

Infection of HTLV-III/LAV IN HTLV-I-Carrying Cell Lines: Virological, Immunological and Cytological Aspects

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A human retrovirus HTLV-III/LAV is most probably a cause of acquired immune deficiency syndrome (AIDS)(1,10). HTLV-III/LAV infects human helper T lymphocytes preferentially (7). Consequently, the infected cells are killed off and release virus progeny. Thus the retrovirus is considered to destroy and reduce T lymphocytes in vivo inducing immune deficiency in the patients. However, precise mechanism of the infection, replication, and release of the virus had not been studied simply because no highly permissive cell line for HTLV-III/LAV has existed so far. In this paper, we describe that cell lines carrying HTLV-I, a causative virus of adult T-cell leukemia, are highly permissive for HTLV-III/LAV infection leading to complete cellular death. This infection system allowed us to develop the quantitative assay for the virus which is very valuable for the study on virus-host interactions, neutralizing antibodies to the virus, and various factors affecting virus replication.

HTLV-III/LAV infection in MT-4 cells

When a representative HTLV-I-positive cell line MT-4 were infected with HTLV-III/LAV (m.o.i. of 0.002) almost 2.7% of the cells were HTLV-III/LAV-specific antigen-positive cells 24 hr post infection (p.i.)(2,3). Then, these antigen-positive cells gradually increased in number with time. After 4 days p.i., more than 95% of the cells were positive. Twenty-four hr p.i., 21.2% positive cells were observed when m.o.i. of 0.02 was used. When m.o.i. was 1.0, 100% of the observed cells were positive. There were no detectable positive cells in uninfected MT-4 cells during any stage of the culture. Viable growth of the virus-infected cells was markedly suppressed on day 2 p.i. and thereafter when cytopathic effect (CPE) of the infected cells and increased pH of the medium were also observed. The addition of cytosine arabinoside completely inhibited the induction of HTLV-III/LAV-specific antigen as shown by immunofluorescence (IF). This finding was further confirmed by radioimmunoprecipitation method. 120K, 46K, 24K, and 17K

polypeptides in HTLV-III/LAV-infected MT-4 cells were specifically precipitated by HTLV-III/LAV antibody-positive serum. But they were not precipitated at all by antibody-negative serum.

Quantitation of HTLV-III/LAV

We then tested whether MT-4 cells could be used as target cells for an HTLV-III/LAV-induced plaque assay (2). Since MT-4 cells were not adherent to the culture vessel, we used plates coated with poly-L-lysine (PLL)(molecular weight 90,000; Sigma) to make a monolayer of cells. The dilution experiment data suggest that a single infectious virus particle is sufficient for infection and plaque formation. To determine whether plaques are formed by HTLV-III/LAV, we incubated portions of the virus preparation with various dilutions of sera from two seronegative controls and two patients with hemophilia A who were both seropositive to HTLV-III/LAV. Serum from patient 1, who had a high titer of antibody detected by IF, completely inhibited plaque. In contrast, sera from two healthy donors (negative IF) did not remarkably reduce plaque formation, although some reduction in the number of plaques was observed. This result may reflect experimental variation due to small number of PFU used as virus controls or the sensitivity of the assay. To confirm that plaque formation was specific to HTLV-III/LAV infection, we compared plaque forming (PFU), median tissue culture infectious doses (TCID₅₀), and the activity of reverse transcriptase (RT) from different preparations of virus. There was a parallel relation between PFU titer and RT activity, suggesting that PFU reflected the amount of the retrovirus. Also, PFU was well correlated with TCID₅₀, as determined by the CPE of the infected MT-4 cell cultures. Plaque formation was also observed when HTLV-III/LAV-infected cells were used instead of cell-free virus (4).

Interaction between HTLV-III/LAV and MT-4 cells

HTLV-III/LAV-infected cells were washed twice at 5, 15, 30, 60, 90 and 120 min after inoculation of the virus, then cultured for 2 days and examined for antigen-positive cells by IF. Result showed that 60 min incubation with the virus was enough for the virus adsorption to the cells since maximum fluorescence-positive cells were observed at this point (3).

Supernatant fluids from HTLV-III/LAV-infected and -uninfected MT-4 cell cultures were collected every day after inoculation of the virus until 5 days p.i. They were used for the RT activity assay and assessment for the PFU of HTLV-III/LAV. The RT activity peaked on day 4 and 5 p.i., suggesting that maximum amounts of retrovirus(es) were released on these days corresponding to the time when CPE was also observed. Since

the MT-4 cells were an HTLV-I-carrying cell line, the RT activity might indicate the total amounts of both HTLV-I and HTLV-III/LAV. To titer only HTLV-III/LAV quantitatively, we used the HTLV-III/LAV-induced plaque-forming assay. A maximum number of plaques (2.2×10^5 and 3.0×10^5 PFU/ml) was observed on day 4 and 5 p.i., respectively. Thus, the RT activity was parallel to the titers of HTLV-III/LAV which were expressed by PFU. The virus was also titrated by the TCID₅₀ method and was assessed by the appearance of CPE in HTLV-III/LAV-infected MT-4 cells. A correlation between TCID₅₀ and PFU was also observed. All data obtained suggested that retroviral activities released into culture fluids from the HTLV-III/LAV-infected MT-4 cells were mainly ascribable to the progenies of the HTLV-III/LAV (3). Up to now data suggesting neither the release of infectious HTLV-I nor the occurrence of phenotypic mixing between HTLV-I and HTLV-III/LAV have been obtained under such experimental conditions.

HTLV-I-positive cells as the target for HTLV-III/LAV

Subsequent studies were devised to prove or disprove that efficient replication of HTLV-III/LAV and resulting cytotoxicity was generally seen in HTLV-I-positive, but not -negative cells. We used 14 HTLV-I-carrying cell lines including 2 of the simian HTLV-I-related virus-carrying lines as well as 6 HTLV-I-negative T- and B-cell lines (8). Very rapid induction of HTLV-III/LAV antigen was observed in MT-4, TOM-JIM and 467 cell lines at a m.o.i. of 0.63. In these cells between 75% and nearly 100% of cells expressed the antigen of HTLV-III/LAV by 3 days p.i. The second group consisted of MT-2, OIK9, HUT102, HIMI, OIK10, TOM-1, M4C-28 and M12P16 cell lines. This group appeared to be moderately susceptible to the induction of HTLV-III/LAV. Ten to 35% of cell expressed the antigen 3 and 5 days p.i. and almost all cells became positive for HTLV-III/LAV at 8 days. An HTLV-I-negative cell line H9 which had been reported to be the most permissive for HTLV-III/LAV among the virus-free cell lines, possessed comparable susceptibility. A final group consisting of KAN and TCL-As cells allowed much less efficient replication of HTLV-III/LAV. By 8 days after infection less than 0.1% of the cells were induced to synthesize HTLV-III/LAV antigen but they disappeared from the cultures upon further cultivation. HTLV-I-negative cells Molt-4, TALL-1 and Jurkat, were also less permissive of antigen synthesis than the second group of HTLV-I-carrying cell lines described above. The EBV-positive P3HR-1 cells also allowed replication of infected HTLV-III/LAV to some extent but Raji did not under our experimental conditions. When lower m.o.i. was applied for the infection, differences in the susceptibility to HTLV-III/LAV between HTLV-I-positive and -negative cells became more prominent.

Next, we studied the influence of HTLV-III/LAV infection on the survival and growth of some HTLV-I-positive cell lines (8). In all cases, the induction of HTLV-III/LAV antigens and CPE, e.g. enlargement of cell size and the appearance of ghost cells, both most probably due to cell fusion, were observed upon further cultivation, especially in MT-2 cells. No further passage of the cells could be achieved in any HTLV-I bearing cells except those of 2 lines, KAN and TCL-As. Interestingly, these 2 types of cells were resistant to the cytopathic effect of HTLV-III/LAV.

In contrast, HTLV-I-negative but OKT-4-positive cell lines H9, Molt-4, Jurkat and TALL-1 infected by HTLV-III/LAV could be subcultivated serially although they showed transient inhibition of cell growth and cytotoxic response 1 or 2 weeks after infection. The growth of 2 EBV-transformed cell lines was not significantly affected by the HTLV-III/LAV. These cells were studied with an electron microscopy (8). Type-C virus particles were detected in the cytoplasmic vacuoles or extracellularly on the cytoplasmic membranes of occasional cells. These particles were moderately pleomorphic and measured about 60-120 nm in diameter with a central nucleoid. Cytoplasmic disruptions were rarely found in the cells containing a large number of intracytoplasmic viral particles. Infected MT-2 cells were generally formed by multinucleated giant cells. These cells showed marked disruption of the cellular organelles with abundant vacuoles, lysosomal dense granules and several lipid droplets in their cytoplasm. A larger number of viral particles were detected in the cytoplasm of the giant cells than in that of the non-infected cells. Most of these viral particles were 100-140 nm in diameter and had bar-shaped nucleoids or condensed circular, eccentric cores consistent with HTLV-III/LAV.

The possibility that the high susceptibility and preferential cytotoxic response to HTLV-III/LAV infection observed in the HTLV-I-bearing cells might be associated with the gene expression of pXs and gag of HTLV-I was examined. However, we observed no significant relationship between susceptibility to HTLV-III/LAV and expression of resident HTLV-I gag and pXs genomes (8). The 467 cells were highly susceptible to and permissive for HTLV-III/LAV without expressing the pXs antigen of HTLV-I. While KAN and TCL-As cells were not permissive for HTLV-III/LAV, all cells of these lines expressed the pXs and gag proteins.

We also examined the influence of HTLV-III/LAV infection on the gene expression of HTLV-I pXs (9). For this purpose, we used the 3 cell lines, of which 2 (TOM-JIM and 467) were negative for pXs protein by the IF procedure and one (TOM-1) was positive for this antigen at about 8% in all cells. We compared the expression of pXs antigen of HTLV-I pre- and post-infection with HTLV-III/LAV in these 3 cell lines. In 467 and TOM-JIM cells, pXs proteins were not induced to a

detectable level even though HTLV-III/LAV antigen was detected by 5 days after infection in almost all cells. In TOM-1 cells no change in pXs expression could be found although the presence of HTLV-III/LAV antigen was observed.

We then investigated quantitatively by radioimmunoassay whether the susceptibility to HTLV-III/LAV in these cell lines was restricted by OKT-4 molecule expression (9). The amount of OKT-4 molecules correlated excellently with the susceptibility to HTLV-III/LAV. MT-4, TOM-JIM, 467 and M4C28 cells which were most permissive for HTLV-III/LAV, possessed large amounts of OKT-4 molecule. Other cells with moderate susceptibility to HTLV-III/LAV among HTLV-I-positive cell lines such as M12P16, MT-2, OIK9, OIK10, TOM-1 and HUT102 cells also contained comparable amounts of receptors. KURI and OCH cells with lower susceptibility to the virus expressed OKT-4 antigens only slightly. KAN, MT-1 and TCL-As cells that were resistant to HTLV-III/LAV replication contained very small amounts of OKT-4 molecule, or none. Several control T cells were studied to elucidate further the relationship between AIDS virus susceptibility and OKT-4 expression. Interestingly, Molt-4 and TALL-1 cells, which required as long as 2 weeks before viral antigen synthesis became evident, contained large amounts of surface antigens comparable to those of highly susceptible HTLV-I-positive cell lines such as MT-4 and 467 cells. The H9 cells (most susceptible among the HTLV-I-negative lines) contained a moderate amount of OKT-4. Our CCRF-CEM cells which were totally resistant to HTLV-III/LAV replication, contain a very small amount of surface antigens.

Neutralizing antibody to HTLV-III/LAV

Recently, it has reported that neutralizing antibodies (NA) are present in sera from patients with AIDS and individuals exposed to the retrovirus but who are subclinically infected. Low titers of NA were detected using plaque-formation induced by a pseudotype of vesicular stomatitis virus (12), syncytium induction (12) and the reduction of infectivity which was assayed by RT (11) or IF (11). However, more sensitive and simpler assay system are required to evaluate the significance of elevated or low titers of NA and the clinical status of patients and to estimate the effectiveness of antiviral therapy and vaccines which may become available.

MT-4 cells were infected with HTLV-III/LAV which was pre-incubated with 1:80, 1:240, 1:720 or 1:2160 dilutions of four preselected sera. After 5 days incubation, CPE of infected MT-4 cells were measured by ³H-thymidine uptake (the proliferation-assay). This was done because an inhibition of DNA-synthesis in infected MT-4 cells was observed when the CPE was microscopically seen. The linear and reverse relationship between the dose of the virus and the amount of

incorporated radioisotope in infected MT-4 cells suggested that this assay system was useful for measuring the amount of the infective virus. When the virus was treated with anti-HTLV-III/LAV positive sera, the count of ^3H -thymidine represented the intact cell growth of infected MT-4 cells which almost paralleled the amount of biologically neutralized virus after the treatment. Thus, titers of NA were tentatively calculated by ^3H -thymidine uptake induced by 25% amount (75% reduction) of the virus used in this experiment. This was estimated from the control curve of 1/4-diluted virus. Healthy controls showed no or little non-specific reactions with the virus in low dilution ($<1/80$) of their sera. Consequently, NA-titer of $<1:80$ was judged as negative. All sera, including 21 patients with AIDS, 10 individuals with AIDS-related complex (ARC), 20 healthy male homosexuals and 10 healthy male controls, were examined by both IF and the proliferation-assay to compare the titers of IF and NA. Of 61 sera, 28 gave negative reactions in both IF and NA tests, 32 were positive in both tests and one showed 1:40 of IF titer but weak positive ($<1:80$) in the NA-test (5). Titers of NA were significantly correlated with those detected by IF using fixed H9/HTLV-III-cells, as indicated by the high coefficient of correlation ($\gamma=0.64$, $p<0.001$). All individuals who had antibody to HTLV-III/LAV, detected by IF, showed fairly high titers of NA against HTLV-III/LAV, when the proliferation assay was applied.

Serum samples were further analysed for antibodies to HTLV-III/LAV by IF to clarify the serological status regarding HTLV-III/LAV-infection (5). Whereas 19 of 21 (90.5%) of the patients with AIDS, 8 of 10 (80.0%) of the patients with ARC and 5 of 10 (50.0%) of the healthy homosexual males in an endemic area (New York) were positive, 1 of 10 (10.0%) of the healthy homosexual males in non-endemic area (Omaha, Neb) and none of 10 healthy controls were positive. All of the samples from the same individuals were also examined by the proliferation assay using HTLV-III/LAV-infected MT-4 cells for the NA to HTLV-III/LAV. The result shows that without exception samples which were positive by the IF were also positive by the NA-assay. Geometric mean titers (GMT) of NA were about 5 times higher than those detected by IF. Among individuals who had antibodies to HTLV-III, GMTs of NA from patients with ARC and healthy male homosexuals were 1318 and 1009, respectively, which were 2 times higher than that (475) from patients with AIDS. High antibody titers in patients with ARC were also obtained when antibodies to HTLV-III/LAV were titrated by IF.

Effect of heat and fresh human serum on the infectivity of HTLV-III/LAV

HTLV-III/LAV was treated with heat at 56°C for 1, 2.5, 5, 15, 30 and 60 min. The percentage of IF-positive cells in MT-4

cells infected with nontreated virus (0 min) was 12.2% 2 days p.i., whereas the percentage of those infected with the virus heated for 2.5 min was 1.5%. Reduction of viral infectivity after heat treatment for 2.5 min was 88%. Infected MT-4 cells were monitored daily for the appearance of IF-positive cells until 6 days p.i. The sensitivity of detection of the virus became higher, because infected MT-4 cells released virus progeny which infected neighboring uninfected MT-4 cells in the cultures. On day 6 p.i., no IF-positive cells were observed when the virus was treated for 30 min or more. Thus, heating the virus at 56°C for 30 min appeared to be sufficient to inactivate its infectivity (6).

Next, to further confirm virus inactivation by heating, a proliferation assay was used, because this assay was more quantitative and objective than IF (6). The viral dose response was linear when the proliferation assay was performed 5 days p.i. Reduction of viral activity by heating was calculated to be 50 and 90% at 1 and 2.5 min, respectively. Thus, the virus was rapidly inactivated by heating at 56°C. A proliferation assay was also performed 7 days p.i. to detect the effect of a minute amount of living virus. Inhibition of DNA synthesis detected by the proliferation assay was also observed in the MT-4 cells infected with virus treated for 10 min. If both assays (IF and proliferation) were performed on the same day p.i., the sensitivity of the proliferation assay was lower than that of IF, because appearance of the cytopathic effect was always preceded by the production of virus-specific antigen(s).

Fresh serum (mainly complement) did not affect the infectivity of the virus if IF and proliferation assays were performed 2.5 and 5 days p.i., respectively (6). The IF positivity and radioactivity of infected MT-4 cells were again linearly dependent on the amount of virus. Fresh antibody-negative serum was not seen by either method to inactivate the virus. However, anti-HTLV-III/LAV-positive serum used as the positive control neutralized 90% of the virus even at a 1/20 dilution.

Conclusion

AIDS has become increasing threat for many countries. Since the viral etiology of the disease is already established following problems should be studied extensively: [1] Development of the vaccine and the establishment of the test system to evaluate it. [2] Development of drugs that interfere with the growth of virus and virus-infected cells. [3] Change of virus and virus-infected cells during the course of the disease with respect to their amounts and characters. Our newly developed system for the AIDS virus infection provides useful tool for the resolution of above problems confronted.

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