Research on Retroviruses: Implication in the Effects of the Viral and Host-Cellular Factors on Retroviral Infection.

（レトロウイルスに関する研究：ウイルス側及び宿主細胞側因子のレトロウイルス感染における影響）

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I Review of retrovirus

I-1 History of retrovirus

The history of retroviruses is mainly as that of RNA tumor viruses. Rous et al. found an infiltrable agent from avian leukosis in 1911, which was later designated as a Rous sarcoma virus (1). In 1914 Fujinami et al. also reported similar tumors which could be transmitted by ultrafiltrates to healthy chickens and ducks (2). However, the significance of the observation was not recognized until much later. Thereafter, a variety of viruses shown in Table 1 were found between 1960 - 1980. During this period, there were two important discoveries for retroviral research; one was the identification of the reverse transcriptase (RT, RNA-dependent DNA polymerase), which was found from RNA tumor viruses by Temin et al. (3) and Baltimore et al. (4), independently, and another was the detection of oncogenes in RNA tumor viruses, which was found in RSV by Vogt and Duesberg in the early 1970s. Afterward, retroviral research progressed with the help of the immunological and molecular biological techniques. Then, retroviral researchers' interests became centered on an enigma as to whether human retroviruses exists or not. In spite of the labors of many researchers, the works had been in vain until 1980. The first human retrovirus found was human T-cell leukemia virus type I (HTLV-I) isolated

from adult T-cell leukemia (ATL) by Poiesz et al. and Hinuma et al., independently (5-7). This finding and further studies contributed to the understanding of the cause of the acquired immunodeficiency syndrome (AIDS), pandemically prevailing all over the world from 1980s; in 1982, Montagnue et al. isolated a retrovirus from lymphocytes of the AIDS patients and proved the virus to be the causative agent of AIDS (8), then it was named as human immunodeficiency virus type 1 (HIV-1).

I-2 Morphological properties of retroviruses

The retrovirus characteristically has a reverse transcriptase (RT) within the virion. The spherical virions, 80 to 100 nm in diameter, have glycoprotein surface projections approximately 8 nm in diameter on the envelope derived from the plasma membrane of the host cell. The glycoprotein is named *env* protein and the precursor is cleaved by host-cell enzymes into the transmembrane portion (TM) and the surface external glycoprotein (SU). Within the envelope of the virion, the group-specific antigens (*gag* protein) form two distinct constituents, the membrane-associated matrix protein (MA) layer and the viral core, which is made up of the inner shell or capsid protein (CA). The CA contains two identical genomic viral RNAs. The RNAs, together with the nucleic-acid-associated basic proteins (NC), make up the

ribonucleoprotein (RNP). The genomes form the inverted dimer of linear, single-stranded, positive RNAs. Fig. 2 shows two-dimensional diagram of HIV-1, as a representative of retroviruses. The processed *gag* and *pol* proteins form proper mature virions, and the virions bud from cells, coated by the cellular ripid-bilayer membrane. This life cycle of the retrovirus is illustrated in Fig. 2.

I-3 Genetics

The genome of the retrovirus consists of positive-stranded RNA molecules. The prototype genomic RNA contains three genes: *gag*, *pol* (protease, RT, and integrase), and *env*. These replicative genes are flanked at both ends by regulatory sequences (R and U5 at 5'; U3 and R at 3'). During the reverse transcription, these sequences are duplicated in a way resulting in the presence of identical units of U3-R-U5, now called long terminal repeats (LTR), at both ends of the genome (Fig. 3). Some of the oncoviruses contain a variety of oncogenes (*v-onc*) in the genome. Other oncoviruses, like HTLV-I and simian T-cell leukemia viruses (STLV), possess a trans-activator gene named *tax* and a regulatory gene called *rex* instead of *v-onc*. The *tax* product transactivates not only 3 copies of 21 base pair repeats which serve as a transcriptional enhancer in the U3 region of the LTR (9,10) but also genes

for both IL-2 (11,12), the IL-2 receptor chain (12,13), oncogenes (14,15), and others (16-20), which may be the reason for the induction of malignant leukemia. The *rex* protein enhances transport of unspliced or partially spliced transcripts from the nucleus to the cytoplasm (21). The net effect of high levels of *rex* expression is to induce the accumulation of *gag* and *env* mRNA expression of viral structural genes while diminishing levels of doubly spliced small mRNA encoding *tax* and *rex* (22). This negative feedback mechanism allows *rex*-regulated viral gene expression to be divided into early and late phases. During the early phase, low levels of *tax* and *rex* are produced. The *tax* product increases the total rate of transcription. In the late phase, increasing levels of *rex* products accumulate the cytoplasmic unspliced or singly spliced mRNA leading to the expression of viral structural genes, and to the diminution of *tax* and *rex* genes' expression. This regulation system of gene expression could be critical for governing viral latency. Also, transient bursts of viral expression resulting from *tax* and *rex*-mediated regulation could effectively minimize immune surveillance by not allowing sufficient time for immune mediated clearance of infected cells (23). The genomic organization of HTLV-I is shown in Fig. 3. As for lentiviruses, similar regulatory genes are also found as *tat* and *rev* genes, furthermore, they have several other

regulatory genes, which might be important for the pathogenicity of the viruses.

I-4 Replication cycle

Replication cycle of the retrovirus is unique among RNA viruses, because of the presence of the RT. First, mature virions attach to the target cell surface in the interaction of the viral *env* protein and cellular receptor like CD4 molecule for HIV-1. Then fusion between viral and cellular membrane occurs and internalization of viral core follows. The core may be internalized by pinocytosis. The core is uncoated in the cytoplasm where the viral genome is exposed. Then genomic RNA is reverse-transcribed by viral RT and the product DNA is integrated into chromosomal DNA. The integrated viral DNA is called proviral DNA. Transcription from proviral DNA depends on cellular RNA polymerase II. Transcribed products are genomic RNA, full length mRNA coding *gag-pol* frame and spliced subgenomic mRNA coding *env*. In some of the retroviruses, small mRNAs spliced two or three times are transcribed. They code various regulatory genes such as *rev*, *tat*, *nef* and others, which may regulate viral replication and pathogenicity, as mentioned above. Viral particles are made of *gag*, *pol* and *env* products translated from mRNAs described above. In this step, the genomic RNA interacts with *gag-pol* precursor and is packaged into

viral particle and simultaneously budding of virion occurs from the surface of the infected cells. In the post-budding step, *gag-pol* precursor is processed by viral protease and the processed *gag* and *pol* proteins form proper mature virion. The life cycle of the retrovirus is illustrated in Fig. 2.

I-5 Taxonomy of retrovirus

Retroviridae family is classified into three subfamilies, *Oncovirinae*, *Lentivirinae*, and *Spumavirinae*.

The subfamily *Oncovirinae* is divided into types A, B, C, and D, according to the morphologic, antigenic, and enzymatic differences. For example, HTLV-I is classified into type C oncovirus.

Lentivirinae was not classified clearly before, but known as slow viruses. *Lentivirinae* is morphologically and chemically like other members of the *Retroviridae* family. It does not show any oncogenicity but induce immunodeficiency (HIV-1, HIV-2, simian immunodeficiency viruses (SIVs), feline immunodeficiency (FIV), and bovine immunodeficiency virus (BIV)), anemia (equine infectious anemia virus (EIAV)), and disorders of central nervous system (visna virus and caprine arthritis encephalitis virus (CAEV)).

Spumavirinae has a characteristic of forming syncytia *in vitro* so that it is called the foamy virus. However,

the virus does not induce tumor or other lentivirus-causing diseases but shows persistent asymptomatic infections *in vivo*. To date the pathogenesis of foamy viruses is not found. Table 1 summarizes the taxonomy of retroviruses.

I-6 Pathogenesis of retroviral infection

I-6-1 Acquired immunodeficiency syndrome (AIDS)

AIDS is the disease that the patients become highly susceptible to many kinds of infectious diseases, including opportunistic infections, tumors, or sometimes disorders of the central nervous system. HIV-1, HIV-2, SIVs, FIV and BIV are the causative agents of AIDS in humans and animals. Here, the AIDS caused by HIVs is explained below. The course of AIDS virus is characterized by a long phase of asymptomatic periods and by the terminal development of immunodeficiency. In acute phase, transient antigenemia occurs for weeks and soon disappears. At the same time, anti-viral antibodies appear after about 3 to 12 weeks, and virus-specific cytotoxic lymphocytes also arise. In the course of infection, the titer of the anti-core antibodies and the number of $CD4^+$ T-lymphocytes gradually decline, which are known as the marker of the beginning of the symptoms. During the periods of AIDS-related complex (ARC) or AIDS, the number of $CD4^+$ T cells decreases usually below 300 cells per μl

and the viral load in the blood is much higher than that in the asymptomatic state.

I-6-2 Oncogenesis

As described above, oncoviruses cause neoplasia, including leukemias, lymphomas, and sarcomas. Oncoviruses containing oncogenes generate sarcomas, or sometimes leukemias in cat, mouse, rat, chicken, and others. The period of transformation by these viruses is generally acute. An enhanced production of transformation proteins might also be the mechanism by which chronic leukemia viruses cause leukemia. Oncoviruses which do not contain oncogenes (HTLV-I, HTLV-II, STLVs and BLV) usually possess trans-activator genes and the products activate many cellular genes as mentioned above. For example, HTLV-I causes malignant and chronic leukemia, ATL. It has been estimated that the incidence of T cell malignancies in HTLV-I-infected persons is about 1 to 2 in 1000.

I-6-3 Other diseases

A. Anemia

Equine infectious anemia (EIA) caused by EIAV is a chronic relapsing anemia with fever. Periodic viral replication leads to an immunologically mediated disease with fever and severe anemia. The hallmark of EIA is periodic remissions. EIAV persists for life in the

infected animals.

B. Arthritis and disorders in CNS

CAEV primarily causes inflammation of the synovia and arthritis in goats. The most common and severe localizations are in the carpal joints. In young animals neurologic disease may be dominant. The disease develops insidiously, and the course is slowly progressive as in other lentiviral infections.

In case of HIV-1, disorders in CNS sometimes occur on ARC or AIDS stages. HTLV-I develops not only ATL but also HTLV-I-associated myelopathy (HAM). So far the cause of these neural diseases is unclear.

Ⅱ General introduction -Aim of this research-

In the early to middle of 20th century the retrovirus was found as the tumor-generating RNA virus, however, definition of retrovirus was not established until the discovery of RT in 1970. In those days immunology and molecular biology rapidly developed, which influenced the development of retrovirology very much. The informations for animal retroviruses had been accumulated, but the existence of the human retroviruses had been suspicious even in 1970s. In the early 1980s, HTLV-I was found in Japan and USA independently as the causative agent of ATL (5-7). Pandemic of AIDS concurrently occurred, however, HIV-1 could be isolated and identified as an AIDS virus in 1982 owing to the previous accumulation of the knowledges on retroviruses, especially those on HTLV-I. Nowadays much has been understood about AIDS and HIV-1, or other retroviruses-related diseases, whereas they have been incurable so far. In this regard, much more studies should be required for the elucidation of them. Especially viral and the host or the host cell interaction still remains unclear. As to HIV-I, for example, the cellular receptor (ie, CD4 molecule) and viral interaction has been studied well, however, the function of the regulatory genes' products *in vivo* remains to be elucidated. Moreover, the fact is that the cellular receptor(s) for HTLV-I or many

other retroviruses have not identified yet. Therefore, the interaction between primate retroviruses and the host cells was brought into focus in this research.

First, the effects of viral and cellular factors on early stages of HTLV-I infection, especially on the binding and fusion steps, were studied as a representative of primate leukemia viruses because HTLV-I has already been studied much so that virus-producer cell lines and monoclonal antibodies against viral antigens are easily available. (i) To detect the specific binding of HTLV-I to the cells, a simple assay system was established by using monoclonal antibodies (mAbs) and flowcytometry, and was applied to see if the antibodies in sera could inhibit the HTLV-I binding. (ii) In the application of the system, envelope of HTLV-I was examined to know the presence of the cell-membrane proteins on the virions. (iii) For further application of the system, it was investigated that envelope of HTLV-I was altered or degenerated on the target cells in the binding and post-binding steps.

Second, the effects of viral factors were examined on the infectivity of HIV-1 and HIV-2 as primate immunodeficiency viruses because of the availability of materials, infectious clones and cell lines. (i) Several mutants in the regulatory genes of the infectious molecular clones of HIV-1 and HIV-2, were infected to the peripheral blood mononuclear cells (PBMC) of humans to see

the phenotypes of the mutants, in comparison to those in the established cell lines. (ii) To evaluate the function of *nef* gene as a negative factor, the potential role of *nef* gene expression in transcription directed by the LTRs of various primate immunodeficiency viruses was examined. (iii) It was found from these experiments that a variety of interaction among viral and cellular factors may be specific and selective for sufficient infection of viruses.

Ⅲ Effects of viral and cellular factors on early steps of the primate leukemia virus infection.

Ⅲ-1 Establishment of a simple assay system for detecting HTLV-I-binding cells and binding inhibitory antibodies.

Ⅲ-1-1 Introduction

HTLV-I is a causative agent of ATL and HAM/tropical spastic paraparesis (TSP) as mentioned above. In order to explain the diverse clinical manifestations of HTLV-I infection, much information about events or consequences of interaction of the viral infection with the host immune response is needed. Unlike other retroviruses, HTLV-I seems to have difficulty in infecting target cells in its cell-free form, although cocultivation of various target cells with HTLV-I-producing cells induces syncytia (24-26). To analyze HTLV-I infection, especially the virus binding to the target cells, the use of labeled HTLV-I (27-29), or vesicular stomatitis virus (VSV) pseudotype with HTLV-I envelope (30) have been reported. However, these methods involve laborious techniques and therefore are not widely used.

In this study, a simple, rapid, and quantitative assay system is demonstrated to detect HTLV-I specific binding to human cells, using HTLV-I concentrate, mAbs and flowcytometry. In application of this system, the

antibodies in sera from HTLV-I-infected individuals including ATL and HAM patients were shown to inhibit the binding of HTLV-I.

Ⅲ-1-2 Materials and methods

A. Cells

The following established human cell lines were used: MT-2, a T cell line from umbilical cord blood lymphocytes exogenously infected with HTLV-I (31); ILT-8M2, an HTLV-I positive T cell line (32); MOLT-4#8 (33), A3.01 (34), and H9 (35), $CD4^+$ lymphoma cell lines; BJAB (36) and Raji (37), Burkitt lymphoma lines; K562, a chronic myelogenous leukemia cell line (38); U937, a promyelocytic cell line (39), U105MG (40) and U251MG (41), glioma cell line; HeLa, an epitheloid carcinoma line (42); and FL, an amnion cell line (43). They were maintained in RPMI1640 medium supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (FBS). Human peripheral blood lymphocytes were separated from healthy donor blood on Ficol-Hypaque gradients, stimulated with phytohemaglutinin-M for 3 days before use.

B. Virus preparation

The culture supernatant of MT-2 cells was spun at 300 × g for 5 minutes to remove the cells, filtered with 0.8 μm filter and centrifuged at 30,000 × g for 2 hours at

4℃. The viral pellets were resuspended in RPMI 1640 medium to give a 100 times concentrated virus and stored in aliquots at -80℃ until use.

C. Virus binding assay

Cells (5×10^5) were washed with RPMI 1640 medium containing 2% FBS (washing medium), resuspended in a 100 μl of virus preparation, and incubated at 37℃ for 1 hour with occasional agitation. Then the cells were washed twice with washing medium and reacted with 100 μl of 1:250 diluted REY-7, rat anti-HTLV-I gp46 mAb (44), on ice for 30 minutes. They were again washed twice with washing medium and resuspended in 100 μl of 1:100 diluted goat fluorescence isothiocyanate (FITC)-conjugated anti-rat Ig (Tago, Inc., California, USA). They were incubated on ice for 30 minutes, washed once with washing medium and resuspended in 0.5 ml of 1% formaldehyde in phosphate-buffered saline (PBS). The stained samples were analyzed for fluorescence intensity on FACScan (Becton & Dickinson, California, USA).

D. Sera

Sera were obtained from normal healthy adults and HTLV-I seropositive individuals including asymptomatic carriers, ATL, and HAM patients. They were inactivated at 56℃ for 30 minutes and stored in aliquots at -20℃ until

use. Dilutions of sera were made with RPMI 1640 medium containing 10% FBS.

E. Binding inhibition assay

Binding inhibition assay was carried out by pretreatment of the virus with serially diluted sera. The diluted sera (100 μl) were mixed with the same volume of the virus and incubated on ice for 1 hour. The mixture (200 μl) was added to 5 $\times$ 10^5 of MOLT-4#8 cells and incubated at 37℃ for 1 hour. Then, the viral binding assay was carried out as described above.

F. Indirect immunofluorescence (IF) assay

Indirect IF assay was carried out as previously described (45). Smeared and fixed MT-2 cells were reacted with 2-fold diluted sera at 37℃ for 30 minutes. After washing with PBS, the cells were incubated with FITC-conjugated rabbit anti-human IgG (Daco, Inc., Glostrup, Denmark) at 37℃ for 30 minutes. The stained cells were examined by fluorescence microscope. Antibody titers were determined by the final dilution of sera which showed positive fluorescence.

G. Syncytium inhibition assay

For the titration of antibodies that inhibit the syncytium formation by HTLV-I, 100 μl of serially diluted

sera were added to 100 μl of a mixture containing an equal number (5×10^4) of MOLT-4#8 and ILT-8M2 cells in a 96 flat well tissue culture plate. Experiments were performed in duplicate. They were cultured at 37℃ for 48 hours and the syncytium formation was evaluated by microscope examination. Titers of syncytium inhibition were determined by final dilutions of sera that inhibited syncytium formation.

Ⅲ-1-3 Results

A. HTLV-I binding assay

Crude HTLV-I preparations (about 100 times concentrated) were obtained from the concentrated supernatant of MT-2 cell cultures. Protein concentration of the viral preparation was about 1 mg/ml. The virus-treated cells were then stained with REY-7 mAb (anti-HTLV-I gp46) and subsequently with FITC-labeled anti-rat Ig. A flow cytometer was used for the detection of HTLV-I binding MOLT-4#8 cells (Fig. 4A). Approximately 80% of the virus-treated MOLT-4#8 cells were detected to be positive for fluorescence. The staining was specific as evidenced by the background level of fluorescence when RPMI 1640 medium was used instead of the virus preparation or REY-7 mAb. Treatment of the virus-adsorbed MOLT-4#8 cells with 0.025% of trypsin for 3 minutes at 37℃ decreased the fluorescence intensity of the positive cells to the

background level. Thus, the viral-binding was restricted on the cell membrane.

To determine whether fluorescence-positive cells are specific to the viral binding, the relationship between the viral dose and the fluorescence-positive cells detected by flowcytometry was studied. Figure 5 demonstrates the results of a 1-hour incubation at 37℃ after adding the serially diluted virus. The frequency of the positive cells decreased with increasing viral dilution. Viral binding to MOLT-4#8 cells yields a linear increase in the percentage of positive cells and fluorescence intensity between 1/16 and 1/2 dilution (50 μl) of the virus, reaching a maximum (80%) at 1/1 dilution of the virus.

Figure 6 shows the kinetics of fluorescence-positive cells at 37℃ (triangle) and 4℃ (circle) on the viral-binding. After 5 minutes postinfection at 37℃, almost 50% of the cells became positive for fluorescence. After 30 minutes postinfection, 80% positive cells were recognized. Thereafter, the positivity plateaued until 8 hours. Incubation of the virus with the MOLT-4#8 cells at 4℃ reduced the frequency of the positive cells. The maximum of positive cells at 4℃ was about 20%. Thus, approximately 60% of the inhibition of fluorescence increase was observed by the incubation at 4℃.

The difference in viral binding at 37 and 4℃ was

further studied by a temperature-shift experiment. MOLT-4#8 cells were treated with the virus at 4℃ for 30 minutes, washed twice to remove the excess virus, and transfered to 37℃. Incubation of the cells at 37℃ was terminated at 5, 15, and 30 minutes by the addition of ice-cold washing medium. The viral-binding cells incubated for 30 minutes at 37℃ showed nearly 3.5-fold increase in the percentage of positivity (open) and mean fluorescence intensity (closed) (Fig. 7).

Pretreatment of the cells with puromycin, a translational inhibitor, did not affect the viral binding or the increase in fluorescence positivity (See Table 6), suggesting that the *de novo* synthesis of gp46 after viral binding is not likely. However, cytochalasin B which blocks membrane motility by inhibiting the polymerization of actin filaments markedly reduced viral binding at 37℃ (see Fig. 15). Thus, membrane fluidity might affect the affinity of the viral binding or fusion process of the viral envelope glycoprotein.

B. Inhibition of the HTLV-I binding by the pretreatment of the virus with sera from HTLV-I-infected individuals.

Figure 8 illustrates the procedure used to determine the titer of binding inhibitory antibodies (BIA) against HTLV-I. MOLT-4#8 cells were treated with HTLV-I after a 1-hour preincubation at 4℃ with 1:20, 1:200, or 1:2000

dilutions of four preselected sera from a healthy (HTLV-I noninfected) donor, an asymptomatic carrier (AC) and patients with ATL or HAM. Fluorescence-positive cells were then assessed by flowcytometry. Sera from HTLV-I-infected individuals inhibited viral binding at various levels. Titers of BIA were tentatively calculated by 50% inhibition of the viral binding (about 40% positive cells indicated by horizontal dashed line in Fig. 8). Titers of BIA were less than 1:20, 1:80, 1:70, and 1:240 in cases of a seronegative donor (closed circle), an AC (closed square) , and patients with ATL (open circle) or HAM (open square), respectively. Thus, Fluorescence-positivity detected with this method was exclusively inhibited by the HTLV-I-positive sera, suggesting that HTLV-I was specifically adsorbed to the MOLT-4#8 cells.

To rule out the possibility that the epitope of REY-7 mAb on the HTLV-I virion was occupied by the patient's antibodies during the pretreatment of the virus with the serum, the virus-adsorbed cells were reacted with or without the serum and then stained with REY-7 mAb. No difference in number of positive cells was observed. Thus, occupancy of the mAb epitope by human serum in our experiments was unlikely.

Using the same method, an additional 8 sera including 2 healthy HTLV-I noninfected donors, 2 AC, 1 patient with ATL and 3 patients with HAM were titrated. All sera were

also examined by IF and syncytium inhibition in order to compare with BIA titers. Table 2 summarizes the data. All sera from 3 healthy donors showed negative in all three assays (IF, syncytium inhibition, and BIA). The range of the titers of BIA by the treatment of the virus with 9 HTLV-I infected individuals was from 1:50 to 1:800. No correlation between clinical category and titers of BIA was observed albeit the cases examined were limited. However, titers of BIA were roughly correlated with those of IF and syncytium inhibition.

C. HTLV-I binding on various human cells

To assess the relationship between HTLV-I and host cells, the viral binding assay was performed to 7 floating cell lines, 4 adherent cell lines, and peripheral blood lymphocytes from a noninfected donor (Table 3 and Fig. 4). Among floating cell lines, T cell lines (MOLT-4#8, H9, and A3.01 cells), K562 and U937 cells had a high percentage (61 to 83%) of positive cells. However, relatively low binding level (40 and 5%) of HTLV-I was observed in B cell lines (Raji and BJAB, respectively). Figure 4B shows the case of Raji cells. Adherent cell lines had a lower (2 to 33%) percentage of positive cells. U105MG cells showed only 1.8% of positive cells (Table 3 and Fig. 4C). About half of phytohemaglutinin-activated peripheral blood lymphocytes were also able to adsorb the virus. Thus,

HTLV-I is seemingly adsorbed to a wide variety of cells.

Ⅲ-1-4 Discussion

The results of these studies demonstrate the HTLV-I specific binding to the target cells by the simple and quantitative assay system using flowcytometry. Secondly, inhibition of the viral binding to the MOLT-4#8 cells was observed by the pretreatment of the virus with sera from HTLV-I-infected individuals, suggesting that sera neutralized the HTLV-I. Thirdly, a rather broad range of host cell populations was shown to be susceptible to HTLV-I binding.

HTLV-I adsorption was confirmed by dose-response relationship (Fig. 5), kinetics of the fluorescence-positivity and its temperature dependency (Figs. 6 and 7), trypsin treatment, and blocking by seropositive sera (Fig. 8 and Table 2). The mechanism of viral adsorption may be different from the antigen-antibody reaction, because the increase in fluorescence-positivity was inhibited by 4℃ (Figs. 6 and 7). The same inhibitory effect of 4℃ incubation was reported in the case of murine leukemia virus binding (46). The parameters of the HTLV-I membrane binding and the penetration process have been difficult to establish because of the inability of the productive infection by cell-free viruses, and lack of a reliable biological assay. The approach described here should be

readily adaptable for elucidating the characteristics of HTLV-I membrane events.

The viral binding detected by the present method may also be useful for predicting the infectibility of cells by different strains of HTLV-I. The viral binding on various cells was also reported by using FITC or rhodamine-labeled HTLV-I (27-29) or VSV-HTLV-I pseudotype virus (30). However, these assays need a large amount of highly purified HTLV-I particles and special techniques to prepare the labeled virus or pseudotype virus. The present method is simple and can be quantitatively available for rapid and mass screening of HTLV-I receptor and neutralizing antibody.

Pretreatment of the virus with sera from HTLV-I-infected individuals inhibited the viral adsorption (Fig. 8 and Table 2). This finding further confirmed that HTLV-I particles were responsible for the induction of fluorescence-positivity in this system. It is reported that sera from ATL patients and healthy carriers contain antibodies against cells-specific glycopolypeptides, gp68 and gp46, using radioimmuno-precipitation assay (47). Sera from patients with ATL or HAM, and healthy carriers inhibited the HTLV-I-binding to the MOLT-4#8 cells by the pretreatment of the virus, however, neither sera from HTLV-I-uninfected controls (Fig. 8 and Table 2) nor GIN-14 mAb (anti-*gag* p19 matrix protein) inhibited the viral

binding. The inhibition phenomenon is probably due to antibodies directed against the viral envelope glycoprotein. Although further studies are needed to confirm that the antibodies detected by this assay are specific to the epitope on viral envelope glycoprotein, this assay system using flow cytometry is useful for rapid screening.

In order to delineate the relationship between HTLV-I and target cells, the viral-binding cells after treatment with the cell-free virus has been studied. When FITC-labeled, highly purified HTLV-I was used to detect the binding to various cell lines, most of the cell lines from various species had HTLV-I binding activity (27). These findings are more or less compatible with ours. However, BJAB and U105MG cells showed low binding activity, suggesting that the cell lines may express less amount of cellular receptor for HTLV-I. To date, the receptor has not been identified. It should be ascertained whether true cellular receptors are present on these cells, and further the mechanism of viral infection should be studied.

Ⅲ-1-5 Conclusions

A simple and rapid assay system for the detection of HTLV-I-binding cells was developed to assess the virus specific receptor and to titrate the antibodies that block the viral binding. The specificity of the viral-binding

was shown by dose-response relationship, kinetics of the binding, and temperature dependency. HTLV-I was adsorbed on a wide range of human cell lines and peripheral blood lymphocytes at various levels. Antibodies to inhibit the viral-binding were also quantitatively detected in sera from HTLV-I infected individuals, including AC and patients with ATL or HAM, but not from healthy seronegatives. This assay system is useful in screening the virus specific receptor and neutralizing antibodies to HTLV-I and in the analysis of virus-cell interaction.

Ⅲ-2 Detection of cellular membrane proteins on HTLV-I envelope.

Ⅲ-2-1 Introduction

In the budding stages of viral replication, enveloped viruses obtain the envelope consisting of viral glycoproteins and lipid-bilayer from the cellular membrane. Simultaneously, the cellular membrane proteins may also be taken up onto viral envelope. The host-derived membrane proteins might function as the cofactor(s) of viral infection, especially, in the initial attachment or binding steps. Actually, it was reported that antisera to β2 microglobulin, MHC-I, and MHC-IIDR neutralized the HIV-I infection (48), and also that β2 microglobulin enhances the infectivity of cytomegalovirus (49). However, the presence of the host-derived membrane proteins on HTLV-I is unknown.

As described above, a simple assay system was established for the detection of HTLV-I binding to the target cells. It is shown here that not only human T cells but also mouse T cells were able to adsorb HTLV-I, and that in application of this phenomenon the presence of the host cell-derived membrane proteins were demonstrated on HTLV-I virions, by using mAbs which react to cellular proteins of human but not to those of mouse, and flowcytometry.

Ⅲ-2-2 Materials and methods

A. Cells

BW5147 (50) and EL-4 (51) cells, mouse T cell lines, and MOLT-4#8 cells (33), a human T lymphoma cell line, were used for the target cells of HTLV-I binding. MT-2 cells (31), an HTLV-I producer cell line, was also used for the determination of the cell-surface molecules.

B. Detection of the cellular membrane proteins on HTLV-I envelope.

The detection of HTLV-I binding to the cells is described above. In the application of this system, the HTLV-I-binding cells were treated with mAbs against the cellular membrane molecules of human, Leu4 (CD3), Leu3a (CD4), and MHC-IIDR (Becton & Dickinson, California, USA); LFA-1, ICAM-1 (Biodesign, Kennebunkport, ME); LFA-3, MHC-I (Cosmobio); and CD2 (Nichirei), respectively, at 4℃ for 40 minutes. The cells treated with HTLV-I and each mAbs were then stained with FITC-labeled anti-mouse or rat Ig at 4℃ for 40 minutes, and analyzed by flowcytometry.

C. Inhibition of HTLV-I binding by sera

For the inhibition of HTLV-I binding to the cells, 50 μl of HTLV-I concentrates were pretreated with the same volume of the serially diluted sera from HTLV-I sero-positives at 4℃ for 1 hour, thereafter the mixture was

treated with BW5147 cells.

Ⅲ-2-3 Results

Preliminarily, the expression levels of the cellular membrane molecules on MOLT-4#8 cells were compared with those on MT-2 cells (Table 4). Both MOLT-4#8 and MT-2 cells expressed high levels of CD4, LFA-1, LFA-3, and MHC-1 molecules, as shown by mean channel fluorescence, however, the expression of CD2 and CD3 molecules was almost undetectable on both cells. Furthermore, ICAM-1 and MHC-IIDR molecules were strongly detectable on MT-2 cells but not on MOLT-4#8 cells. These results prompted that if ICAM-1 or MHC-IIDR molecules are present on the HTLV-I envelope, they could be detected on MOLT-4#8 cells adsorbing the HTLV-I. Therefore, HTLV-I was added to MOLT-4#8 cells at 37℃ for 1 hour, washed, and the cells were treated with mAbs to ICAM-1, MHC-IIDR, and as a control, LFA-1. Figure 9 shows the histograms of the stained cells; the expression levels of ICAM-1 and MHC-IIDR on MOLT-4#8 cells were very low (line b) compared with the background (line a) as shown in Table 4, however, the fluorescence of the HTLV-I-treated MOLT-4#8 cells to these molecules became obviously positive (line c). As for LFA-1, MOLT-4#8 cells originally expressed the molecule so strongly (line b to a) that the increase in fluorescence intensity was little even after the virus treatment

(line c).

It is reported that strong induction of ICAM-1 was seen in HTLV-I-positive T-cell lines (52). Therefore, it is still possible that the stimulation of HTLV-I binding might induce the increase in the expression levels of ICAM-1 and MHC-IIDR molecules. To exclude this possibility, MOLT-4#8 cells were pretreated with the translational inhibitor cycloheximide at a concentration of 1 μg/ml for 1 hour at 37℃, and then HTLV-I was added to the cells. The detectable levels of ICAM-1 or MHC-IIDR molecules on HTLV-I-treated MOLT-4#8 cells were not affected with or without cycloheximide treatment, suggesting that the stimulation of HTLV-I binding did not affect the expression levels of the molecules.

From these results, ICAM-1 and MHC-IIDR were seemingly present on HTLV-I envelope, however, the presence of other molecules was still undetectable by the method above. To investigate the presence of the other cellular proteins, mouse T cell lines were applied for the target cells of HTLV-I because possibly the mAbs to the cellular membrane proteins of human do not react to those of mouse so that any human proteins may be detectable specifically. Therefore, the efficiency of HTLV-I binding to mouse T cell lines, BW5147 and EL-4 cells, was investigated. Figure 10 demonstrates that HTLV-I gp46 was detectable on both HTLV-I-treated mouse T cell lines, indicates that

HTLV-I was able to bind to the cells. Thus, BW5147 cells were selected for further experiments.

Preliminarily the cross-reactivity of the mAbs, which react to the membrane proteins of human, to the membrane molecules on BW5147 cells was examined. No cross-reaction of the mAbs to the molecules on BW5147 cells was observed. Then, the detectable levels of cellular membrane molecules of human on HTLV-I-binding BW5147 cells were tested. Fig. 11 and Table 5 describes the results on HTLV-I-binding BW5147 cells. Table 5 also compared the results with the expression levels on MT-2 cells, shown as mean channel fluorescence. All the molecules expressed on MT-2 cells were also detectable on viral-binding BW5147 cells. Furthermore, the levels of the mean channel fluorescence of these molecules on HTLV-I-treated BW5147 cells were correlated with those on MT-2 cells. These results suggest that the host cell-membrane proteins may be transferred to the viral envelope according to the expression levels of them on MT-2 cells, although the variety of molecules screened was limited.

There remains the possibility that the soluble cellular proteins in HTLV-I concentrate might attach to the cells so that they became detectable. To exclude this possibility, it was determined if pretreatment of HTLV-I with sera from HTLV-I-seropositive individuals, which is shown to inhibit the HTLV-I binding to the target cells,

affect the detectable levels of cellular membrane-derived molecules of human on BW5147 cells. The HTLV-I concentrate was pretreated with the same volume of the serially diluted sera from HTLV-I-seropositive individuals or seronegative controls at 4℃ for 1 hour, then the mixture was added to BW5147 cells and stained as mentioned above. Figure 12 demonstrates the representative result of the experiments. The data were presented as percentage compared with the positive controls (pretreatment with serum-free medium). As expected, HTLV-I pretreatment with the serum from a seropositive individual (asymptomatic carrier) reduced the detectable levels of ICAM-1 and MHC-IIDR molecules on BW5147 cells (open and closed triangle, respectively). The rate of inhibition was dependent on the concentration of the serum. On the other hand, the serum from a seronegative individual did not affect the detectable levels of the molecules on the cells even at a concentration of 1:40 dilution (open and closed circle). Similar results were obtained in the sera from different individuals. These results indicated the presence of HTLV-I-transferred host cell-membrane proteins on BW5147 cells.

Ⅲ-2-4 Discussion

In this study, first, the expression levels of the membrane molecules on MOLT-4#8 cells were compared with

those on MT-2 cells (Table 4). Secondly, ICAM-1 and MHC-IIDR, which were merely expressed on MOLT-4#8 cells, became detectable on HTLV-I-binding MOLT-4#8 cells (Fig. 9). Thirdly, HTLV-I binding to mouse T lymphoma cell lines was demonstrated (Fig. 10), thus, a batch of molecules, including adhesion molecules (LFA-1, ICAM-1, and LFA-3), MHC-I, MHC-IIDR, and CD4 molecules, was shown to become detectable on viral-binding BW5147 cells (Fig. 11 and Table 5). It was also demonstrated that the detectable levels of the molecules on viral-binding BW5147 cells were correlated with those expressed on MT-2 cells (Table 5). Finally, the pretreatment of HTLV-I with the serum from an HTLV-I seropositive individual, which was shown to inhibit the viral binding (Fig. 5 and Table 2), reduced the detectable levels of the cellular proteins of human on BW5147 cells (Fig. 12). From these results, it is concluded that the HTLV-I envelope carries cellular membrane proteins derived from the host cells.

Previously, many groups have reported the presence of host cell-membrane proteins on enveloped viruses, including HIV-1 (48, 53-55), SIV from macaque monkey (56), CMV (57), Friend leukemia virus (58), avian leukosis virus (59), VSV (60), murine leukemia virus (60), Sindbis virus (61), and influenza virus (62). However, it is controversial so far whether the uptake of cellular membrane proteins on virions is selective or not. The results in

this section suggest non-selective uptake of cellular proteins to the viral envelope. The discrepancy might be the differences in budding system of these viruses, interaction of the molecules with viral proteins, or in the assay system used for the detection of the membrane proteins.

It is possible that adhesion molecules or MHC molecules on virions may function as secondary molecules for viral attachment or binding. In fact, Auther et al. (48) reported that antisera to β2 microglobulin, MHC-I and MHC-IIDR neutralized the HIV-1 infection. In this regard, it should be elucidated whether mAbs against some of the cellular proteins observed could affect the early steps of HTLV-I infection.

Ⅲ-2-4 Conclusion

A batch of cellular membrane proteins of human became detectable on HTLV-I-binding mouse T cells by mAbs and flowcytometry, suggesting the presence of host cell-derived membrane proteins on HTLV-I virions.

Ⅲ-3 Alteration of the envelope and exposure of *gag* p19 matrix protein of HTLV-I on the target cells in the post-binding (fusion) steps

Ⅲ-3-1 Introduction

HTLV-I is the causative agent of ATL/HAM. *In vivo*, ATL shows a preferential incidence in $CD4^+$ T lymphocytes (63). However, the cellular receptor(s) for HTLV-I is seemingly distinct from CD4 molecule (32,64). HTLV-I is usually transmitted to uninfected cells by co-culture of the virus-carrying cells with peripheral blood lymphocytes from healthy donors (7,65). Because of the inability of the cell-free HTLV-I to infect cells, little is known about the viral receptor and the early steps of viral infection such as adsorption, penetration and uncoating.

In the previous section, the assay system for the detection of HTLV-I binding to the target cells was described, in which MOLT-4#8 and U937 cells were shown to specifically adsorb high levels of HTLV-I (Table 3). Kuroda et al. reported that MOLT-4#8 cells showed marked cell-fusion after co-cultivation with MT-2 cells but U937 cells did not, and also that fluorescence to envelope glycoprotein gp46 and *gag* p19 matrix protein (MA) was detected on HTLV-I-treated MOLT-4#8 cells, whereas virus-treated U937 cells were only gp46-positive, using the same assay system (66). Based on these observations, it was

hypothesized that MA of HTLV-I may be exposed during the post-binding steps on the plasma membrane of MOLT-4#8 cells but not on U937 cells. The present results showed that MA was exposed on MOLT-4#8 cells in post-binding steps but not on U937 cells.

Ⅲ-3-2 Materials and methods

A. Cells

MOLT-4#8 (33), U937 (39) and MT-2 (31) cells were used for the target cells of HTLV-I as mentioned above.

B. Inhibitors

As translational inhibitors, cycloheximide and puromycin were used at the final concentration of 0.1 or 1 μg/ml, and 5 or 50 μg/ml, respectively. Cytochalasin B, an inhibitor of polymerization of cytoskeletal actin filaments, was used at the concentration of 10 μg/ml.

C. Detection of gp46 and MA on HTLV-I-binding cells

Detection of gp46 and MA on viral-binding cells is described above, except for the use of Gin-14 mAb (anti-MA) (66) for the detection of MA.

D. Detection of intracellular gp46 and MA.

For the detection of intracellular gp46 and MA, MT-2 cells or HTLV-I-binding MOLT-4#8 and U937 cells were

treated with 70% methanol at 4℃ for 3 minutes, washed, then stained by mAbs as described above.

Ⅲ-3-3 Results

For detection of envelope gp46 and MA on the surface of plasma membrane after adsorption of cell-free HTLV-I, flowcytometric analyses using mAbs to the antigens were applied as described. Preliminarily, MOLT-4#8 and U937 cells were treated with HTLV-I, then stained as mentioned above, except for the use of REY-7 (anti-gp46) or GIN-14 (anti-MA) mAbs for the first labeling, followed by flowcytometric analysis. Figure 13 showed that both cell lines treated with virus and REY-7 mAb became positive for fluorescence. On the other hand, MOLT-4#8 cells treated with virus and REY-7 mAb became positive for fluorescence but U937 cells did not (Fig. 13), as reported previously (67).

Then, the kinetics of % positivity for fluorescence of MOLT-4#8 and U937 cells treated with HTLV-I at 37℃ (Fig. 14). As for the fluorescence to gp46 on MOLT-4#8 cells, nearly 40% positivity became detectable 5 minutes after incubation, then 80% positivity was observed after 30 minutes (panel A, open circle). The kinetics of fluorescence positivity of virus-treated U937 cells was similar to that of MOLT-4#8 cells, although maximum positivity was about 60% (panel B, open circle). When MA

on virus-treated MOLT-4#8 cells was stained, positivity for fluorescence increased to 50% after 60 minutes incubation, though the increase in positivity was slower than that for gp46 (panel A, closed circle). However, virus-treated U937 cells were almost negative for MA even after 60 minutes (panel B, closed circle). These results indicate the inability of U937 cells to present MA on their surface in the post-adsorption steps, suggesting that MA might be exposed by degeneration or alteration of the intact viral envelope or that *de novo* synthesis of MA might occur in MOLT-4#8 cells but not U937 cells. To assess the possibility that MA was expressed by *de novo* protein synthesis after the viral inoculation, MOLT-4#8 cells were pretreated with cycloheximide (0.1 or 1 μg/ml) or puromycin (5 or 50 μg/ml) as translational inhibitors at 37℃ for 1 hour. Then HTLV-I was added to the pretreated cells and stained as described above. Compared with the negative control (without pretreatment), the rate of fluorescence positivity for gp46 and MA was not affected by the cycloheximide or puromycin-pretreatment (Table 6), indicates that *de novo* synthesis after the viral inoculation is not likely.

In the section Ⅲ-1, increase in fluorescence positivity for gp46 was shown to be temperature-dependent (Figs. 6 and 7). It was also demonstrated that viral binding occurred even at 4℃, however, increase in

fluorescence positivity for gp46 on HTLV-I-binding cells required 37℃ incubation (Fig. 7).

Here, it was studied whether MA was exposed in the post-binding steps at 37℃ (possibly fusion step) or already MA was exposed in the binding step. MOLT-4#8 cells were treated with HTLV-I at 4℃ for 30 minutes, washed twice, and resuspended in warm medium at 37℃ (Fig. 15). Incubation of the virus-treated MOLT-4#8 cells at 4℃ for 30 minutes induced less than 20% positivity for both gp46 and MA. The incubation of the cells at 37℃ was terminated at 5, 15, 30 and 60 minutes by adding cold medium. The temperature shift from 4℃ to 37℃ resulted in the gradual increase in positivity, and the rate of positivity increased from 18 to 65% for gp46 and from 16 to 40% for MA after 60 minutes (Fig. 15). Alternatively, HTLV-I-binding MOLT-4#8 cells at 4℃ for 30 minutes were treated with 10 μg/ml of cytochalasin B and incubated at 37℃ for 60 minutes. Cytochalasin B treatment completely inhibited the increase in fluorescence positivity for both molecules, similar to that of virus-treated cells without incubation at 37℃ (Fig. 15, open and hatched bar). These data demonstrate that the increase in fluorescence positivity for gp46 and MA on HTLV-I-binding MOLT-4#8 cells required 37℃ incubation and cytochalasin B-dependent phenomenon, i.e. polymerization of cytoskeletal actin filaments. It may be possible that the envelope of

HTLV-I was degenerated or broken on MOLT-4#8 cells and the inner matrix proteins resultantly were exposed.

If this is the case, HTLV-I virion or at least envelope and MA structure may stay intact on U937 cell membrane, so that MA might not become detectable on HTLV-I-treated U937 cells. Thus, to confirm whether U937 cells treated with HTLV-I were able to adsorb intact viral particles sufficiently and yet exposure of MA did not occur, intracellular gp46 and MA in HTLV-I-treated MOLT-4#8 and U937 cells were monitored. For the detection of intracellular antigens, the cells were treated with 70% methanol at 4℃ for 3 minutes, washed, then stained with mAbs as described. As for MT-2 cells without methanol treatment, gp46 was strongly detectable but MA was merely detectable, whereas more than 90% of fluorescence-positivity for both gp46 and MA was observed in methanol-treated MT-2 cells (Fig. 16). The result indicates that methanol fixation of the cells allow antibodies to penetrate the membrane into the cells. Figure 17A displays that methanol fixation of the HTLV-I-adsorbed MOLT-4#8 cells decreased the gp46-positivity from 85% to 5% but increased MA-positivity from 55% to 95%. In case of the virus-treated U937 cells, fluorescence for MA of the fixed cells was clearly detectable, reached to 93% positivity, however, fluorescence of the unfixed cells was merely detectable (Fig. 17B). These data indicated that the

intact viruses truly bound to U937 cells but MA was not exposed on the surface of the unfixed cells. Alternatively, fluorescence-positivity for gp46 on viral-binding MOLT-4#8 and U937 cells decreased to the background level by methanol treatment (Figs. 17A and B), which was probably due to the degeneration of the epitope of gp46 on the cell surface.

Ⅲ-3-4 Discussion

Previously, Kuroda et al. demonstrated that co-culturing MT-2 cells with MOLT-4#8 cells led to syncytium-formation but not with U937 cells (67). By cell-free HTLV-I adsorption, fluorescence for gp46 became detectable on the surface of both cells, however, that for MA became positive on MOLT-4#8 cells only (67, Table 3 and Fig. 13). These data necessitated studies to compare these two types of cells in terms of the properties of the plasma membrane after the viral adsorption. The data presented suggest that MA may be exposed on the cell membrane in consequence to the lysis of the envelope and that the increased fluorescence-intensity and positivity after 37℃ incubation may be due to the spread of the envelope on the cell surface, as shown by cytochalasin B treatment (Fig. 15). Lysis of viral envelope might be necessary for the next steps of HTLV-I infection such as fusion and penetration. In fact, U937 cells induce no fusion when co-

cultured with MT-2 cells (67). This finding is similar to the case of HIV-1, in which V3 region of envelope gp120 is cleaved on the cellular membrane by proteolysis (68). In cases of orthomyxoviruses, paramyxoviruses and coronaviruses, activating proteolytic cleavages catalyzed by cellular proteases late in the secretory pathway are critical for the fusion activity (69). In this regard, several available anti-protease agents (amastatin, phosphoramidon, E-64, arphamenine A, leupeptin, antipain and pepstatin A) were tested to block the exposure of MA on the virus-adsorbed MOLT-4#8 cells. However, no effective agent has been found so far. It still remains to be elucidated whether the exposure of MA is followed by successive uncoating of the virus. Further studies would be needed to identify the mechanism.

Ⅲ-3-5 Conclusion

This study demonstrated that MA was exposed on HTLV-I-binding MOLT-4#8 cells in the post-binding steps, probably in fusion step, but not on viral-binding U937 cells, suggesting that the phenomenon may affect the ability of syncytium formation or infectivity of HTLV-I.

Ⅳ Effects of viral factors on the infection of primate immunodeficiency viruses

Ⅳ-1 Introduction

Major disease of lentiviruses is acquired immunodeficiency syndrome (AIDS), shown by FIV, BIV, SIV_{mac}, HIV-1 and HIV-2. AIDS is the disease that the patients become highly susceptible to a variety of opportunistic infections, tumors, and sometimes disorders of central nervous system as mentioned above.

The primate immunodeficiency viruses are classified into four groups; HIV-1 and SIV from chimpanzee (SIV_{CPZ}) group, SIV from african green monkey (SIV_{AGM}) and from african white-crowned mongabey monkey (SIV_{WCM}) group, HIV-2, SIV_{MAC} and SIV from sooty mongabey monkey (SIV_{SM}) group, and SIV from mandrill (SIV_{MND}) group. These four groups are genetically equidistant from each other, having 55% to 60% homology, although more than 80% homology within the group.

Both HIV-1 and HIV-2 cause AIDS in humans, but pathogenicity of SIVs to humans is unknown. However, some of the SIVs (SIV_{MAC} and SIV_{SM}) induce AIDS in the macaque monkeys (70,71), and others (SIV_{AGM} and SIV_{MND}) do not induce AIDS to monkeys (72-74). Although difference of the genes between pathogenic and non-pathogenic viruses is not so obvious as the grouping by the homology of the nucleic-

acid sequences (74), the determinant(s) of the pathogenicity is still unknown. On the other hand, HIV-1 can infect the chimpanzee but shows no AIDS-like disease, furthermore, HIV-1 can not establish infections in other monkeys. Similarly, SIVMAC causes AIDS in macaque monkeys but not in other monkeys like the african green monkey.

These findings suggest that virus-host interaction is exactly regulated for establishing the infection or developing the AIDS. Especially, these viruses have several small regulatory genes other than structural genes, *gag*, *pol* and *env*, different from other retroviruses, suggesting that these regulatory genes may play important roles for the infection and pathogenicity of viruses. From this point of view, data from functional analyses of the regulatory genes have been accumulating. The functions of two trans-activator genes, *tat* and *rev*, are known well and are indispensable for viral replication (75-78), but the other regulatory genes, named *vif*, *vpr*, *vpu*, *vpx* and *nef*, are not always essential for infectivity (79-94). The *tat* and *rev* genes are present in the genomes of all these viruses. HIV-1 genome contains *vif*, *vpr*, *vpu* and *nef* genes. HIV-2/SIVMAC/SIVMND have a *vpx* gene instead of the *vpu* gene in HIV-1. SIVAGM genome is similar to HIV-2/SIVMAC/SIVMND except for the deficient of the *vpr* gene. Fig. 18 shows the genomic organizations of these four groups of primate immunodeficiency viruses.

To analyze the function of these regulatory genes, infectious molecular cloned viruses introducing gene-specific mutations have been used for *in vitro* infection experiments. A recent report showed that *vpx* mutants of HIV-2 was able to propagate normally in T cell and monocytic lines, but did not establish productive infection in primary lymphocytes (95). The behavior of the mutants in primary cell cultures, therefore, may reflect the functions of the genes *in vivo* more precisely than those in established cell lines.

Here, phenotypes of the gene-specific infectious mutants of HIV-1 (*vif*, *vpr*, *vpu* and *nef* mutants) (79) and HIV-2 (*vif*, *vpx*, *vpr* and *nef* mutants) as representatives of the primate immunodeficiency viruses (89) in human primary blood mononuclear cells (PBMC) were compared with those in established cell lines. The results demonstrate that some of the mutants display different growth potentials in PBMC from those in established cell lines.

Ⅳ-2 Materials and methods

A. Cells

Human PBMC were separated from blood samples obtained from healthy seronegative individuals on Ficol-Hypaque gradients, stimulated with 5 μg/ml of Concanavalin A and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS for one day. The culture medium was then

replaced by the same medium containing 3% recombinant human IL-2. The $CD4^+$ human T cell lines, A3.01 (34) and MOLT-3 (33) cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. A human colon carcinoma cell line, SW480 (96), was maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS.

B. DNA construct

The infectious molecular clones pNL-432 (96) and pGH-123 (89) were wild type (wt) proviral DNAs of HIV-1 and HIV-2, respectively. The HIV-1 mutants designated as pNL-Nd (*vif* mutant), pNL-Af2 (*vpr* mutant), pNL-Ss (*vpu* mutant) and pNL-Xh (*nef* mutant) were derived from pNL-432. The HIV-2 mutants named pGH-Xb (*vif* mutant), pGH-St (*vpx* mutant), pGH-Ec (*vpr* mutant) and pGH-Ml (*nef* mutant) were derived from pGH-123. Table 7 shows the mutations in these DNA clones.

Some biological properties of the constructs under the control of viral LTRs were made by placing LTR fragments derived from HIVs/SIVs in front of the CAT gene of pHd-CAT (89,97). The *nef* expression plasmid, pcD-SRα*nef*460, and the control plasmid, pcD-SRα*nef*ΔKpnI473 were used in this experiment. Briefly, the HinfI (nucleotides 8623)-HindIII (nucleotides 9606) fragment, containing the *nef* gene was excised from the pNL-432 DNA and was repaired with Klenow

fragment. After adding BamHI linkers to both ends, the fragment was inserted into dephosphorylated BglⅡ site of the pcDL-SRαBglⅡ456 (98), generating the pcD-SRα*nef*473 plasmid, in which the *nef* gene was expressed under the control of the SRα promoter (98). In the pcD-SRα*nef*Δ KpnI473, the KpnI site (nucleotides 9605) in the *nef* gene was deleted by T4 DNA polymerase, creating a frame-shift mutation in the *nef* gene.

C. DNA transfection

Twenty μg of plasmid DNA were mixed with 50 μl of 2.5M $CaCl_2$ for 30 minutes. The mixture was added to SW480 cells, followed by the incubation at room temperature for 5 to 7 hours. Then the cells were treated with 30% glycerol for 3 minutes, washed, and culture medium was added to the cells.

D. Infection

The growth kinetics of the mutants was determined in PBMC and $CD4^+$ T cell lines. Culture supernatants of transfected SW480 cells was filtered with 0.45 μm pore size and appropriate volume of filtrates which contains same amount of virions adjusted by RT activity were infected to 1×10^6 cells. The culture supernatants were harvested every 3 or 4 days to check the rate of viral replication and then new medium was supplemented to the

culture.

E. Reverse transcriptase (RT) assays

Virion-associated RT activity was assayed for screening the amount of progeny virions in culture supernatant. Fifty μl of the RT cocktail containing 50 mM Tris-HCl, 75 mM KCl, 10 mM dithiothreitol, 4.95 mM $MgCl_2$, 10 μg/ml poly A, 5 μg/ml Oligo dT, 0.05% NP-40, and 0.37 MBq of ^{32}P-dTTP were incubated with 10 μl of the culture supernatant at 37℃ for 3 hours. Ten μl of the mixture were spotted onto DE81 filter, followed by several washing. The radioactivity of the filter was measured by scintillation counter.

F. Syncytium formation

Syncytium formation was monitored at intervals by microscopic examination.

G. Chloramphenicol acetyl-transferase assays

Chloramphenicol acetyl-transferase (CAT) assays were carried out as previously described (99). 20 μl of the cell lysates from transfected SW480 cells were mixed with 170 μl of the reaction buffer containing 3.7 KBq of ^{14}C-chloramphenicol, 0.25 M Tris-HCl (PH 7.8), and 4 mM of acetyl-coenzyme A, at 37℃ for 1 hour. One ml of ethyl acetate was added to the mixture, shaked vigorously, then

spinned down. The supernatant was dried, resuspended in 15 μl of ethyl acetate. It was spotted onto the thin-layer chromatography (TLC) filter, then allow the solvent, chloroform/methanol (95:5), front to move to the top of the filter. The filter was dried, exposed to X-ray film.

Ⅳ-3 Results

A. Biological characterization of mutants

For assessment of the biological activities of HIV-1 and HIV-2 mutants in PBMC, equivalent amounts of cell-free viruses (5×10^4 cpm of RT activity) prepared from transfected SW480 cells were inoculated into PBMC and $CD4^+$ T cell lines. Virus replication and syncytium formation were monitored by measuring RT production and by microscopic examination, respectively. Both of the wt viruses replicated well in PBMC, A3.01 (HIV-1) or MOLT-3 (HIV-2) cells.

Figure 19 shows the growth kinetics of HIV-1 mutants in A3.01 cells (panel A) and PBMC (panel B). The mutants of the *vpu* and *nef* genes exhibited different growth patterns in the two cell types. Virus replication of the *vpu* mutant was clearly less efficient than that of wt virus in A3.01 cells but replicated equally to wt virus in PBMC. In contrast, growth of the *nef* gene mutant was similar to that of the wt virus in A3.01 cells, but was slightly slower than that of the wt virus in PBMC. The *vif*

mutant did not grow in either type of the cells. The *vpr* mutant and the wt virus replicated similarly in both types of cells when infected at a relatively high input dose (Fig. 19), but 10-fold decrease in the input dose of the former delayed the virus growth markedly in A3.01 cells (Fig. 20A) and slightly in PBMC (Fig. 20B).

Figure 21 shows the growth kinetics of HIV-2 mutants in MOLT-3 cells (panel A) and PBMC (panel B). The growth characteristics of the *vif* and *vpx* mutants in PBMC were different from those in MOLT-3 cells. The *vpx* mutant replicated well in MOLT-3 cells but very poorly in PBMC. To examine whether the retarded growth of the *vpx* mutant in PBMC was a general feature, a similar infection experiment was carried out by using PBMC samples prepared from another individual. As shown in Fig. 22, the *vpx* mutant propagated poorly but the reduction in its growth rate to one-third of that of the wt virus was not so remarkable as that shown in Fig. 19. The *vif* mutant grew poorly in MOLT-3 cells, and showed no productive infection in PBMC. The kinetics of growth and the levels of virus production of the *vpr*, *nef* mutants and wt virus were similar in MOLT-3 cells and PBMC. A 10-fold reduction in the input dose had no significant effect on the relative growth kinetics of the wt virus and the *vpr* mutant in MOLT-3 cells and PBMC (Figs. 20C and D).

Results of the syncytium formation by the wt virus and

the mutants are summarized in Table 8. The wt virus and all mutants of HIV-1 except the *vif* mutant induced syncytium formation in both of the cell types. On the other hand, neither HIV-2 wt virus nor any of its mutants induced syncytium formation in PBMC, which was in sharp contrast to their effects on MOLT-3 cells.

B. Effects of expression of HIV-1 *nef* on HIV/SIV LTRs

Earlier studies showed that mutations of the *nef* gene lead to more efficient viral replication (80,86,94) or transcription directed by the HIV-LTR (80,94). However, the possible function of the *nef* gene is still controversial (85,87,100,101). As described above, the *nef* gene product of HIV-1 showed no negative effect on virus replication (Fig. 19). For further examination of the potential role of HIV-1 *nef* gene expression in transcription directed by the LTRs of various primate immunodeficiency viruses, cotransfection experiments were carried out; SW480 cells were transfected with a series of LTR-CAT constructs and a plasmid expressing either the HIV-1 *nef* gene (pcD-SRα*nef*460) or the mutated *nef* gene (pcD-SRα*nef*ΔKpn473), and the CAT expression was monitored. As shown in Fig. 23, pcD-SRα*nef*460 did not repress CAT expression of any of the HIV/SIV LTR-CAT constructs compared with that by pcD-SRα*nef*ΔKpn473. Essentially the same results were obtained in repeated

experiments.

Ⅳ-4 Discussion

The regulatory genes of HIVs (*vif, vpr, vpu* and *nef* of HIV-1, and *vif, vpx, vpr* and *nef* of HIV-2) are known to be dispensable for viral replication in established T cell lines (79-94). Recently, Guyader et al. reported that *vpx* mutants of HIV-2 replicated normally in T and promonocytic cell lines, but not in peripheral blood lymphocytes (95), suggesting that the *vpx* gene product may have an important role *in vivo*. Therefore, to see if the mutants of these genes might exhibit distinct characteristics in PBMC, the virus replication and syncytium formation in PBMC were compared with those in established T cell lines. The results are summarized in Table 8.

The *vpu* gene is unique to the HIV-1 genome. The results of the *vpu* mutant (Fig. 19A) and previous data (79,92,93) showed that the *vpu* gene is necessary for efficient viral replication in established T cell lines. Unexpectedly, the *vpu* mutant showed similar growth kinetics to that of wt virus in PBMC (Fig. 19B). This observation suggests that the requirement of the *vpu* product for viral replication may differ depending on the origins or character of the cells. Further investigations should be carried out for the understanding of the function of the vpu gene *in vivo*.

The *vpx* gene is conserved in all primate lentiviruses except HIV-1. This gene of HIV-2 is reported to be necessary for viral replication in human PBL but not in established cell lines (95). The results demonstrated here (Fig. 21) is consistent with the reported findings. However, the phenotype of the *vpx* mutant in PBMC was found to vary individually (Figs. 21 and 22). These results also indicate the importance of the character of target cells for infectivity of mutant viruses.

The *vif* genes of HIV-1 and HIV-2 have been demonstrated to be required for efficient replication in established cell lines, but the effect of mutation in the *vif* gene varies depending on the cells used (79,84,89-91). In this experiment, mutants of the *vif* genes of neither HIV-1 nor HIV-2 replicated in PBMC (Figs. 19 and 21), suggesting that *vif* gene product may be indispensable for virus replication *in vivo*.

There are reports that the *vpr* product of HIV-1 increases the rate of replication and accelerates the cytopathic effect of the virus in established T cell lines (82,88). In case of HIV-2, however, *vpr* mutants exerted no effect on the growth kinetics (83,89). The results presented on PBMC (Figs. 19-21) are essentially consistent with these previous reports, therefore, the function of *vpr* genes is seemingly not obvious, at least *in vitro*.

Earlier studies showed that *nef* mutants multiply

faster than the wt virus in established cell lines (80,86,94). However, the present investigation demonstrated that the *nef* genes' products did not affect the rate of viral replication (Figs. 19 and 21). The *nef* product has also been reported to reduce HIV-1 LTR-mediated transcription (80,87), but more recent data indicated the absence of the negative influence of the gene (85,100,101). Cheng-Mayer et al. found that the *nef* products from several isolates had different effects on HIV replication *in vitro* (80). Moreover, the *nef* protein of HIV-1 NL-432 did not repress CAT activity directed by LTRs derived from HIV-1, HIV-2, SIVMAC, SIVMND and SIVAGM, which are representatives of major primate immunodeficiency viruses, in the present experiments (Fig. 22). This finding is consistent with the results of the infection experiments showed above. Lately, Kestler Ⅲ et al. showed the importance of the *nef* gene of SIVMAC for maintenance of high virus loads and for development of AIDS (102).

So far, the real function of these regulatory genes *in vivo* has been unknown, however, biological functional analyses using animal models for AIDS such as the report of Kestler Ⅲ et al. (102) are required to evaluate the function and implication of the genes for the pathogenesis of immunodeficiency viruses.

Ⅳ-5 Conclusion

The phenotypes of the mutants of several regulatory genes in HIV-1 and HIV-2 were studied in PBMC and established cell lines. The results showed that some of the mutants displayed different growth potentials in PBMC from those in established cell lines, suggesting that the functions of some genes could be detected only in PBMC. Studies on the roles of these genes in PBMC may provide a better understanding of their functions *in vivo*. Furthermore, infection experiments of these mutants *in vivo* should be required for analyzing the pathogenesis of HIV and AIDS.

V Final conclusion

The main object of the present study was to determine the significance of the interaction among viral and cellular factors in the retroviral infection.

First, the effects of viral and cellular factors on early stages of HTLV-I infection, especially on the binding and fusion steps, were studied as a representative of primate leukemia viruses. (i) A simple assay system for detecting HTLV-I-binding cells was established by using mAbs and flowcytometry. (ii) The system was applicable for the determination of the antibodies in sera inhibiting the HTLV-I binding, (iii) and for the detection of the host cell-derived membrane proteins on the envelope of HTLV-I virions. (iv) As for further application of the system, it was found that the envelope of the virions altered or degenerated on the surface of the cells, and in this process MA was exposed on the cells, dependent on the temperature and probably cytoskeletal actin polymerization.

Secondly, the effects of viral factors on the infectivity of HIV-1 and HIV-2 as primate immunodeficiency viruses were examined, and it was found that; (i) several mutant viruses of the infectious molecular clones of HIV-1 and HIV-2, directed to the portions of the several regulatory genes, showed distinct replication patterns in

PBMC from those in established cell lines, (ii) and the potential role of the *nef* gene expression in transcription directed by the LTRs of various primate immunodeficiency viruses was examined.

These findings suggest that the infection of primate retroviruses is established by the interaction among viral and cellular factors at each step of the life cycle of the viruses. The interaction may restrict not only the target cells, organs and the host but also pathogenesis of the viruses. The understanding of these phenomena would be meaningful for the elucidation of the diseases caused by retroviruses and also of the mechanism of the functions of the cells.

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Table 1. Taxonomy of retroviruses

Family and Subfamily	Virus Group	Common Species (Abbreviation)
Retroviridae		
Oncovirinae	Type C oncovirus group	
	Mammalian	Murine C-type viruese
		Murine leukemia viruses (MuLV)
		Murine sarcoma viruses (MuSV)
		Murine endogenous viruses
		Rat C-type viruses
		Hamster C-type viruses
		Feline C-type viruses
		Feline leukemia viruses (FeLV)
		Feline sarcoma viruses (FeSV)
		Ferine endogenous viruses (RD114)
		Bovine C-type leukemia viruses (BLV)
		Porcine C-type viruses
		Primate C-type viruses
		Gibbon ape leukemia viruses (GaLV)
		Simian sarcoma viruses (SiSV)
		Baboon endogenous viruses (BaEV)
		Simian T-lymphotropic viruses (STLV)
		Human T cell leukemia viruses (HTLV-I ,-II)
	Avian	Avian C-type viruses
		Avian leukemia viruses (ALV)
		Avian sarcoma viruses (ASV)
		Avian reticuloendotheliosis viruses (REV)
		Avian endogenous viruses
	Reptilian	Viper C-type virus
	Type B oncovirus group	Mouse mammary tumor virus (MMTV)
	Type D oncovirus group	Mason-Pfizer monkey virus (MPMV)
		Simian immunodeficiency retroviruses (SRV-1, -2)
		Langur endogenous virus (PO-1-Lu)
		Squirrel monkey endogenous virus (SMRV)
Lentivirinae		Maedi-Visna virus
		Caprine arthritis encephalitis virus (CAEV)
		Equine infectious anemia virus (EIAV)
		Bovine immunodeficiency virus (BIV)
		Ferine immunodeficiency virus (FIV)
		Simian immunodeficiency viruses (SIV)
		Human immunodeficiency viruses (HIV-1, -2)
Spumavirinae		Foamy viruses of several animal species
		Human foamy virus (HFV)

Table 2. Antibody titers detected by indirect immunofluorescence (IF), syncytium inhibition and HTLV-I-binding inhibition in sera from HTLV-I infected individuals and uninfected controls.

Serum*	Titers detected by:		
	IF	Syncytium inhibition	Binding inhibition
Nor-1	<1:10	<1:10	<1:20
Nor-2	<1:10	<1:10	<1:20
Nor-3	<1:10	<1:10	<1:20
AC-1	1:80	1:40	1:80
AC-2	1:640	1:320	1:700
AC-3	1:80	1:80	1:50
ATL-1	1:80	1:40	1:70
ATL-2	1:160	1:40	1:400
HAM-1	1:160	1:160	1:240
HAM-2	1:320	1:320	1:600
HAM-3	1:80	1:320	1:80
HAM-4	1:1280	1:640	1:800

*Nor, normal uninfected control; AC, asymptomatic carrier; ATL, adult T-cell leukemia; HAM, HTLV-I-associated myelopathy.

Table 3. Binding of HTLV-I to various human cell lines and peripheral blood lymphocytes.

Cells	Origin	% positive cells*
I Floating cell lines		
MOLT-4#8	T-cell leukemia	82.7 ± 5.4
H9	T-cell leukemia	70.9 ± 2.1
A3.01	T-cell leukemia	61.4 ± 9.1
Raji	Burkitt's lymphoma	39.7 ± 1.0
BJAB	Burkitt's lymphoma	4.6 ± 3.1
K562	Myelogenous leukemia	64.2 ± 1.9
U937	Promyelocytic cell line	63.6 ± 5.2
II Adherent cell lines		
FL	Amnion cell line	29.8 ± 4.3
U105MG	Glioma cell line	1.8 ± 0.2
U251MG	Glioma cell line	33.3 ± 9.2
Hela	Epithelioid carcinoma	12.7 ± 5.0
III Peripheral blood lymphocytes		47.5 ± 0.4

*Percentage represents the average ± s.d. from three independent experiments.

Table 4. The expression levels of the molecules on the surface of MOLT-4#8 and MT-2 cells.

mAbs to:	Mean channel fluorescence	
	MOLT-4#8	MT-2
CD3	5.6	4.0
CD4	62.4	80.7
LFA-1	75.5	46.9
ICAM-1	8.8	126.0
LFA-3	33.7	101.6
CD2	6.2	5.7
MHC-1	74.5	136.8
MHC-IIDR	1.9	71.0

Mean channel fluorescence on each cellular molecules was averaged from the results of several experiments. The values are shown as the mean channel fluorescence of stained cells minus that of the background control (MOLT-4#8; about 33, MT-2; about 35, in average).

Table 5. Detection of cellular membrane molecules of human on HTLV-I-binding BW5147 cells and its correlation with the producer MT-2 cells.

mAbs to:	Mean channel fluorescence	
	HTLV-I-treated BW5147	MT-2
CD3	3.8	4.0
CD4	35.8	80.7
LFA-1	15.5	46.9
ICAM-1	58.3	126.0
LFA-3	38.3	101.6
CD2	5.2	5.7
MHC-I	87.3	136.8
MHC-IIDR	33.0	71.0

Mean channel fluorescence on each cellular membrane molecules was averaged from the results of several experiments. The values are shown as the mean channel fluorescence of stained cells minus that of background control (BW5147; about 30, MT-2; about 35, in average).

Table 6. Inhibition of *de novo* synthesis by translational inhibitors.

Pretreatment of MOLT-4#8 cells		% positive cells	
		REY-7	GIN-14
untreated		84.0	61.0
cycloheximide	0.1 μg/ml	88.9	67.0
	1 μg/ml	85.0	67.3
Puromycin	5 μg/ml	84.8	56.2
	50 μg/ml	86.8	58.1

Table 7. HIV-1 and HIV-2 mutants used in this study

Plasmid	Region of mutation	Description of mutation[a]	predicted size of mutated protein[b]
pNL-432	wild type (HIV-1)	none.	
pNL-Nd	*vif*	2 bp insertion at Nde I (5122).	28(192)
pNL-Af2	*vpr*	4 bp insertion at Afl II (5634).	26(96)
pNL-Ss	*vpu*	8 bp linker insertion at Ssp I (6153).	32(81)
pNL-Xh	*nef*	4 bp insertion at Xho I (8887).	35(206)
pGH-123	wild type (HIV-2)	none.	
pGH-Xb	*vif*	4 bp insertion at Xba I (5064).	67(215)
pGH-St	*vpx*	4 bp insertion at Sty I (5518).	60(112)
pGH-Ec	*vpr*	4 bp insertion at Eco RI (5756).	42(120)
pGH-Ml	*nef*	4 bp insertion at Mlu I (8637).	34(246)

[a]Nucleotide sequence data were from Los Alamos data bank (Los Alamos, NM, USA) for pNL-432 and pGH-123. Values in parentheses are the first nucleotide no. of the enzyme recognition sites.
[b]Values indicate no. of Wild type (wt) amino acid residues from N-terminus of mutated proteins. No. of amino acid residues of wt are also shown in parentheses.

Table 8. Characterizations of HIV-1 and HIV-2 mutants

Mutants	Mutated gene	Viral growth in		Syncytium formation in	
		cell line[a]	PBMC	cell line[a]	PBMC
pNL-432 (HIV-1)	None	+	+	+	+
pNL-Nd	*vif*	−	−	−	−
pNL-Af2	*vpr*	+[b]	+	+	+
pNL-Ss	*vpu*	+[c]	+	+	+
pNL-Xh	*nef*	+	+[b]	+	+
pGH-123 (HIV-2)	None	+	+	+	−
pGH-Xb	*vif*	+[c]	−	−	−
pGH-St	*vpx*	+	+[b]	+	−
pGH-Ec	*vpr*	+	+	+	−
pGH-Ml	*nef*	+	+	+	−

[a] A3.01 cells (HIV-1) and MOLT-3 cells (HIV-2) were used as target cell lines for infection. [b] Delayed kinetics of viral growth. [c] Reduction of viral growth.

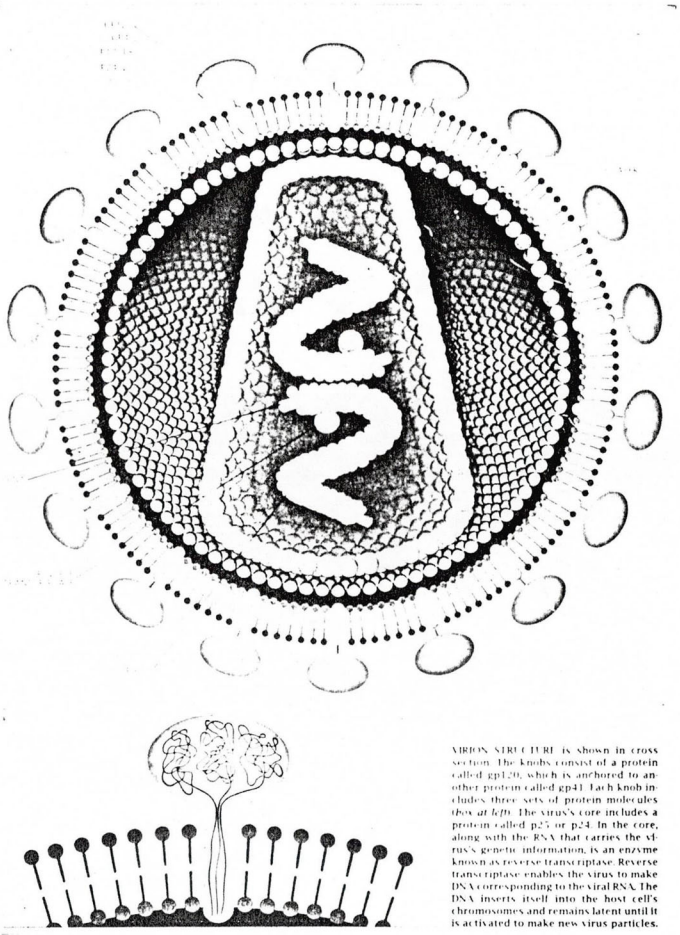

Fig. 1. Two dimensional diagram of HIV-1.
(Referred from ref. 103)

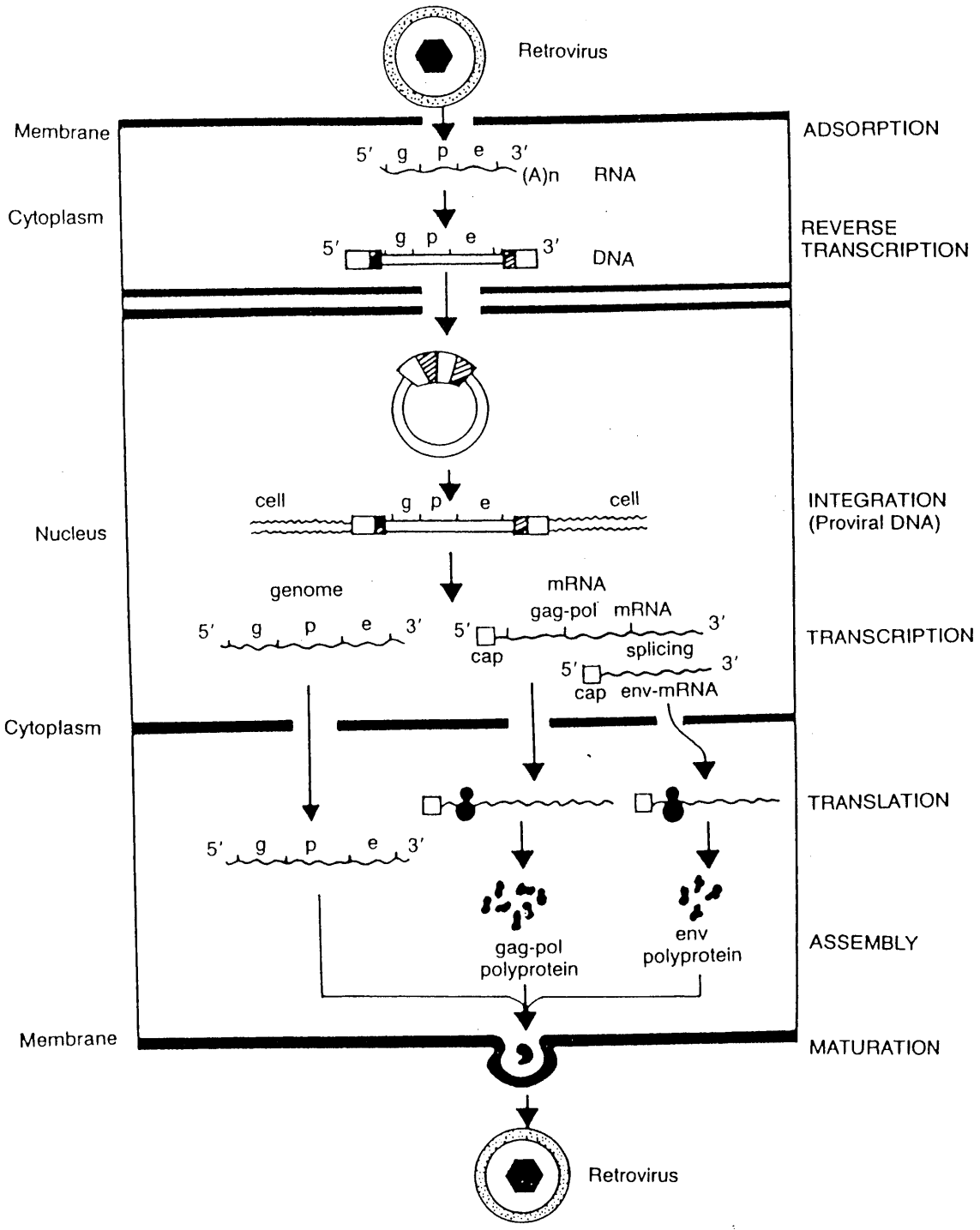

Fig. 2. The life cycle of retroviruses.

(Referred from ref. 104)

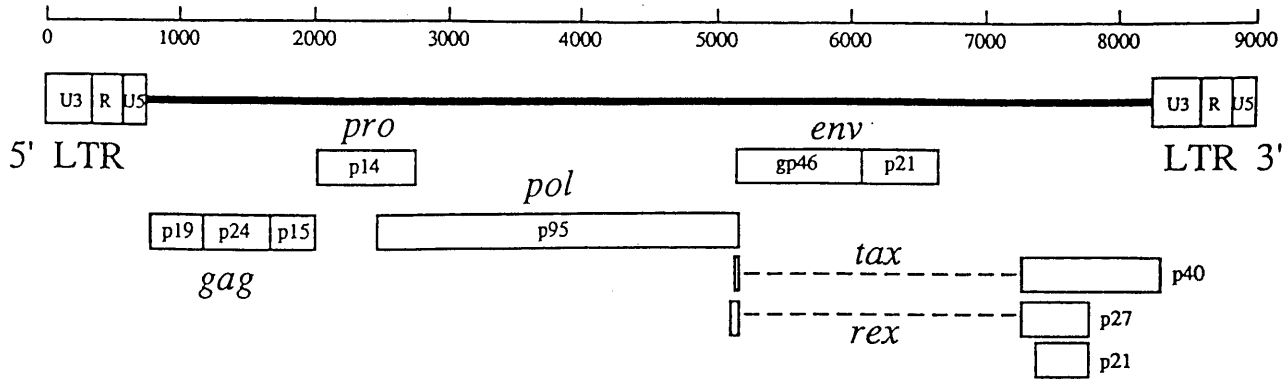

Fig. 3. The genomic organization of HTLV-I.

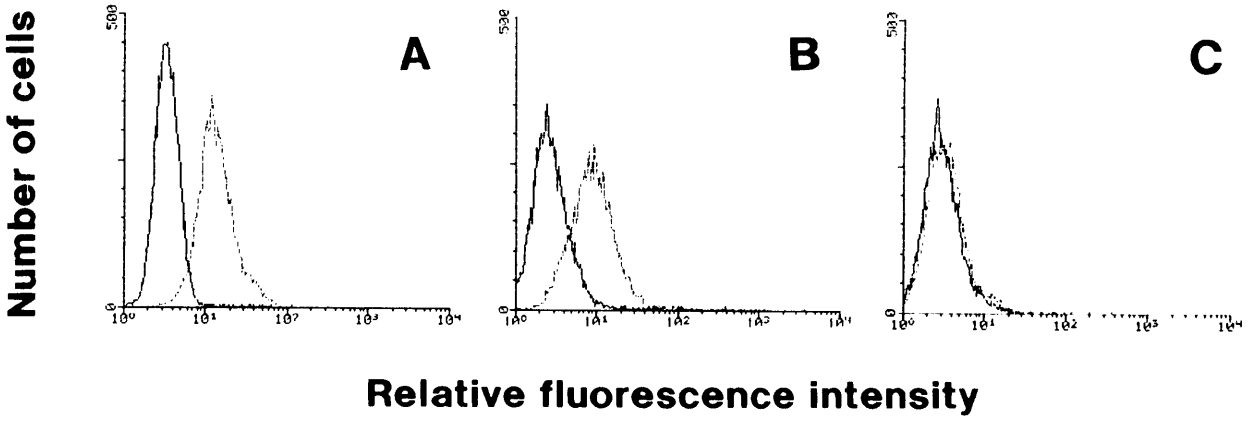

Fig. 4. HTLV-I binding to MOLT-4#8 (A), Raji (B), and U105MG (C) cells.

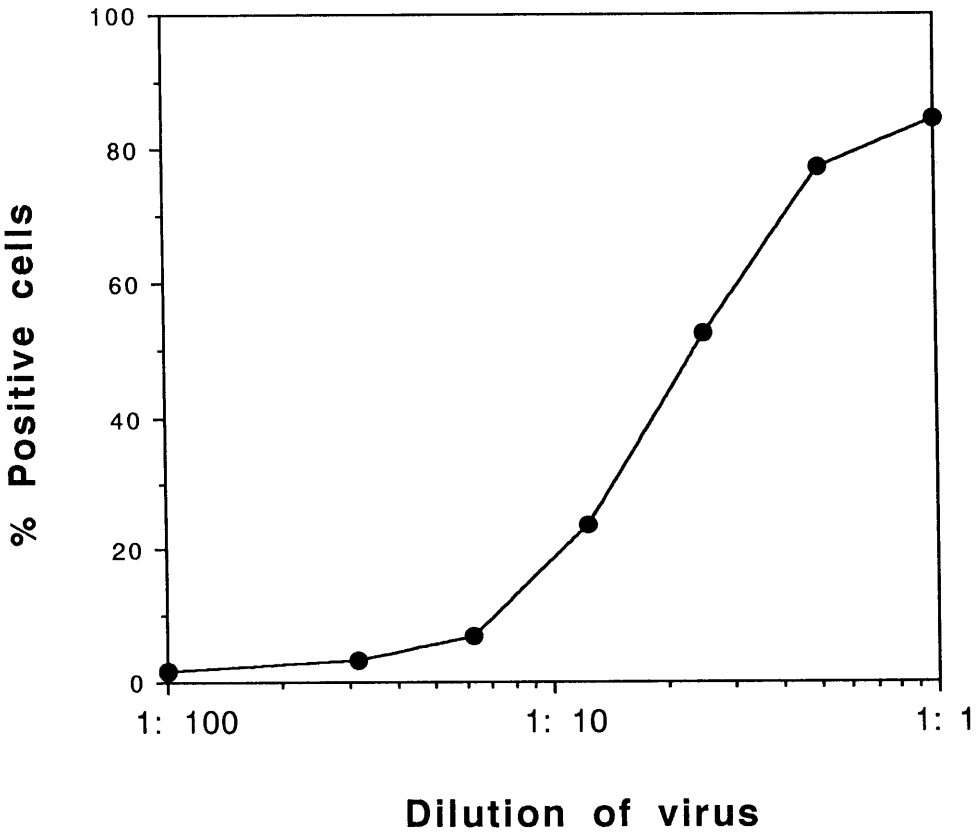

Fig. 5. Concentration-dependent HTLV-I binding to MOLT-4#8 cells.

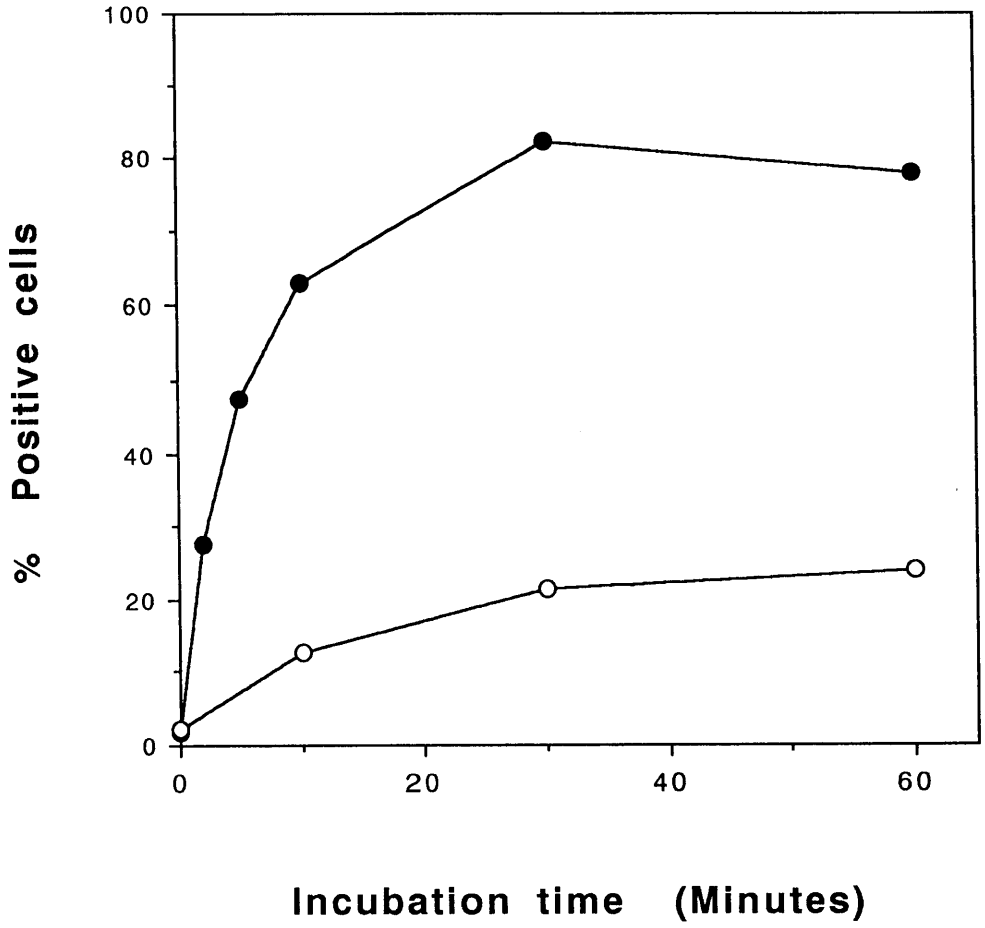

Fig. 6. The kinetics of HTLV-I binding to MOLT-4#8 cells at 37℃ (closed circle) or 4℃ (open circle).

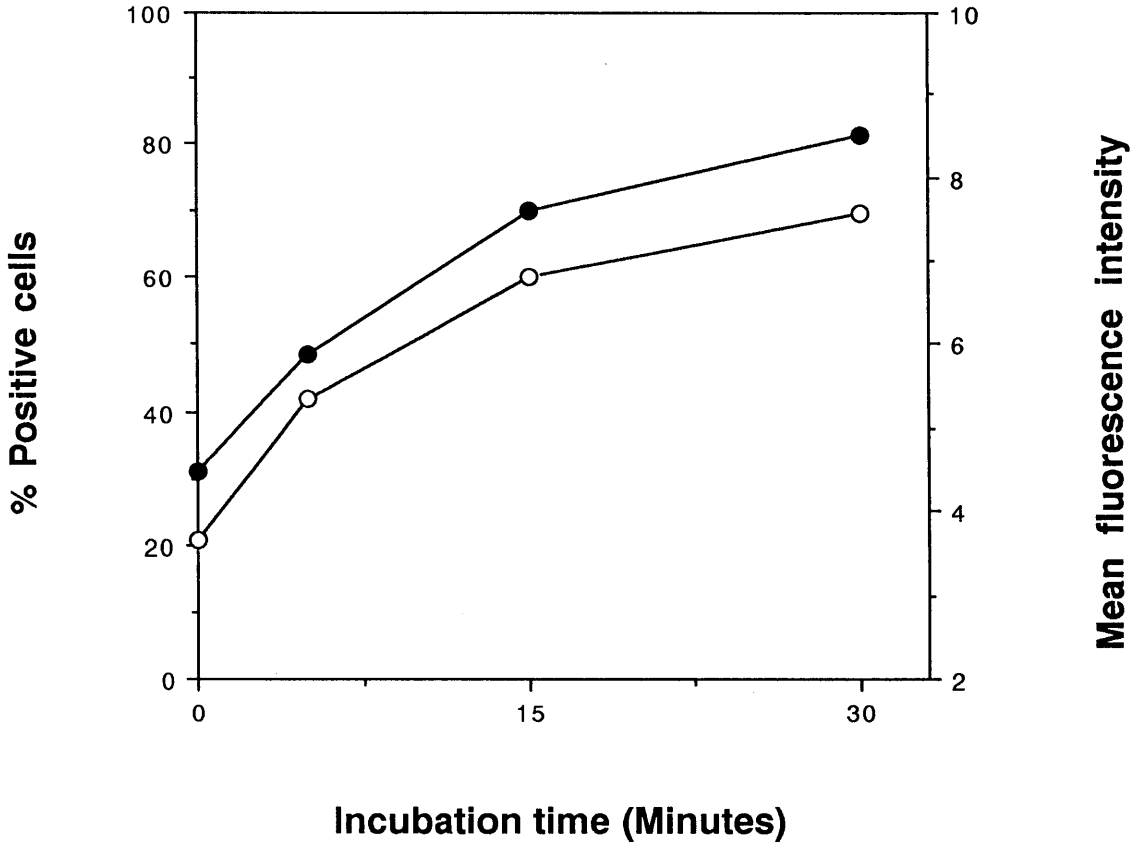

Fig. 7. Fluorescence positivity (open circle) and mean fluorescence intensity (closed circle) were increased by the 37℃ incubation of MOLT-4#8 cells treated with HTLV-I at 4℃.

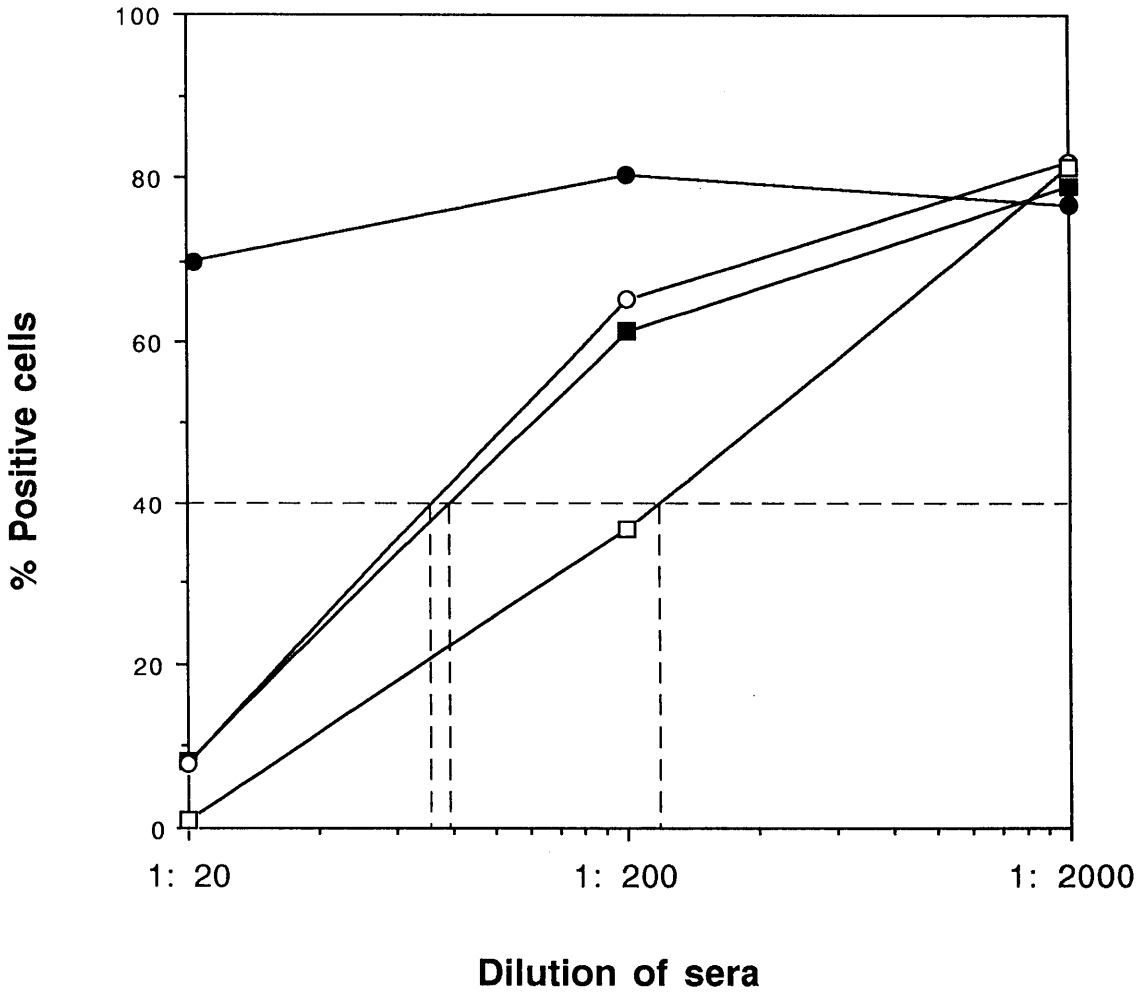

Fig. 8. Titration of binding inhibitory antibodies in sera from HTLV-I-infected individuals. The virus was pretreated with 1:20, 1:200, or 1:2000 diluted sera from a seronegative donor (closed circle), an asymptomatic carrier (closed square),an ATL patient (open circle), and a HAM patient (open square).

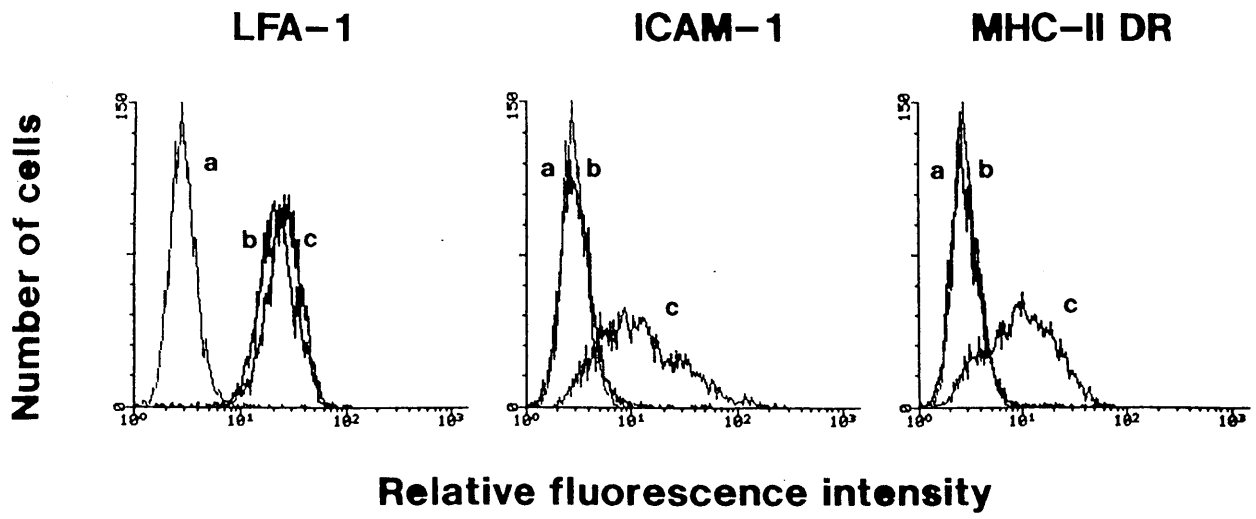

Fig. 9. Detection of the host cell-membrane proteins on HTLV-I-binding MOLT-4#8 cells. MOLT-4#8 cells were treated with medium (lines a and b) or HTLV-I (line c) at 37℃ for 1 hour. The cells were washed and reacted with medium (line a) or each mAb (lines b and c) or medium (thin line) at 4℃ for 40 min. The washed cells were stained with goat anti-mouse IgG-FITC and the fluorescence intensity was measured by FACScan.

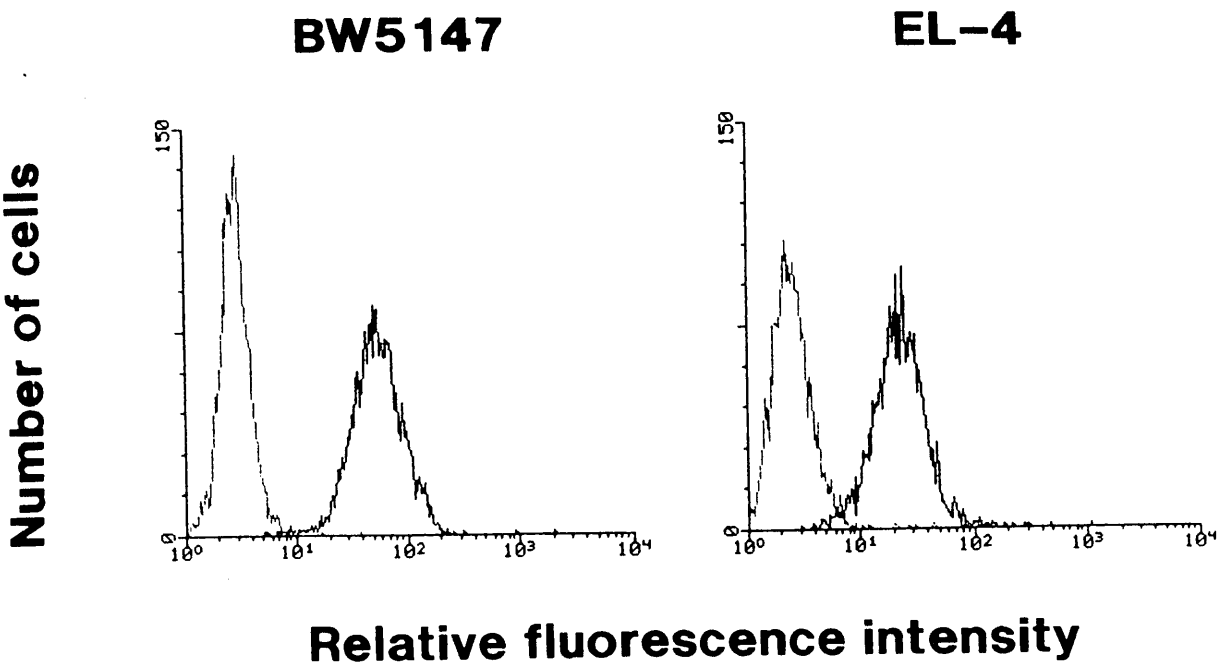

Fig. 10. Binding of HTLV-I to mouse T-lymphoma cell lines, BW5147 or EL-4 cells.

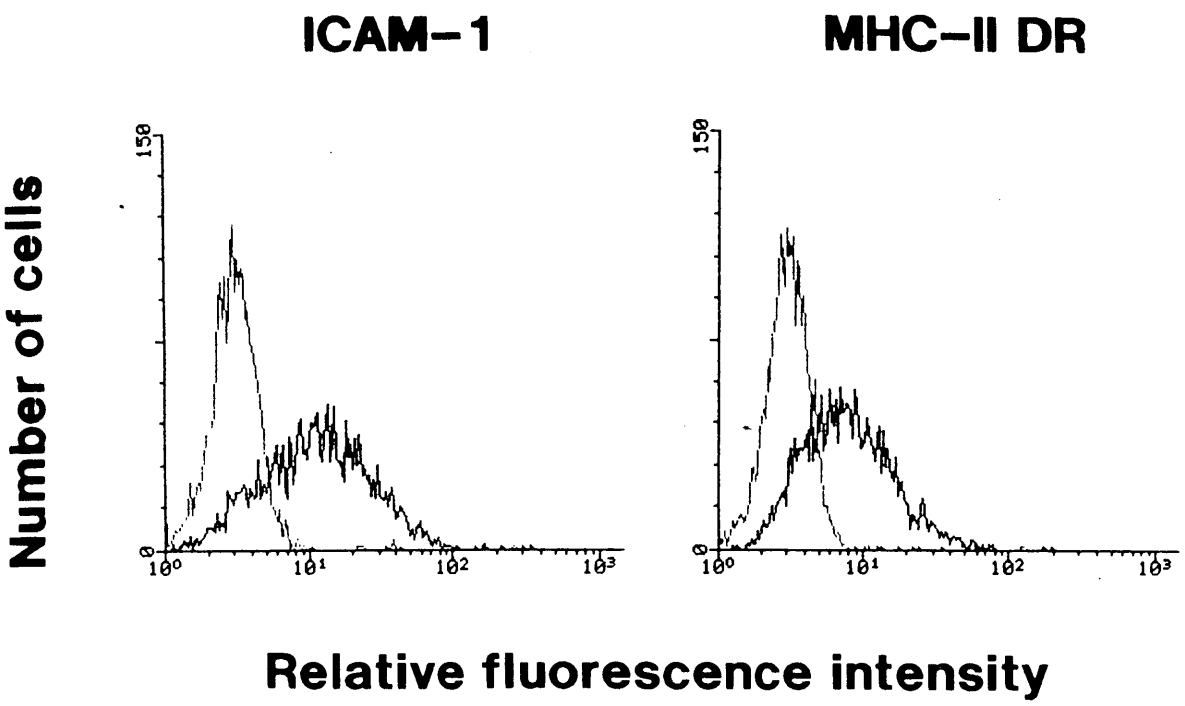

Fig. 11 Detection of cellular molecules of human (ICAM-1 and MHC-IIDR) on HTLV-I-binding BW5147 cells.

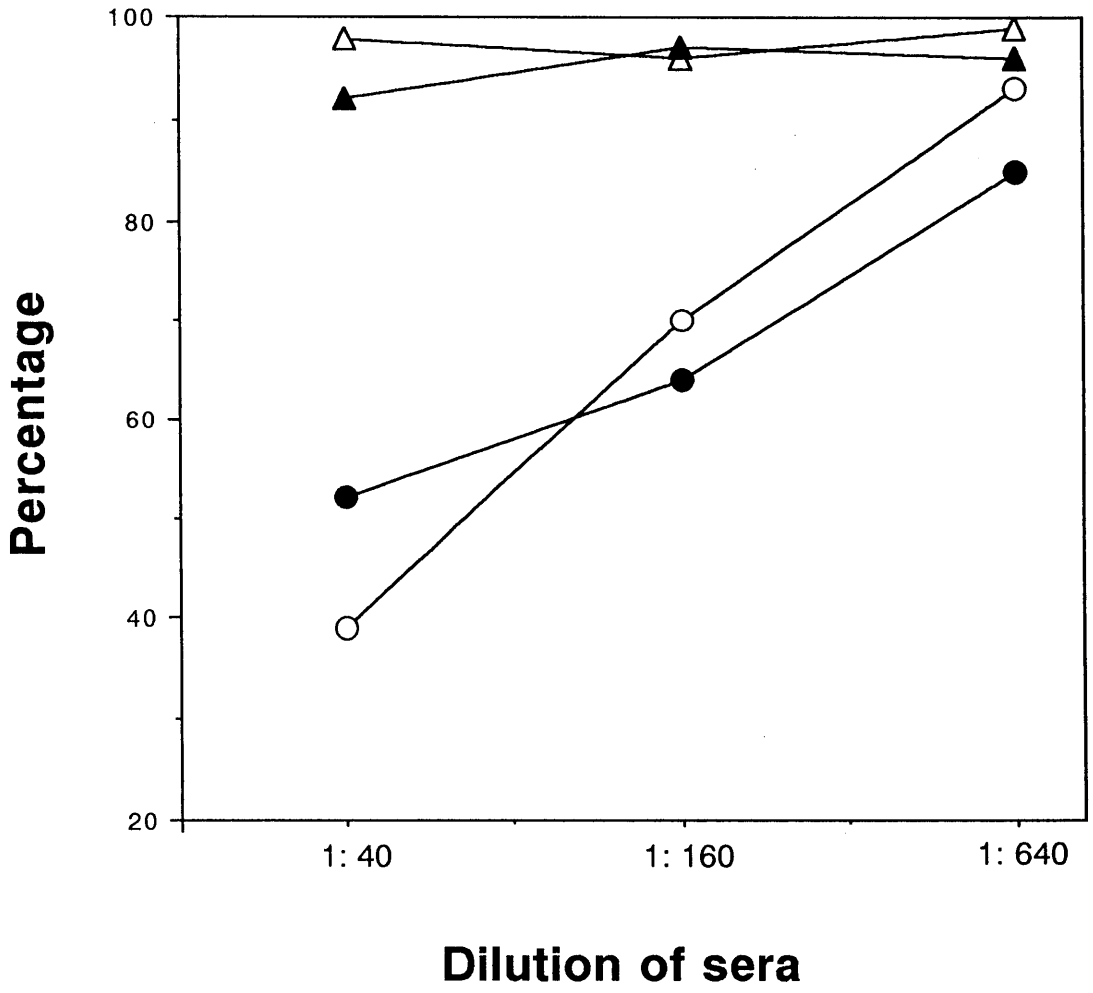

Fig. 12. The detectable levels of cellular membrane molecules on BW5147 cells were reduced by pretreatment of the serum from an HTLV-I seropositive individual (circle) but not from a seronegative control (triangle) with HTLV-I at 4℃ for 1 hour. The rate of reduction by serum pretreatment is shown for ICAM-1 (open) and MHC-II DR (closed) molecules as percentage of mean channel fluorescence against positive controls (pretreatment of serum-free medium).

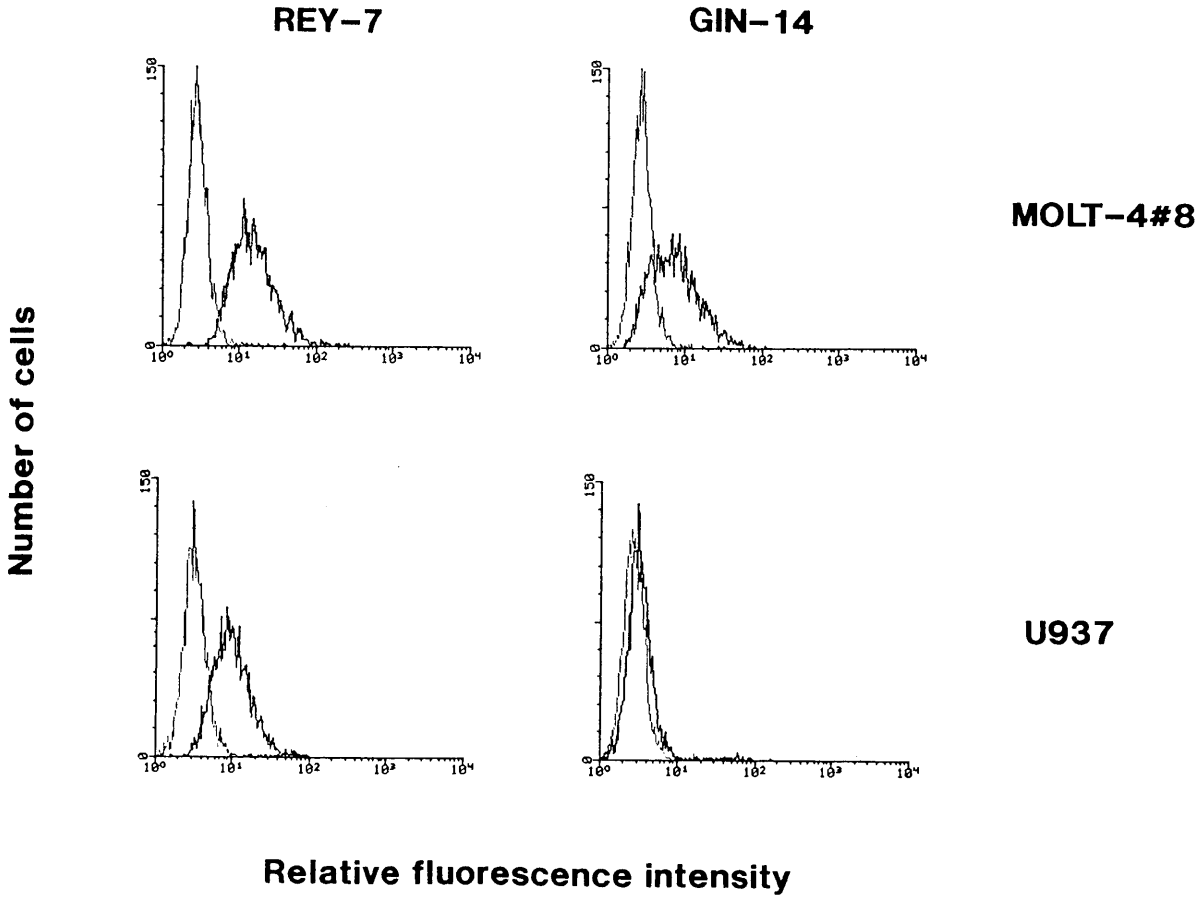

Fig. 13 Detection of HTLV-I gp46 (REY-7 mAb) and MA (GIN-14 mAb) on viral-binding MOLT-4#8 and U937 cells .

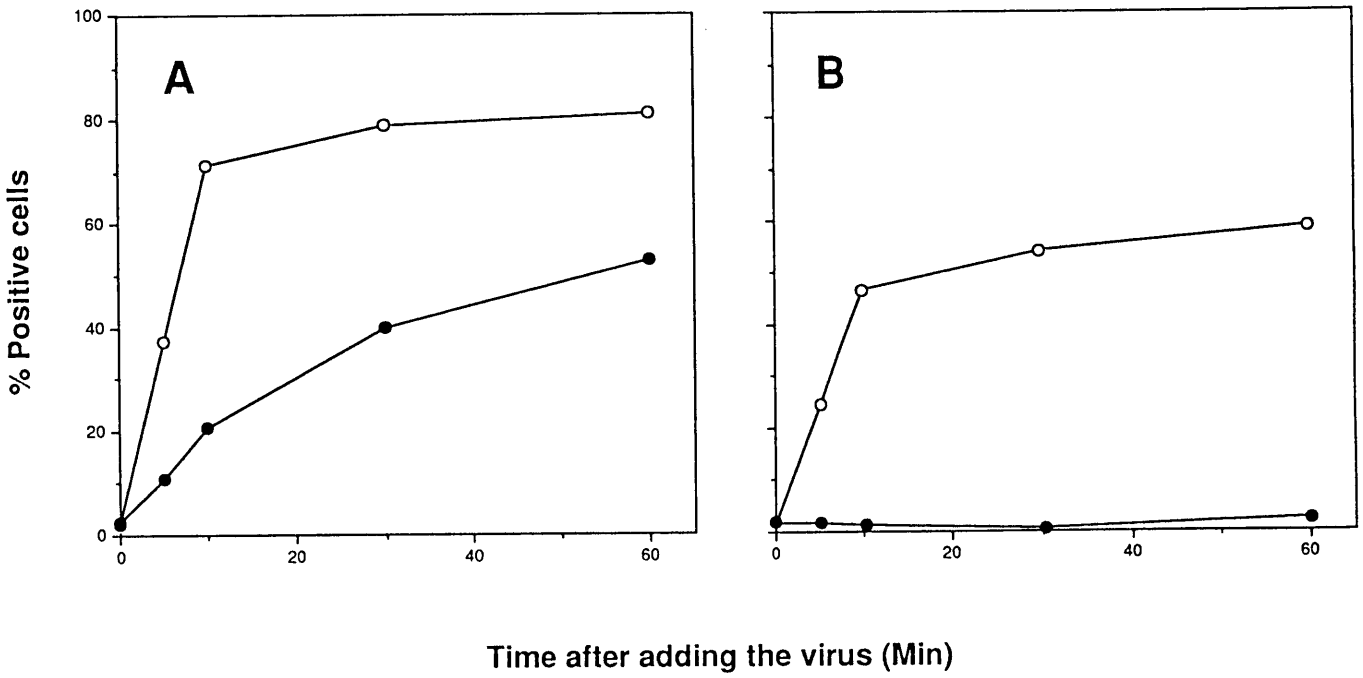

Fig. 14. Kinetics of fluorescence positivity for gp46 (open circle) and MA (closed circle) on HTLV-I-binding MOLT-4#8 (A) and U937 (B) cells.

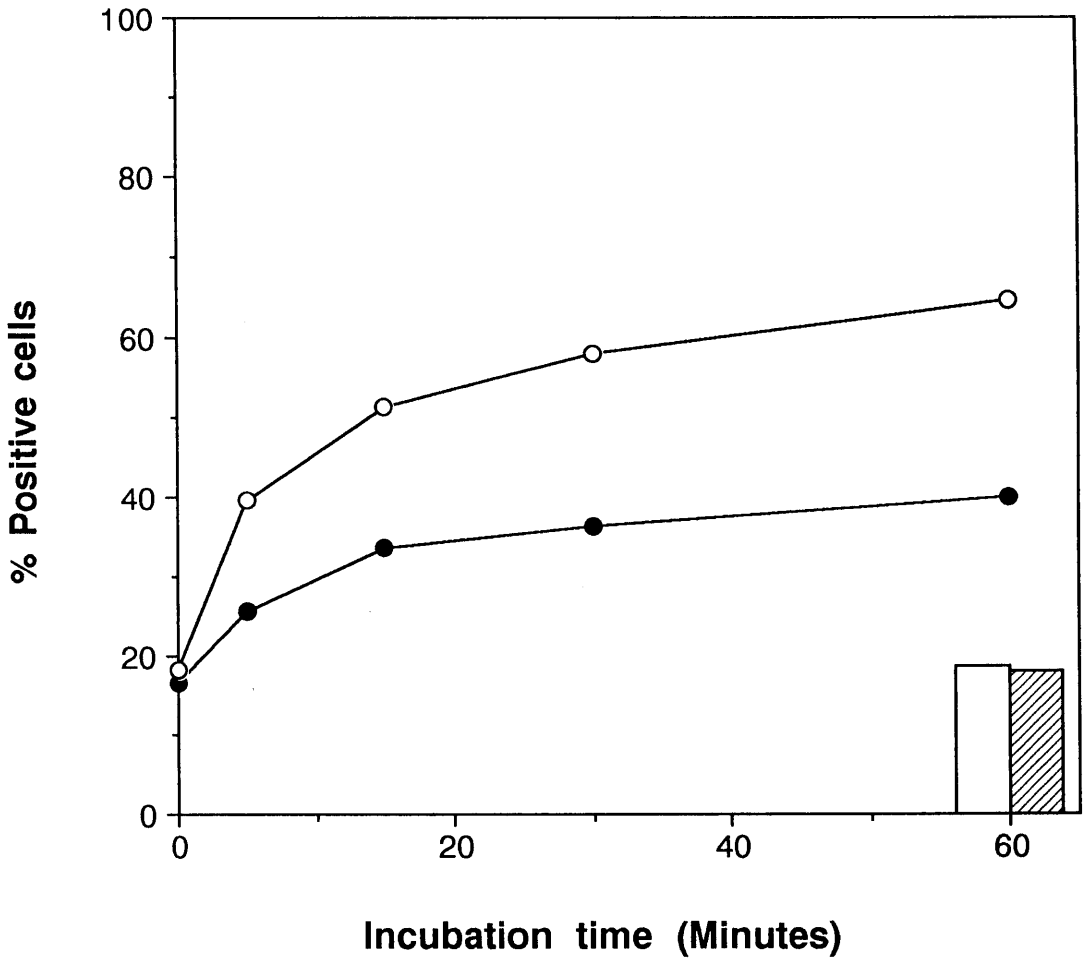

Fig. 15. Fluorescence positivity for gp46 (open circle) and MA (closed circle) were increased by the 37℃ incubation of MOLT-4#8 cells treated with HTLV-I at 4℃. However, cytochalasin B treatment completely inhibited the increase in fluorescence positivity for both gp46 (open bar) and MA (hatched bar) after 60 minutes incubation at 37℃.

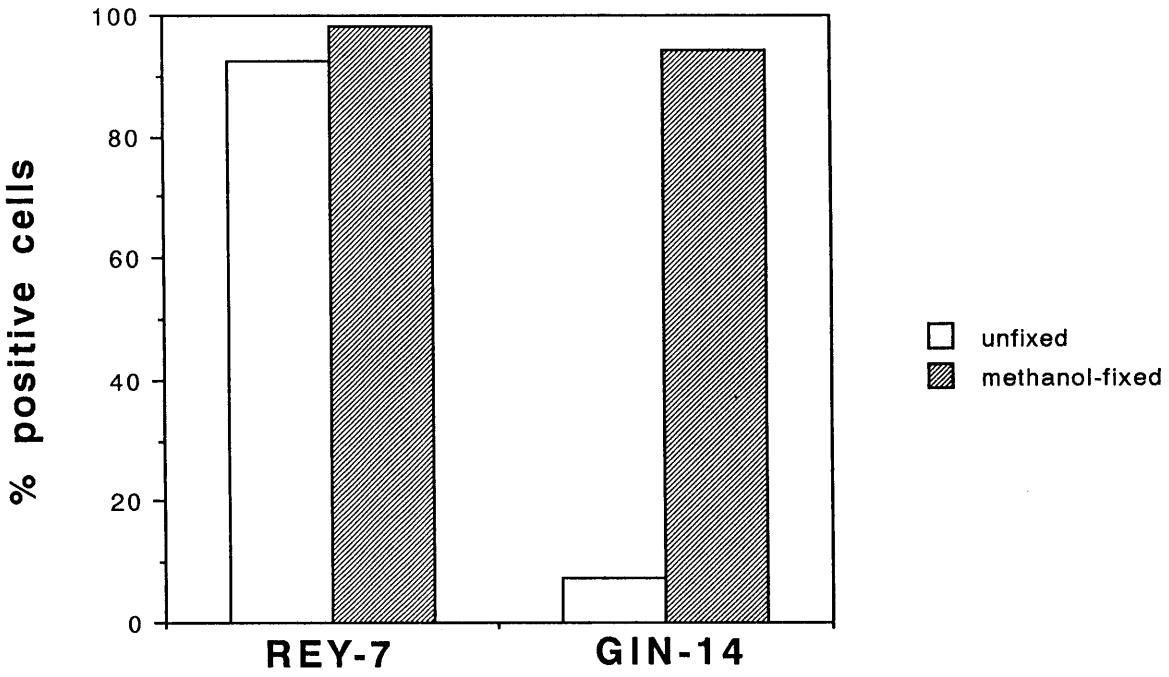

Fig. 16. Effects of methanol fixation on fluorescence positivity for gp46 (REY-7 mAb) and MA (GIN-14 mAb) of MT-2 cells.

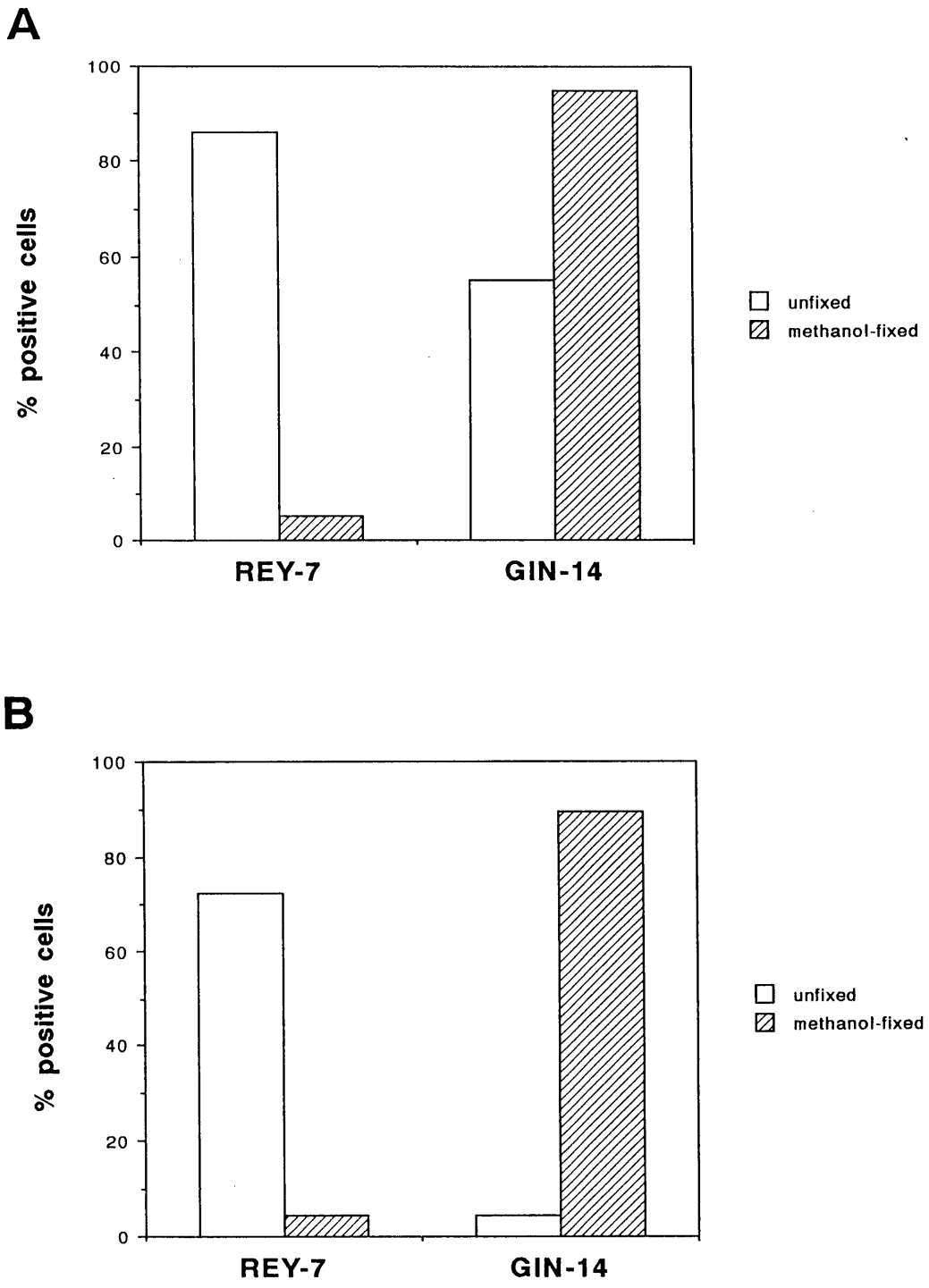

Fig. 17. Effects of methanol fixation on fluorescence positivity for gp46 (REY-7 mAb) and MA (GIN-14 mAb) on HTLV-I-binding MOLT-4#8 and U937 cells.

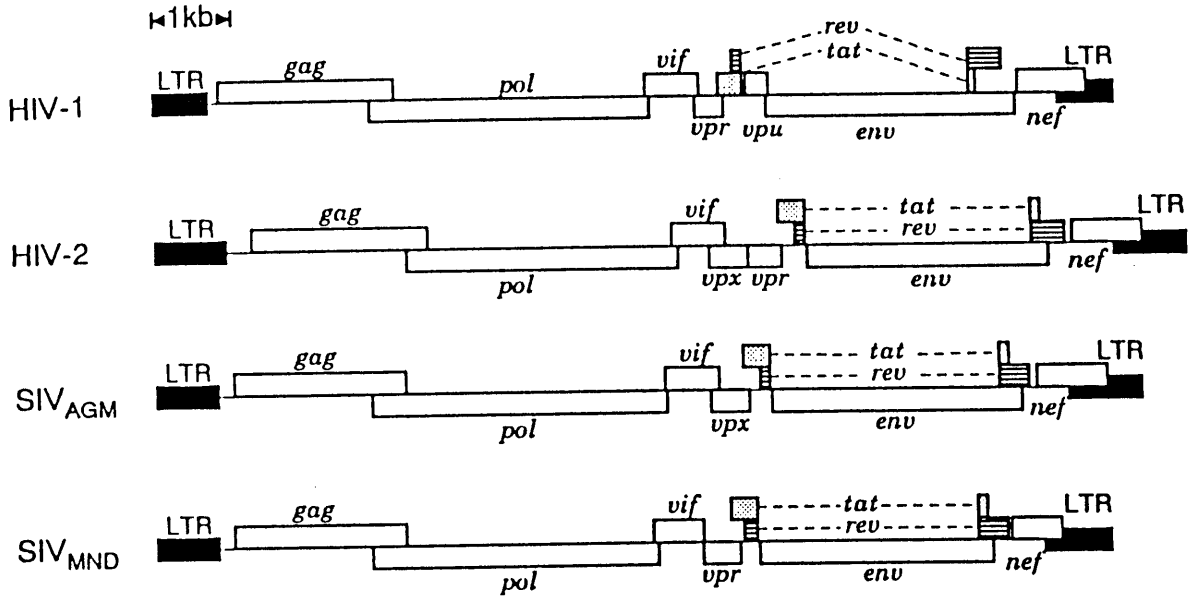

Fig. 18. Genomic organization of the four groups of primate immunodeficiency viruses.

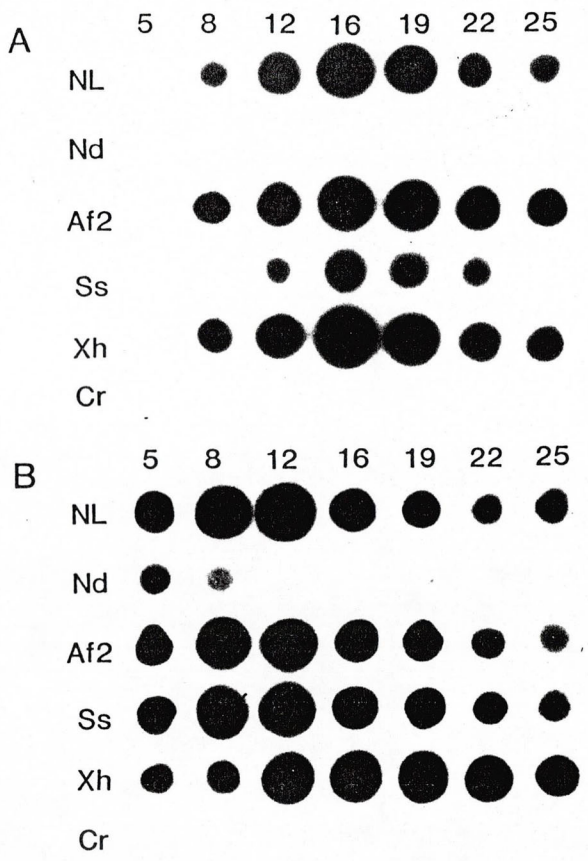

Fig. 19. Growth kinetics of various HIV-1 mutant clones in A3.01 cells (A) and PBMC (B). 1 x 10^6 cells were infected with 5 x 10^6 cpm of RT activity of cell-free virus from transfected SW480 cells or medium (mock infected; Cr), and progeny virus production was monitored by RT production at every 3 to 4 days. NL virus is derived from pNL-432 wild type clone of HIV-1. Nd (*vif* mutant), Af2 (*vpr* mutant), Ss (*vpu* mutant), and Xh (*nef* mutant) are mutants of wild type virus.

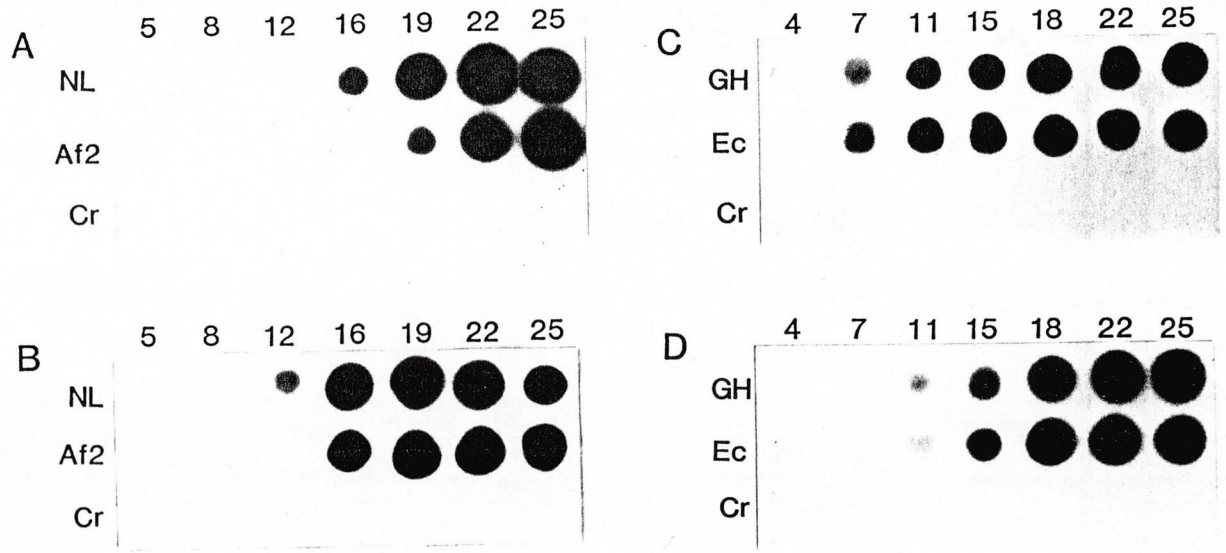

Fig. 20. Growth kinetics of wild type viruses and *vpr* mutants of HIV-1 (A, B) and HIV-2 (C, D) in A3.01 (A), MOLT-3 cells (C) and PBMC (B, D). 1 x 10^6 cells were infected with 5 x 10^5 cpm of RT activity of cell-free virus or medium (mock infected; Cr). NL and Af2 viruses from the pNL-432 wild type and *vpr* mutant clones of HIV-1; GH and Ec from pGH-123 wild type and *vpr* mutant clones of HIV-2, respectively

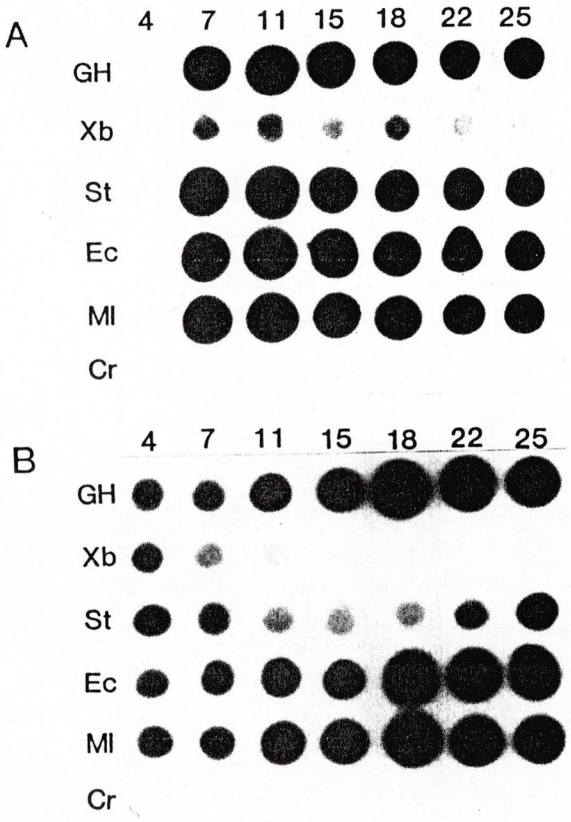

Fig. 21. Growth kinetics of various HIV-2 mutant clones in MOLT-3 cells (A) and PBMC (B). 1 x 10^6 cells were infected with 5 x 10^6 cpm of RT activity of cell-free virus from transfected SW480 cells or medium (mock infected; Cr), and progeny virus production was monitored by RT production at every 3 to 4 days. GH virus is derived from pGH-123 wild type clone of HIV-2. Xb (*vif* mutant), St (*vpx* mutant), Ec (*vpr* mutant), and Ml (*nef* mutant) are mutants of wild type virus.

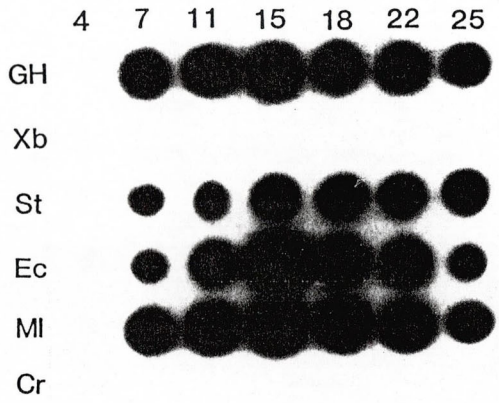

Fig. 22. Growth kinetics of various HIV-2 mutant clones in another PBMC preparation. 1 x 10^6 of PBMC separated from a different individual were infected with 5 x 10^6 cpm of RT activity of cell-free virus from transfected SW480 cells or medium (mock infected; Cr), and progeny virus production was monitored by RT production at every 3 to 4 days.

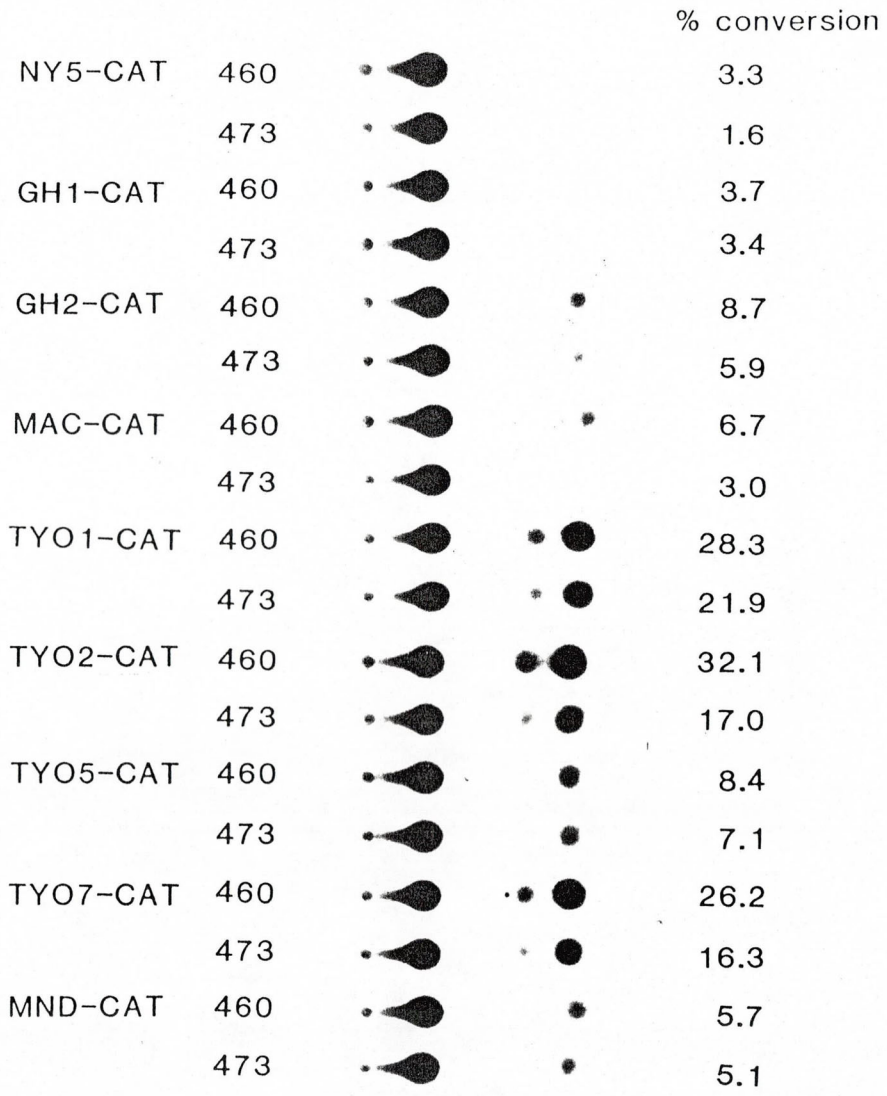

Fig. 23. Effect of *nef* gene expression on various LTRs. 10 μg of expression vector of the *nef* gene from pNL-432 HIV-1 clone pcD-SRα*nef*460 (460) or its mutant pcD-SRα*nef*ΔKpnI473 (473) were cotransfected into SW480 cells with 10 μg of each of HIV/SIV LTR-CAT constructs, NY5-CAT (HIV-1), GH1-CAT (HIV-2), GH2-CAT (HIV-2), MAC-CAT (SIV$_{MAC}$) TYO1-CAT to TYO7-CAT (SIV$_{AGM}$) and MND-CAT (SIV$_{MND}$). CAT activity in the cell lysates was determined at 48 hours post-transfection. The percent conversion of chloramphenicol to its acetylated forms is indicate on the right.