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Morphologic and immunoelectronmicroscopic identification of human T-cell lymphotropic virus type III (HTLV-III)

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Surnmary. The AIDS associated HTLV-III virus infected H9 cells were extensively studied using light, scanning and transmission electronmicroscopy. It was demonstrated that the morphological features of HTLV-III are different from the C-type particles and are similar to those of lentiviridae. For immunological identification high titer pre-AIDS patient sera served as the anti-HTLV-III envelope antibody source. The immunoelectronmicroscopic method was able to identify the viral envelope antigen on the surface of infected cells and in certain areas of the viral envelope. This is the first application of immunoelectronmicroscopy for the identification of the HTLV-III virus.

Key words: TEM - SEM - Immunogold - AIDS virus

Introduction

The role of retroviruses in the pathogenesis of human malignant diseases has only very recently been confirmed. Human T-cell leukemia virus type I (HTLV-I) is a C-type retrovirus closely associated with adult T-cell leukemia-lymphoma (Poiesz et al., 1980). HTLV-I is able to transform T-lymphocytes, resulting in indefinite proliferation of infected cells (Miyoshi et al., 1981; Popovic et al., 1983). A distinct group of human retroviruses HTLV-III/LAV has been isolated from patients with acquired immune deficiency syndrome (AIDS) or AIDS related complex (ARC) and from individuals of high risk groups (Barré-Sinoussi et al., 1983; Levy et al., 1984; Popovic et al., 1984). Sera of the majority of these patients contain specific antibodies reactive with HTLV-III/LAV but not with HTLV-I (Sarngadharan et al., 1984). Serological, restriction endonuclease and DNA hybridisation studies (Arya et al., 1984; Schüpbach et al., 1984) indicate that the AIDS virus is related to other members of the HTLV family. Recently, however, there is new evidence which suggests that the AIDS virus represents the prototype of a new class of human retroviruses, possibly a lentivirus (Gonda et al., 1985; Wain-Hobson et al., 1985). HTLV-III/LAV is extremely T-cell tropic, infecting only lymphocytes expressing T4 antigen, unlike HTLV-I (Dalgleish et al., 1984; Nagy and Weiss, 1985). In contrast to HTLV-I, HTLV-III/LAV has a cytopatic effect on infected cells (Popovic et al., 1984). Moreover, HTLV-III/LAV has unique electronmicroscopic morphology differing from that of HTLV-I and other C-type retroviruses (Barré-Sinoussi et al., 1983). In the present work, we demonstrate that, besides the characteristic ultrastructure, HTLV-III/LAV can also be identified by immunoelectronmicroscopy using an anti-HTLV-III antibody positive patient sera and immunogold technique.

Materials and rnethods

Viruses

Human T-cell lymphotropic virus type III (HTLV-III) is produced by infected H9 human T-cell line (kindly provided by R.C. Gallo, NCI, NIH, Bethesda).

Cells

Uninfected H9 human T-cell cells and H9/HTLV-III_B cells were maintained as suspension culture in RPMI 1640

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medium supplemented with 15% heat inactivated fetal calf serum and antibiotics. Cells showing positive fluorescence for viral antigens were used for electronmicroscopy.

Antisera

Human serum reactive with viral component of HTLV-III_B was obtained from a British patient (D.K.) with extended lymphadenopathy (provided by R.A. Weiss, ICR, London). Specificity of the serum was confirmed by Western-blott technique (R.A. Weiss personal communication). 1:20 dilutions of the serum were used for IFA and 1:100 dilutions for immunoelectronmicroscopy.

Indirect immunofluorescence assay for virus antigens

Steps of procedure have been previously published (Nagy et al., 1983). Briefly: 5 µl H9/HTLV-III_B cells, at a density of 10^7/ml, were smeared on a slide and air-dried for 2 hrs. The cells were then fixed in acetone. 20 µl human serum, at a dilution of 1:20, was incubated with the fixed cells at 37° C for 30 min. 20 µl of FITC-conjugated goat anti-human IgG (1:15 dilution) was added and incubated at 37° C for 30 min. After washing the slides were mounted with coverslips and the presence of fluorescent cells was observed using a Leitz UV-Microscope.

Transmission electron microscopy

The HTLV-III-infected H9 cell suspension was fixed in 1% glutaraldehyde (TAAB, UK) buffered with 0,2 M phosphate buffer (pH = 7,2) for 30 min, at room temperature. After washing in buffer, an OsO_4 (Merck) postfixation was performed for 1 hr at room temperature. The specimens were then dehydrated in greaded series of alcohol and embedded in Polybed 812 (Polyscience). The semithin and ultrathin sections were made by LKB Ultrotome, the semithin ones being stained by methylene blue while the ultrathin ones were contrasted with uranyl-lead sequence. The electronmicrographs were taken with a Jeol-100B transmission electron microscope at an accelerating voltage of 60 kV.

Scanning electron microscopy

The glutaraldehyde/OsO_4 fixed and dehydrated specimens were critical point dried in a Balzers apparatus using CO_2, then Sputter coated (Balzers) with gold. The photographs were taken with a Phillips 501 stereoscan at an accelerating voltage of 20 kV.

Immunoelectronmicroscopy

The labeling protocol used here was essentially the same as we previously used for HTLV-I (Timár et al., 1984). The "normal" or HTLV-III-infected H9 cells were kept at 4° C during the entire labeling period. The HTLV-III envelope antigen was demonstrated by a multistep prefixation process. First, an anti-HTLV-III antibody-positive pre-AIDS patient sera was incubated with cells, diluted 1:100 with PBS for 15 min. After extensive washings, a second layer of mouse monoclonal antihuman IgG (Miles) was added for 15 min (1:200 in PBS). After another series of multiple washings, a third layer of goat-anti-mouse IgG conjugated to 5 nm gold (GAMG5-Janssen, Belgium) diluted 1:10 in PBS was added for 15 min. After a final series of washings, the cells were processed as for transmission electronmicroscopy. Controls: H9 cells, non-infected by HTLV-III, were used in the complete staining procedure. HTLV-III-infected cells were stained in multiple staining steps, either by using normal human serum as a first layer or by having no first layer of any kind. No background staining on cells or particles could be observed in any controls.

Results

The semithin sections demonstrated HTLV-III-infected H9 cells are dominantly mononuclear with prominent nucleoli (Fig. 1). The samples prepared for SEM contained clumps, in which few bigger cells were surrounded by small ones (Fig. 2). The characteristic surfaces features of these cells, revealed by SEM, was the abundance of round particles with a diameter of 200 nm (100 nm gold coating). Besides these structures, microvilli and surface ruffles were present on these lymphoid cells (Fig. 3). Transmission electronmicroscopy revealed that the cytoplasm of infected H9 cells

Fig. 1. High magnification light microscopic picture of HTLV-III-infected H9 cells in semithin section. Note prominent nucleoli of the cells. Methylene blue staining.

Fig. 2. Stereoscan appearance of infected H9 cells at low power. Note presence of cell clumps dominantly containing small cells and a few larger ones. × 600

Fig. 3. Typical scanning image of a HTLV-III producing H9 cell. The cell surface is covered by numerous spherical structures besides few microvilli and ruffles. × 4,000

Fig. 4. Cell surface and part of cytoplasm of infected H9 cell at low power in TEM. Note appearance of numerous associated viral particles (arrow). × 12,500

Fig. 5. a. High power stereoscan of infected H9 cell surface. Numerous round profiles (arrow) are in close connection with plasmamembrane. × 48,000. **b.** High power TEM picture of the plasmamembrane of infected H9 cell. Two budding event (arrow) and two mature particles are visible (double arrow). × 10,000

Fig. 6. TEM appearance of extracellular mature HTLV-III particles in infected H9 cell culture. Note electrondense core located excentrically (arrow), or forming rod-shape (double arrow). × 80,000

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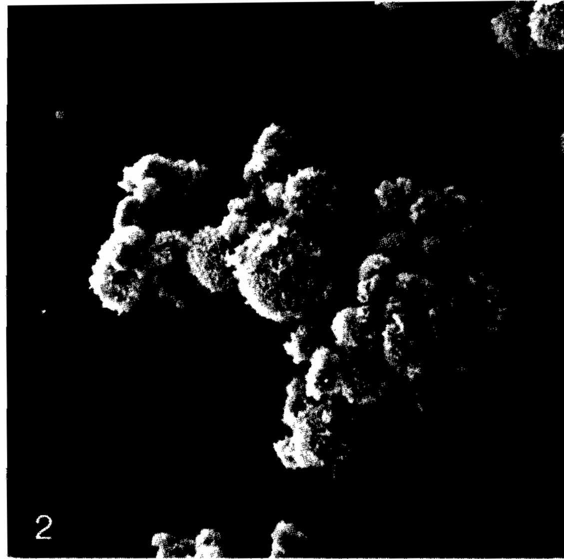
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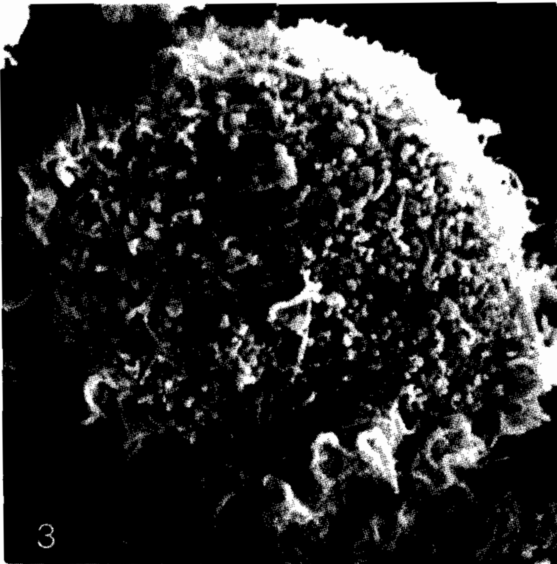
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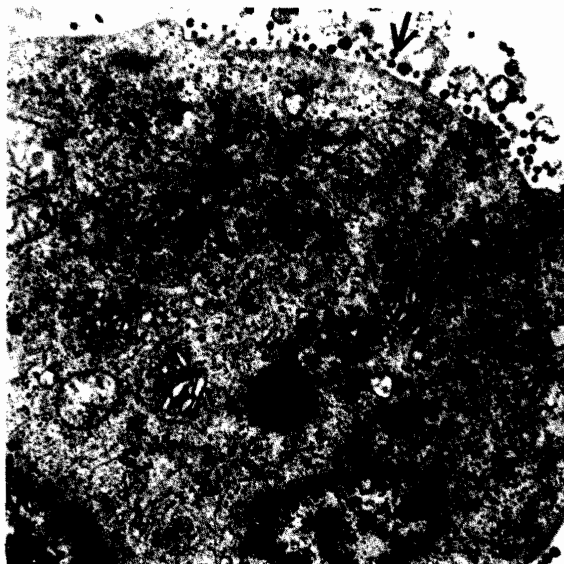

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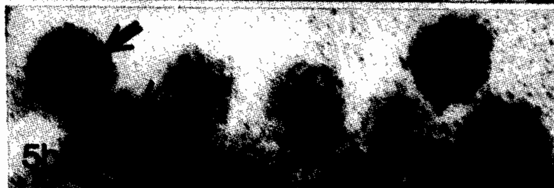

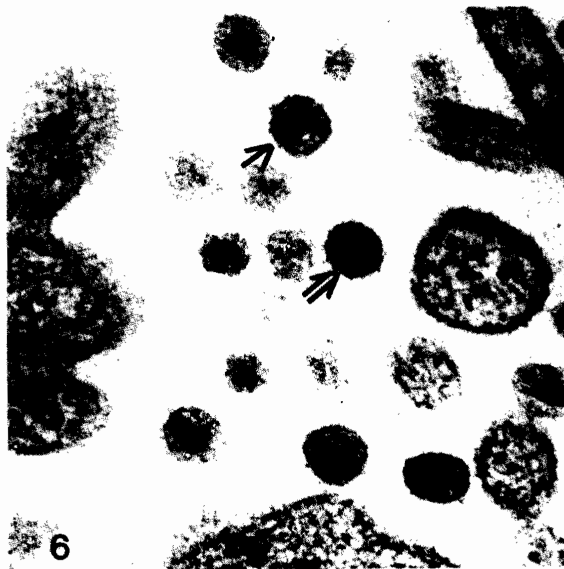
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contained well-developed rER, Golgi region, mitochondria and lysosomes, no cytoplasmic viral elements being detected. The nuclei contained expanded nucleolar zones. At the cell surfaces, a large number of viral particles were present (Fig. 4) usually having a dominant diameter of 80-90 nm although bigger particles could also be seen. The scanning EM was able to demonstrate frequent semicircular profiles on the cell surfaces (Fig. 5a) probably corresponding to viral buddings. This was proved by high magnification TEM pictures because crescent core elements were present at the plasmamembrane. The double layer of these membrane areas were hardly distinguishible from electrondense cores (Fig. 5b). The matured intracellular particles have a dominant 80 nm diameter, an electrondense core situated in an excentric position, with an electronlucent zone between the outer lipid layer and the inner core. Sometimes the core profiles were bar-shaped or circular (Fig. 6).

Immunocytochemistry

The smears of HTLV-III, produced by H9 cells grown in culture, were consequently stained for membrane fluorescence using anti-HTLV-III antibody containing pre-AIDS patient sera (Fig. 7). As a positive reaction bright patches could be demonstrated at the cell membranes. Using a multistep immunoelectronmicroscopic approach (see materials and methods) it was possible to localise the anti-HTLV-III antibody on infected H9 cells. These are plasmamembrane determinants frequently localised into coated pits (Fig. 8a), consisting of few gold particles (2-5). Accidentally 80nm surface particles contained gold label (Fig. 8b), but only at a certain sector of the envelope. In the extracellular space typical viral particles containing sectorially localised gold tracer could be demonstrated (Fig. 8c).

Discussion

Our transmission and scanning electronmicroscopic studies confirmed the results of Gonda et al. (1985), that the budding process and the morphology of HTLV-III virus is different from that of the HTLV-I, the latter being a typical C-type virus. There was no typical C-type budding observed and the morphology of the particles was much more similar to lentiviridae than to that of the C-type particles, having a lipid layer, no electronlucent halo, an excentric core with frequent circular or rodshaped profile and uniform sized (80-90 nm diameter).

The SEM was able to demonstrate that the majority of the infected H9 cells were actively producing particles and also revealed some surface features of viral maturation in the cell membrane. Clinical diagnosis of AIDS is based

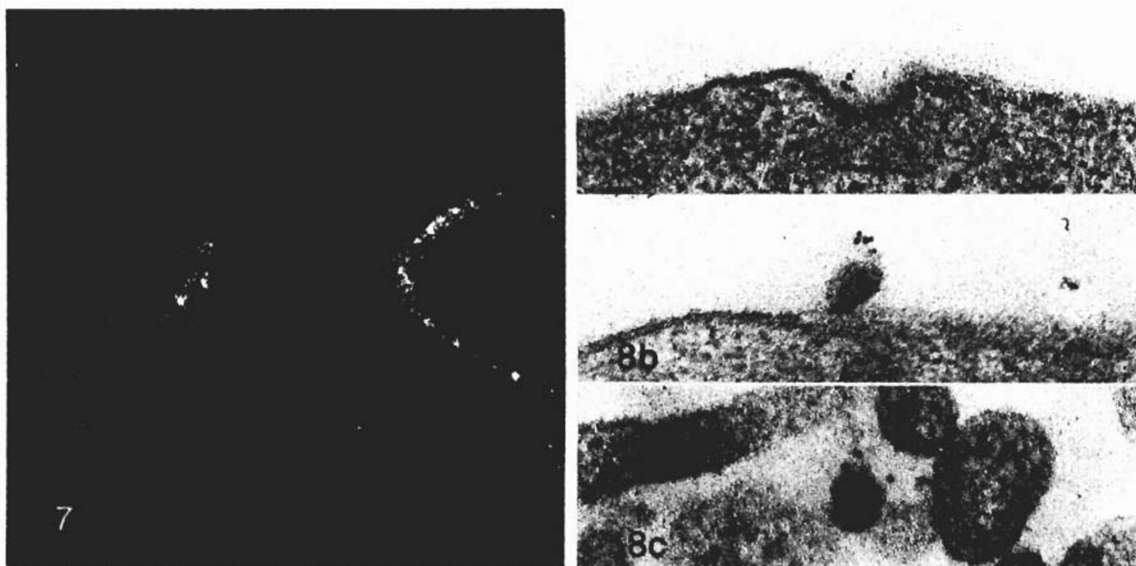

Fig. 7. High power cell surface immunfluorescence of HTLV-III infected H9 cells after staining with pre-AIDS patient sera according to rnaterials and methods. Note bright patches of the cell membrane, indicating presence of HTLV-III antigen.

Fig. 8. Immunoelectronmicroscopic detection of HTLV-III antigen on infected H9 cells. The labeling protocol is included in rnaterials and methods. a. Coated pit of infected H9 cell membrane containing few tracer granules. x60,000. b. Cell surface-connected round particle labeled by colloidal gold on infected H9 cell. x60,000. c. Extracellular matured HTLV-III virus containing few tracer granules, as found in certain envelope areas. x60,000

on specific symptoms and laboratory findings (Francis et al., 1983) but exact virological diagnosis is much more difficult, being based on isolation and identification of viral particles, DNA hybridization techniques, nucleotide sequencing and Western-blott analysis. Immunoelectronmicroscopy either using ferritin-labelled antibodies (Ohtsuki et al., 1983) or colloidal gold labeled ones (Tanaka et al., 1984) is able to demonstrate and identify HTLV-I viruses in T-cell lymphoma cultures (Timár et al., 1984). In our present paper we also used the simple but highly specific immunogold method for the precise identification and localisation of HTLV-III envelope antigen on infected T-cells and on the surface of particles. As anti-HTLV-III monoclonals were not available at the time, high titer anti-HTLV-III antibody from pre-AIDS patient sera was used as primary antibody source. The high dilution of the antibody made it possible to avoid background staining preserving high specificity. The AIDS patient sera contain dominantly anti-HTLV-III envelope antibody (p41) (Sarngadharan et al., 1984) and this was probably the cause of the succesful use of patient sera for our purposes. Similarly to the HTLV-I envelope antigen demonstrated by us using patient sera (Timár et al., 1984) the envelope antigen of HTLV-III is also localised sectorially on the surface of particles.

The present methodological approach is sensitive, simple and is even able to distinguish between HTLV-I and III viruses produced by the same cell. The method could be valuable in diagnosis of AIDS or AIDS-related syndromes by detecting and identifying viral particles of AIDS and their envelope antigen.

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