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# Isolation of Virus-producing Transformants from Human Gastric Cancer Cell Line, HGC-27, Infected with Human T-cell Leukemia Virus Type I

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**A human anaplastic gastric cancer cell line, HGC-27, showed marked degeneration with formation of multinucleated syncytia and cell detachment of nearly all cells which began 24 hr after and reached a maximum 2 to 3 days after co-cultivation with X-irradiated MT-2 cells, HTLV-I producing human cord leukocytes. Less severe degeneration without formation of syncytia was also observed in the cultures inoculated with cell-free MT-2 culture media. Morphologically altered cells began to proliferate and formed piled up colonies in some of the cultures co-cultivated with X-irradiated MT-2 cells after a long culture period. The two clones designated HGC/MT2 (Cl-1) and HGC/MT2 (Cl-2) were separated by cell cloning. HGC/MT2 (Cl-1) and HGC/MT2 (Cl-2) cells were positive for HTLV-I *gag* proteins (p19 and p24) and pX gene products, $p40^x$, as demonstrated by immunohistochemistry and immunoblotting analysis, contained HTLV-I provirus DNA, and consistently produced type C virus particles.**

Key words: Human T-cell leukemia virus type I — Transformation — Gastric cancer — Cell line

Human T-cell leukemia virus type I (HTLV-I) is a human retrovirus etiologically associated with human adult T-cell leukemia (ATL), which is endemic in southwest Japan.[1-3] On co-cultivation, HTLV-I is able to infect and immortalize human T cells[4-7] and lymphocytes of animals such as monkey,[8] rabbit,[9] rat,[10] cat[11] and hamster.[12] HTLV-producing cells also induce cell fusion leading to the formation of multinucleated syncytial giant cells when co-cultivated with a wide variety of human and animal non-lymphoid cells.[13-19]

However, productive infection has been restricted to only a small proportion of the infected cell lines.[15] Morphologically transformed cell lines consistently producing HTLV-I have not been reported. In the present study, we examined the effect of HTLV-I-producing human T cells on a human gastric cancer cell line, HGC-27 and succeeded in establishing two HTLV-producing, morphologically transformed clones derived from HGC-27.

## Materials and Methods

**Cells** An HTLV-I-producing lymphoid cell line, MT-2, was established by co-cultivating normal human cord blood lymphocytes with leukemic T-cells from a patient with ATL.[4] TALL-1, a human T-cell acute leukemia cell line not associated with HTLV, was used as a negative control. HGC-27 is a human anaplastic gastric cancer cell line established from a metastatic lymph node.[20] All cultures were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum at 37° in a humidified 5% $CO_2$ atmosphere.

**HTLV-I Infection** HGC-27 cells grown subconfluently in 25 $cm^2$ plastic flasks (Corning 25100) were inoculated with $5\times10^6$ X-irradiated (10,000 R) MT-2 cells or 6 ml of cell-free supernatant fluid from MT-2 cultures. Culture media were exchanged 2 days later, and thereafter the cultures were fed twice a week. In order to examine the clonal difference of susceptibility to HTLV-I infection, 55 clones of HGC-27 cells were isolated in 96-well microtest plates by limiting dilution. Isolated clones were seeded into 24-well plates (Corning 25820) and co-cultivated with $1\times10^6$ X-irradiated MT-2 cells.

**Antisera** Sera from patients with ATL had a titer of 1:1,000 against HTLV-I associated antigens

(HTLV-A). Mouse monoclonal antibodies (MAbs) to HTLV-I *gag* proteins, p19 or p24, were obtained from Seralab Inc. MAb to pX gene products, NCC-pX-1G, supplied by S. Watanabe (Epidemiology Division, National Cancer Center Research Institute), had been produced against a fused protein of a part of pX protein and a bovine growth hormone synthesized in *Escherichia coli*.[21] MAbs to human T-cell antigens (OKT 3 and OKT 11) were obtained from Ortho Diagnostic Systems, Inc., New Jersey. MAbs to human and rat IL-2 receptors were obtained from Becton-Dickinson and Serotec Inc., respectively. Leukocyte common antigens (LCA) and human T-cell antigens were also examined by using MAbs available for paraffin-embedded specimens (anti LCA, DAKO, Copenhagen and MT-1, Bio-Science, Emmenbrücke, respectively). Fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti mouse and human IgG were obtained from Tago, Inc. (Burlingame, California).

**Immunohistochemistry** Monolayer cells were fixed in acetone for 10 min. For indirect immunofluorescence, fixed cells were first treated with a 1:80 dilution of anti-p19, a 1:80 dilution of anti-p24, a 1:1,000 dilution of anti-pX, a 1:20 dilution of anti-human T-cell MAbs, a 1:10 dilution of anti-IL-2, or a 1:100 dilution of ATL patient's sera. After washing of the cells in phosphate-buffered saline, the respective FITC-conjugated anti IgG reagent was added at a 1:10 dilution.

HTLV-I core antigens, pX gene products, LCA, and MT-1 antigens were also examined by the avidin-biotin-peroxidase complex (ABC) method as described by Hsu *et al.*[22] using an ABC Kit (Vector Lab., Burlingame, California).

**Electron Microscopy** Monolayer cells were scraped off with a rubber policeman, fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in an epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and then examined under a Hitachi H-500 electron microscope.

**Southern Blot Hybridization** The assay was performed as described previously.[11] Cellular DNAs were digested with an excess of *Eco*RI (Takara Shuzo, Kyoto), which does not cut the HTLV-I provirus genome internally, and hybridized with probe DNA containing a mixture of proviral fragments which covered the whole genome sequence of HTLV-I (obtained from M. Hatanaka, Institute for Virus Research, Kyoto University).

**Western Blot Analysis** Cells were lysed with extraction buffer (1% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol, 10m*M* tris-HCl, pH 8.0). A soluble extract was electrophoresed on a 12.5% polyacrylamide slab gel containing 0.1% sodium dodecyl sulfate as described by Laemmli.[23] The separated proteins were electrophoretically transferred to nitrocellulose sheets.[24] Polypeptides on the nitrocellulose sheets were then detected by the ABC method using MAbs to HTLV-I p19, p24 or pX proteins and ATL patient's sera as the first antibodies, biotinylated anti-mouse IgG or anti-human IgG as the second antibody, avidin-biotinylated horseradish peroxidase complex as an indicator of bound second antibodies, and diaminobenzidine tetrahydrochloride and hydrogen peroxide as substrates.

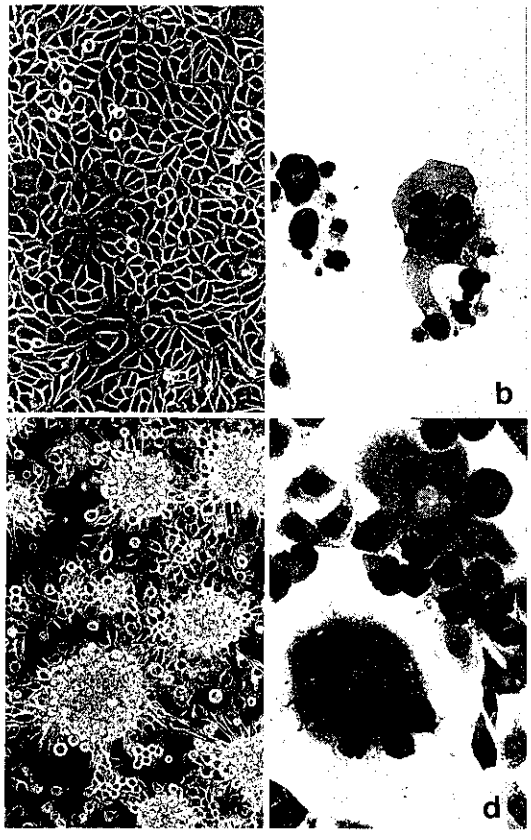
**Cell Cloning** Trypsinized cells were seeded in 21 $cm^2$ plastic dishes (Falcon, 3002) at 50 cells per dish. After 10 to 14 days of incubation, some colonial clones were isolated and expanded by transfer to 24-well tissue culture plates. The expanded cells were recloned by limiting dilution, plating in 96-well microplates (Corning, 25860).

## Results

**Cytopathic Effects (CPE)** HGC-27 cells co-cultivated with X-irradiated MT-2 cells showed marked degeneration beginning 24 hr after co-cultivation and reaching a maximum 2 to 3 days later. Almost all cells became small and round-shaped and were detached from the culture vessels. Some of the remaining cells formed multinucleated syncytia containing between 4 and 12 nuclei (Fig. 1b). No CPE were observed in HGC-27 cells co-cultivated with control TALL-1 cells or infected with virus fractions sedimented from MT-2 culture fluid. HTLV-A, HTLV-I *gag* proteins (p19 and p24), and pX gene products ($p40^x$) were detected in all of the surviving HGC-27 cells including multinucleated syncytia by indirect immunofluorescence and the ABC method when inoculated with MT-2 cells but not when inoculated with MT-2 culture fluid. All of the 55 clones isolated from HGC-27 cells were susceptible to CPE induced by co-cultivation with MT-2 cells.

Morphologically altered cells began to proliferate, forming piled up colonies, in some of the cultures co-cultivated with X-irradiated MT-2 cells after a long culture period. Two clones were separated from such morphologically transformed cells by cell cloning and designated HGC/MT2 (Cl-1) and HGC/MT2 (Cl-2).

**Characterization of Morphologically Transformed Clones of HGC-27 Cells Infected with HTLV-I** The cells of HGC/MT2 (Cl-1) and HGC/MT2 (Cl-2) showed a growth pattern




quite different from that of the original HGC-27 cells, which were short spindle-shaped or polygonal and grew forming a monolayer (Fig. 1a). They were loosely arranged, and formed piled up colonies with a tendency to be detached in the old cultures (Fig. 1c). The shape of the cells became rounder, and many multinucleated giant cells were observed (Fig. 1d). Ultrastructurally, HGC/MT2 (Cl-2) cells showed more irregularly outlined cell boundaries with outstanding formation of microvilli or slender cytoplasmic processes compared with the smooth surfaces of the original HGC-27 cells. The nuclei had smooth nuclear envelopes, prominent nucleoli, and abundant euchromatin. The cytoplasm contained abundant mitochondria and ribosomes

Fig. 1. a. Phase contrast micrograph of normal HGC-27 cells. ×70. b. HGC-27 cells co-cultivated with X-irradiated MT-2 cells, 43 hr after the start of coculture. Note the multinucleated syncytia and degenerated cells. Giemsa stain. ×180. c. Phase contrast micrograph of HGC/MT2 (Cl-2) cells. Note the many piled-up cell clusters. ×70. d. HGC/MT2 (Cl-2) cells. Note the multinucleated syncytia. Giemsa stain. ×270.

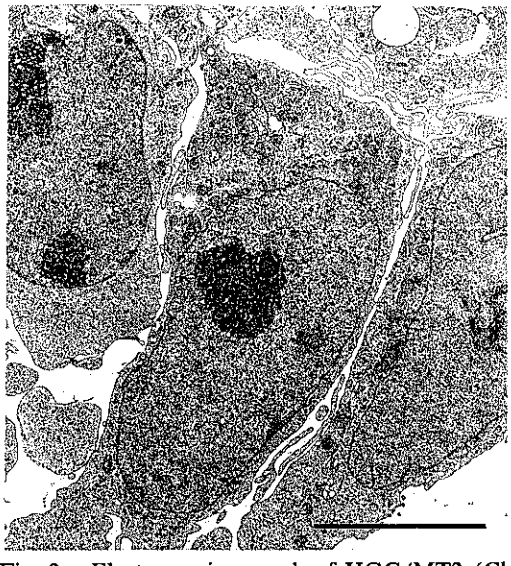


Fig. 2. Electron micrograph of HGC/MT2 (Cl-2) cells. The nuclei have prominent nucleoli and abundant euchromatin. The cytoplasm contains abundant mitochondria and ribosomes. In this area, no virus particles were observed. The bar represents 5 $\mu$m.

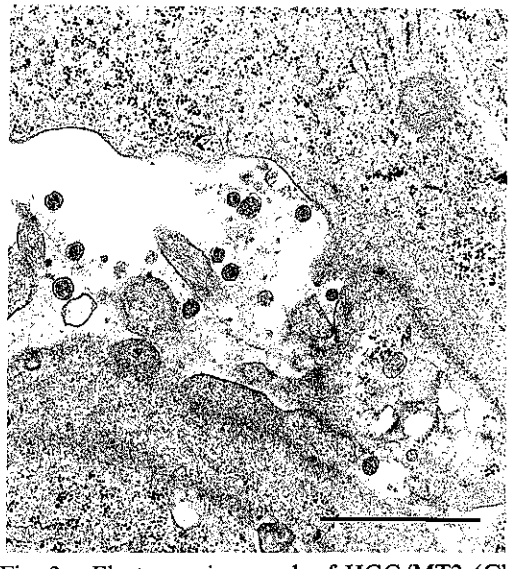


Fig. 3. Electron micrograph of HGC/MT2 (Cl-2) cells. Many type C virus particles with variable sizes were observed in the extracellular space. The bar represents 1 $\mu$m.

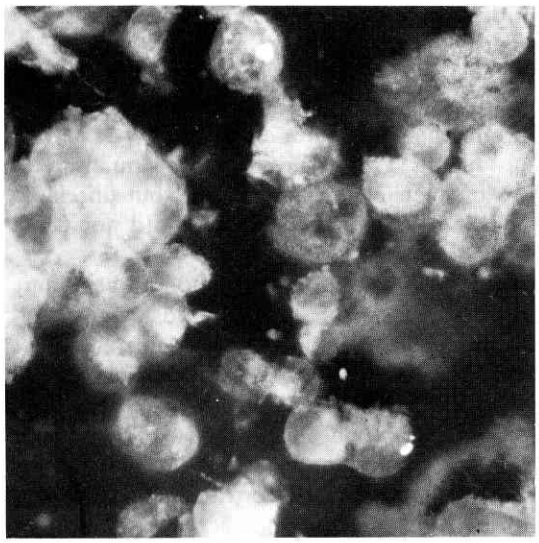


Fig. 4. HTLV-I-associated antigens (HTLV-A) of HGC/MT2 (Cl-2) cells demonstrated by indirect immunofluorescence using ATL patient's sera. ×340.

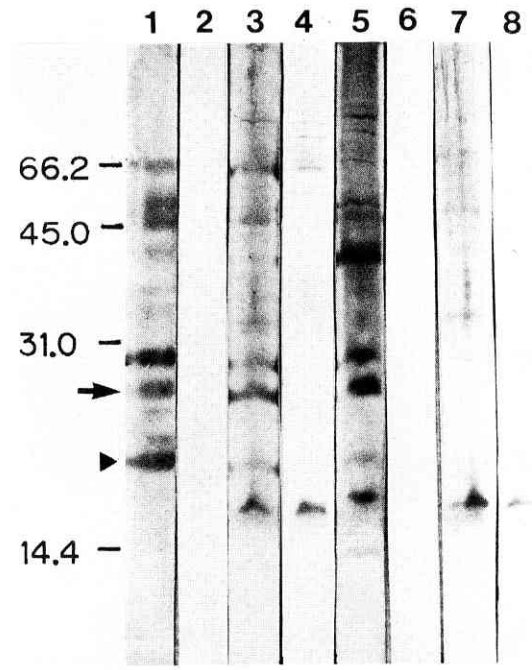



Fig. 5. Western blot analysis of proteins recognized by ATL patient's sera in the cell extracts. Lanes 1 and 2, MT-2; lanes 3 and 4, HGC/MT2 (Cl-1); lanes 5 and 6, HGC/MT2 (Cl-2); lanes 7 and 8, normal HGC-27. Lanes 1, 3, 5, and 7, ATL patient's sera; lanes 2, 4, 6, and 8, control normal human sera. The arrow and arrowhead indicate p24 and p19, respectively.

mostly taking the form of polysomes, but the endoplasmic reticulum was inconspicuous. Desmosomes, secretory granules, and intracytoplasmic canaliculi were never observed, these morphological features being compatible with those of HGC-27 cells (Fig. 2). In the extracellular spaces, some type C virus particles with variable sizes were observed (Fig. 3). These cultures were not contaminated with mycoplasma.

Human T-cell antigens (OKT 3, OKT 11, MT-1), LCA, and human IL-2 receptor antigen were not detected.

**Detection of HTLV-I-related Antigens** Almost 100% of HGC/MT2 (Cl-2) cells and 10–20% of HGC/MT2 (Cl-1) cells were positive for HTLV-A, HTLV-I *gag* proteins (p19 and p24), and pX gene products ($p40^{x}$) as demonstrated by indirect immunofluorescence and the ABC method (Fig. 4). Western blot analysis using ATL patient's sera revealed that p19 and p24 were present in the extracts of the cells from both cell lines (Fig. 5), and this was also demonstrated by using MAbs to p19 and p24. MAbs to pX gene products revealed the p40 band.

**Detection of Proviral DNA** Southern blot hybridization using DNA probe containing a whole genome of HTLV-I revealed that *Eco*RI digests of HGC/MT2 (Cl-1) and HGC/MT2 (Cl-2) cellular DNAs contained hybridizable fragments. The sizes of fragments were approximately 21.7 kilobases (kb) for HGC/MT2 (Cl-1) line and 20.8 and 19.0 kb for HGC/MT2 (Cl-2) line (Fig. 6).

## Discussion

HGC-27 cells severely degenerated and were detached from culture vessels shortly after co-cultivation with HTLV-I-producing MT-2 cells, forming multinucleated syncytia. Syncytia induction by HTLV-I has been described for other human and animal non-lymphoid cells.[13-19] This phenomenon is thought to be virus-dependent, presumably being mediated by viral envelope glycoproteins, because syncytia formation was inhibited by the addition of ATL patient's

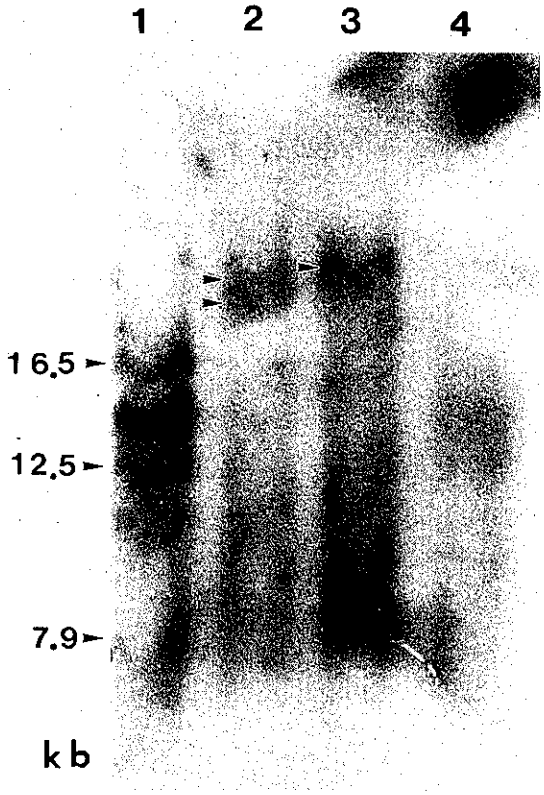



Fig. 6. Detection of HTLV-I provirus genome in HGC/MT2 (Cl-1) and HGC/MT2 (Cl-2) cells. *Eco*RI digests of cellular DNAs were analyzed by Southern blot hybridization. Lane 1, MT-2,; lane 2, HGC/MT2 (Cl-2); lane 3, HGC/MT2 (Cl-1); lane 4, HGC-27. Arrows indicate the hybridizable bands.

sera[14-18] which contained antibodies reacting against HTLV-I *env* gene products, gp46 and gp68.[25]

Marked degeneration with cell detachment was a prominent feature in the cultures of HGC-27 cells co-cultivated with MT-2. Human endothelial cells infected with HTLV-I showed similar degenerative alterations which appeared much later.[17] Cell detachment was also induced by the addition of cell-free MT-2 culture media. However, the degree of degeneration was less severe, and syncytia formation and expression of HTLV-I related antigens did not occur. Virus fractions sedimented from MT-2 culture media by ultracentrifugation did not have CPE-inducing capacity, but virus-free supernatant induced CPE. These results strongly suggest that CPE may not be induced by HTLV-I virions themselves, but may be mediated by a kind of lymphokine produced by HTLV-I-producing T-cell lines because other HTLV-I-producing T-cell lines showed similar cytotoxic effects of variable degree, but unrelated cell lines including T-cell lines did not (data not shown).

A wide variety of non-lymphoid cells may be permissive for HTLV-I adsorption and penetration, resulting in the formation of multinucleated syncytia, but establishment of cell lines producing HTLV-I virions continuously has rarely been achieved.[13] The amount of virus production might be limited and not be sufficient for effective transmission to non-infected new cells. Productive infection of HGC-27 cells was only achieved by co-cultivation with MT-2 cells, but not with cell-free culture media. Effective infection may be achieved by direct association or intimate contact with HTLV-I producing T-cells, but not via heterokaryons by cell fusion between target cells and HTLV-I producing cells, because the transformed cells did not have human T-cell markers and Tac antigens.

It may be argued that morphologically altered cells were obtained by the selection of infected HGC-27 cells resistant to CPE induced by MT-2 cells which were present in the cultures from the first. However, this may not be the case, because HGC/MT2 (Cl-2) cells were also susceptible to CPE by MT-2 cells (data not shown), and selected clones from HGC-27 cells were all susceptible to CPE. Some of the clones showed the regrowth of morphologically altered cells together with variable numbers of syncytia.

HGC/MT2 is an adherent cell line continuously producing HTLV-I virions and is easy to manipulate, so it may prove to be a useful tool for elucidation of the function of the HTLV-I genome.

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