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Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection

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The human T-lymphotropic virus type I (HTLV-I) is etiologically linked to adult T-cell leukemia (ATL). To develop a vaccine against ATL, we constructed recombinant vaccinia viruses containing the envelope gene of HTLV-I in the vaccinia virus hemagglutinin (HA) gene, a new site where foreign genes can be inserted. A single inoculation of the recombinant virus induced antibodies to the env proteins of HTLV-I in rabbits and had a protective effect against HTLV-I infection.

Key words: envelope protein/hemagglutinin gene/HTLV-I/vaccinia virus recombinants/vaccine

Introduction

The human T-lymphotropic virus type I (HTLV-I) has been shown to be etiologically associated with human adult T-cell leukemia (ATL) (Poiez *et al.*, 1980; Hinuma *et al.*, 1982; Hinuma, 1985). HTLV-I is known to be endemic in several parts of the world, especially in Japan where approximately one million people are estimated to be carriers. The env glycoprotein of HTLV-I is the major antigen recognized by the serum of the individuals infected with HTLV-I (Schupbach *et al.*, 1984). The antibody to HTLV-I env protein was demonstrated to neutralize the pseudotypes of vesicular stomatitis virus with HTLV-I envelope (Clapham *et al.*, 1984; Hoshino *et al.*, 1985) and to inhibit fusion activity of HTLV-I by which HTLV-I is considered to be transmitted from cell to cell (Hoshino *et al.*, 1983, 1985; Nagy *et al.*, 1983; Kiyokawa *et al.*, 1984). Moreover, most neutralizing antibodies against several other retroviruses (Taniyama and Holden, 1979; Flyer *et al.*, 1983) are directed towards their env proteins. Thus, to develop an effective vaccine against HTLV-I, the env protein should be the first candidate to be considered.

Potential new vaccines based on the expression of foreign genes in vaccinia virus have been developed (Panicali and Paoletti, 1981; Smith *et al.*, 1983). This type of vaccine offers potential advantages for the control of diseases. It induces not only humoral antibodies against the foreign antigens expressed by the recombinant vaccinia virus but also cellular immunities (Bennink *et al.*, 1984; Yewdell *et al.*, 1985). Since vaccinia virus has the capacity to retain multiple foreign genetic elements in its genome, it is possible to make a polyvalent vaccine capable of eliciting immunity against a number of heterologous infectious diseases (Perkus *et al.*, 1985). Recently several sites where foreign genes can be inserted have been demonstrated (Perkus *et al.*, 1986).

The vaccinia virus hemagglutinin (HA) gene is also one of the candidates for the sites to insert foreign genes because it is non-essential for virus replication in cultured cells as well as in laboratory animals (Ichihashi and Dales, 1971). In addition, HA^- recombinant viruses can be easily isolated because HA^+ plaques of wild-type virus are stained red by chicken erythrocytes whereas HA^- plaques are not (Oda, 1965). A large number of HA^- mutants have already been isolated by this technique (Shida and Matsumoto, 1983). The nucleotide sequence of the HA gene has been determined (Shida, 1986).

We describe here the construction of the recombinant vaccinia virus harboring the HTLV-I env gene in the vaccinia virus HA gene and the effects of the inoculation of the recombinant vaccinia viruses on the HTLV-I infection.

Results

Construction of recombinant vaccinia viruses containing the gene coding for the HTLV-I envelope proteins

The full sized proviral genome of HTLV-I has been cloned into phage from TLOm1 cells, an ATL cell line established from the peripheral lymphocytes of an ATL patient (Sugamura *et al.*, 1984). The 1.6-kb fragment containing the env gene of HTLV-I was subcloned into pBR322, designated pKHe (K.Hirayoshi and M.Hatanaka, unpublished). To construct the recombinant vaccinia virus, the HA gene (Shida, 1986) was chosen as a site to insert the foreign gene.

To compare the efficiency of the env gene expression, we constructed two types of recombinant vaccinia virus containing the env gene flanked by segments of the vaccinia virus HA gene, WR-env17 and WR-proenv1. In WR-env17, the env gene was introduced into the region in the HA gene encoding the signal peptide sequence of the vaccinia HA protein in such a way that the reading frame from the HA protein continued to that for the env protein in phase (Figure 1a). Expression of this env gene was expected to be directed by the promoter of the HA gene that is located immediately upstream of the HA coding region. The HA promoter has characteristic sequences common to a group of late promoters of vaccinia virus (Shida, 1986). The translation initiation would occur at the first AUG codon of the HA gene to produce the fusion protein in a nascent state. However, the amino-terminal region of the translation product containing the extra amino acid residues and the signal sequence of the env protein should be cleaved off by the signal peptidase upon its insertion into the rough endoplasmic reticulum (Blobel and Dobberstein, 1975). Therefore, it was expected that the final product would be the same as the authentic env protein produced by HTLV-I. In WR-proenv1, the env gene was first placed downstream from a vaccinia 7.5K promoter element (Venkatesan *et al.*, 1981). Then this promoter–env element construct was inserted within the HA gene (Figure 1b). This chimeric gene contains the transcription start site of the 7.5K gene juxtaposed with the env translational initiation codon so as to produce the authentic protein. These plasmid constructs were transfected into vaccinia

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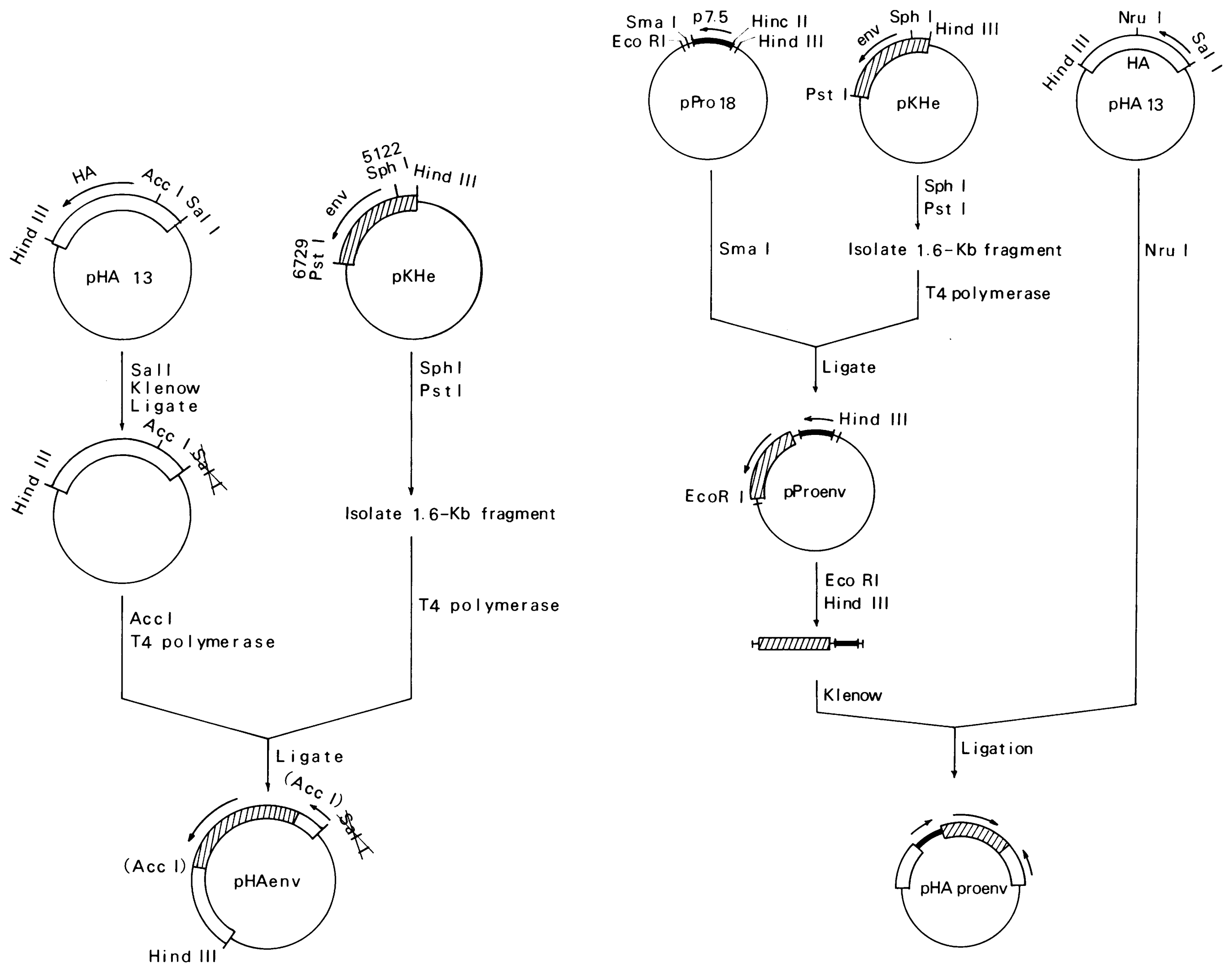

Fig. 1. Construction of plasmid vectors for insertion of the HTLV-I envelope protein coding sequence into vaccinia virus. The arrows show the direction of transcription.

virus-infected cells to prepare recombinant viruses that contain the HTLV-I env DNA inserted into the HA locus.

Expression of HTLV-I env in recombinant virus-infected cells

To determine whether the HTLV-I env gene was expressed by the recombinant vaccinia viruses, rabbit kidney cells (RK13) infected with these viruses were metabolically labeled with [^{3}H]-leucine. Env proteins were immunoprecipitated with the ATL serum followed by polyacrylamide gel electrophoresis. In the lysate of cells infected with either WR-env17 or WR-proenv1 virus, four major bands were detected by autoradiography (Figure 2). The apparent mol. wts of these proteins were estimated to be ~62 000 (62 kd), ~46 kd, ~20 kd and ~16 kd, all of which co-migrated with corresponding HTLV-I envelope glycoproteins synthesized in the TL-Su cells that produce HTLV-I (Sugamura *et al.*, 1984) (data not shown). The 16-kd peptide might be derived from gp46 as reported recently (Copeland *et al.*, 1986). Digestion with endoglycosidase H revealed that the gp46 possessed complex type of oligosaccharide chains but that gp62 had high mannose type carbohydrates (Figure 3). These four proteins were also recognized by the anti-HTLV-I env antiserum that had been obtained by injecting the env polypeptide produced in *Escherichia coli* that harbor the plasmid containing the HTLV-I env gene fragment (Kiyokawa *et al.*, 1984) (data not shown). The total amounts of the env proteins synthesized in the cells infected with WR-env17 and WR-proenv1 were similar. However, the processing from the precursor gp62 to gp46 and p20 occurred more efficiently in WR-proenv1-infected cells than WR-env17-infected cells. In addition, WR-proenv1-infected cells shed larger amounts of the gp46 (approximately one-fifth of the total env proteins) into the medium than did WR-env17-infected cells (Figure 2). Indirect immunofluorescence staining showed that the surface of the viable cells infected with either of the recombinant vaccinia viruses was stained with the ATL serum (Figure 4) whereas the surface of the wild-type vaccinia virus-infected cells was not (data not shown). These results demonstrate that both of the recombinant viruses are able to express the HTLV-I env gene whose products can be transported to the cell surface.

Immunization of rabbits with the recombinant viruses and their effects on HTLV-I infection

To examine the immunogenicity of proteins expressed by the recombinant vaccinia viruses, rabbits were intradermally inoculated with each of the purified viruses. Plasma samples were collected at 2-week intervals for 8 weeks post-inoculation and were analyzed by the particle agglutination method for titration

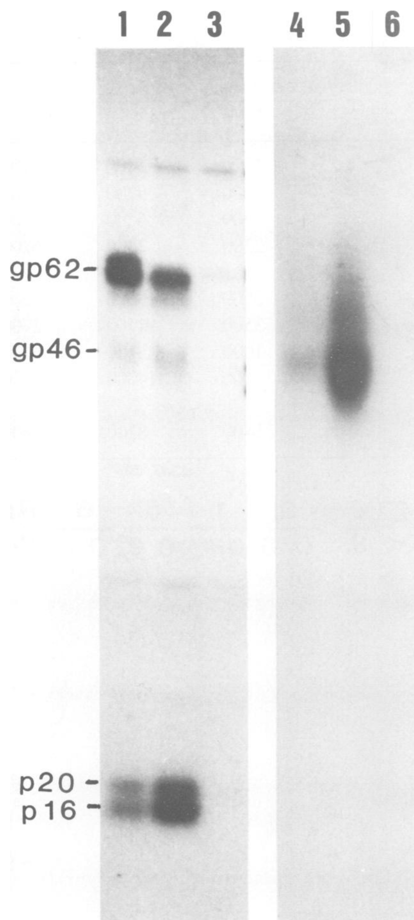

Fig. 2. Immunoprecipitation of HTLV-I env proteins from cells infected with vaccinia virus recombinants. RK13 cells were infected with standard vaccinia virus (**lanes 3** and **6**) or recombinant vaccinia virus, WR-env17 (**lanes 1** and **4**) or WR-proenv1 (**lanes 2** and **5**). The cells were labeled with [^{3}H]leucine 1 h post-infection (PI). At 20 h PI, the cells (**lanes 1–3**) and media (**lanes 4–6**) were subjected to immunoprecipitation as described in Materials and methods. The intensities of the bands of the cell lysates and those from the medium cannot be compared quantitatively since the latter samples were exposed five times longer than the former.

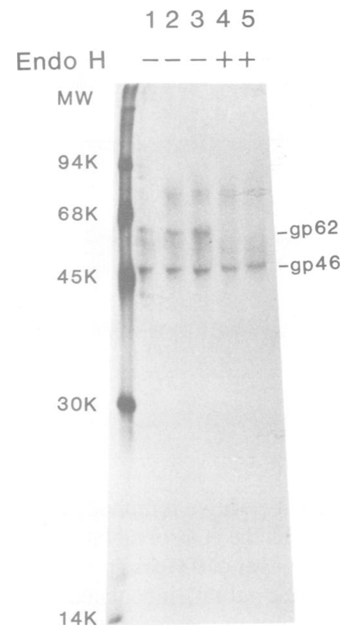

Fig. 3. Endo H digestion of the env proteins produced by the recombinant vaccinia viruses. Cells were labeled overnight with [^{3}H]glucosamine as described in Materials and methods. The env proteins were isolated by immunoprecipitation then digested with Endo H (+). Controls were incubated without Endo H (−). TL-Mor cells were run in **lane 1**; WR-env17-infected cells were in **lanes 2** and **4**; WR-proenv1-infected cells were in **lanes 3** and **5**.

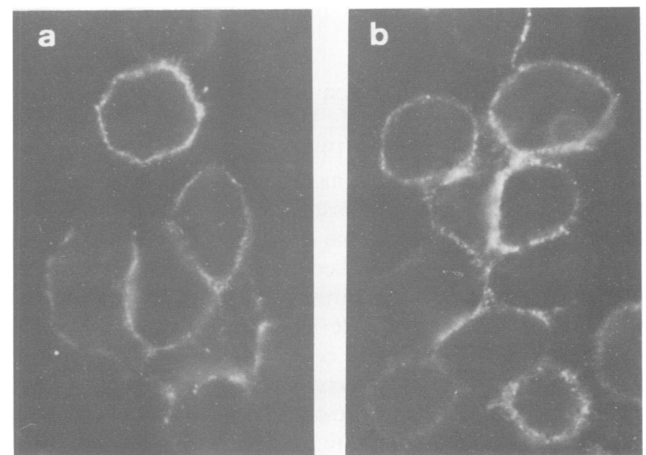

Fig. 4. Detection of the env proteins on the surface of the recombinant vaccinia virus-infected cells by immunofluorescence method. At 24 h PI WR-env17 (**a**) or WR-proenv1 (**b**) infected cells were stained by the sera from an ATL patient followed by FITC-conjugated anti-human IgG antibody as described by Shida *et al.* (1982). 600×.

of anti-HTLV-I antibodies (Ikeda *et al.*, 1984). Both of the recombinant vaccinia viruses induced high titers of anti-HTLV-I antibodies in all animals inoculated (Table I). Anti-HTLV-I antibodies in these plasma samples were also confirmed by the immunofluorescence staining using MT-1 cells that express HTLV-I env proteins (data not shown). WR-proenv1 induced anti-HTLV-I antibodies more rapidly than WR-env17 although both the recombinant and wild-type viruses induced similar titers of anti-vaccinia neutralizing antibodies. This may relate to the more efficient processing of env proteins synthesized in WR-proenv1-infected cells than those in WR-env17-infected cells. Figure 5 demonstrates that the representative plasmas obtained 8 weeks post-inoculation reacted with all the four envelope glycoproteins. Furthermore, the rabbits (nos 2, 4, 6 and 8) maintained a high titer of anti-env antibodies for 49 weeks after vaccination (data not shown).

Two rabbits each immunized with either recombinant or parental vaccinia virus were selected to be challenged with HTLV-I and the residual rabbits (nos 2, 4, 6 and 8) were kept without challenge to follow the persistence of the anti-env antibodies. HTLV-I-producing cells (MT-2) were intravenously injected 11 weeks post-vaccination since it is thought that HTLV-I infects men through contact of the infected cells with the uninfected cells (Yamamoto *et al.*, 1982). To ascertain the establishment of the HTLV-I infection in the rabbits challenged, the rabbit blood specimens were collected at 1-week intervals post-challenge and the peripheral blood lymphocytes were cultured in the presence of interleukin 2. Then, the appearance of the lymphocytes that possess the HTLV-I antigen was examined by the indirect immunofluorescence technique using the ATL serum or the monoclonal antibody to HTLV-I p19. The lymphocytes examined were not the MT-2 cells inoculated, since karyotype analysis showed that they were rabbit cells but not human (data not shown). None of the rabbits vaccinated with either of these recombinant viruses

Table I. Antibody response of vaccinated rabbits

Vaccine	Rabbit no.	Titers of anti HTLV-I on week					Titers of anti-vaccinia on week				
		0	2	4	6	8	0	2	4	6	8
WR-proenv1	1	<2	256	512	256	256	<4	1000	5400	3100	9300
	2	<2	256	2048	1024	2048	<4	2000	9300	6200	4100
	3	NT	128	1024	256	1024	NT	2300	6200	3100	4100
	4	NT	128	512	512	1024	NT	2300	2300	6200	5400
WR-env17	5	<2	128	512	128	1024	<4	3500	16000	8200	19000
	6	<2	32	1024	128	512	<4	3500	4700	2700	8200
	7	NT	16	128	64	512	NT	1000	5400	6200	4100
	8	NT	32	32	64	512	NT	3100	14000	3500	57000
WR	9	2	4	4	2	2	<4	5400	8200	9300	28000

had detectable HTLV-I-positive lymphocytes by 31 weeks post-challenge. In contrast, all the control rabbits demonstrated HTLV-I-positive lymphocytes within 9 weeks post-challenge (Table II). We could use only one rabbit, inoculated with wild-type WR strain as control, because of the unfortunate accidental death of another rabbit (no. 10). However, in separate experiments all seven rabbits that had been vaccinated with several strains of the wild-type vaccinia viruses and challenged subsequently with MT-2 cells demonstrated HTLV-I antigen-positive lymphocytes until 9 weeks post-challenge (data not shown). Thus, these results indicate that the recombinant vaccinia viruses capable of expressing HTLV-I env proteins are able to induce immunity against HTLV-I infection.

Discussion

The HTLV-I env gene has been expressed in animal cells using papilloma vectors (Eiden *et al.*, 1985), *E.coli* (Kiyokawa *et al.*, 1984), or yeast (Kuga *et al.*, 1986). Formation of the authentic HTLV-I env proteins with full modification such as glycosylation, however, has not been demonstrated in any of these systems. Incomplete modification of the env proteins may reduce their antigenicities. Here, we described that the env proteins expressed in the recombinant vaccinia viruses were fully glycosylated and had mol. wts indistinguishable from those of the env proteins produced in HTLV-I-infected cells. Thus, the env proteins expressed by vaccinia virus recombinants were expected to be highly immunogenic. Indeed we observed that a single vaccination induced a high titer of anti-env antibodies which lasted for 49 weeks and that it seemed to have protective effects on HTLV-I infection into rabbits. These results suggest a promising approach for vaccine development against HTLV-I.

A similar amount of the env proteins was synthesized in the cells infected with each of the two recombinant vaccinia viruses, suggesting that the strength of the HA and 7.5K promoters is similar. The precursor gp62 expressed from the WR-proenv1 was processed to gp46, p20 and p16 more efficiently than that from the WR-env17. The signal sequences of the nascent gp62 expressed from WR-proenv1 is cleaved off efficiently, unlike that of the WR-env17 (data not shown). This may affect the subsequent maturation events of the gp62. Furthermore, the efficacy of the processing of the env proteins seemed to affect their immunogenicities in rabbits; the rabbits inoculated with WR-proenv1 produced anti-HTLV-I antibodies more rapidly than those inoculated with WR-env17.

To examine the establishment of HTLV-I infection in the rabbits, we cultured their lymphocytes *in vitro* and then tested the presence of viral antigens in the cells by immunofluorescence techniques. This is so far the most sensitive method to detect the cells harboring HTLV-I since the *in vitro* cultivation prompts the viral spread (Hinuma *et al.*, 1982; Miyoshi *et al.*, 1985). *In situ* hybridization techniques using the HTLV-I genome as a probe failed to detect the lymphocytes containing the HTLV-I genome.

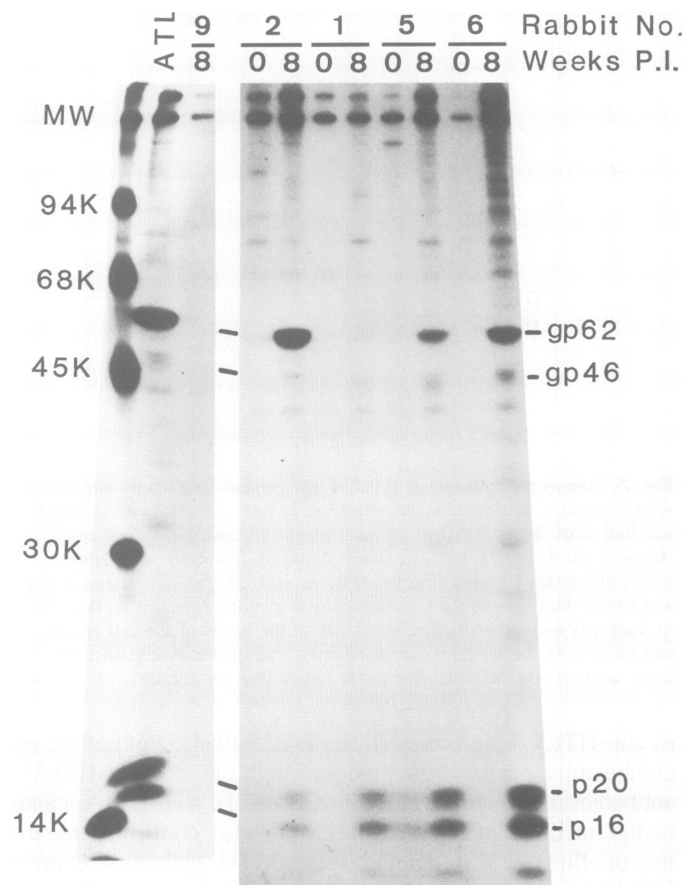

Fig. 5. Immunoprecipitation of HTLV-I env proteins by serum samples from rabbits inoculated with recombinant vaccinia viruses. TL-Mor cells were labeled with [^{3}H]leucine overnight. The cell lysates were incubated with the sera from an ATL patient, or from representative rabbits that had been inoculated 0 or 8 weeks before with WR-proenv1 (rabbits nos 1 and 2), WR-env17 (rabbits nos. 5 and 6), or wild-type virus (rabbit no. 9).

Table II. Detection of HTLV-I antigen in lymphocytes of vaccinated rabbits after challenge with HTLV-I

Vaccine	Rabbit no.[a]	HTLV-I antigen in cultured lymphocytes on week																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19	21	23	27	31
WR-proenv1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	[b]				
	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
WR-env17	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	7	–	–	–	–	–	–	–	–	[b]													
WR	9	–	–	–	–	–	–	–	–	+	+	+	–	+	+	+	[b]						
–	11	–	+	–	–	–	–	–	–	+	+	+	+	+	–	+	–	–	[c]				
	12	–	–	–	–	–	+	+	+	+	+	+	+	+	–	+	–	–	[c]				

[a]Rabbit numbering as in Table I.
[b]Died by accident.
[c]Killed.

We have demonstrated that the HA gene is a useful site to accept and express foreign genes besides the thymidine kinase gene, the F fragment, and other genes described more recently (Mackett *et al.*, 1981; Panicali and Paoletti, 1981; Perkus *et al.*, 1985, 1986). Use of the HA site to insert foreign genes is advantageous over other sites since the HA site accompanies the selection marker to pick up the recombinant viruses and the promoter of the HA gene is as strong as the 7.5K promoter. The availability of several insertion sites may facilitate the development of polyvalent vaccines based on the vaccinia virus.

Materials and methods

Cells and viruses

RK13 and CV-1 cells were grown at 37°C in Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS). TL-Su and TL-Mor cells were cultured in RPMI 1640 medium supplemented with 10% FCS. Vaccinia virus WR strain was propagated and titrated on monolayers of the RK13 cells and purified essentially as described by Joklik (1962).

Construction of chimeric donor plasmids for in vivo recombination

Plasmids were constructed, analyzed and purified using standard techniques (Maniatis *et al.*, 1982). In order to insert the HTLV-I env gene into the HA site of vaccinia virus, donor plasmids were constructed as follows. (i) One type of the recombinant plasmid contains the env gene flanked by the segments of the sequence encoding vaccinia HA (Figure 1a). Plasmid pKHe (K.Hirayoshi and M.Hatanaka, unpublished) contains HTLV-I-specific sequences from the *Hin*dIII site at nucleotide 4990 to the *Pst*I site at 6729 (Seiki *et al.*, 1983) inserted at the corresponding restriction enzyme sites of plasmid vector pBR322. The plasmid was digested with *Sph*I and *Pst*I, and a 1.6-kb fragment was isolated and blunt-ended with T4 polymerase. This fragment contains HTLV-I-specific DNA (nucleotides 5127–6728), including the sequences that encode the entire envelope proteins and 53 bp or 86 bp of 5′ or 3′ untranslated sequences respectively. Plasmid pHA13, which contains sequences encoding the vaccinia virus HA protein inserted between the *Sal*I and *Hin*dIII sites of plasmid vector pUC13 (Shida, 1986), was digested with *Sal*I, blunt-ended with Klenow enzyme and ligated by T4 ligase to disrupt the *Sal*I recognition sequence. The plasmid that lost the *Sal*I site was isolated and digested with *Acc*I followed by T4 polymerase treatment. The linearized plasmid was ligated with the isolated 1.6-kb fragment and transformed *E. coli* strain JM103. Plasmid DNA from ampicillin-resistant transformants was tested for the orientation of the insert. The resultant plasmid was named pHAenv. (ii) Another type of recombinant plasmid contains the env that is fused to the vaccinia 7.5K promoter and flanked by the segments of the HA gene locus (Figure 1b). The vaccinia virus transcriptional control element (7.5K promoter) was cloned into the pUC18 plasmid by the method described by Venkatesan *et al.* (1981), and the resultant plasmid was designated pPro18. The 1.6-kb fragment isolated from pKHe followed by blunt-ending with T4 polymerase was ligated with the plasmid pPro18 that had been linearized with *Sma*I. Recombinant plasmid with the structure indicated was isolated and designated pProenv. This plasmid was digested with *Eco*RI and *Hin*dIII and the 1.9-kb fragment containing the 7.5K promoter as well as the env sequence was isolated. After being blunt-ended with Klenow enzyme, the fragment was ligated with the plasmid pHA13 linearized with *Nru*I. The resultant plasmid, pHAproenv, contained the env sequences placed downstream from the 7.5K promoter and flanked by the segments of the HA gene.

Construction and isolation of recombinant vaccinia viruses

To obtain the recombinant vaccinia viruses containing the env gene in the HA gene locus, the cells infected with vaccinia virus WR strain at 0.1 p.f.u./cell were transfected with the plasmid pHAenv or pHAproenv. Resultant HA^- recombinant viruses were picked as described previously (Shida and Matsumoto, 1983). The HA^- viruses were plaque-purified twice more before use. Induction of synthesis of env proteins was examined by overlaying the plaques successively with serum from an ATL patient (ATL serum) and peroxidase-conjugated anti-human IgG antiserum followed by coloring with 4-chloro-1-naphthol and hydrogen peroxide (Mackett *et al.*, 1985). The recombinant virus produced by transfection with pHAenv or pHAproenv was named WR-env17 or WR-proenv1 respectively.

Immunoprecipitation of HTLV-I env proteins

RK13 cells infected with 10 p.f.u./cell of vaccinia recombinant virus or TL-Mor cells that produce HTLV-I continuously were labeled with 200 μCi of [^{3}H]leucine overnight. The cells were lyzed with a detergent mixture (Shida and Dales, 1982) and incubated successively with the ATL serum or rabbit sera, and formalin-fixed *Staphylococcus aureus*. Proteins were eluted from the bacteria, resolved on SDS–polyacrylamide gels and autoradiographed. The culture medium of the vaccinia virus-infected cells was subjected to immunoprecipitation in a similar manner.

Endo H treatment of env proteins

TL-Mor cells were cultured overnight in sugar-free RPMI 1640 supplemented with 1/10th the amount of glucose, 5 mM Na pyruvate, 10% FCS and 100 μCi of [^{3}H]glucosamine. The recombinant vaccinia virus-infected cells were labeled with 20 μCi of [^{3}H]glucosamine overnight. The env proteins were immunoprecipitated as described above, and the pellets were washed once with 1 mM Tris–HCl, pH 7.4. The env protein bearing bacteria were digested overnight at 37°C with 1 mU of endo H (Seikagaku Kogyo Co. Ltd) in 0.15 M Na citrate (pH 5.3) containing 2 mM PMSF, 0.5 μg/ml pepstatin A, 0.75 kU/ml trasylol.

Immunization of rabbits and quantitation of antibody response

Rabbits were injected intradermally at three sites on the back with a total of 2–4 $\times 10^8$ p.f.u. of WR-proenv1, WR-env17 or wild-type (WR) vaccinia virus. The rabbits were bled at 2-week intervals, and their plasmas were tested for antibodies to HTLV-I with a commercially available particle agglutination kit (Serodia® -ATLA, Fujirebio Inc., Tokyo, Japan). Titers are expressed by the reciprocal of plasma dilution that can agglutinate the gelatin particles coated with HTLV-I antigens (Ikeda *et al.*, 1984). The anti-vaccinia virus antibodies of vaccinated rabbits were measured by plaque-reduction assays monitoring the reduction of vaccinia virus (LC16mO strain) infectivity. The reciprocal of serum dilution giving 50% reduction in plaque number on RK13 cell monolayers is shown.

Challenge of immunized rabbits with HTLV-I

MT-2 cells (2×10^8) were injected intravenously into the rabbits 11 weeks post-vaccination and 5 ml of the peripheral blood was collected at 1-week intervals post-challenge. The lymphocytes obtained by Ficol-Conray gradient centrifugation were seeded to 12-well tissue culture plates by adjusting the cell number to 2×10^6/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum. After stimulation with Concanavalin A (25 μg/ml) overnight, the medium was replaced by interleukin 2-containing medium and the culture was continued for 4 or 5 weeks. The cultured cells were harvested every week to detect HTLV-I

antigen by an indirect immunofluorescence method using the ATL serum or the monoclonal antibody to HTLV-I p19 (GIN 14).

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