**DETECTION OF ADULT T-CELL LEUKEMIA-ASSOCIATED
ANTIGENS BY DIRECT IMMUNOPEROXIDASE MICRO-
SCOPY WITH Fab'-PEROXIDASE CONJUGATES
PREPARED WITH A MALEIMIDE COMPOUND¹**

HIROSHI SUMII, SEIKO WATANABE AND TAKUZO ODA²

*Department of Biochemistry, Cancer Institute, Okayama University
Medical School, 2-5-1 Shikata-cho, Okayama 700*

Received for publication December 16, 1986 and in revised form January 19, 1987

Direct immunoperoxidase staining was applied to the detection of adult T-cell leukemia (ATL)-associated antigens (ATLA) in human T-cell leukemia virus (HTLV-I)-producing MT-2 cells. We successfully conjugated Fab' to peroxidase using a maleimide compound to perform highly sensitive immunohistochemistry with a low background and specific binding. Antisera from three healthy carriers containing antibodies reacting with ATLA were analyzed by Western blotting. ATLA was observed at the light and electron microscopic levels. Positive staining of ATLA was demonstrated in the plasma membrane, endoplasmic reticulum, nuclear membrane, and viral particles, but not in the nucleus nor the mitochondria in frozen-sectioned specimens of MT-2 cells fixed with periodate-lysine-paraformaldehyde (PLP) fixative. Control cells (Molt-4) were not stained. The staining in MT-2 cells and HTLV-I represents the distribution of the viral antigens or their precursors.

Adult T-cell leukemia (ATL) is recognized as being endemic in the southwest area of Japan. The ATL virus (ATLV) has been demonstrated in human T-cell lines derived from patients with ATL (4, 12, 28), and human T-cell leukemia virus (HTLV) has been isolated from patients with cutaneous T-cell lymphoma (17, 18). ATL has been shown to be identical or closely related to HTLV (19, 22, 25), and is now termed human T-cell leukemia virus type I (HTLV-I). Adult T-cell leukemia (ATL)-associated antigens (ATLA) was detected by indirect immunofluorescence with sera from ATL patients and healthy carriers in MT-1 (4), and MT-2 (12) cell lines and in short-term cultured cells from peripheral lymphocytes of ATL patients (1, 5). Type-C virus particles in these cells were detected by electron microscopy (1, 4, 12, 15). Adult T-cell leukemia virus (ATLV)-specific antibodies in sera from ATL patients and healthy carriers have been determined by the immunoprecipitation analysis (26, 27). ATLA in HTLV-I-producing MT-2 cells have been detected by electron microscopy using the immunoferritin method (2, 13, 15, 16). Indirect immunoperoxidase staining of ATLA with sera of ATL

¹ Supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

² To whom correspondence should be addressed.

patients, the monoclonal antibody to ATLV p19 and p28 (14, 23), and antisera to p24 in frozen-sectioned MT-2 cells were observed in our previous study (14).

Recently, a mild and efficient procedure for conjugating Fab' to peroxidase using a maleimide compound was developed for the labeling of antibodies in enzyme immunoassay and immunohistochemistry (6). In an attempt to determine the precise location of ATLA in more detail at the light and electron microscopic levels, we studied ATLA in HTLV-I-producing MT-2 cells by direct immunoperoxidase staining using Fab' conjugated to peroxidase with a maleimide compound.

MATERIALS AND METHODS

Cells

An ATLA-positive MT-2 cell line, established from normal human cord lymphocytes that had been co-cultivated with leukemia cells from a patient with ATL, was used (12). An ATLA-negative cell line, Molt-4, was used as a control cell line (11). Both cell lines were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum and fed twice a week.

Antisera

Antisera against ATLA were obtained from healthy carriers with moderate anti-ATLA titers. All these sera were titrated against MT-2 cells by indirect immunofluorescence (4). Anti-ATLA negative sera were used as control sera. The demonstration of ATLA reacted with the anti-sera was performed by Western blot analysis (24). For this procedure, cells were lysed with extraction buffer (0.14 M NaCl, 3 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-HCl (pH 8.0), 0.5% Nonidet P-40) and electrophoresed on a 12.5% polyacrylamide-sodium dodecyl sulfate (SDS) slab gel (SDS-PAGE) (9). The ATLA was detected by the enzyme antibody method using the avidin-biotin complex system (Vectastain Co.).

Preparation of Fab'-peroxidase conjugates with a maleimide compound

Immunoglobulin G (IgG) was obtained by precipitation of antisera with 50%-(NH₄)₂SO₄ saturated followed by chromatography on a dimethylaminoethyl (DEAE) cellulose (Pharmacia Fine Chemicals AB, Uppsala) column equilibrated with 0.01 M sodium phosphate buffer (pH 7.2). F(ab')₂ was prepared by digestion of the IgG with pepsin (Sigma, 2×cryst., (1:60,000)) and separation on a Sephacryl S-200 (Pharmacia Fine Chemicals) column. Fab' was prepared by the reduction of the F(ab')₂ with 2-mercaptoethylamine (Nakarai Chemicals Ltd., Kyoto) at 37°C for 90 min. The Fab' was conjugated to horseradish peroxidase (HRP) (EC 1.11.1.7) (Toyobo Ltd., Tokyo (grade 1-C)) using a maleimide compound (6). HRP (6 mg/ml) in 0.1 M phosphate buffer (pH 7.0) was incubated with an N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl) maleimide (CHM) (Zieben Chemicals Co., Tokyo) at 30°C for 60 min to introduce maleimide groups to the HRP, and subjected to gel filtration on a column of Sephadex G-25 using 0.1 M sodium phosphate buffer (pH 6.0). Then, the maleimide-HRP was allowed to react with thiol groups of Fab' at 4°C for 16 hr. The conjugate was separated from unbound HRP and Fab' by gel filtration with a Sephacryl S-200 column. The amount of concentration of Fab' and HRP were calculated from the absorbance at 280 nm and 403 nm, respectively.

Immunoperoxidase staining for light microscopy

Cells were washed with PBS (0.01 M phosphate-buffer saline, pH 7.2), and fixed with acetone for 10 min at 4°C or PLP (0.01 M periodate-0.075 M lysine-2% paraformaldehyde (PFA)-0.035 M phosphate buffer, pH 6.2) (10) at 4°C for 1 hr. The fixed cells were pretreated in 1 : 10 diluted normal human serum for 30 min at 37°C, and then incubated with the Fab'-peroxidase conjugates in PBS containing 1% BSA (bovine serum albumin) for 60 min at 37°C in a moist chamber. After washing with PBS, they were incubated in Graham-Karnovsky's substrate solution containing 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% H₂O₂ in 0.05 M Tris-HCl buffer, pH 7.6 (3) for 10 min at 37°C.

Immunoperoxidase staining for electron microscopy

For detecting antigens on the cell surface by electron microscopy, the cells were washed with PBS, fixed with PLP, and pretreated with 1 : 10 diluted normal human serum in cell suspension as described above. Following this, they were incubated with the Fab'-peroxidase conjugates overnight at 4°C, washed with PBS, postfixed with 1% glutaraldehyde and then incubated in the Graham and Karnovsky's solution for 5 min at room temperature. After fixing with 2% OsO₄ for 1 hr, they were dehydrated in a graded ethanol series and embedded in epoxy resin.

For detecting intracellular antigens, frozen-sectioned specimens were prepared as follows. Cells were washed in PBS, pelleted by centrifugation, and fixed with PLP at 4°C for 1 hr. The pellets were washed gradually with PBS containing 10, 15 and 20% sucrose, and immersed in 20% sucrose plus 5% glycerol and then in OCT compound (AMES Co.). The specimens were frozen-sectioned and mounted on albumin-coated slide glasses, and dried. The specimens were washed with PBS and incubated with Fab'-HRP conjugates at 4°C overnight. After the immunological reaction, the specimens were washed with PBS, fixed in 1% glutaraldehyde, washed with PBS and incubated in diaminobenzidine tetrahydrochloride (DAB) solution without H₂O₂ (pre-Graham-Karnovsky's solution) for 30 min and then in DAB solution with H₂O₂. After the reaction, the specimens were fixed with 2% OsO₄, dehydrated in ethanol and propylene oxide, and embedded in epoxy resin on slides. The embedded specimens were removed from the slides by brief heating, ultrathin-sectioned, and examined with a Hitachi H-700 transmission electron microscope.

RESULTS

Antisera

The sera from healthy carriers (HC) gave titers of 1 : 40 (HC-1), 1 : 40 (HC-2), 1 : 160 (HC-3), respectively, as determined by indirect immunofluorescence with MT-2 cells (Table 1). The three sera reacted specifically with three polypeptides with molecular weights of 68,000 (gp68), 46,000 (gp46), and 24,000 (p24) in MT-2 cell extracts. As shown in Table 1 and Fig. 1, three polypeptide bands of gp68, gp46, and p24 were specifically observed on reaction with HC-1, HC-2, and HC-3 in Western blotting. In the negative control, Molt-4 cell extract, none of the three bands were detected with the sera (Fig. 1).

Light microscopy of immunoperoxidase staining

Immunoperoxidase staining with Fab'-peroxidase conjugates prepared with

TABLE 1. *The reactivity of sera from HC with ATLA: indirect immunofluorescence titers and Western blotting analysis*

Serum	Titer of anti-ATLA	Reactivity of sera with ATLA by Western blotting analysis		
		gp 68	gp 46	p 24
HC-1	40	+	+	++
HC-2	40	+	+++	++
HC-3	160	+++	+	+++

HC: healthy carriers

ATLA: adult T-cell leukemia-associated antigens

FIG. 1. Western blotting analysis of ATLA with antisera from healthy carriers (HC-1, HC-2, and HC-3) and sera from ATL patients. Cell extracts of MT-2 and Molt-4 cells were separated by 12.5% SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose paper. In immunostaining, the antisera (HC-1, -2, -3) reacted specifically with gp68, gp46, and p24. Molecular weight markers are indicated on the right-hand side (daltons).

FIGS. 2a-d. Immunoperoxidase staining with Fab'-peroxidase conjugates of MT-2 and Molt-4 cells fixed with acetone at the light microscopic level. Cells: MT-2 cells (a, b, c), Molt-4 cells (d). Antisera: HC-1 (a), HC-2 (b, d), HC-3 (c). $\times 330$

FIGS. 3a-d. Immunoperoxidase staining with Fab'-peroxidase conjugates of MT-2 and Molt-4 cells fixed with PLP at the light microscopic level. Cells: MT-2 cells (a, b, c), Molt-4 cells (d). Antisera: HC-1 (a, d), HC-2 (b), HC-3 (c). $\times 300$

FIGS. 4a-d. Immunoelectron micrographs of staining of MT-2 and Molt-4 cells fixed with PLP and incubated in cell suspension with Fab'-peroxidase conjugates. Cells: MT-2 cells (a, b, c), Molt-4 cells (d). Antisera: HC-1 (a), HC-2 (b), HC-3 (c, d). $\times 22,000$ Bars = 1 μm

FIGS. 5a-c. Immunoelectron micrographs of staining of MT-2 and Molt-4 cells fixed with PLP, frozen-sectioned and incubated with Fab'-peroxidase conjugates prepared with serum of a healthy carrier (HC-1). Cells: MT-2 cells (a, b), Molt-4 cells (c). N: nucleus. a, $\times 6,600$; b, $\times 27,000$; c, $\times 6,500$. Bars (a, c) = $1\ \mu\text{m}$. Bar (b) = $0.5\ \mu\text{m}$.

FIGS. 6a-c. Immunoelectron micrographs of staining of MT-2 and Molt-4 cells fixed with PLP, frozen-sectioned and incubated with Fab'-peroxidase conjugates prepared with serum of a healthy carrier (HC-2). Cells: MT-2 cells (a, b), Molt-4 cells (c). N: nucleus. a, $\times 6,000$; b, $\times 59,000$; c, $\times 7,900$. Bars (a, c) = $1\ \mu\text{m}$. Bar (b) = $0.1\ \mu\text{m}$.

FIGS. 7a-c. Immunoelectron micrographs of staining of MT-2 and Molt-4 cells fixed with PLP, frozen-sectioned and incubated with Fab'-peroxidase conjugates prepared with serum of a healthy carrier (HC-3). Cells: MT-2 cells (a, b), Molt-4 cells (c). N: nucleus. a, $\times 8,000$; b, $\times 17,200$; c, $\times 7,900$. Bars = $1\ \mu\text{m}$.

the sera from healthy carriers showed a positive reaction by light microscopy in all the MT-2 cells fixed with acetone (Fig. 2) and PLP (Fig. 3). Positive staining was observed at the periphery of MT-2 cells and diffusely in the cytoplasm of the cells fixed with acetone and PLP. Control cells, Molt-4 cells, were not stained (Figs. 2, 3). Immunoperoxidase staining of MT-2 cells with Fab'-peroxidase conjugates prepared with control sera was negative.

Electron microscopy of immunoperoxidase staining

The surface of HTLV-I particles and the plasma membrane of MT-2 cells fixed in suspension with PLP were positively stained with HC-1, HC-2, and HC-3 Fab'-peroxidase conjugates (Fig. 4). The plasma membranes were stained diffusely for the most part, but as patches in some places. Intracisternal HTLV-I and the adjacent cisternal membranes were also positive. In the control Molt-4 cells fixed in suspension with PLP, plasma membranes were not stained with the Fab'-peroxidase conjugates. On the other hand, MT-2 cells frozen-sectioned and incubated with Fab'-peroxidase conjugates were positively stained on the cisterna of the endoplasmic reticulum, and the outer and inner leaflets of nuclear membranes (Fig. 5b), but not in the nucleus nor mitochondria (Figs. 5-7). The plasma membranes, the intracisternal HTLV-I, and adjacent cisternal membranes were also positive. However, the preservation of the ultrastructural morphology of the periphery of the cells could not always be obtained in the frozen-sectioned specimens. The reaction on the plasma membrane and intracellular membranes differed from case to case. Among the sera from three healthy carriers (HC), the immunostaining of the nuclear envelope and the endoplasmic reticulum was strongest in HC-3, in which the immunostaining of gp68 was strongest by Western blotting (Fig. 7). However, the difference of the sites stained with HC-1, HC-2, and HC-3 could not be demonstrated clearly. Endogenous peroxidase staining was not observed in the MT-2 cells under the present conditions of the reaction. In the Molt-4 cells, no reaction was detected on the plasma membranes or in the cytoplasm.

DISCUSSION

Although immunofluorescence is useful for the detection of ATLA positive cells and for the titration of anti-ATLA positive serum, detailed distribution of ATLA in cells is not revealed by this method. Electron microscopy with the immunoferritin method revealed that the ferritin-labeled antibodies could not penetrate the cellular and viral membranes (2, 13, 15, 16). Indirect immunoperoxidase staining has been employed for the observation of the detailed distribution of ATLA in MT-2 cells and HTLV-I (14).

In our previous study (14), the location of ATLA in HTLV-I-producing MT-2 cells was observed by light and electron microscopy with the indirect immunoperoxidase method using sera of ATL patients, the monoclonal antibody to HTLV p19 and p28, and antisera to p24. ATLA was demonstrated on some areas of the cisternal membrane of the endoplasmic reticulum and the nuclear membrane, but not in the nucleus. Staining with the monoclonal antibody to HTLV p19 and p28 and antisera to p24 was observed in the viral core and envelope and some parts of plasma membranes adjacent to virus clusters.

Recently, a mild and efficient conjugation of Fab' to peroxidase using

a maleimide compound was developed for enzyme immunoassay and immunohistochemistry (6). The Fab'-peroxidase conjugates prepared with the maleimide compound allowed a more sensitive immunohistochemistry, with a low background and highly specific binding, than conjugates prepared by the glutaraldehyde method (6). Since the conjugates were less polymerized by the maleimide compound, the conjugates easily penetrated the cytoplasm and reached the intracellular organelles in the frozen-sectioned specimens.

In an attempt to clarify the distribution of ATLA in MT-2 cells and HTLV-I in more detail, direct immunoperoxidase staining with Fab'-peroxidase conjugates prepared with a maleimide compound was successfully employed in this study. The sera of healthy carriers were examined by radioimmunoprecipitation for various species of antibodies to ATLA including the envelope glycoproteins (gp68 and gp46) and internal structural proteins (p24 and p19) (7, 8, 20, 21, 27). In the present study, specific antibodies to ATLA were detected by Western blotting analysis (24). Various ATLA in MT-2 cells have been detected as gp68, gp46, and p24 in the cell extract and HC-1, -2, and -3 possessed antibodies to p24, gp46, and gp68 of ATLA.

By this direct immunoperoxidase staining with sera from ATL healthy carriers, the plasma membranes, the cisternae of the endoplasmic reticulum, the inner and outer leaflets of the nuclear membranes and the virus particles were successfully stained. The nuclear chromatin and mitochondria, however, were not stained. Molt-4 cells were not stained in the control experiments. In our previous study, immunoperoxidase staining with the antisera to p24 and with monoclonal antibody to HTLV p19 and p28 was not observed on the inner and outer leaflets of the nuclear membrane (14). In this study, the immunoperoxidase staining was demonstrated for the first time on the both inner and outer leaflets of the nuclear membrane using the direct immunoelectron microscopy with sera from ATL healthy carriers. It is therefore possible to assume the two mechanisms of the staining on both inner and outer leaflets of the nuclear membrane. First, the antibodies to ATLA, except for p19 and p28, and p24, gave the staining of the nuclear membrane. Second, Fab'-peroxidase conjugates prepared with a maleimide compound could penetrate deeply and reacted the ATLA on the nuclear membrane. Since gp46 is a envelope protein of HTLV-I (27), the immunostaining of the viral envelope and the plasma membrane is supposed to be due to gp46. Thus, the immunostaining of the nuclear envelope and the endoplasmic reticulum in MT-2 cells is deduced to be due to gp68. In fact, the immunostaining of the nuclear envelope and the endoplasmic reticulum was strongest with HC-3, in which the immunostaining of gp68 by Western blotting was strongest among sera from three healthy carriers.

However, in order to clarify the specific location of each ATLA in more detail, it will be necessary to examine direct immunoperoxidase staining by electron microscopy using Fab'-peroxidase conjugates prepared with a maleimide compound using monospecific polyclonal and monoclonal antibodies. The biological significance of the distribution of various components of ATLA has to be elucidated in relation to the mechanism of leukemogenesis by this retrovirus.

ACKNOWLEDGEMENTS

We are grateful to Professor Gozo Tanabe, Department of Orthopedic Surgery,

Okayama University Medical School, for giving his kind guidance and encouragement to H. Sumii. We also thank Dr. Isao Miyoshi for generously providing MT-2 cells, Dr. Taiji Kunitomi for Molt-4 cells, Dr. Kanji Miyamoto for antisera from healthy HTLV-I carriers, Dr. Koichi Kitajima for sera from ATL patients. The skillful technical assistance of Mr. Takashi Nakamura and Dr. Yusei Arakaki is kindly acknowledged.

REFERENCES

1. Akagi, T., Ohtsuki, Y., Takahashi, K., Kubonishi, I. and Miyoshi, I.: Detection of type C virus particles in short-term cultured adult T-cell leukemia cells. *Lab. Invest.* 47; 406-408, 1982.
2. Aoki, T., Hamada, C., Ohno, S., Miyakoshi, H., Koide, H., Robert-Guroff, M., Ting, R. C. and Gallo, R. C.: Location of human T-cell leukemia virus (HTLV) p19 antigen on virus-producing cells. *Int. J. Cancer* 33; 161-165, 1984.
3. Graham, R. C. and Karnovsky, M. J.: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14; 291-302, 1966.
4. Hinuma, Y., Nagata, K., Hanaoka, M., Nakai, M., Matsumoto, T., Kinoshita, K., Shirakawa, S. and Miyoshi, I.: Adult T-cell leukemia: Antigens in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA* 78; 6476-6480, 1981.
5. Hinuma, Y., Komoda, H., Chosa, T., Kondo, T., Kohakura, M., Takenaka, T., Kikuchi, M., Ichimaru, M., Yunoki, K., Sato, I., Matsuo, R., Takiuchi, Y., Uchino, H. and Hanaoka, M.: Antibodies to adult T-cell leukemia-virus-associated antigen (ATLA) in sera from patients with ATL and controls in Japan: a nation-wide seroepidemiologic study. *Int. J. Cancer* 29; 631-635, 1982.
6. Imagawa, M., Yoshitake, S., Hamaguchi, Y., Ishikawa, E., Niitsu, Y., Urushizawa, I., Kanazawa, R., Tachibana, S., Nakazawa, N. and Ogawa, H.: Characteristics and evaluation of antibody-horseradish peroxidase conjugates prepared by using a maleimide compound, glutaraldehyde, and periodate. *J. Appl. Biochem.* 4; 41-57, 1982.
7. Kalyanaraman, V. S., Sarngadharan, M. G., Nakao, Y., Ito, Y., Aoki, T. and Gallo, R. C.: Natural antibodies to the structural core protein (p24) of the human T-cell leukemia (lymphoma) retrovirus found in sera of leukemia patients in Japan. *Proc. Natl. Acad. Sci. USA* 79; 1653-1657, 1982.
8. Kalyanaraman, V. S., Jarvis-Morar, M., Sarngadharan, M. G. and Gallo, R. C.: Immunological characterization of the low molecular weight gag gene proteins p19 and p15 of human T-cell leukemia-lymphoma virus (HTLV) and demonstration of human natural antibodies to them. *Virology* 132; 61-70, 1984.
9. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of the bacteriophage T₄. *Nature* 227; 680-685, 1970.
10. McLean, I. W. and Nakane, P. K.: Periodate-lysine-paraformaldehyde fixative, a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22; 1077-1083, 1974.
11. Minowada, J., Ohnuma, T. and Moore, G. E.: Rosette-forming human lymphoid cell lines. 1. Establishment and evidence for origin of thymus-derived lymphocytes. *J. Natl. Cancer Inst.* 49; 891-895, 1972.
12. Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraiishi, Y., Nagata, K. and Hinuma, Y.: Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T-cells. *Nature* 294; 770-771, 1981.
13. Nakai, M., Goto, T., Miyoshi, H., Sano, K., Chosa, T. and Hinuma, Y.: Reactivity of sera from patients with adult T-cell leukemia (ATL) with type C virus particles associated with ATL cell lines: immunoelectron microscopic study. *Gann* 73; 511-513, 1982.
14. Oda, T., Watanabe, S., Arakaki, Y., Hatsushika, M., Sumii, H., Nakamura, T., Suma, F. and

- Hisatake, K.: Immunoelectron microscopy of adult T-cell leukemia-associated antigen by the peroxidase-labeled antibody method. *Cell. Mol. Biol.* 31; 49-56, 1985.
15. Ohtsuki, Y., Akagi, T., Takahashi, K. and Miyoshi, I.: Detection of antibodies to adult T-cell leukemia (ATL)-associated virus in ATL patients' sera by immunoelectron microscopy. *J. Cancer Res. Clin. Oncol.* 106; 240-243, 1983.
 16. Ohtsuki, Y., Miyoshi, I., Taguchi, H., Takeda, I. and Akagi, T.: Immunoelectron microscopic study on the cross-reactivity of antibodies to adult T-cell leukemia-associated antigens in human and monkey sera. *Acta Pathol. Jpn.* 35; 863-870, 1985.
 17. Poiesz, B. J., Ruscetti, F. W., Gazdar, A. F., Bunn, P. A., Minna, J. D. and Gallo, R. C.: Detection and isolation of type-C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA* 77; 7415-7419, 1980.
 18. Poiesz, B. J., Ruscetti, F. W., Reiz, M. S., Kalyanaraman, V. S. and Gallo, R. C.: Isolation of a new type C retrovirus (HTLV) in primary uncultured cells of a patient with S'ezary T-cell leukemia. *Nature* 294; 268-271, 1981.
 19. Popovic, M., Reiz Jr, M. S., Sarngadharan, M. G., Robert-Guroff, M., Kalyanaraman, V. S., Nakao, Y., Miyoshi, I., Minowada, J., Yoshida, M., Ito, Y. and Gallo, R. C.: The virus of Japanese adult T-cell is a member of the human T-cell leukemia virus group. *Nature* 300; 63-66, 1982.
 20. Robert-Guroff, M., Fahey, K. A., Maeda, M., Nakao, Y., Ito, Y. and Gallo, R. C.: Identification of HTLV p19 specific natural human antibodies by competition with monoclonal antibody. *Virology* 122; 297-305, 1982.
 21. Schneider, J., Yamamoto, N., Hinuma, Y. and Hunsmann, G.: Sera from adult T-cell leukemia patients react with envelope and core polypeptides of adult T-cell leukemia virus. *Virology* 132; 1-11, 1984.
 22. Seiki, M., Hattori, S., Hirayama, Y. and Yoshida, M.: Human adult T-cell leukemia virus: Complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proc. Natl. Acad. Sci. USA* 80; 3618-3622, 1983.
 23. Tanaka, Y., Koyanagi, Y., Chosa, T., Yamamoto, N. and Hinuma, Y.: Monoclonal antibody reactive with both p28 and p19 of adult T-cell leukemia virus-specific polypeptides. *Gann* 74; 327-330, 1983.
 24. Towbin, H., Staehelin, T. and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76; 4350-4353, 1979.
 25. Watanabe, T., Seiki, M. and Yoshida, M.: Retrovirus terminology. *Science* 222; 1178, 1983.
 26. Yamamoto, N. and Hinuma, Y.: Antigens in an adult T-cell leukemia virus-producer cell line. Reactivity with human serum antibodies. *Int. J. Cancer* 30; 289-293, 1982.
 27. Yamamoto, N., Schneider, J., Koyanagi, Y., Hinuma, Y. and Hunsmann, G.: Adult T-cell leukemia (ATL) virus-specific antibodies in ATL patients and healthy virus carriers. *Int. J. Cancer* 32; 281-287, 1983.
 28. Yoshida, M., Miyoshi, I. and Hinuma, Y.: Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA* 79; 2031-2035, 1982.