codon changes, producing a synthetic protein (40). The same study revealed that despite the cytoplasmic mRNA concentration is similar in the groups receiving the synthetic protein and the wild-type control, the first will generate a higher humoral and cellular immune response (41). We found that HIV-1 subtype B sequences registered in the genebank were different from those found in Mexican patient samples (42). We believed that it might be possible to have a different immune response based on the differences on codons. In fact, the compounds that are being tested in North America, Europe and Thailand, in phase III clinical trial, AIDSVAX B/B and AIDSVAX B/E described above, are the result of a recombinant gp120 bivalent preparation. The major problem observed with these vaccines is that they confer limited protection (43), which also prompted our present research using autochthonous recombinant gp120.

In summary, we are reporting the construction of two recombinant baculovirus, using one reference (HXB2) and one Mexican patient DNA sequence from the foreign HIV-1 subtype B gp120 gene. We demonstrated the gp120 expression of both recombinant baculovirus in an IC-BEVS system using Western blot analysis. The production, purification and antigenic activity test, to validate the differences in immune response among the two recombinant gp120 glycoproteins, are being currently evaluated in our laboratory.

Abstract

The aim of the present study was the implementation of an insect cell-baculovirus expression vector system (IC-BEVS) for the expression of the human immunodeficiency virus (HIV) type 1 gp120 recombinant protein. For this, we isolated the DNA sequences from the gp120 protein from a Mexican AIDS patient, and from the reference plasmid HXB2, which were independently cloned in the plasmid pFastBac1. We used the plasmids to transform *E. coli* DH10Bac containing the AcMNPV baculovirus bMON14272 bacmid, and then induced a site-directed transposition to transfer the HIV-gp120 genome to the baculovirus´s bacmid. Baculovirus recombinant was detected using PCR analysis and colony phenotyping, with both the Bac-69-1 and the reference plasmid Bac-HX-4. The baculoviral naked genome was then encapsulated

within liposomes to transfect the insect cell culture *Sf9*. Western blot analysis, developed by chemiluminescence and hydrogen peroxide hydrolysis was then utilized to identify the recombinant gp120 expression. Results demonstrated the generation of two recombinant baculoviruses expressing either gp120 from a Mexican AIDS patient or as reference plasmid. The potential of using the gp120 recombinant baculovirus as HIV vaccine in Mexico is discussed.

Key words: HIV, gp120, AIDS, Mexican patient, baculovirus, insect cell culture.

Resumen

El objetivo principal del presente estudio fue la implementación de un sistema vector de expresión en baculovirus en células de insecto (IC-BECS), para la expresión de la proteína recombinante gp120 del virus de la inmunodeficiencia humana (VIH). Para esto, se aislaron secuencias de la proteína gp120 de una paciente mexicano con SIDA, y del plásmido de referencia HXB2, los cuales se clonaron en forma independiente en el plásmido pFastBac1. Posteriormente, se utilizaron estos plásmidos para transformar *E. coli* DH10Bac conteniendo el bacmido bMON14272 del baculovirus AcMNPV, para luego inducir una transposición sitio dirigida para transferir el genoma de gp120 del VIH al bacmido del baculovirus. Se detectó el baculovirus recombinante de Bac-69-1 (paciente) y el plásmido de referencia Bac-HX-4, utilizando la técnica de PCR y la fenotipificación de colonias. Después se encapsuló el genoma desnudo baculoviral en liposomas para su transfección al cultivo de células de insecto *Sf9*. Se utilizaron, además, análisis de Western blot (que se revelaron por quimioluminiscencia e hidrólisis de peróxido de hidrógeno) para la identificación de la expresión de la proteína gp120. Los resultados obtenidos demostraron la generación de dos baculovirus recombinantes que expresaron la gp120 del paciente mexicano de SIDA o el plásmido de referencia. Se discute el potencial de utilizar a los baculovirus recombinantes de gp120 como vacunas del VIH en México.

Palabras claves: VIH, gp120, SIDA, pacientes Mexicanos, baculovirus, cultivo de células de insecto.

Acknowledgments

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