Localization of Adult T-Cell Leukemia-associated Antigens by the Immunocolloidal Gold Method

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ABSTRACT

Adult T-cell leukemia-associated antigen (ATLA) and adult T-cell leukemia virus (ATLV) antigens were localized in the MT-2 cell system by the immunocolloidal gold method using 71 human sera having various anti-ATLA titers and rabbit anti-ATLV antisera. In the thin-section method with anti-ATLA-positive human sera and rabbit antisera, protein A-gold particles were preferentially observed on and around sectioned adult T-cell leukemia (ATL) virions located in pericellular aggregates and within the cytoplasmic vacuoles but nowhere else in a significant number. The number of gold particles per ATL virion was statistically well correlated with the anti-ATLA titers of human sera applied (p < 0.005). The preembedding method showed that pericellular ATL virions were specifically tagged with protein A-gold, but the antigens in question were not expressed on the plasma membrane of MT-2 cells. The absence of ATLA and ATLV antigens on the plasma membrane constitutes a unique pathobiological feature of ATL and ATLV as compared with other retrovirus systems.

INTRODUCTION

ATL is an endemic disease observed in restricted areas in Japan, and was established as a unique clinical entity by K. Takatsuki in 1977 (26). The disease is now subject to an increasing number of investigations following the discovery of a retrovirus called ATLV that was found associated with this disease by Hinuma et al. (12) in 1981. Recent studies have shown that ATLV is closely related to HTLV (21), which was reported in 1980 by Gallo and his coworkers to be associated with other types of human T-cell cancer (for review, see ref. 8).

ATL is an antigen(s) found initially in MT-1 cells (15) by the IF test using ATL patients' sera. It is widely accepted that most, if not all, of the anti-ATLA activities of human sera are directed to structural components of ATLV (31) and HTLV (8).

Although the IF test is a powerful technique for screening a large number of human sera, it does not permit precise intracellular localization of ATLV at the ultrastructural level. Thus, the present study was carried out by using the immunocolloidal gold method; the superiority of this method over other methods of immunoelectron microscopy has been well discussed (11, 13). A part of the present study was reported in preliminary form elsewhere.

MATERIALS AND METHODS

Materials. Protein A was purchased from Pharmacia Fine Chemicals, Inc., New Market, NJ. Tetrachloroauric acid was obtained from Wako Junyaku Co., Osaka, Japan.

Human Sera. Seventy-one serum samples were collected from in- and outpatients of Kumamoto University Hospital and their relatives. Kumamoto district is one of the endemic areas of ATL. The classification of these sera is listed in Table 1, together with the results of IF test for anti-ATLA activities.

IF Test. This was carried out by the method of Hinuma et al. (12). In brief, acetone-fixed smear of MT-1 cells (15) was sequentially treated with an appropriately diluted human serum and fluorescein isothiocyanate-conjugated IgG of rabbit anti-human IgG antiserum, each for 30 min at 37°C. IF titers were expressed by the reciprocals of the highest serum dilution that gave positive results.

Rabbit Antisera against Purified ATLV. ATLV was purified from culture medium of MT-2 cells (16, 17) by CsCl density gradient centrifugation. Anti-ATLV sera were raised in rabbits by 4 weekly i.m. injections of Triton X-100-disrupted ATLV mixed with the same volume of complete Freund's adjuvant.

Microimmunodiffusion Test. Human sera with high IF titers were examined by the microimmunodiffusion test in 1.2% agarose in PBS using Triton-X-100-disrupted ATLV as an antigen and rabbit anti-ATLV antisera as positive references. Since reactivities of human sera were considerably weak, each human serum well was filled twice with the same serum, with the second filling 1 hr after the first.

Cells. MT-1 cells were established by Miyoshi et al. (15) in 1980 from peripheral blood leukocytes of a male ATL patient. These cells were used for screening and titration of human sera for anti-ATLA activity in IF test. Only a small proportion (1 to 5%) of MT-1 cells are ATLA positive (12).

MT-2 cells were subjected to the immunocolloidal gold method for localization of ATLV. These cells were also established by Miyoshi et al. (16, 17) in 1982 from umbilical cord leukocytes of a healthy male infant cocultivated with leukemic cells derived from a female ATL patient. The male nature of these cells was confirmed by the karyotype analysis (16, 17). In contrast to MT-1 cells, MT-2 cells are producing continuously an abundance of ATLV, and practically 100% of them are ATLA positive (17). In our laboratory, MT-2 cells are transferred every 3 to 7 days according to the seeding cell densities, and cells used for immunocolloidal gold investigation were harvested usually in the second-half stage of their growth.

Immunocolloidal Gold. Colloidal gold was prepared by reducing tetra-
chloroauric acid by sodium ascorbate (24), and coated with either Protein A or IgG of rabbit antisera against human IgG. Protein-gold was centrifuged at 55,000 × g for 40 min on a 3-ml cushion of 5% glycerol containing 0.05% polyethylene glycol and 0.01% sodium merthiolate. At the bottom of the centrifuge tubes, approximately 2 ml of condensed sol and small, densely packed pellets of protein-gold were formed from 100 ml of the original colloidal gold. Condensed sol was stored in a refrigerator until used. Immediately before use, the sol was diluted 10-fold with 1% BSA in PBS (hereafter referred to as BSA-PBS) and centrifuged at 4000 rpm for 10 min to remove aggregates. Addition of BSA-PBS reduced nonspecific adsorption of protein-gold to the section surface without any interference with the sensitivity or specificity of protein-gold reaction. The average size of gold particles was about 10 nm.

**Thin-Section Immunocolloidal Gold Method.** MT-2 cells were fixed with 2% glutaraldehyde in PBS for 1 hr at 4° and embedded in Vestopal W (22). Thin sections were picked up on celluloid-covered grid meshes. These were floated, thin section side down, on a drop of the following reagents sequentially at room temperature in the order: (a) BSA-PBS for 5 min; (b) serum appropriately diluted with BSA-PBS for 30 min; (c) BSA-PBS 3 times, each for 5 min; (d) protein-gold sol for 30 min; (e) BSA-PBS 3 times, each for 5 min; and (f) distilled water 3 times, each for 5 min. These were finally stained with uranyl acetate and lead citrate. Rabbit anti-ATLV antisera and some human sera with high IF titers were used at multiple dilution. For comparison of a number of human sera, however, a 100-fold dilution was used.

**Preembedding Immunocolloidal Gold Method.** Glutaraldehyde-fixed MT-2 cells were treated with 0.1 M glycine, pH 7.2, for 1 hr to block the remaining free aldehyde residues. After washing, the cells were further treated with appropriate serum followed by protein-gold, each for 1 hr at room temperature, with occasional pipeting. Then the cells were post-fixed with 1% OsO₄ in PBS and embedded. Rabbit antisera were used at multiple dilutions. After these sera were confirmed to agglutinate sheep RBC at dilutions up to 80-fold, the sera were diluted 10-fold with BSA-RPMI and absorbed with the same volume of packed sheep RBC for 1 hr at room temperature. In some cases, the sera were further absorbed with acetone powders (100 mg/ml) (10) of human liver and/or spleen. Human sera were routinely used unabsorbed at a 30-fold dilution. For comparison of antibody activities of various human sera and for estimation of the reaction specificity on a quantitative basis, the following parameters were introduced. In the thin-section method, gold/virion (G/V) ratios represent the number of gold particles per sectioned ATL virion. Since binding between ATL virions and gold particles was mediated by 2 protein molecules (the first antibody IgG, and the second antibody IgG or protein A), we assumed that protein-gold within 10 nm from ATL virions was also reacting with the virion antigens as were those found on the virions. One sq µm of pericellular virion aggregates contains 143 ± 25 (S.D.) sectioned ATL virions, and therefore, this value is a multiplying factor to convert the G/V ratios into those directly comparable with G/C and G/NC ratios described below. G/C ratios are the number of gold particles per sq µm of the cytoplasm of MT-2 cells, excluding the ATLV-containing vacuoles, while the G/NC ratios are the number of gold particles per sq µm of the noncellular space which consists solely of polymerized Vestopal W. Therefore, G/NC ratios are parameters representing contamination or nonspecific adsorption of protein-gold to the section surface. In the preembedding method, the number of gold particles reacting with the plasma membrane per µm length of the plasma membrane (G/M ratios) was calculated. In this case, only those parts of the plasma membrane that were sectioned perpendicularly to the membrane surface were examined.

All ratios were calculated from at least 10 electron micrographs for each serum which included 1000 or more sectioned ATL virions.

**RESULTS**

**Microimmunodiffusion Test.** At least 2 precipitin lines were discernible between purified ATLV and rabbit anti-ATLV antisera (Fig. 1). One human serum with a high IF titer (×640) showed completely identical reactions with rabbit antisera (Fig. 1, Well a), and some of the other sera formed a single precipitin line. A single precipitin line continuously to the rabbit precipitin line located closer to the antigen well (Fig. 1, Wells b, d, and g). Other sera showed no reaction. These observations indicate that at least some of anti-ATLV activities of human sera are directed toward ATLV antigens, although the levels of such activities are in general much lower than those of rabbit antisera.

**Distribution of ATLV within and around MT-2 Cells.** The ultrastructural architecture of MT-2 cells was already reported (19). Approximately 50% of cells are associated with pericellular ATLV aggregates of various sizes (Fig. 2). These ATL virions could not be dissociated from MT-2 cells by washing 3 times with PBS or PBS containing Polybrene (16 µg/ml). ATL virions are also seen mixed with cellular debris within the cytoplasmic vacuoles in 10 to 20% of cells (Fig. 3). These vacuoles are not phagocytic vacuoles, since MT-2 cells are not phagocytic (17), which we also confirmed by electron microscopy using ferritin and polylysine latex beads, 96 nm in diameter, as tracers. This suggests that these intravacuolar ATL virions somehow assembled at the membrane of cytoplasmic vacuoles, which presumably were derived from autophagosomes. Nevertheless, we could not obtain a convincing profile of budding ATLV at the vacuole membrane or at the plasma membrane, although other...
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3.01
2.5
2.0
1.5
1.0
0.5
0.0
G/V ratios

10^3-0
10^3-1
10^3-2
10^3-3
10^3-4
10^3-5
10^3-6
10^3-7
10^3-8
Serum dilution

Chart 1. G/V ratios of rabbit anti-ATLV antiserum and some IF-positive human sera examined at serial dilution in the thin-section method. O, rabbit anti-ATLV serum; @, @, @, human sera, their IF titers are 1280, 640, and 40, respectively. The typical concentration effect is seen with rabbit serum with a reaction peak at a 10 x 3^-fold dilution. Similar patterns are confirmed with 2 human sera (@ and @).

authors reported rare instances of budding ATLV (12) and HTLV (21).

Thin-Section Method with Rabbit Anti-ATLV Antisera. Since MT-2 cells used for the thin-section method were fixed with glutaraldehyde alone, identification of structural detail of cells and ATL virions is somewhat difficult. However, protein A-gold particles are readily found to be distributed on and very close to sectioned ATL virions located in pericellular virion aggregates (Fig. 4) and within the vacuoles (Fig. 5).

G/V ratios varied remarkably with antiserum dilutions. A peak of reaction (G/V ratio, approximately 2.0) was seen at a 10 x 3^-fold dilution (Chart 1). Higher dilutions resulted in almost linearly decreasing G/V ratios. Unexpectedly, however, higher serum concentrations also gave reduced G/V ratios (Chart 1). This paradoxical phenomenon which we refer to as the concentration effect was confirmed repeatedly with rabbit antisera and even with human sera with high IF titers (Chart 1).

Few gold particles were observed on the cytoplasm of MT-2 cells or on the noncellular space (Figs. 4 and 5). G/C and G/NC ratios at serial serum dilutions are presented in Chart 2. Significantly low levels of these ratios as compared with G/V ratios (the values obtained by multiplying the G/V ratios by 143 are comparable with the G/C and G/NC ratios) seem to support the reaction specificity of Protein A-gold to ATLV. In addition, G/C ratios varied in an almost identical manner with G/NC ratios at various serum dilutions (Chart 2), suggesting that ATLV antigens are not expressed in a detectable amount in the cytoplasm of MT-2 cells, since G/NC ratios represent nonspecific staining.

In control experiments in which Protein A-gold was applied without pretreatment with serum, G/V, G/C, and G/NC ratios were 0.19, 1.0, and 0.5, respectively. In absorption test, G/V ratio was reduced to 0.18, while nonspecific staining increased considerably (G/NC ratio, approximately 30).

Thin-Section Method with Human Sera. Because colloidal gold coated with protein A gave nearly identical results with that coated with IgG of rabbit anti-human IgG antiserum, only Protein A-gold was used in the following experiments. As shown in Figs. 6 and 7, findings with IF-positive human sera were essentially the same as those with rabbit antisera; gold particles appeared exclusively on and around sectioned ATL virions. However, G/V ratios varied from serum to serum. As mentioned above, the concentration effect was also observed with human sera with high IF titers but not with low IF titers (Chart 1). This makes comparison of various sera at a single dilution difficult. For convenience, however, we decided to use a 100-fold dilution in the examination of a number of human sera because: (a) at this dilution, differences between reactivities of various sera were manifest; (b) higher serum concentrations increased the chance of nonspecific staining, and reduced the reactivities of sera with high IF titers (concentration effect); and (c) higher dilutions reduced reactivities of sera with low IF titers.

In Chart 3, G/V ratios of 71 human sera were plotted against their IF titers. Although a clear linear relationship was not observed, correlation between these 2 parameters (those of 4 sera with borderline IF results were omitted) was statistically significant (p < 0.005). Since 29 of 34 IF-negative sera showed G/V ratios less than 0.2 (mostly lower than 0.1) and, as mentioned above, G/V ratio of Protein A-gold alone was 0.19, we regarded 0.2 as the upper limit of G/V ratios of sera judged as negative in the thin-section immunocolloidal gold method. Thus, of 33 IF-positive sera, 2 sera with an IF titer of 20 were negative. The
other 31 IF-positive sera showed G/V ratios higher than 0.4. Therefore, we used this value as the lowest limit of G/V ratios of positive sera in the thin-section method. Based on this criterion, 3 of 34 IF-negative sera were judged positive (Chart 3), 2 of which were from 2 brothers of a family in which 2 other brothers were ATL patients. Of the 4 sera with borderline IF results, one was positive and 2 were negative. As a result, the 3 remaining cases show G/V ratios between 0.2 and 0.4 as borderline cases in the thin-section method (2 from IF-negative sera and one from a serum with borderline IF results).

Chart 3 shows G/C ratios of human sera plotted against their IF titer. Correlation between these 2 parameters are statistically significant. •, sera from ATL patients; ◦, sera from other subjects.

Chart 4 shows G/C ratios of human sera plotted against their G/NC ratios. G/C ratios are statistically not independent from G/NC ratios for IF-positive (•) and IF-negative (◦) sera, suggesting that no ATLA is expressed in the cytoplasm of MT-2 cells.

Preembedding Method with Rabbit Antisera. To confirm whether ATLA and ATLV antigens are expressed on the plasma membrane of MT-2 cells, the preembedding method was carried out. Results with rabbit antisera are shown in Figs. 8 and 9. Rabbit antisera, when used unabsorbed and at high concentrations, showed marked protein A-gold reaction to pericellular ATL virions (Fig. 8), although, in contrast to the thin-section method, only envelope antigens were assumed to participate in the reaction. G/V ratios in this case, however, could not be taken as reliable parameters indicative of reaction levels, because the reaction did not occur evenly; virions located in the central part of aggregates (Fig. 8, arrows) were hardly accessible to antibody molecules and protein A-gold particles due to steric hindrance. For the same reason, ATL virions contained in the cytoplasmic vacuoles were not tagged with gold particles (not shown).

In contrast, the plasma membrane was readily accessible to these reagents and was covered extensively and evenly with gold particles (Fig. 8). Chart 5 shows that G/M ratios of unabsorbed sera sharply decreased with serum dilution, which contrasts to the concentration effect in G/V ratios when the same sera were applied to thin sections (Chart 1). Absorption of rabbit anti-sera with various materials resulted in a stepwise and measurable reduction of G/M ratios while retaining specific but slightly reduced reactivity to ATL virions as compared with unabsorbed sera (Fig. 9). G/M ratios of sera exhaustively absorbed with sheep RBC and acetone powders of human liver and spleen were 10 times lower than that of unabsorbed sera at a 30-fold dilution, and G/M ratio at a 90-fold dilution was only 1.6, a level
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we expected to obtain similar observations by applying the thin-section method to the ATL virus. In this method, however, only antigens which are accidentally exposed at the section surface are detectable, while many others that are covered by embedding material are not. Therefore, antigens expressed presumably at a low level are easily overlooked; a disadvantage of this otherwise very useful method.

On the other hand, the preembedding method made it possible to detect all antigens throughout thin sections as long as they were accessible to the reagents applied, because reactions were completed before embedding and thin sectioning. Thus, it seems plausible that the failure to detect ATLA and ATL virus antigens on the plasma membrane of MT-2 cells (Figs. 9 and 10; Charts 5 and 6) is not due to technical limitation. In contrast to our present observations are 2 reports (2, 18) which dealt with ATL virus or HTLV p19 protein localization by means of ferritin-antibody technique. These 2 reports claimed that the antigens in question were expressed on the plasma membrane. However, in both reports, reacting ferritin molecules appeared in aggregates. These aggregates are "sticky," causing nonspecific staining, and their removal by a prior centrifugation at 18,000 rpm for 15 min (25) would make the conclusion more persuasive. Consistent with our observations is a report by Ohtsuki et al. (20) who cultured MT-2 cells in the presence of ATL patients' sera, and found heavy antibody deposits around pericellular ATL virus but none on the plasma membrane.

However, the present findings are not in agreement with those on other retroviruses. In the murine leukemia virus system, for example, 2 types of virus-coded antigens are expressed on the plasma membrane; one is the envelope antigen called G\(\text{a}\), and the other is glycosylated core antigen represented by Gross cell surface antigen (for review, see Ref. 7). Expression of these antigens was readily visualized by various methods of immunoelectron microscopy (1, 3, 23) regardless of whether budding viral particles were observed at the cell surface. Therefore, the absence of virus-related antigens on the plasma membrane constitutes a unique pathological feature of ATL and ATL virus.

Other techniques conventionally used to examine antigens on the plasma membrane such as membrane immunofluorescence and immunoprecipitation following external cell labeling are unsuitable for the ATL virus system, because many cells exhibit pericellular ATL virus aggregates which cannot be removed by thorough washing. The only other method that shows promise for investigating the ATL virus system is the cytotoxic test, a sensitive test which has been very useful in the murine leukemia virus system (7). Unfortunately, no data are available yet.

A major drawback of the immunocolloidal gold method is that it apparently contributes little to the understanding of ATL virus maturation process. No accumulation of viral antigens was observed anywhere in MT-2 cells except for mature forms of ATL virus. This should be reinvestigated by the preembedding method applied under a condition under which intracellular antigens are made accessible to antibodies and protein-gold. It is also puzzling that we obtained not a single convincing profile of budding ATL virus. Neither phenothiazines which are shown to stop virus budding by phenothiazines. Virology, 130: 44–55, 1983.


Mindlin, P., and Farr, R. S. Ammonium sulphate method to measure antigen-binding capacity. In D. M. Weir (ed.), Immunology, Ed. 3, pp. 131–
Fig. 2. Untreated MT-2 cell. ATL virions are seen in aggregate at the cell surface. × 63,000.
Fig. 3. Untreated MT-2 cell. ATL virions are also observed within the cytoplasmic vacuoles. × 42,000.
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Fig. 4. Thin-section method. MT-2 cell treated with rabbit anti-ATLV antiserum (dilution, 1000-fold). Protein A-gold particles are seen preferentially on and very close to sectioned ATL virions of pericellular virion aggregate. × 45,000.

Fig. 5. Thin-section method with same serum used in Fig. 4. ATL virions within the cytoplasmic vacuoles are also tagged with gold particles. × 45,000.
Fig. 6. Thin-section method. MT-2 cell treated with a human serum (IF titer, 160; dilution, 100-fold). As in Fig. 4, pericellular ATL virions are specifically tagged with gold particles. × 42,000.

Fig. 7. Thin-section method with same serum used in Fig. 6. Protein A-gold reaction is directed toward ATL virions located within the cytoplasmic vacuole. × 42,000.
Fig. 8. Preembedding method. MT-2 cell treated with unabsorbed rabbit anti-ATLV antiserum diluted 30-fold. Marked gold reaction is observed to pericellular ATL virions and the plasma membrane. In contrast to the thin-section method, no or few gold particles are seen around virions located in the central part of virion aggregate due to steric hindrance (arrows). × 42,000.

Fig. 9. Preembedding method. MT-2 cell treated with rabbit anti-ATLV antiserum exhaustively absorbed with sheep RBC and acetone powders of human liver and spleen and used at a 30-fold dilution. The number of gold particles on the plasma membrane is remarkably reduced as compared with Fig. 8, while reaction to pericellular ATL virions is still retained. × 42,000.
Fig. 10. Preembedding method. MT-2 cell treated with a human serum (IF titer, 1280; dilution, 30-fold). Pericellular ATL virions are tagged moderately as in Fig. 9. Few gold particles are found associated with the plasma membrane. ATL virions in the cytoplasmic vacuole are free of gold particles because they were not accessible to antibodies and protein A-gold. x 42,000.

Fig. 11. Preembedding method. MT-2 cell treated with an IF-negative human serum (dilution, 30-fold). Few scattered gold particles are seen around pericellular ATL virions and on the plasma membrane. x 42,000.
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