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Envelope proteins of human T-cell leukemia virus: Expression in *Escherichia coli* and its application to studies of *env* gene functions

(hybrid proteins/anti-*env* products/syncytium/cytotoxicity)

TETSUYUKI KIYOKAWA*†, HIROSHI YOSHIKURA‡, SEISUKE HATTORI*, MOTOHARU SEIKI*, AND MITSUAKI YOSHIDA*§

*Department of Viral Oncology, Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan; and †Department of Bacteriology, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 131, Japan

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ABSTRACT **The DNA fragments of the 5′ and 3′ halves of the putative *env* gene predicted from the DNA sequence of human T-cell leukemia virus (HTLV) provirus were inserted into expression vectors pORF2 and pORF1, respectively, and two hybrid proteins composed of *env* polypeptides and β-galactosidase were efficiently produced in *Escherichia coli*. The hybrid proteins containing the NH_2-terminal (EH9) and COOH-terminal (EA1) halves were both immunologically reactive with sera from adult T-cell leukemia patients, demonstrating the utility of the hybrid proteins for diagnosis of HTLV infection. Rabbit antisera against these hybrid proteins detected the two glycoproteins gp62 and gp46, which were previously identified as HTLV *env* gene products. With these rabbit antisera, two properties of the *env* gene products were studied. (*i*) The antisera inhibited syncytia formation of cat S^+L^- cells induced by HTLV, suggesting that one or both of the *env* gene products of HTLV, gp62 and gp46, are involved in induction of cell fusion. (*ii*) The *env* product gp62 or gp46 or both products are exposed on the surface of HTLV-infected cells and might modulate the proliferation of HTLV-infected T cells in the host because the antisera against the hybrid proteins were cytotoxic on HTLV-producing cell lines. The latter conclusion also is supported by the fact that adult T-cell leukemia patients and healthy HTLV carriers have antibodies to the *env* gene products.**

Human T-cell leukemia virus (HTLV) (1, 2), previously also known as adult T-cell leukemia virus (ATLV) (3–5), was isolated from human T-cell lymphoma and adult T-cell leukemia (ATL). From molecular and epidemiological studies, HTLV was shown to be closely associated with ATL (3, 6–8) and was further identified as a causative factor in ATL development (8, 9). HTLV was found to be exogenous for humans and to be transmitted by infection (3, 9, 10) and also to replicate by a mechanism similar to those of known animal retroviruses (11).

The glycoproteins encoded by the *env* gene of retroviruses are exposed on the surface of the viral particles (12) and are known to be essential in the early stage of viral infection for interaction with receptors on the surface of target cells (13). However, the proteins encoded by the *env* gene of the HTLV genome had not been well defined (14) and only recently were identified (15, 16). To establish a system for study of the *env* gene function, we constructed expression plasmids containing DNA segments of the *env* gene, and the polypeptides directed with these sequences were efficiently produced in *Escherichia coli*. These hybrid proteins were shown to be useful in the diagnosis of HTLV infection. We also report that antisera against these *env* gene products inhibited syncytia formation induced by HTLV and were cytotoxic to HTLV-producing cell lines.

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MATERIALS AND METHODS

Cells and Media. MT-1 (17), MT-2 (18), and HUT102 (1) are human T-cell lines infected with HTLV; CCRF-CEM and Molt-4 are acute lymphatic leukemia T-cell lines (19); and IMR90 (20) is a normal human diploid fibroblast line. TARL-2 (21) is a rat lymphoid cell line with HTLV infection, and a cat S^+L^- cell line (22) is a MSV-nonproducer kidney cell line containing the murine sarcoma virus (MSV) genome. Lymphoid cell lines were maintained in RPMI-1640 medium containing 10% fetal calf serum; fibroblastic cell lines were maintained in Dulbecco's modified Eagle's medium containing 5% fetal calf serum.

Plasmids and Bacteria. A plasmid pATK100 (4) containing the *env* gene of HTLV provirus was used as the starting material of the *env* gene. Expression vectors for open reading frames pORF1 and pORF2 and their hosts, MH3000 and TK1046 (23), were gifts from T. J. Silhavy (NCI–Frederick Cancer Research Facility). The conditions for culture of the bacteria and transformation were as reported (23).

Detection and Isolation of Hybrid Proteins. *E. coli* strain TK1046 was transformed with the recombinant plasmids pEH9 and pEA1 (Fig. 1) and cultured at 25°C until the absorbance at 600 nm reached 0.2. The culture was then shifted to 37°C and incubated for 1 hr. The cells were collected, sonicated, and then extracted with the loading buffer (125 mM Tris·HCl, pH 6.8/2% sodium dodecyl sulfate/9% glycerol/0.7 M 2-mercaptoethanol/0.002% bromophenol blue). The soluble fraction was loaded on 7% polyacrylamide gel and subjected to electrophoresis by the method of Laemmli (24). The proteins were purified by either preparative gel electrophoresis or HPLC using a gel permeation column and were used for immunization of rabbits. The immunocomplexes were isolated as described by Kessler (25), and antigens fixed on filters were detected by the method of Burnette (26).

Syncytia Formation and Cytotoxicity Tests. Assay of syncytia formation by cat kidney MSV nonproducer (S^+L^-) cells was in principle similar to that described by Hoshino *et al.* (27) and Nagy *et al.* (28). The procedure was modified as follows. Lymphocytes from ATL patients were cocultured with human fibroblasts IMR90 for 10 days, and the subpopulation of IMR90 cells was confirmed to be infected with HTLV by Southern blotting and immunoprecipitation analysis. These IMR90 cells producing viral antigens were plated

Abbreviations: ATL, adult T-cell leukemia; HTLV, human T-cell leukemia virus; MuLV, murine leukemia virus; MSV, murine sarcoma virus; gp, glycoprotein; kbp, kilobase pairs.

†On leave from The Second Internal Medicine, Medical School, Kumamoto University, Japan.
§To whom reprint requests should be addressed.

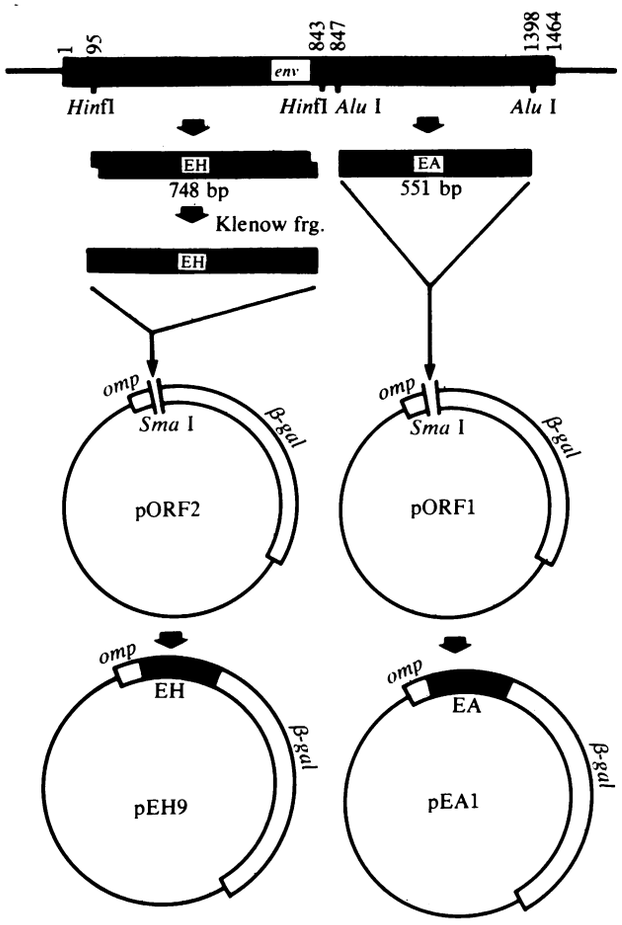

FIG. 1. Construction of plasmids for expression of the HTLV *env* gene in *E. coli*. Fragments of the 748-bp *Hinf*I fragment and 551-bp *Alu* I fragment were isolated from digests of pATK100 (4), and the *Hinf*I fragment was filled in with deoxynucleotides by using the Klenow fragment (frg.) of DNA polymerase I. The fragments were each ligated to *Sma* I-cleaved pORF2 or pORF1 with T4 DNA ligase. Each mixture was used to transform *E. coli* strain MH3000 and was plated on agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside. Then blue colonies were selected. *β-gal*, β-galactosidase.

onto cat S^+L^- cells and cultured for several days. Polykaryons containing more than 10 nuclei were counted after Giemsa staining. The cytotoxic test was carried out as described (29). All sera used were heated at 56°C for 30 min.

RESULTS

Construction of Plasmids Containing *env* Gene Sequences. Digestion of the plasmid pATK100 (4) with *Hinf*I or *Alu* I produced DNA fragments containing the 5′ half (EH fragment, 748 base pairs (bp) from the nucleotide position 95 to 843) or 3′ half (EA fragment, 551 bp from position 847 to 1398) of *env* as illustrated in Fig. 1. These two fragments represent 89% of the *env* gene sequence. The staggered ends of the EH fragment were filled in with deoxynucleotides by the Klenow fragment of DNA polymerase I. The EH and EA fragments were inserted into *Sma* I sites of pORF2 and pORF1 (23), respectively. Thus, the constructed plasmids contained the following three sequences: a short sequence coding for an *E. coli* outer membrane peptide, half of HTLV *env*, and a β-galactosidase-encoding region. The constructed plasmids were used to transform *E. coli* MH3000. Blue colonies on agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactoside were selected, and two plasmids, pEH9 containing the 5′ half and pEA1 containing the 3′ half, were isolated. Both plasmids were confirmed to contain *env* sequences in the correct reading frames by analysis of their DNA sequence.

Production of Hybrid Proteins Containing *env* Polypeptides. When *E. coli* strain TK1046 (23) was transformed with plasmids pEH9 and pEA1, new proteins with molecular weights of about 150 kDa were produced in amounts as much as 15–20% of the total proteins (Fig. 2*A*). The sizes of these main proteins were the same as those expected from the DNA sequences. To demonstrate that these newly produced proteins EH9 and EA1 had antigenicities similar to the native *env* gene products, their cross-reactivities with serum from an ATL patient were tested. EH9 and EA1 both cross-reacted with serum from an ATL patient on electrophoretic immunoblot analysis (Fig. 2*C*), indicating that these hybrid proteins had antigenic sites similar to the native *env* gene products, although they were not glycosylated. In preliminary experiments, these proteins were purified to homogeneity by preparative gel electrophoresis (Fig. 2*B*) and were fixed on nitrocellulose filters. The filters were then subjected to indirect radioimmunoassay to detect *env*-specific antibodies in human sera. Both hybrid proteins were found to detect antibodies in patient sera, but not in sera from noninfected normal adults (data not shown). The two hybrid proteins were almost equally effective in this assay, although EH9 was slightly more sensitive. The procedure with EH9 was at least 10 times more sensitive than the standard indirect immunofluorescence assay (6) with MT-2 cells (data not shown).

Identification of *env* Gene Products with Antibodies to EH9 and EA1. The purified proteins EH9 and EA1 were injected into rabbits to obtain antisera against the hybrid proteins. The rabbit antiserum against EH9 was found to detect bands of 62 and 46 kDa in cell extracts of MT-2 and HUT102 by immunoprecipitation (Fig. 3*A*). Since these bands have been characterized as glycoproteins encoded by the *env* gene (15, 16), these findings were direct evidence that the inserted DNA in pEH9 was translated in the correct frame and that

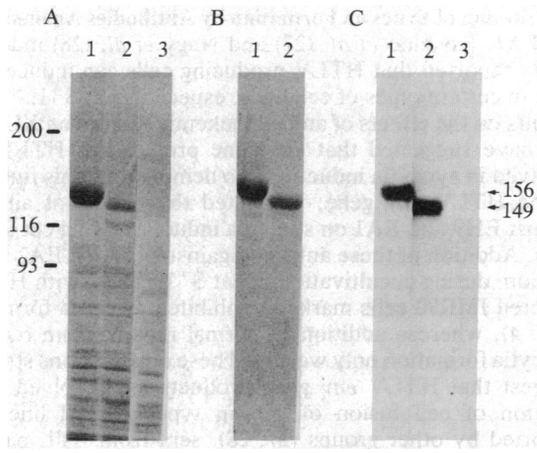

FIG. 2. Characterization of hybrid proteins consisting of *env* sequences and β-galactosidase. (*A*) Total extract of *E. coli* TK1046 containing pEH9 (lane 1), pEA1 (lane 2), or no plasmid (lane 3) were separated by 7% polyacrylamide gel electrophoresis, and the gel was stained with Coomassie blue. (*B*) The hybrid proteins EH9 (lane 1) and EA1 (lane 2) were purified by preparative gel electrophoresis and analyzed by polyacrylamide gel electrophoresis. (*C*) The proteins after electrophoresis as in *A* were transferred electrophoretically to a nitrocellulose filter, and the filter was treated with serum from an ATL patient and then with sheep ^{125}I-labeled anti-human whole immunoglobulins by the standard procedure for electroblot analysis (29). Lanes: 1, *E. coli* TK1046 with pEH9; 2, TK1046 with pEA1; 3, TK1046. Sizes are shown in kDa.

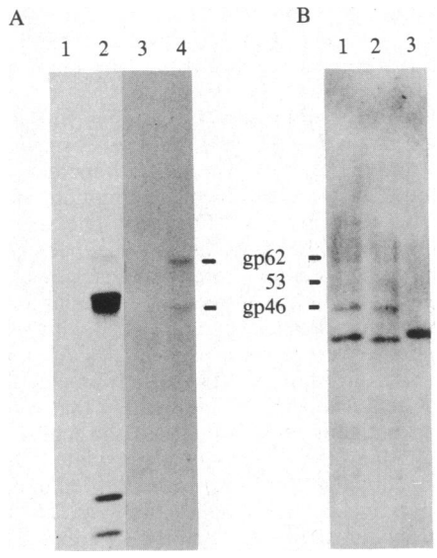

FIG. 3. Detection of *env* gene products in extracts of cells producing HTLV. (*A*) Samples (≈10 μg of proteins) from extracts of MT-2 cells labeled for 16 hr with [^{35}S]cysteine were immunoprecipitated with normal human serum (lane 1), serum from an ATL patient (lane 2), normal rabbit serum (lane 3), and rabbit antiserum to EH9 (lane 4). The immunocomplexes were analyzed by 10% polyacrylamide gel electrophoresis. (*B*) Samples (10 μg) of cell extracts from MT-2 cells (lane 1), HUT102 (lane 2), and normal human lymphocytes (lane 3) were separated by acrylamide gel electrophoresis and transferred to nitrocellulose. The filter was treated with rabbit antiserum to EA1 and then with donkey ^{125}I-labeled anti-rabbit whole immunoglobulins.

gp46 is one of the *env* gene products. The antiserum against EA1 detected bands with apparent molecular masses of 62, 53, and 46 kDa in electroblot analysis (Fig. 3*B*), although it did not detect significant bands in immunoprecipitation assay (data not shown). The two bands corresponding to 62 and 46 kDa seemed to be gp62 and gp46. It is not yet known if the 53-kDa protein is also an *env* gene product or not.

Inhibition of Syncytia Formation by Antibodies Against EH9 and EA1. Hoshino *et al.* (27) and Nagy *et al.* (28) independently reported that HTLV-producing cells can induce syncytia in certain types of cell lines, especially cat S^+L^- cells. Results on the effects of animal leukemia viruses on XC cells (30) have suggested that *env* gene products of HTLV are involved in syncytia induction. To demonstrate this function of the HTLV *env* gene, we tested the effects of antisera against EH9 and EA1 on syncytia induction using cat S^+L^- cells. Addition of these antisera against EH9 and EA1 to the medium during cocultivation of cat S^+L^- cells with HTLV-infected IMR90 cells markedly inhibited syncytia formation (Fig. 4), whereas addition of normal rabbit serum reduced syncytia formation only weakly. These observations strongly suggest that HTLV *env* gene products are involved in induction of cell fusion of certain types of cell lines. As reported by other groups (27, 28), sera from ATL patients also inhibited syncytia formation. These findings are consistent with the presence of antibodies against the *env* gene products of HTLV in sera of ATL patients (15, 16).

Cytotoxicity of Antibodies Against EH9 and EA1. *env* genes of animal retroviruses are exposed on the surface of virus particles (12) or infected cells (31), and antibodies against the *env* glycoproteins neutralize the infectivity of the virus (32) and are cytotoxic to cells expressing these molecules (33). Therefore, HTLV *env* products also may be exposed on the surface of T cells producing HTLV and, thus, may be useful as tumor-specific antigens of ATL cells. To demonstrate these surface *env* antigens, we treated cells producing HTLV

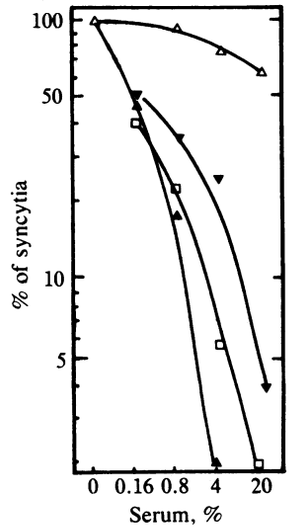

FIG. 4. Inhibition by antisera to EH9 and EA1 of syncytia formation induced by HTLV. IMR90 cells infected with HTLV were plated onto semiconfluent cultures of cat S^+L^- cells and cultured in the continuous presence of various amounts of antisera. On day 5, the cultures were stained and polykaryons containing >10 nuclei were counted under a microscope. ▼, Rabbit antiserum to EH9; ▲, rabbit antiserum to EA1; □, serum from ATL patient; △, normal rabbit serum.

with rabbit antisera against EH9 and EA1 in the presence of rabbit complement. As shown in Fig. 5*A*, sera against the NH_2- and COOH-terminal halves of the *env* product both killed MT-2 cells in the presence of complement. Antisera without complement and normal rabbit serum were not cytotoxic. Antiserum to EA1 was more toxic than anti-EH9. However, this result may be a reflection of the difference in antibody titers and may not imply that the COOH-terminal

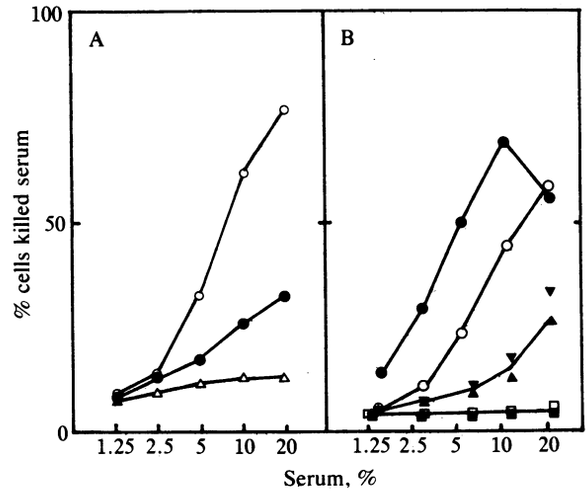

FIG. 5. Complement-dependent cytotoxicities of antisera to EH9 and EA1. (*A*) In 50 μl of minimal essential medium containing 7% heat-inactivated fetal calf serum, MT-2 cells (10^4 cells) were mixed with various amounts of antisera in the presence of 3 μl of rabbit serum as a source of complement and incubated for 30 min at 37°C. The percentage of killed cells was determined by the trypan blue dye-exclusion test. Rabbit antiserum to EA1 in the presence (○) and absence (△) of rabbit complement and rabbit antiserum to EH9 with complement (●). (*B*) Human T-cell lines infected with HTLV [MT-2 (○), MT-1 (▲), and HUT102 (▼)], a rat T-cell line infected with HTLV [TARL-2 (●)], and human T-cell lines not infected with HTLV [CCRF-CEM (□) and Molt-4 (■)] were tested as targets of rabbit antiserum to EA1. The conditions were the same as for *A*.

half of the *env* gene product is more important in cytotoxicity. The cytotoxic effect of anti-EA1 was tested further on other cell lines with and without HTLV infection (Fig. 5*B*). A rat cell line TARL-2 infected with HTLV was highly sensitive to the antiserum against EA1, and two human T-cell lines, MT-1 and HUT102, infected with HTLV were also sensitive, but less sensitive than MT-2. Two human control T-cell lines, CCRF-CEM and Molt-4, which were not infected with HTLV, were completely resistant to this antiserum. Thus, the cytotoxic activities of anti-EH9 and -EA1 were well correlated with infection with HTLV but not with the origin of the cells. Therefore, these observations, especially those with anti-EH9, suggest that an *env* gene product, gp62 or gp46 or both, is exposed on the cell surface and is recognized by these antibodies against the hybrid proteins.

DISCUSSION

The NH_2- and COOH-terminal halves of the *env* gene products were produced in *E. coli* as hybrid proteins, with β-galactosidase constituting 15–20% of the total proteins. The polypeptides encoded by the halves of the *env* were covalently linked at both ends to β-galactosidase and were not glycosylated, but they showed antigenicities cross-reactive with antibodies in sera from ATL patients. Therefore, these proteins can be used for diagnosis of HTLV infection, detecting *env*-specific antibodies in human sera, since they are free from any possible contamination with human proteins. In indirect radioimmunoassay, the purified proteins fixed on nitrocellulose filters were >10 times more sensitive in detecting *env* antibodies in human sera than is the indirect immunofluorescence assay (6) with MT-2 cells.

Antisera raised in rabbits against these hybrid proteins, EH9 and EA1, detected gp62 and gp46, which had been identified as *env* gene products of HTLV (15, 16). These antisera efficiently inhibited cell fusion of cat S^+L^- cells induced by HTLV-producing cells. This result suggests that the *env* gene products of HTLV, like those of other animal retroviruses (30), induce syncytia.

Antisera against EH9 and EA1 were found to be cytotoxic to cell lines producing HTLV in the presence of complement. Since these toxicities were specific to HTLV-infected cells and unrelated to the origin of the cells, it was concluded one or both of the *env* gene products of HTLV, gp62 and gp46, were exposed on the cell surface and were the targets in this system. However, rather high concentrations of antisera are required for detectable cytotoxicity. This result may be explained by low titers of the antisera or by the presence of a limited number of gp62 or gp46 molecules on the cell surface. The presence of antibodies against the *env* gene product (19, 20) in the sera of ATL patients may explain the absence of viral antigens in primary ATL cells. In most cases of ATL, fresh tumor cells in peripheral blood are not expressing the HTLV antigens in amounts detectable by indirect immunofluorescence assay (6), but they can express the viral antigens after short-term (2–3 days) culture with calf serum. These phenomena are reasonably explained now by immunological masking with antibodies to HTLV-positive cells in the serum of patients, as found in other systems (34). These considerations on cytotoxic antibodies against *env* gene products suggest that gp62 or gp46 or both can be targets for modulating the proliferation of the HTLV-infected or -transformed cells.

The cytotoxic activity on HTLV-producing cells of antibodies against *env* gene products suggests that these antibodies might be useful for prevention of viral transmission by killing the virus-producing cells, although further careful studies are required to test this possibility. It is difficult to demonstrate direct inhibition of viral infection by these antisera, because an efficient cell-free system for viral infection is not available. The results of cytotoxicity tests suggest, however, that these antisera would inhibit viral infection, recognizing glycoprotein on the viral particles. Since we reported previously that HTLV has causative roles in the development of ATL (8, 9), these viral antigens or antisera to them could be useful in preventing viral infection and ATL development.

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