Expression and immunogenicity of the entire human T cell leukaemia virus type I envelope protein produced in a baculovirus system


1Immunology Group, The John P. Robarts Research Institute, London, Ontario, Canada N6A 5K8 and 2Duke University Medical Center, Durham, North Carolina 27710, U.S.A.

The entire envelope gene of human T cell leukaemia virus type I (HTLV-I) has been successfully expressed in a baculovirus non-fusion vector system. The HTLV-I envelope protein accumulated within the insect cells as inclusion bodies which allowed efficient recovery of the recombinant protein. In an attempt to study the role of the HTLV-I envelope glycoprotein as an immunogenic target, mice were immunized with the envelope protein inclusion bodies (env-I.B.) in the presence or absence of an adjuvant. Antibodies of broad specificity were produced against the HTLV-I envelope protein in the presence or absence of an adjuvant as detected by Western blotting, radioimmunoprecipitation and peptide ELISA. Neutralizing antibody was detected when env-I.B. immunizations were carried out in the presence of high doses of a new adjuvant composed of a mycobacterial cell wall extract. In a combined immunization regimen, env-I.B. were found to enhance and broaden the antibody response to the HTLV-I envelope glycoprotein, following priming with various recombinant vaccinia virus (RVV) constructs expressing either the entire native HTLV-I envelope (gp46 and gp21) or just the surface envelope protein (gp46). Increased titres of neutralizing antibodies were observed following priming with the RVV expressing gp46 only. Results indicate that immunization regimes that involve priming with RVV expressing HTLV-I envelope followed by boosting with recombinant baculoviral HTLV-I envelope might be useful in eliciting protective immune responses in vivo.

Introduction

Human T cell leukaemia virus type I (HTLV-I) has been firmly established by epidemiological and molecular studies to be the aetiological agent of adult T cell leukaemia (Yoshida et al., 1984; Robert-Guroff et al., 1982) and is associated with a degenerative neurological disorder known as HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Osame et al., 1986). The virus is principally transmitted in a cell-associated manner such that the major routes of transmission involve sexual contact, mother-to-child transmission, or contaminated blood either from transfusion of cellular blood components or sharing of needles by intravenous drug abusers (Blattner et al., 1986; Kinoshita et al., 1987; Satow et al., 1991). Public education, discouraging breast feeding of infants by HTLV-I-positive mothers and screening of blood products are the only methods currently available to prevent transmission of this retrovirus. In developing countries these methods of prevention are ineffective and impractical; thus there is a need for additional interventional strategies such as vaccines to control the spread of HTLV-I infection.

Efforts to develop a subunit vaccine against HTLV-I infection have focused on the envelope glycoprotein. Like those of other retroviruses, the HTLV-I envelope protein appears to play a major role in the infection of target cells (Dickson et al., 1982; Pique et al., 1990) and elicitation of host antiviral immunity (Shida et al., 1987; Nakamura et al., 1987). The HTLV-I-encoded envelope is synthesized as a precursor gp63 protein which is cleaved to form the mature gp46 glycoprotein and a transmembrane protein, gp21. Recent animal protection studies suggest that host immune effector functions directed toward the HTLV-I envelope protein represent an important mechanism for preventing infection and/or the spread of the virus (Tanaka et al., 1991; Clapham et al., 1984).

In an attempt to study the role of the HTLV-I envelope glycoprotein as an immunogenic target and to assess its vaccine potential against HTLV-I infection, we have successfully expressed the entire HTLV-I envelope protein using a baculovirus vector system. The accumulation of the HTLV-I envelope protein as inclusion bodies in the insect cells simplified their isolation and recovery. In this study, attempts were made to enhance the immunogenicity of the HTLV-I envelope inclusion bodies (env-I.B.) using various concentrations of adjuvant in the hope of generating a strong neutralizing
antibody response in mice. In addition, a combined immunization regimen was also assessed. This involved priming with one of three live recombinant vaccinia viruses (RVV) each expressing a different version of the HTLV-I envelope protein (Ford et al., 1992) followed by boosting with env-I.B. The resultant sera have been analysed using Western blotting, radioimmunoprecipitation, peptide ELISA and a syncytium inhibition assay.

Methods

Cell lines. The Spodoptera frugiperda (SF9) cells were obtained from Dr P. Faulkner (Department of Microbiology and Immunology, Queen’s University, Kingston, Ontario, Canada) and grown in TC100 medium (Gibco-BRL) supplemented with 10% foetal bovine serum (FBS; Gibco-BRL) and 100 μg/ml of gentamicin (Gibco-BRL). The human T lymphocyte cell line MT-2 was obtained from Dr L. Arthur (AIDS Vaccine Program, NCI-Frederick Cancer Research and Development Center). Two other T cell lines, HTLV-I-producing C91 PL cells and non-infected indicator C8166 cells, were used in the syncytium inhibition assay. All T cell lines were maintained in RPMI-1640 supplemented with 10% FBS and 100 units/ml of penicillin and streptomycin.

Plasmids and viruses. The envelope fragment was derived from the plasmid pMT-2 (provided by Dr R. C. Gallo, NCI/NIH; Ratner et al., 1985). The baculovirus expression vector pVL1393 (provided by Dr M. Summers, Texas A & M Agricultural Experimentation Station) was chosen as the vehicle for insertion of the HTLV-I envelope glycoprotein into the genome of Autographa californica multiple capsid nuclear polyhedrosis virus (AcNPV, provided by Dr P. Faulkner). The procedures involving the production and isolation of the resultant recombinant baculovirus were as defined by Summers & Smith (1987). The RVV constructs RVV Els, RVV E3s, and the antisense construct RVV Elas used in the combined immunization regimen (see below) have been described in detail (Ford et al., 1991, 1992).

Indirect immunofluorescence of baculovirus-infected cells. Individual monolayers of SF9 cells were infected with wild-type baculovirus and the two recombinant AcNPV clones (VHB5 and VHB6) at a multiplicity of 0.5. Three days post-infection, the cells were harvested, washed and resuspended in PBS (pH 7.3) at a density of 5 x 10^6 cells/ml. Cell suspensions were spotted on tissue-grit (Fischer Scientific) coated glass slides, allowed to air-dry and directly fixed in cold acetone for 10 min. Slides were rinsed twice in PBS (pH 6.8) and rinsed once in distilled water. Non-specific binding was blocked by incubating the washed cells with a 3% solution of BSA (Boehringer Mannheim) in PBS for 1 h at room temperature. Cells were incubated in the appropriate primary antibody, diluted in PBS-MMCW (pH 7.4). Photography was with an Olympus BH-2 fluorescence microscope with a fluorescein filter.

Detection of secreted or cell-associated HTLV-I envelope protein. Monolayers of 2 x 10^7 SF9 cells were infected with either wild-type AcNPV or recombinant baculovirus (infections of both clones VHB5 and VHB6 were tested) at a multiplicity of 0.2. Infected cells were incubated in the presence of serum-free medium (Excell 400, JRH Biosciences). After 46 h, the infected cells and supernatants were harvested by centrifugation at 3000 g for 15 min at 4°C, in the presence of the protease inhibitors 1 mM-PMSF and 1 mM-EDTA. The cell pellets were stored at -70°C. To remove the majority of extracellular virus from the supernatant, the supernatant was centrifuged at 14000 g for 60 min at 4°C. The supernatant was then dialysed against a buffer of 0.1 mM-EDTA, 1 mM-Tris-HCl pH 7.4 for 2 days at 4°C. The volume of the dialysed supernatant was then reduced to 200 μl by lyophilization. Both the supernatants and their corresponding cell pellets were resuspended in an equal volume of Laemmli buffer, boiled for 5 min and electrophoresed on a 12% SDS-polyacrylamide gel containing 6 μg-urea (Hayden et al., 1986). Gels were then processed for Western blot analysis.

Inclusion body isolation. SF9 cells were infected with recombinant baculovirus at a multiplicity of 1:40. Forty-six hours post-infection the cells were chilled for 10 min and pelleted at 10000 g for 20 min at 4°C. The cells were washed once in cell wash buffer consisting of 50 mM-Tris–HCl pH 7.5, 1 mM-EDTA, 1 mM-DDT, 1 mM-PMSF (Nyunoya et al., 1995), dispensed into 1 x 10^6 cell aliquots and the centrifugation was repeated. The crude cell lysate was resuspended in Tbuffer (Nyunoya et al., 1995) and homogenized in a glass homogenizer in the presence of DNase 1 (1 mg/ml) (Boehringer Mannheim). The suspension was centrifuged for 20 min at 10000 g. Pellets were sonicated in T-buffer and incubated as described (Nyunoya et al., 1990). Use of the pellets decided the further processing of the env-I.B. (i) For mouse immunizations, the env-I.B. pellets underwent three washes in PBS pH 7.2, interspersed by centrifugation at 10,000 g at 4°C. Pellets containing 10 μg of env-I.B. were stored at -70°C following the final centrifugation and removal of the supernatants. (ii) For SDS-PAGE, a pellet containing approximately 120 μg of env-I.B. (protein equivalent of 5 x 10^7 cells) was resuspended by sonication, in a Laemmli buffer containing 4 μg-urea, 100 mM-DDT and 1 mM-PMSF. This suspension was incubated for 1 h at 4°C on a nutator, after which it was divided into various required quantities for storage at -70°C.

Western blot assay. Inclusion body extract, solubilized in 4 μg-urea reducing buffer, was electrophoresed and transferred from a 12% SDS/6 μg-urea polyacrylamide gel (Hayden et al., 1986) to an Immobilon P membrane (Millipore). Blots were blocked in a solution of 5% skim milk powder (Johnson et al., 1984) at room temperature. Blots were incubated in the appropriate primary antibody, diluted in blocking buffer, overnight at 4°C with rocking. Blots were then washed in blocking buffer and exposed to goat anti-mouse, goat anti-rabbit or goat anti-human IgG conjugated to alkaline phosphatase (Jackson ImmunoResearch) at a final dilution of 1:1000, for 30 min at room temperature. The blots were washed and exposed to substrate according to the manufacturer’s instruction (Blot Detection Kit: Amersham). Each Western blot assay included three positive controls of (i) 1C11, an anti-gp46 mouse monoclonal antibody (MAb) (Palker et al., 1989), (ii) anti-SP7 rabbit polyclonal serum (SP7 peptide sequence derived from gp21; Palker et al., 1989) and (iii) human HTLV-I patient sera (TSIP).

Immunizations. Three different inbred mouse strains, BALB/c (Charles River), C57BL/6 (Charles River) and CFW/D (Ball & McCarver, 1979) were immunized at 6 to 8 weeks of age by intraperitoneal injection. Two different forms of HTLV-I envelope protein immunogens were studied. For the adjuvant titration studies, mice were injected with 10 μg of env-I.B. in the absence or presence of various amounts of adjuvant formulated from a mycobacterial cell wall extract (MCWE; Bioniche/Vetechem). MCWE is a purified and deproteinized cell wall extract from a non-pathogenic species of mycobacterium. The env-I.B. pellet was resuspended by sonication in either 500 μl PBS (pH 7.2) or in 500 μl of a 1:2 dilution of PBS–MCWE emulsion. For the combined immunization regimen, mice were primed with 4 x 10^6 p.f.u. of the appropriate purified RVV (RVV Els, RVV
E3s or RVV E1as) diluted to 100 μl with RPMI-1640. RVV E1s contains only the portion of the HTLV-I envelope gene encoding gp46. RVV E1a is identical to RVV E1s except that the envelope gene is in the antisense orientation. After 2 and 4 weeks, the primed mice were boosted with either the same RVV preparation or with 10 μg of env-L.B. suspended in 100 μl of 100 μg/ml MCWE adjuvant preparation. Terminal bleeds were recovered 2 weeks after the second boost.

Immunoprecipitation of radiolabelled proteins. HTLV-I-infected human M-J2 cells were labelled with [35S]cysteine for immunoprecipitation of HTLV-I envelope proteins by serum of mice immunized with HTLV-I env-L.B. Cells (2 x 10^5) were washed in cysteine-free RPMI-1640 (Gibco-BRL Selectamine kit) with 1% dialysed FBS, pelleted and incubated for 30 min at 37 °C with gentle mixing in cysteine-free medium plus 1% FBS. The cells were pelleted and resuspended in cysteine-free medium containing 0.5 Ci [35S]cysteine (1000 Ci/mmol; Dupont, NEN) and incubated for 5 h at 37 °C with mixing. Cell lysates were prepared as previously described (Dekaban et al., 1984). The resultant cell lysate was precleared for 2 h at 4 °C by incubating with Protein G Plus/A agarose (Oncogene Science), which had been preincubated for 2 h at 4 °C with preimmune mouse serum. The Protein G Plus/A agarose was pelleted and the resulting supernatant was divided into 5 x 10^6 cells equivalents for immunoprecipitation. Each aliquot of cell lysate was suspended in 30 μl of Protein G Plus/A agarose. The positive control samples consisted of an aliquot of precleared labelled cell lysate incubated with 30 μl of Protein G Plus/A agarose and a mixture of rabbit polyclonal HTLV-I envelope antipeptide sera raised against peptides SP-2, SP-4A, SP-6 and SP-7 (Palker et al., 1989). Immune complexes were allowed to form overnight at 4 °C, washed with cold extraction buffer and then resuspended in an equal volume of 2 x Laemmli buffer before loading onto a 12 % SDS-polyacrylamide gel. Gels were stained with Coomassie blue, fixed at 80 °C under vacuum and exposed for autoradiography at −70 °C.

Peptide ELISA. Binding of serum antibodies to HTLV-I envelope synthetic peptides by ELISA was performed as previously described (Palker et al., 1989) with the following exceptions: 2 μg of peptide per microtitre well was used; and for efficient blocking, the reaction buffer contained 2% dried milk instead of 5% BSA. The following synthetic peptides containing hydrophilic sequences from HTLV-I gp46 or gp21 were chosen for the study: SP-2 (gp46, envelope amino acids 86 to 107), SP-4A (gp46, aa 190 to 209), SP-6 (gp46, aa 296 to 312) and SP-7 (gp21, aa 374 to 392), all of which have been previously described (Palker et al. 1989). The endpoint titre was defined as the serum dilution at which the signal-to-noise ratio was >20; the mean absorbance reading obtained with serum from a mouse injected with 100 μg MCWE alone was used to estimate background readings for ELISA.

Syncytium inhibition assay. Neutralizing antibody titres were determined in a syncytium inhibition assay as previously described (Nagy et al., 1983; Lal et al., 1991), by incubating 45 μl of HTLV-I-producing C91 PL T cells with C8166 T cells (each cell line at 10^6 cells/ml in RPMI with 10% FBS) overnight in a tissue culture incubator (5% CO_2, 37 °C) in the presence of 10 μl of serially diluted test serum (heat-inactivated at 56 °C for 30 min). After 24 h, the presence of syncytia was evaluated in an inverted microscope at 200-fold magnification and the neutralizing titre was determined as the last serum dilution that inhibited syncytium formation by greater than 90%. Routinely, 100 to 200 syncytia could be obtained per microtitre well in the presence of 10% normal mouse serum. All mouse sera were coded prior to testing in the syncytium inhibition assay, and codes were broken only after neutralizing titres had been measured. Neutralizing anti-HTLV-I peptide antiserum (Palker et al., 1992) and preimmune serum served as positive and negative controls, respectively.

Results

Construction and characterization of recombinant baculovirus

The entire HTLV-I envelope gene fragment was isolated from the plasmid pMT-2 (Ratner et al., 1985) by a BamHI–PstI partial digestion. This 1636 bp fragment was inserted into the baculovirus transfer vector pVL1393 downstream from the polyhedrin gene promoter as indicated in Fig. 1. In this pVLHTL construct, the translation initiation codon of the envelope gene is located 123 bp downstream from the non-functional start codon of the polyhedrin gene and thus will result in expression of the complete HTLV-I envelope protein in the absence of additional polyhedrin protein sequences. The pVLHTL plasmid was then transfected together with AcNPV DNA into insect tissue culture cells (Sf9) and virus was isolated from occlusion-negative plaques. Southern blot analysis of digested recombinant viral DNA generated the expected restriction fragments when probed with an HTLV-I envelope-specific fragment.

![Diagram of HTLV-I envelope gene fragment](image-url)
Fig. 2. Indirect immunofluorescence analysis with anti-HTLV-I envelope MAb 1C11. (a) Uninfected Sf9 cells; (b) wild-type baculovirus-infected Sf9 cells; (c) recombinant VHB5 baculovirus-infected Sf9 cells; (d) recombinant VHB6 baculovirus-infected Sf9 cells.

**Indirect immunofluorescence**

To determine whether the envelope gene was expressed by the recombinant baculovirus, indirect immunofluorescence using HTLV-I patients' sera was performed. As illustrated in Fig. 2, normal Sf9 insect cells and those infected with wild-type baculovirus failed to fluoresce, whereas both recombinant baculovirus isolates (VHB5 and VHB6) revealed strong positive fluorescence. There appeared to be aggregates of protein at the poles of several envelope-expressing cells, and other recombinant baculovirus-infected cells were stippled in appearance suggesting that they may have been sequestering the envelope protein within vacuoles.

**Isolation and identification of the HTLV-I envelope glycoprotein**

In light of the indirect immunofluorescence observations, it was important to determine whether any of the HTLV-I envelope protein was being secreted by the Sf9 cells. Western blots of the supernatant and corresponding cell pellet of a recombinant baculovirus infection (Fig. 3b) revealed that the HTLV-I envelope glycoprotein was not being secreted from the infected Sf9 cells but was accumulating within the cells. The recombinant baculovirus-infected cell pellet contained three major size classes of HTLV-I envelope protein with $M_r$ values averaging 43K, 54K and 63K. All three size classes were recognized by both the anti-gp46 1C11 MAb and human HTLV-I patient sera. In addition, several minor protein bands of lower $M_r$ ranging in size from 30K to 39K were also observed. No specific immunoreactive proteins were observed in the supernatant and cell pellet of a wild-type baculovirus infection (Fig. 3a). The results of the Western blots and the indirect immunofluorescence of recombinant baculovirus-infected cells, combined with the requirement for strong denaturing agents (4 M-urea or 4 M-guanidinium hydrochloride) for solubilization of the HTLV-I envelope protein, suggested that the recombinant baculovirus-infected cells stored the HTLV-I envelope protein as inclusion bodies.
The accumulation of the HTLV-I envelope protein as inclusion bodies allowed its isolation from other cellular and viral proteins to greater than 80% purity as determined by SDS-PAGE. A modification of the method developed by Nyunoya et al. (1990) allowed the enrichment of these insoluble protein aggregates as illustrated in Fig. 4(a). In our method, the addition of DNase I was critical in obtaining maximum purification of the env-I.B. Isolation of env-I.B, from an equivalent amount of infected cells (Fig. 4a, compare lanes 3 and 4), resulted in the recovery of the three major HTLV-I envelope protein forms with minimal loss. No HTLV-I envelope proteins were detected by Western blot analysis in the cell lysate supernatants during the inclusion body isolation (data not shown). The 43K, 54K and 63K immunoreactive envelope proteins previously observed in the total cell pellet (Fig. 3 b) were present in the same relative amounts within the inclusion bodies (data not shown).

When an equivalent amount of electrophoresed env-I.B. (Fig. 4a, lane 4) was analysed by Western blotting, the majority of extracted inclusion body material proved to be of HTLV-I envelope origin, as shown in Fig. 4(b).

The three major proteins of 43K, 54K and 63K present in the inclusion bodies proved to be immunoreactive with both anti-gp46 1C11 MAb (Fig. 4b, lane A) and anti-SP-7 peptide sera (Fig. 4b, lane B) and this suggested that they represent different forms of the HTLV-I envelope precursor protein. To determine whether any of these forms were the result of glycosylation, the effects of tunicamycin were studied. Tunicamycin treatment resulted in the disappearance of the 63K protein; however, it had no effect on the production of the 43K and 54K proteins. This suggested that the 63K protein was the glycosylated precursor representing 5 to 10% of the total envelope protein, whereas the 43K and 54K proteins were unglycosylated HTLV-I envelope precursor forms. Further confirmation of their precursor origin came from competition Western blot assays in which SP-7 peptide was found to inhibit binding of the anti-SP-7 sera (SP-7 peptide sequence derived from gp21) to the 43K and 63K proteins (Fig. 4b, compare lanes C and D). The SP-7 peptide did not completely inhibit the binding of the anti-SP-7 serum to the 54K protein (Fig. 4b, lane D). The reason for this was not clear. Control experiments using normal mouse and rabbit sera, or sera raised against Sf9 cells infected with unrelated recombinant baculovirus, did not possess antibodies capable of binding to the 54K protein (data not shown). Conversely, HTLV-I-specific sera did not react with Western blots of unrelated recombinant baculovirus cell pellets (data not shown).

Immunogenicity of inclusion bodies

Radioimmunoprecipitation and Western blot assays revealed that injection of mice with env-I.B., in the absence of adjuvant, could stimulate humoral responses to the HTLV-I envelope protein. Serum from immunized C57BL/6 mice possessed antibodies capable of immunoprecipitating HTLV-I envelope proteins from [35S]cysteine metabolically labelled HTLV-I-infected M12 cells (Fig. 5a). Normal C57BL/6 sera did not immunoprecipitate these HTLV-I envelope proteins. Western blot analysis confirmed the reactivity of the sera from the immunized C57BL/6 mice to HTLV-I envelope proteins (data not shown). Sera from BALB/c and CFW/D mice immunized with env-I.B. alone exhibited similar humoral responses to the HTLV-I envelope protein, as monitored by radioimmunoprecipitation and Western blot assays (data not shown).

To stimulate an elevated humoral response to env-I.B., MCWE (Archambault, 1989) was employed as an adjuvant. Since this was a new adjuvant, a titration experiment was performed to determine the optimal dose (0 to 500 µg) required to give the best antibody response. From Western blot and radioimmunoprecipitation
assays, maximal seroconversion was observed in mice immunized with 50 μg of MCWE adjuvant preparation (Fig. 5b). Exceeding this dose resulted in a gradual decrease in mouse seroconversion with increasing amounts of MCWE adjuvant preparation (Fig. 5c; 500 μg MCWE).

Characterization of the antibody response to env-I.B.

In order to study further the effects of varying amounts of MCWE adjuvant on the antibody response to env-I.B., sera were assayed by ELISA for the ability to bind to four synthetic peptides, SP-2, SP-4A, SP-6 and SP-7. The locations of these peptides within the HTLV-I envelope proteins are shown in Fig. 1(b). The regions of the envelope protein gp46, encompassed by the peptides SP-2 and SP-4A, have been associated with virus neutralization, whereas the SP-6 peptide region of gp46 has been shown to be immunogenic in humans (Palker et al., 1989; Tanaka et al., 1991; Horal et al., 1991). The SP-7 peptide spans another immunogenic region of the HTLV-I envelope and has allowed us to monitor the immune response to the transmembrane envelope protein, gp21. Mice injected with env-I.B. in the presence of 10 μg of MCWE adjuvant preparation produced sera with the highest ELISA titres for all four synthetic peptides (Fig. 6, group 4) and exhibited strong Western blot reactivity (data not shown). As illustrated in Fig. 6, inoculation of higher doses of MCWE resulted in a corresponding decrease of serum reactivity with the various peptides, with some mouse sera from these high dose groups completely failing to recognize any of the synthetic peptides. Those mice which failed to generate antibody capable of recognizing the four synthetic peptides also produced low levels of anti-envelope antibody as detected by Western blotting and radioimmunoprecipitation.

The peptide ELISA data helped to map the immunogenic regions of the recombinant HTLV-I envelope protein present in the inclusion bodies. In all groups, env-I.B. generated the highest ELISA titres to the synthetic SP-6 peptide (Fig. 6). In fact, the level of SP-6-binding antibodies stimulated by env-I.B. injection was influenced only negligibly by the adjuvant dose received. Intermediate antibody titres to the synthetic SP-4A and SP-7 peptides were observed, with the lowest antibody titres directed to the SP-2 peptide (Fig. 6). All env-I.B.-immunized mouse sera were compared to sera from mice injected with MCWE only (group 1) to determine significant ELISA titres.

The various mouse sera were screened in a syncytium inhibition assay to determine whether neutralizing antibodies were generated. Neutralizing antibody titres of 10 to 40 were observed in only a few mice receiving the highest doses of MCWE (250 μg and 500 μg; data not shown). These doses produced unwanted side-effects in the mice, according to observation of their general health.

**Combined immunization regimens**

Previous experiments (Ford et al., 1992) have shown that RVV E1s expressing the native HTLV-I envelope (gp46 and gp21), and RVV E3s expressing only the surface
Fig. 6. Peptide ELISA titres of sera from mice immunized with env-I.B. and various amounts of MCWE. All groups with the exception of group 1 received 10 μg of env-I.B. in the presence of the appropriate amount of MCWE. Group 1, 100 μg MCWE; group 2, 0 μg MCWE; group 3, 5 μg MCWE; group 4, 10 μg MCWE; group 5, 25 μg MCWE; group 6, 50 μg MCWE; group 7, 100 μg MCWE; group 8, 250 μg MCWE; group 9, 500 μg MCWE. ELISA titre is the dilution that resulted in an absorbance equal to or greater than twice background values obtained with control mouse sera injected with 100 μg of MCWE alone (group 1). Titres less than $50^{-1}$ were assigned as values of 0.
Fig. 7. Monitoring anti-envelope seroconversion. Western blot reactivity of six C57BL/6 mouse sera (sera 7 to 12, denoted above the lane) screened on days 14, 28 and 37 during the course of combined immunization with RVV Els and env-I.B. (group B of Table 1). Samples were taken prior to each env-I.B. boost and upon termination. Reactivity was graded: +, weak; ++, moderate; ++++, strong.

Table 1. Characterization of the immune response induced in mice immunized with a combined RVV and env-I.B. regime

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunogen</th>
<th>Day serum obtained</th>
<th>Western blot assay*</th>
<th>Peptide ELISA (titre⁻¹)†</th>
<th>Neutralizing antibody (titre⁻¹)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SP2</td>
<td>SP4A</td>
<td>SP7</td>
</tr>
<tr>
<td>A</td>
<td>RVV-Els</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RVV-Els</td>
<td>14</td>
<td>14</td>
<td>+ (2/4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RVV-Els</td>
<td>28</td>
<td>28</td>
<td>+ (4/5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Termination</td>
<td>37</td>
<td>37</td>
<td>+ (4/4)</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>RVV-Els</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>14</td>
<td>14</td>
<td>+ (1/6)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>28</td>
<td>28</td>
<td>+ (6/6)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Termination</td>
<td>37</td>
<td>37</td>
<td>+ + (6/6)</td>
<td>100 (6/6)</td>
</tr>
<tr>
<td>C</td>
<td>RVV-Els</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RVV-Els</td>
<td>14</td>
<td>14</td>
<td>- (4/4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RVV-Els</td>
<td>28</td>
<td>28</td>
<td>+ (2/5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Termination</td>
<td>37</td>
<td>37</td>
<td>+ (5/5)</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>RVV-Els</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>14</td>
<td>14</td>
<td>+ (2/6)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>28</td>
<td>28</td>
<td>+ (6/6)</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>RVV-Els</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>14</td>
<td>14</td>
<td>- (4/4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>28</td>
<td>28</td>
<td>+ (5/5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Termination</td>
<td>37</td>
<td>37</td>
<td>+ + (3/3)</td>
<td>50 (3/4)</td>
</tr>
<tr>
<td>F</td>
<td>env-I.B.</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>14</td>
<td>14</td>
<td>- (4/4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>env-I.B.</td>
<td>28</td>
<td>28</td>
<td>+ (4/4)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Termination</td>
<td>37</td>
<td>37</td>
<td>+ (4/4)</td>
<td>460 (4/5)</td>
</tr>
<tr>
<td>G</td>
<td>RVV-Els</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RVV-Els</td>
<td>14</td>
<td>14</td>
<td>- (3/3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RVV-Els</td>
<td>28</td>
<td>28</td>
<td>- (6/6)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Termination</td>
<td>37</td>
<td>37</td>
<td>- (4/4)</td>
<td>-</td>
</tr>
</tbody>
</table>

* Western blot reactivity was graded as the following: +, weak; ++, moderate; ++++, strong.
† Peptide ELISA titres depicted in the table are calculated means of positive samples only. Individual ELISA titres were determined as the dilution that resulted in an absorbance equal to, or greater than, twice background values obtained with control mouse sera (injected with 10 µg of MCWE alone).
‡ Neutralization titre: the highest dilution that inhibited HTLV-I syncytium formation by greater than 90% relative to normal mouse serum controls.
§ ND, Not determined.
glycoprotein (gp46) were capable of inducing neutralizing antibodies. In an effort to enhance neutralizing antibody titres directed to the HTLV-I envelope, env-I.B. was injected in combination with RVV Els and RVV E3s. RVV Elas which contains the antisense version of the native envelope gene was used as a control. Following priming with either RVV Els or RVV E3s, the mice were boosted twice with env-I.B. in the presence of 10 μg of MCWE adjuvant. This dose of adjuvant was chosen because it generated optimal antibody titres to the biologically significant SP-2 and SP-4A regions of gp46 (Fig. 6). The resulting sera were characterized by Western blot assay, peptide ELISA and syncytium inhibition assay (neutralization assay) and the results are summarized in Table 1. Western blot reactivity was recorded using a grading (+, + +, + + +) system as illustrated in Fig. 7.

Priming with RVV Els (Table 1, group B) and RVV E3s (group D) when combined with boosts of env-I.B. increased the overall antibody response to the HTLV-I envelope protein as determined by the Western blot assay, when compared to immunization with either RVV Els or RVV E3s alone (groups A and C), or env-I.B. alone (groups E and F). This did not translate into increased ELISA antibody titres to the SP-2 and SP-4A regions of gp46, when compared to the titres elicited by env-I.B. alone (compare groups B and D with E and F). We did not examine SP-6 since it is not associated with virus neutralization. Priming with RVV Els did enhance neutralizing antibody titres when used in combination with env-I.B. (compare groups C, D, E and F). As previously demonstrated (Ford et al., 1992), RVV Els (group A) efficiently induced neutralizing antibody in the absence of significant antibody titres to the SP-2 and SP-4A regions.

Discussion

In this study the entire HTLV-I envelope glycoprotein was successfully expressed in a baculovirus system. It was hoped that the presence of the complete envelope sequence, in a non-fusion form, would permit the presentation of both linear and conformational epitopes to the immune system. Only truncated or fusion proteins with smaller gene segments have been successfully expressed in Escherichia coli (Kiyokawa et al., 1984; Samuel et al., 1984; Chen et al., 1989). Expression of full or nearly full length gp63 in E. coli has proven to be toxic to the host cells. HTLV-I envelope protein expression in the baculovirus system has been limited to recombinant polyhedrin fusion proteins (Nyunoya et al., 1990). Synthesis of the HTLV-I envelope glycoprotein by a mammalian expression vector has also proven to be toxic to the host murine cells (Vile et al., 1991). Indeed, only very low levels of expression of the entire HTLV-I envelope protein have been obtained in Saccharomyces cerevisiae by Kuga et al. (1986).

Efficient expression of the HTLV-I envelope protein at levels of up to 6 mg/l of cell culture medium was observed in our baculovirus system. The protein was not secreted from the host cells but was stored intracellularly as inclusion bodies. The envelope protein obtained from purified inclusion bodies consisted of three major size classes of protein averaging about 43K, 54K and 63K. The 63K protein was equivalent in size to the expected glycosylated envelope precursor gp63 of HTLV-I (Pique et al., 1992). The glycosylated nature of the recombinant 63K protein was suggested by its disappearance upon tunicamycin treatment. Western blot analysis and tunicamycin treatment suggested that the 54K and 43K proteins represented variant unglycosylated forms of the envelope precursor. As predicted from the HTLV-I nucleotide sequence (Ratner et al., 1985), the 54K protein may represent the non-glycosylated envelope precursor with its leader peptide still attached. Unfortunately, the precursor origin of the recombinant 54K protein cannot be confirmed despite its ability to bind with anti-SP-7 peptide serum (SP-7 peptide sequence is derived from gp21), since SP-7 peptide could not completely inhibit the binding of the anti-SP-7 serum to the 54K protein. The reason for this lack of inhibition is not known but it may be due to a heteroclitic response to the SP-7 peptide. The identity of the non-glycosylated 43K protein is uncertain; however, we have demonstrated that the protein contains the epitopes recognized by the 1C11 MAb (specifically binds SP-4A peptide) and anti-SP-7 polyclonal sera. Amino-terminal sequencing of the 43K protein has been hindered by its lack of solubility. The identities of the immunoreactive low Mr proteins of 30K to 39K observed on Western blots have yet to be deduced. Similar low Mr proteins were seen upon expression of the human immunodeficiency virus (HIV) envelope glycoprotein in a similar baculovirus system (Hu et al., 1987). They could represent proteolytic degradation products of the envelope precursor protein or variant glycosylated forms of the mature protein since some forms disappeared when cells were treated with tunicamycin.

The accumulation of the HTLV-I envelope protein in the form of inclusion bodies could be the result of several related factors. The baculovirus expression system is inefficient in the processing of viral glycoproteins such as the haemagglutinin of influenza virus (Kuroda et al., 1986) and the envelope glycoprotein of HIV (Hu et al., 1987; Rusche et al., 1987), since these precursor proteins failed to be processed into the mature form. It is likely that the inefficient cleavage of the baculovirus-produced HTLV-I envelope precursor protein may be due to
incomplete or improper glycosylation. Sf9 cells are capable of N-linked glycosylation but are unable to perform the complex sugar linkages normally found on the HTLV-I envelope proteins (Miller, 1988). This may lead to the accumulation of the envelope precursor protein since efficient proteolytic cleavage appears to depend on proper glycosylation (Pique et al., 1992). Another contributing factor may be that, like HIV-1 gp160/120, HTLV-I gp63/46 may have a sequence that causes the retention of large amounts of the envelope protein in the secretory pathway (Bonifacino et al., 1991; Li et al., 1992). It may be that when Sf9 cells are made to express large amounts of HTLV-I envelope precursor, they retain more protein than is physiologically compatible, and hence the envelope proteins are stored in inclusion bodies to maintain cell viability. Even in mammalian expression systems, non-glycosylated and partially glycosylated forms of the HTLV-I envelope protein have been shown to accumulate within the cells to significant levels (Pique et al., 1992).

The HTLV-I env-I.B, isolated from our baculovirus system proved to be immunogenic in three strains of mice. Efficient stimulation of envelope-specific antibodies by env-I.B. was observed in animals immunized in the absence of adjuvant. The significant anti-envelope humoral response observed suggests that the packing of the HTLV-I envelope protein into inclusion bodies may mediate the slow release of the immunogen at the injection site and cause a prolonged stimulation of the animal’s immune effector cells.

The injection of an emulsion of env-I.B, and MCWE adjuvant preparation proved to elevate the HTLV-I envelope-specific response significantly in immunized mice. An MCWE dose of 10 μg produced maximum antibody titres to the four regions of envelope represented by the synthetic peptides used in the peptide ELISA. However, 50 μg of MCWE produced the best overall antibody response as determined by Western blot and radioimmunoprecipitation assays. Exceeding the 10 to 50 μg dose range of MCWE adjuvant resulted in a decrease in peptide ELISA titres and a drop in the absolute number of mice that seroconverted.

These results suggest that MCWE can serve as an effective adjuvant in immunization regimens as it stimulated good antibody responses against important regions of the HTLV-I envelope. Recent studies have shown that the carboxy terminus of gp46 is highly immunogenic in humans (Copeland et al., 1986; Palker et al., 1989). Similar results are shown here, particularly in the presence of MCWE. The carboxy-terminal SP-6 region elicited the highest antibody titres of the four regions tested by peptide ELISA. Immune responses against the SP-4A region are particularly significant since this region encompasses a B cell epitope (Palker et al., 1989), a T cell epitope (Kurata et al., 1989), a cytotoxic T cell epitope (Jacobson et al., 1991) and a virus-neutralizing epitope (Tanaka et al., 1991). In the presence of MCWE, significant titres to the SP-4A region were elicited. The SP-2 region has also been associated with virus neutralization; however, this region within the env-I.B. did not elicit as high an antibody response as did other regions in the presence or absence of MCWE.

Neutralizing antibodies, as detected by the syncytium inhibition assay, were elicited by env-I.B. only at high adjuvant doses of MCWE; thus, there was no correlation between the titre of antibody capable of binding to the SP-4A and SP-2 peptides and the ability of the sera to inhibit syncytium formation. This lack of correlation suggests either that the HTLV-I envelope glycoprotein possesses other epitope(s) capable of eliciting neutralizing antibody, or that the generation of neutralizing antibodies requires that the epitopes contained within SP-2 and SP-4A be presented in a specific conformation that does not occur in the env-I.B. to a significant extent.

Recently we have shown that RVV expressing different versions of the HTLV-I envelope could induce neutralizing antibody against HTLV-I (Ford et al., 1992). In an effort to increase the neutralizing antibody titres, a combined immunization regimen was devised employing both env-I.B. and RVV. Priming with RVV Els or E3s clearly enhanced the anti-envelope antibody response as compared to RVV alone or env-I.B. alone. Most notably, the combined immunization with RVV E3s, expressing gp46, and env-I.B. generated higher neutralizing antibody responses in comparison to mice immunized with E3s alone. Interestingly, the RVV Els, which expresses the native HTLV-I envelope of gp46 and gp21, did not prime mice to induce higher levels of neutralizing antibody. The reason for this is not clear since RVV Els was capable of inducing neutralizing antibody on its own. Perhaps the manner in which the gp46 was presented to the immune system by RVV Els and RVV E3s was different.

Previous studies (Ford et al., 1992) revealed that RVV Els-infected human H9 T cells properly process and express the native HTLV-I envelope proteins on the cell surface to the same extent as HTLV-I-infected T cells, as determined by fluorescence-activated cell sorting (FACS) analysis. Thus we would anticipate that the majority of the RVV Els-encoded envelope protein would be presented to immune effector cells in association with major histocompatibility complex (MHC) class I antigen (Teyton et al., 1990). RVV E3s-infected H9 T cells do not appear to retain HTLV-I gp46 envelope protein on their surfaces as determined by FACS analysis, although RVV E3s expresses higher levels of gp46 than RVV Els in infected cells. This is in agreement with studies describing
the expression of HTLV-I gp46 (Pique et al., 1990) and HIV gp120 (Kieny et al., 1988) in the absence of their respective transmembrane proteins, which demonstrated that the majority of the HTLV-I gp46 and HIV gp120 is released from the cell surface. This suggests that the exogenous gp46 secreted by RVV E3s-infected cells would be processed and presented through the MHC class II pathway (Teyton et al., 1990), the same pathway expected to process and present the recombinant baculovirus-produced env-I.B. An MHC class II presentation pathway shared by the RVV E3s- and baculovirus-produced HTLV-I envelope-produced HTLV-I envelope proteins suggests that similar envelope protein epitopes would be presented to the immune system and thus may explain why boosts of recombinant baculovirus envelope protein (env-I.B.) enhanced the neutralizing response in RVV E3s-primed mice.

In summary, we have successfully expressed the entire HTLV-I envelope protein in the baculovirus expression system. The envelope proteins were produced as inclusion bodies which could be efficiently recovered. When env-I.B. were combined with an adjuvant, MCWE, antibodies were raised to important regions of the HTLV-I envelope, but this did not translate into significant neutralizing antibody titres. The combined immunization with RVV E3s, expressing gp46, and env-I.B. did result in increased neutralization titres. It will be interesting to determine whether this combined immunization approach will prove effective in animal challenge experiments.

We wish to thank Dr J. K. Ball for her initial advice and help with the animal experiments. This work was financially supported by the MRC of Canada, NIH/NCI CA40660 of the U.S. and the Leukemia Society of America. J.A. is supported by an MRC of Canada Studentship, G.D. is supported by an Ontario Ministry of Health Career Scientist Award, and T. J. P. is supported by a Leukemia Society of America Scholar Award.

References


(Received 29 May 1992; Accepted 14 October 1992)