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Invited Review

Immunoelectron microscopy of human retroviruses

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Summary. The ultrastructural detection and identification of human retroviruses - HTLV (human T-cell lymphotropic virus) and HIV (human immunodeficiency virus) - have become an everyday task for pathologists and virologists as well as for cell and molecular biologists. The development of better and conventionally available immunocytochemical techniques, such as pre- or postembedding immunocytochemical methods, cryofixation-variants and low temperature embeddings, have made it possible to use them in this field. With the help of these methods the structural proteins of HTLV-I and HIV have been identified in infected cells. The virus assembly at the cell membrane has also been described in detail. Using these methods the incorporation of human transplantation antigens into the envelope of these viruses can be followed. Future studies should establish the pathological significance of this process.

Key words: Immunocytochemistry, Retrovirus, Human

Introduction

The localization of antigens at ultrastructural level became a routine method in laboratories involved in studies of normal or altered cell types. In the field of virus research — especially in the area of human viruses — electron microspy is essential for the identification of viral particles in the infected cells or in cell-free isolates. The pathological significance of a series of viruses has increased in the past decade including the herpes family, the hepatitis viruses and more recently the retrovirus family (Table I). The more precise identification of viral particles at ultrastructural level has become an everyday task for pathologists as well as for biologists. With the development of the sophisticated immunomorphological techniques it is now possible to identify viral particles or viral antigens in infected cells. This review attempts to describe the basic morphological techniques suitable for immunodetection of viruses at ultrastructural level. It also demonstrates applications in the field of human retroviruses HTLV-I-responsible for ATL- and HIV-I -the causative agent of the acquired immunodeficiency syndrome.

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1. Techniques for studies of viral antigens at ultrastructural level

The development of better and more sophisticated preservation techniques as well as the availability of smaller non-dislocating electrondense probes provide a wide range of methods for the location of viral antigens on the surface of, or inside, the infected cells. The small probes are also useful in mapping the different antigens on, or in the viral particle itself for the better understanding of the viral structure and thus provide basic information for the design of more efficient diagnostic probes (Carrascosa, 1988).

1.1 Surface labelling of cells and viruses

Antigens present on the cell surface could be most easily labelled by probes at 4° C, where the antigenicity of the proteins was the strongest. The probes could be particulate ones such as ferritin (7-10 nm), colloidal gold of different sizes (3-40 nm) (Figs. 1, 2), or non-particulate ones, such as the peroxidase-DAB-OsO_4 loose precipitate. Other methods for the visualization of DAB-reactions are also available using Ag or Ni-ammonium-chloride/Co-chloride (Graham and Karnovsky, 1966). Several antigens are stable after «minimal fixation» using 1-4% paraformaldehyde/0.01-0.1% glutaraldehyde mixture. In such cases immunoreactions can be performed at room temperature. Considering the dimensions of viral particles, the smallest particulate

probes have the mayor advantage in the localization of viral envelope proteins and the spatial interactions between host antigens and the viral ones.

1.2 Intracellular and intraparticular detection of antigens

Contrary to the surface-exposed antigens it is usually much more difficult to detect antigens inside the cell or in the viral particle.

1.2.1. Permeabilization

The oldest techniques for such purposes is the permeabilization of cells after «minimal fixation» using detergents to make the intracellular proteins available for the antibody and the probe. Because the particulate probes are poorly permeable, the peroxidase/DAB is used almost exclusively (Fig. 3) (Timár et al., 1988). The problem with using this technique is usually to find the balance between the ultrastructural and the immunocytochemical quality.

1.2.2 Cryo-techniques

The advantage of the cryo-techniques is the good preservation of the antigenicity of the structure studied, but the most frequent disadvantage is the loss of good ultrastructural quality. One of the most popular methods is the cryoultramicrotomy (Tokuyashu, 1983; Griffith et al., 1984) often preceded by «minimal fixation». With the development of new water-soluble resins it is now possible to perform low temperature embeddings (Carlemalm et al., 1984). The loss of antigenicity is frequently caused by dehydration procedures. The cryo-substitution provides a solution for such problems (Sitte, 1986) when the dehydration takes place at low temperature. This method is combined with the low temperature embedding. It is a new version of cryo-technique when the biological sample is not fixed chemically but with the help of cryofixation (Monaghan and Robertson, 1989) (Fig. 4). In all of these cases the immunocytochemical reaction is performed on ultrathin sections using almost exclusively particulate probes.

1.2.3. Microinjection

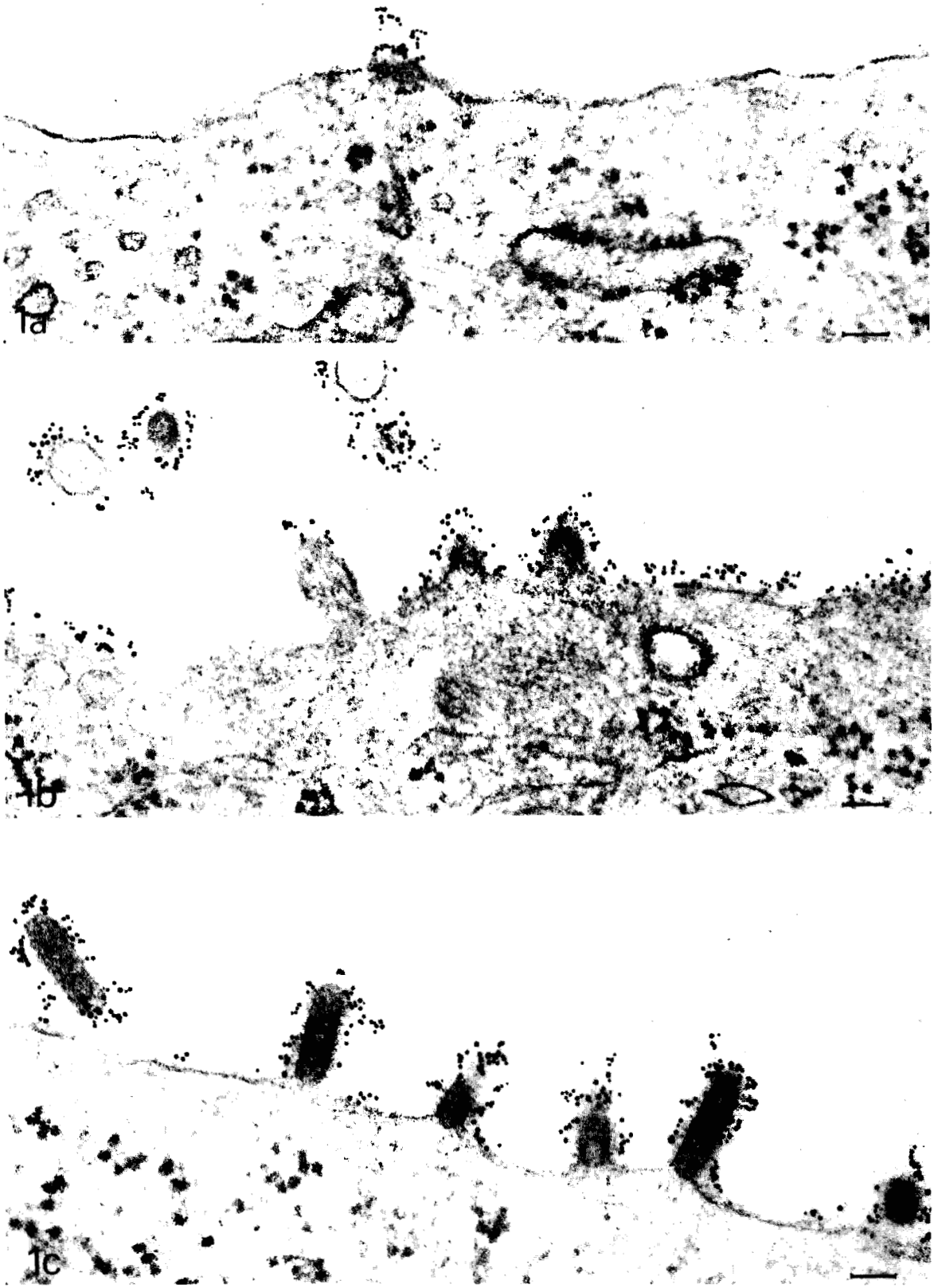
The precise localization of intracellular antigens has also become possible by using the sophisticated technology of microinjection. This technique helps to administer the primary and/or the secondary probes into the living cells to make the antigen/antibody reactions more probable (Timár et al., 1987c). The disadvantage of this technique is that only a limited amount of cells can be loaded by the probes, so it is very difficult to obtain a large amount of cells for immunocytochemical studies (Fig. 5).

2. Immunoelectron microscopy of human T-cell-lymphoma-leukemia virus I. (HTLV-I)

HTLV-I is a C-type (RNA-containing) retrovirus with average diameter of 100 nm (from 80 to 150), characterized by 10nm electron-lucent space between the envelope and the core (Fig. 6). This virus is the aetiological agent of adult T-cell lymphoma-leukemia (ATL) (Poiesz et al., 1980; Hinuma et al., 1981). The viral genom consists of gag, pol, env genes necessary for viral growth, and of pX, the putative transforming gene and of LTR sequences, but there is no oncogene present in it (Yoshida et al., 1987; Oda et al., 1988). The virus has a clear T4 + cell tropism (Mioshi et al., 1981). The transacting activator of LTR is the protein p40 (tat-1) (Felber et al., 1985), which also induce IL-2 and IL-2R in the infected cells (Inoue et al., 1986). The p27 protein of the virus regulates the gag gene-derived mRNA (Inoue et al., 1986). The env gene's products are gp 46 and gp20E (Hattori et al., 1984). The product of the gag gene is the p19 protein which is present in the envelope as a lipid-binding protein and as an RNA binding one as well. Other gag gene products are the major phosphoprotein, the capsid protein p24, and the p15, another RNA-binding protein in the viral core (Oda et al., 1988).

In HTLV-I-infected patients there are several types of antibodies against different viral components such as gp46, gp62, p40, p24, p19, p15 (Schneider et al., 1984). In ATL cells the patient's sera recognized the viral envelope, the plasma membrane (Timár et al., 1987a) the endoplasmic reticulum and the nuclear envelope. Using monospecific antibodies it was possible to localize the viral constituents at ultrastructural level (Oda et al., 1988). The p24 and p19 were present in the viral core, while in the envelope two proteins were found: the gp46 and p19. In the infected cells p19 was detected in the plasma membrane and in the cytoplasm (Timár et al., 1987a). The gp62, the precursor of gp46, was present in the nuclear envelope, and in the endoplasmic reticulum, while the gp46 was detectable in the plasma membrane. The p40 (pX) was localized in the nucleus only. The assembly of the virus takes place on the cell surface in the form of budding (Fig. 7). During this process the gp46-containing plasma membrane areas protrude and the core-components of the virus attach to them. Finally the particle separates. Interestingly, gp46 is not the only envelope constituent, because it has an atypical sectorial localization (Fig. 7). The other envelope protein, p19, could be detected only in a small amount, on the cell surface (Fig. 8b). It is present in the cytoplasm (Fig. 8a). After permeabilization the p19 became strongly accessible to the antibodies, demonstrating the lipophilic nature of the protein (Fig. 8c).

It is a well-known phenomenon during the maturation of viral particles, that some human membrane antigens are included in the viral envelope (McKeating et al., 1986). In the case of HTLV-I virus-infected cells the beta2-microglobulin, a structural constitutent of HLA-class I epitopes, is continuously present in the envelope of mature particles (Fig. 9a. - Timár et al., 1987). The specificity of this phenomenon was proved by demonstrating that another HLA-class I-related epitope Fib-75, although expressed on the host, was not associated with the viral envelope (Fig. 9b).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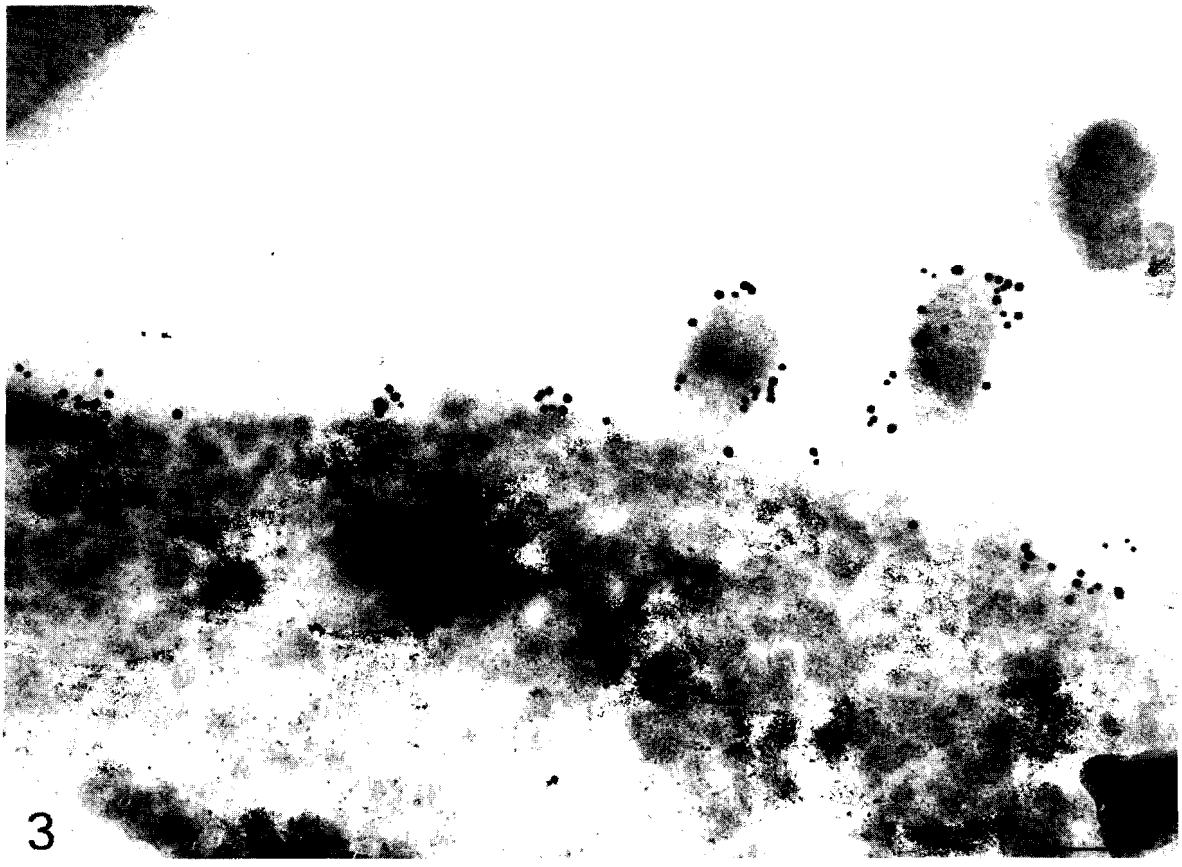

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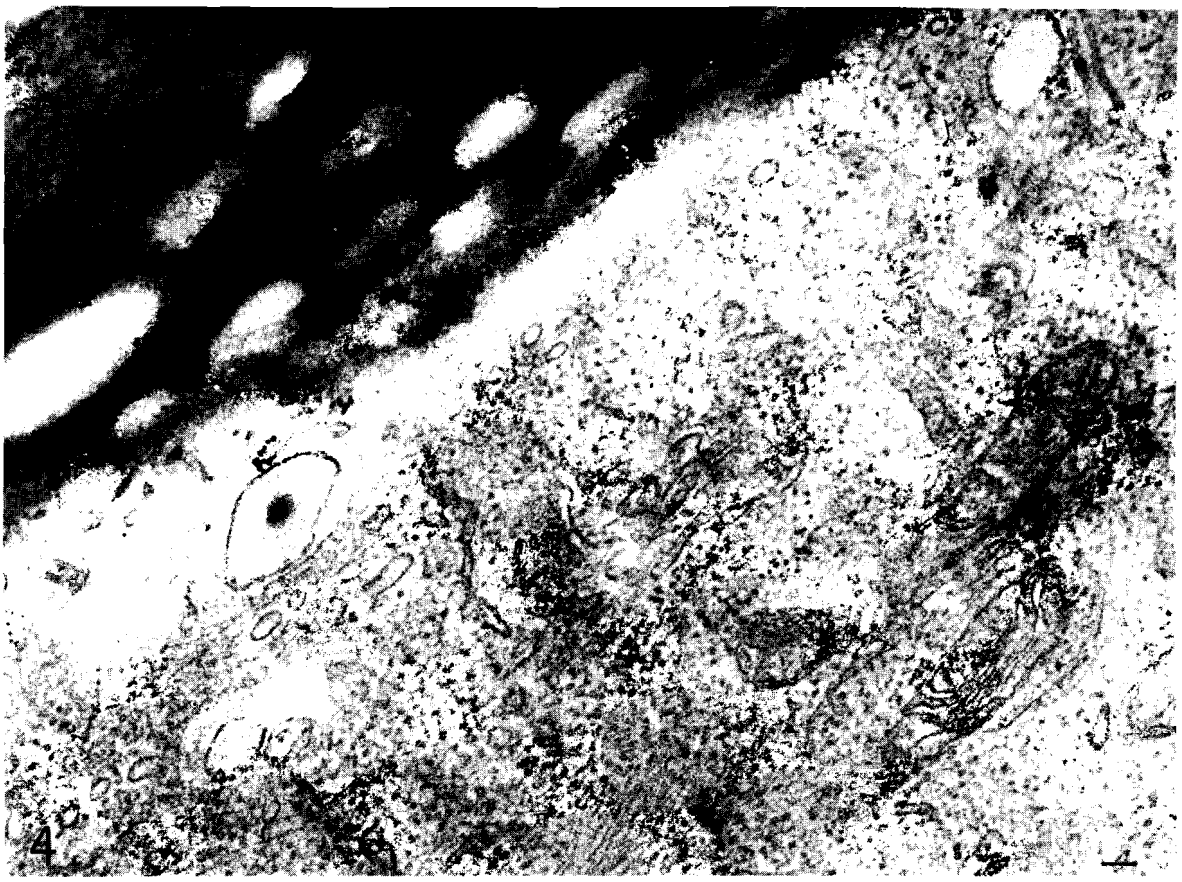
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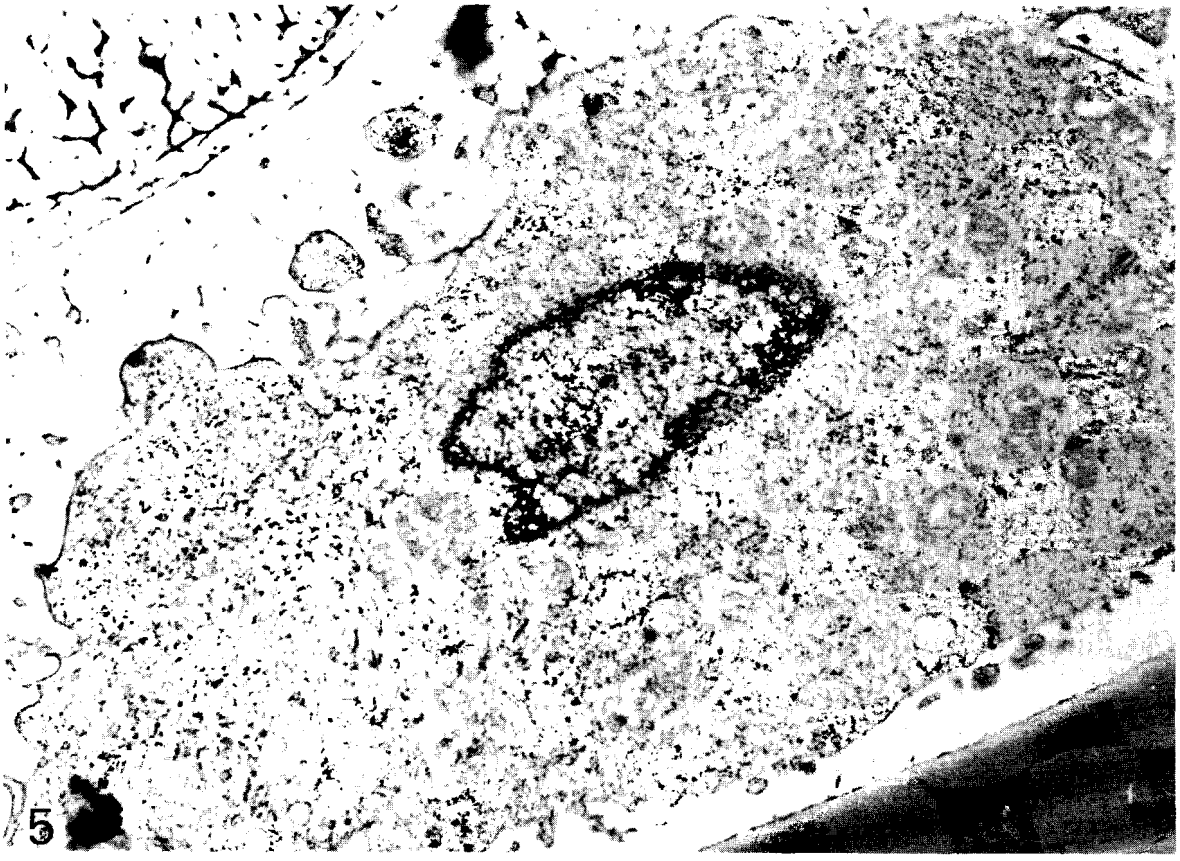
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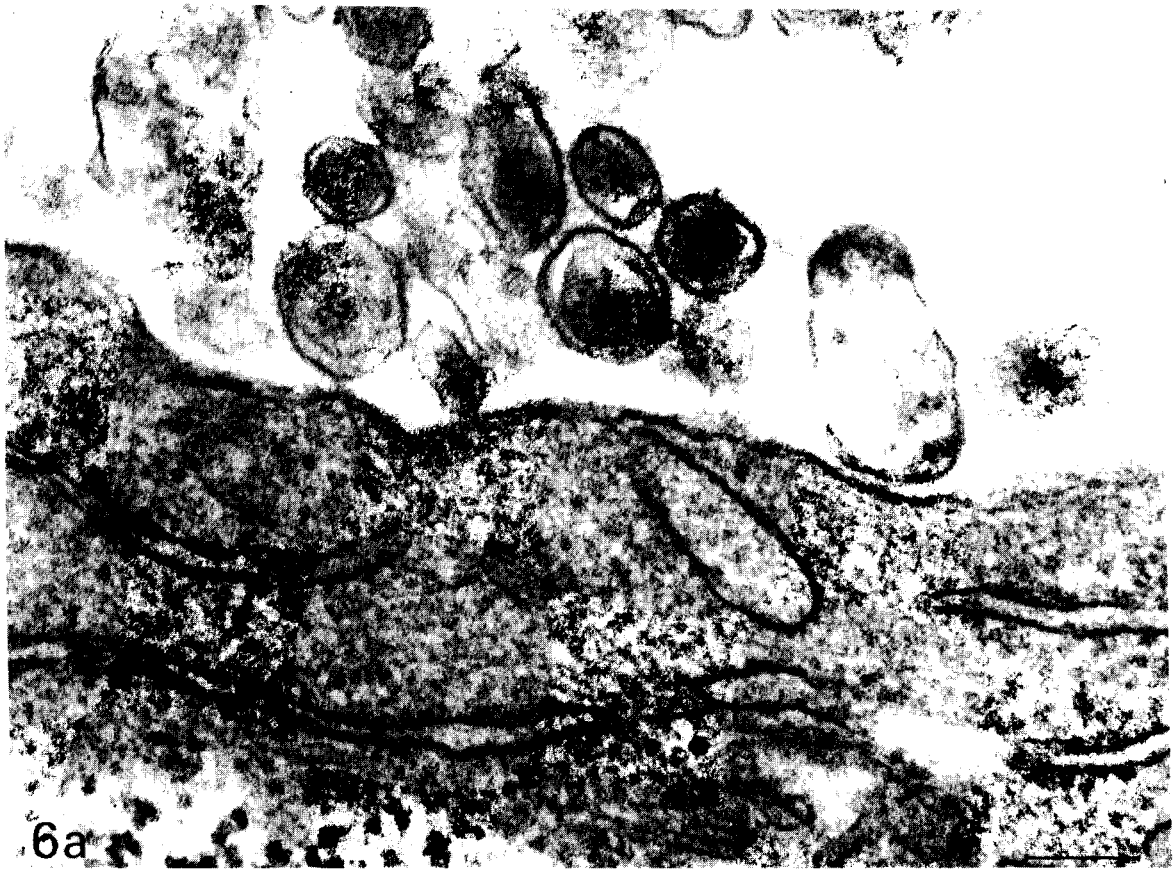

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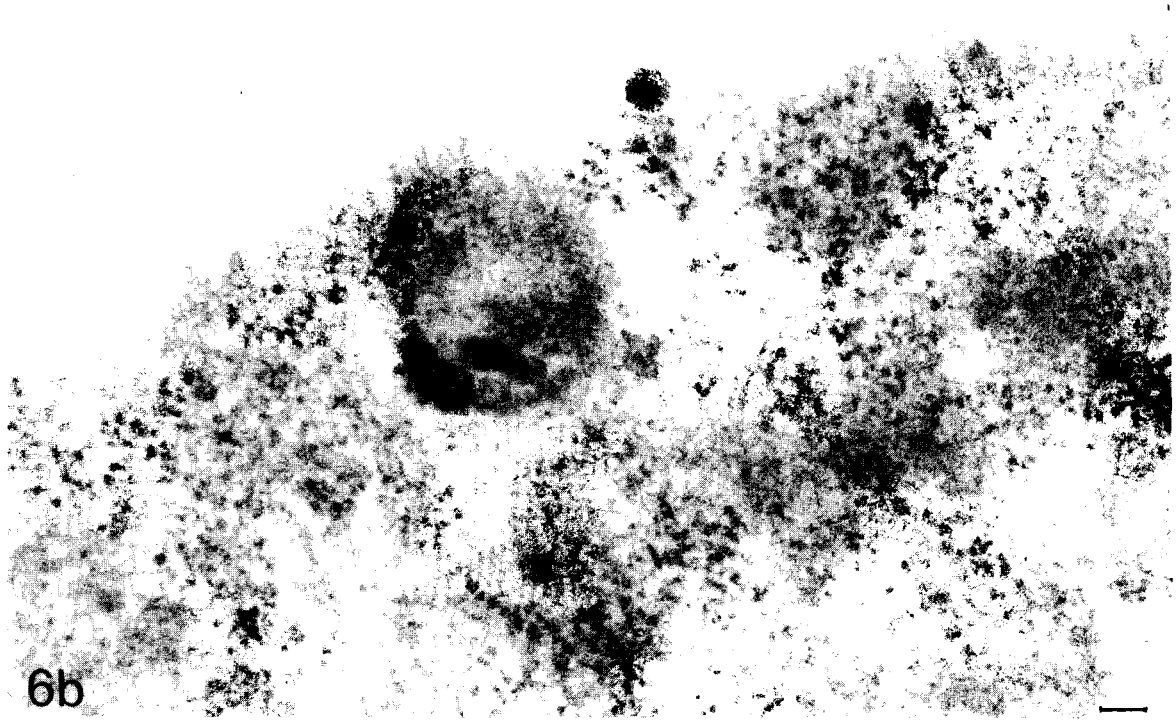
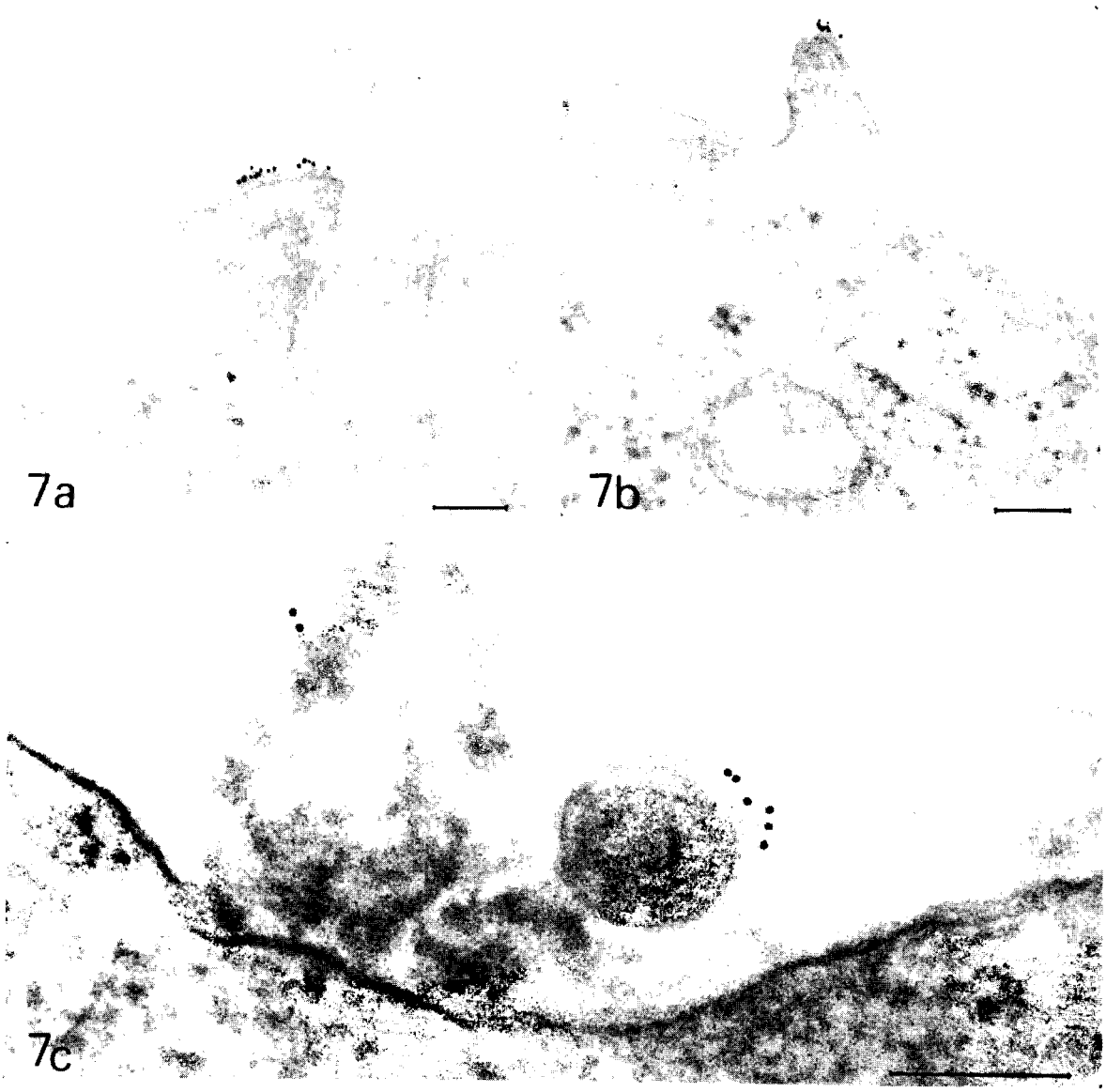
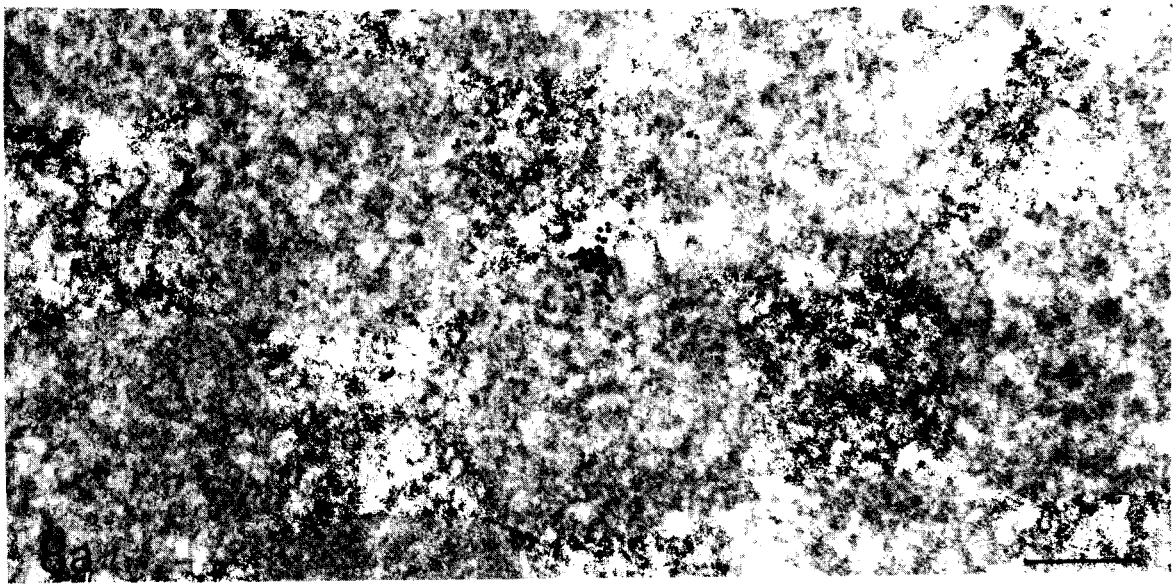


6b

Table 1. Human retroviruses.

1. Human T-cell lymphoma-leukemia virus (HTLV)
   - A. Type I. (ATL)
   - B. Type II. (Sezary syndrome ?)
2. Human immunodeficiency virus (HIV) - (AIDS)
   - A. Type I.
     - Lymphadenopathy associated virus type I. (LAV-I)
     - synonyms: Human T-cell lyumphoma-leukemia virus type III. (HTLV-III.)
     - Adenopathy related virus (ARV)
   - B. Type II. (LAV-II.)
3. Human T-cell lymphoma-leukemia virus type IV. (HTLV-IV.)
   (non cytopathic)





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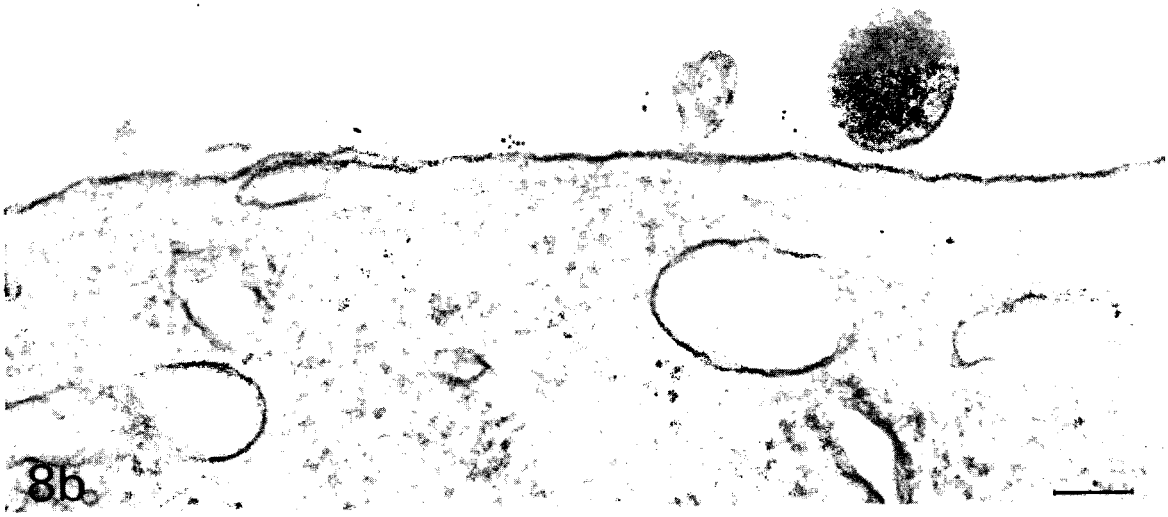

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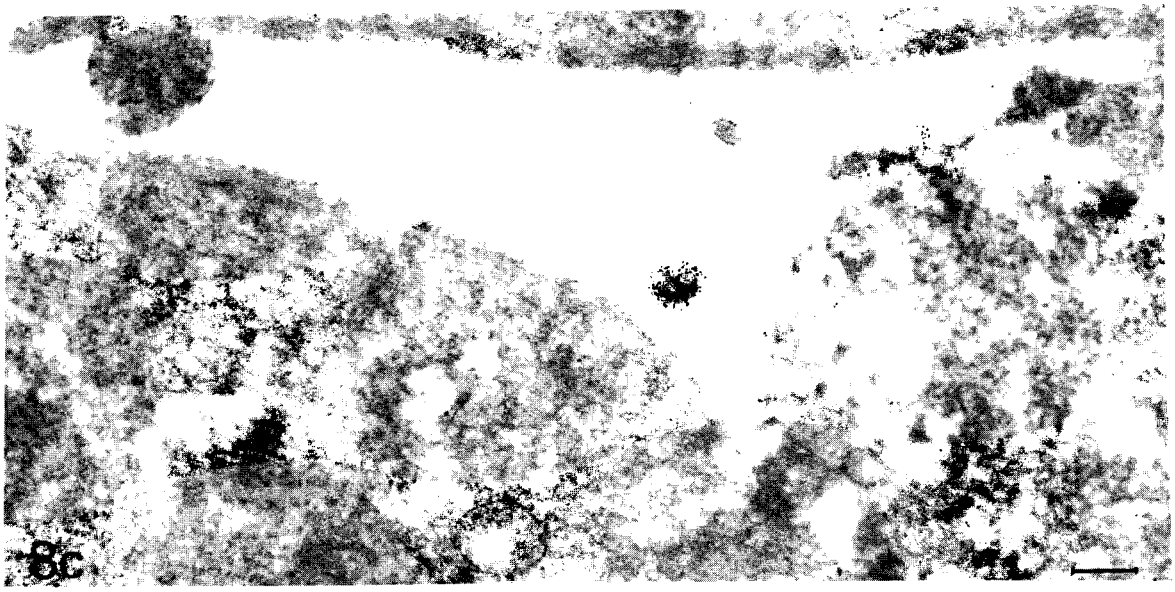

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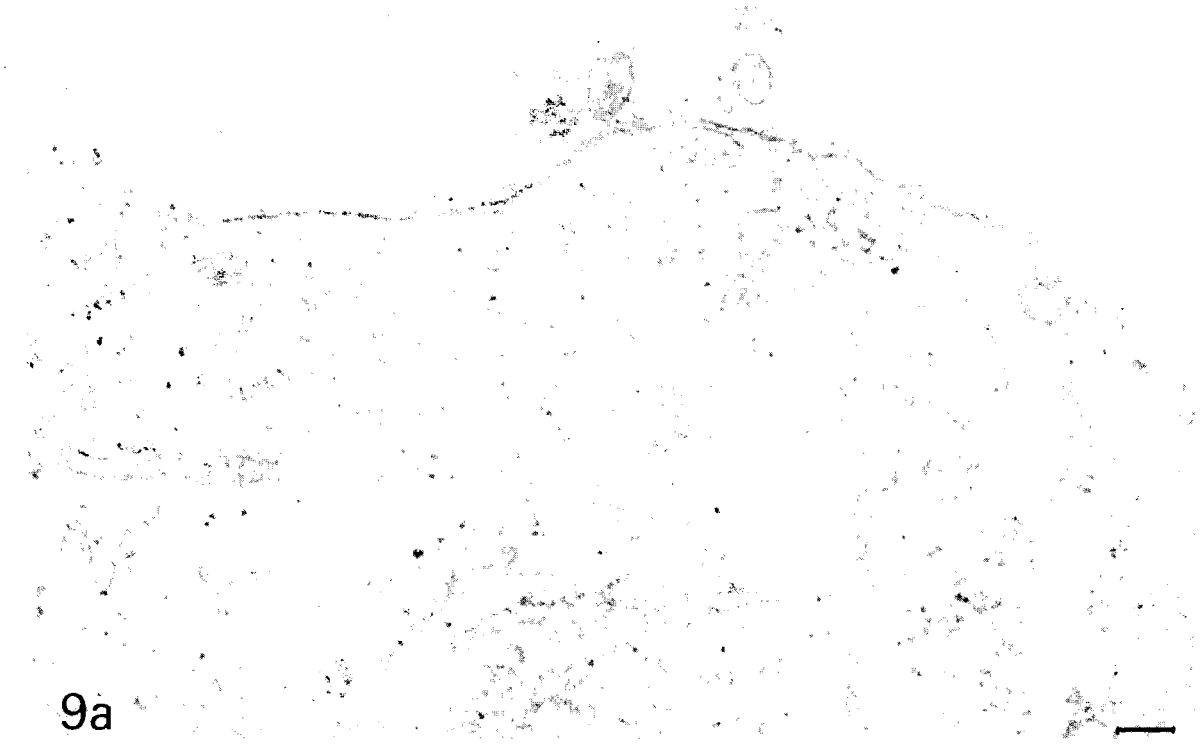

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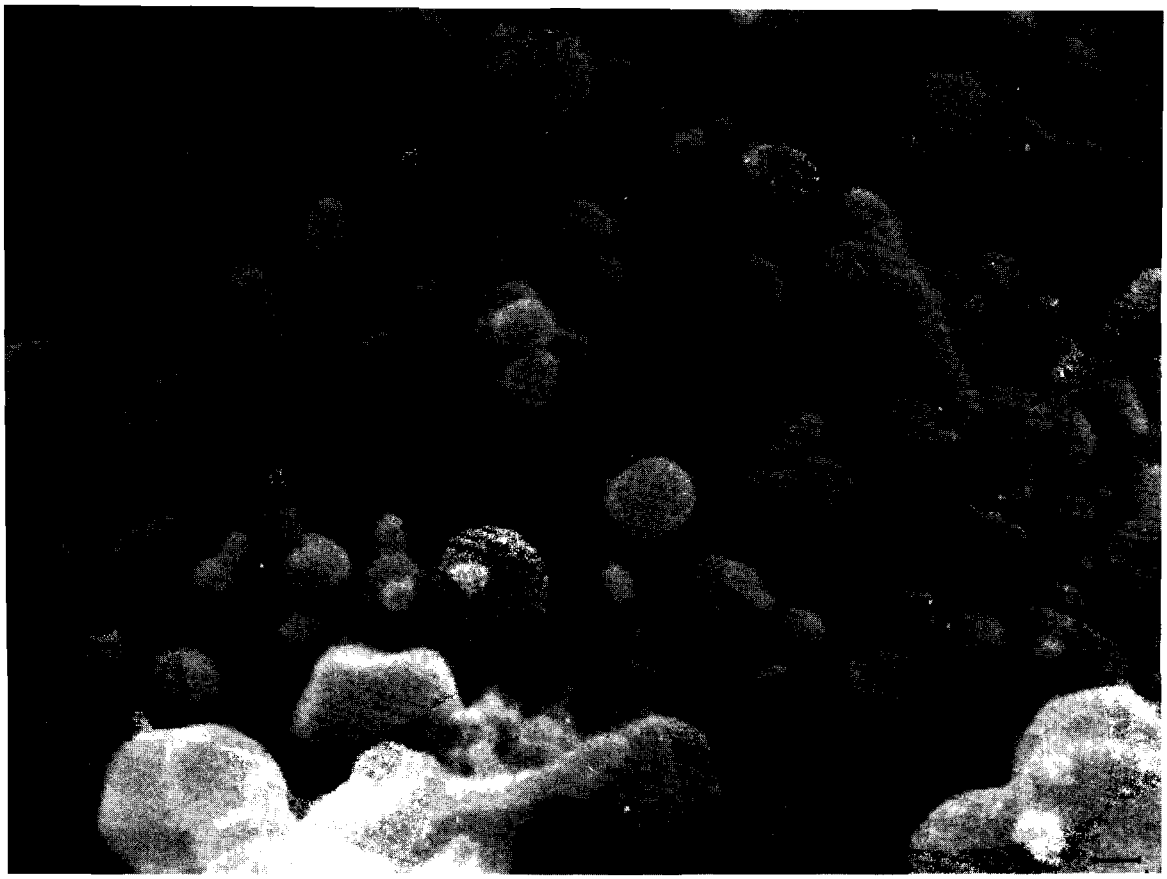

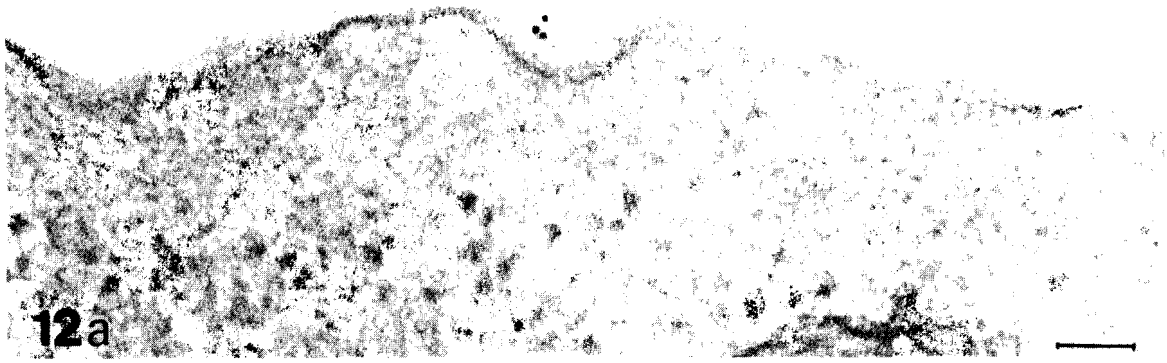
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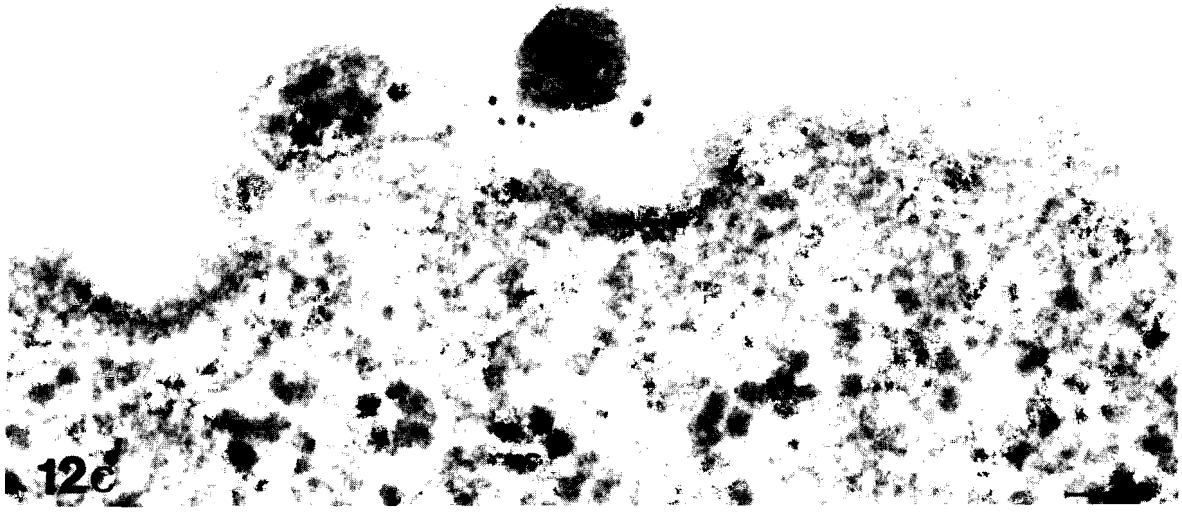
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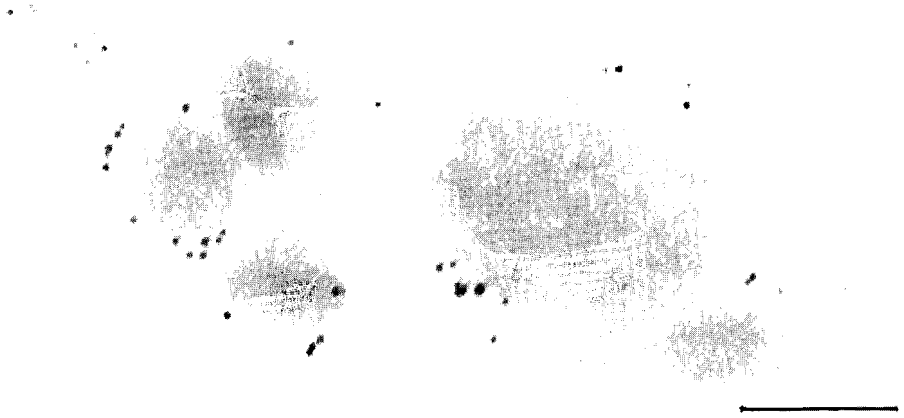

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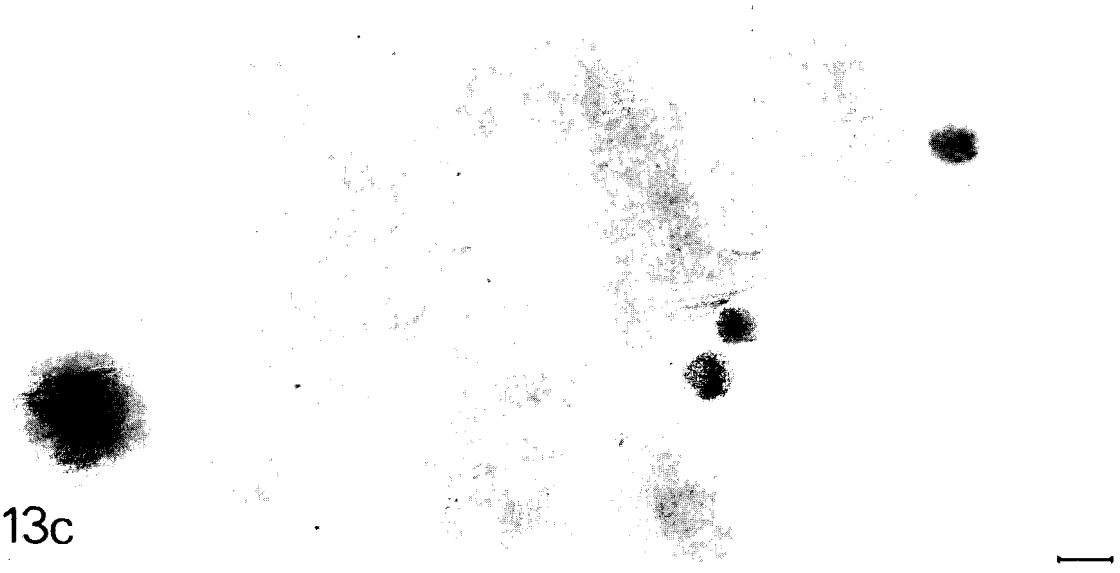

13c

Fig. 1. Involvement of VSV-envelope (G) protein in the budding process of rhabdovirus particles in Mink-lung cells. Detection of G protein on the surface of infected cells: indirect immunolabelling at 4° C. Method: mouse monoclonal anti-G (4AI-H. Paterson, ICR, London, UK) in 1:200 in PBS for 15 min, goat-anti-mouse IgG-G5, diluted 1:10 in PBS for 15 min, conventional double-fixation and counterstaining. Sequential steps of budding: **a.** G-protein containing area in the cell membrane. Note the cytoplasmic filaments directed towards the involved area. × 75,000. **b.** Protrusion of G-containing plasma membrane segment. × 75,000. **c.** Mature rhabdovirus particles are almost continuously covered by G protein in close association with the cell surface. × 75,000

Fig. 2. Parallel detection of VSV-envelope (G) and core (N) protein on the surface of infected cells. Method: prefixation indirect immunolabelling. Mouse monoclonal anti-G diluted 1:200 in PBS at 4° C for 15 min, goat-anti-mouse IgG diluted 1:25 in PBS for 15 min, rabbit-anti-goat IgG-ferritin diluted 1:20 in PBS for 15 min, normal mouse serum for 15 min, mouse monoclonal anti-N (2A4-I-H Paterson, ICR, London, UK) diluted 1:400 in PBS for 15 min at 4° C, goat-anti-mouse IgG-G5 diluted 1:10 in PBS. Conventional double fixation and counterstaining. × 50,000

Fig. 3. Parallel detection of VSV-envelope (G) and core (N) proteins on the surface of infected cells. Method: «Minimal fixation»- 1% paraformaldehyde + 0.5% glutaraldehyde for 15 min at 4° C, permeabilization with 0.5% saponin/PBS for 30 min at 4° C. Incubations at room temperature. Mouse monoclonal anti-N diluted 1:400 in PBS for 1hr, goat-anti-mouse Ig G-POX diluted 1:20- in PBS for 30 min, normal mouse serum for 30 min, monoclonal anti-G diluted 1:200 in PBS, goat-anti-mouse IG-G5 diluted 1:10 in PBS for 15 min. Postfixation in glutaraldehyde. DAB reaction, OsO_4 postfixation, not contrasted. DAB-reaction (N) was found in well-defined cytoplasmic areas near the cell membrane as well as in the budding profiles. The gold tracer demonstrating the envelope (G) protein can be seen only at the cell surface and on the viral particles. × 100,000

Fig. 4. Detection of VSV-core (N) protein in infected cells using microinjections technique. Method: 0.2 µl of mouse monoclonal-anti-N is microinjected in the cytoplasm of 100 living infected adherent Mink cells, then reinjected with the same amount of goat-anti-mouse IgG-G5. The cells are incubated at 37° C for 40 min and are fixed and embedded in situ. Conventional sectioning and contrasting. Note the gold-tracer-containing areas in the cytoplasmic matrix. × 45,000

Fig. 5. Cryosubstituted human fibrosarcoma cell. Method: cryofixation without chemical fixation (slamming), cryosubstitution at – 80° C with metOH, low temperature embedding into Lowicryl HM-20 at –39° C. Conventional room temperature sectioning and double counterstaining. Although there is no chemical fixation used, note the excellent ultrastructural preservation and contrast of subcellular organelles. × 8,200

Fig. 6. Electron microscopy of HTLV-I. **a.** Virus particles near the surface of C91PI human T-lymphoma cells. Note the variable sizes of the mature particles (80-150 nm), the electron-lucent zone under the lipoprotein membrane and the homogeneous core. Glutaraldehyde/ OsO_4 fixation, conventional embedding and counterstaining. × 150,000. **b.** Demonstration of the RNA content of the HTLV-I, using uranyl-acetate-EDTA-lead technique. The mature particle at the cell membrane has a highly electron-dense core. × 60,000

Fig. 7. Incorporation of the HTLV-I-envelope antigen into the mature particle during the budding process. Method: Prefixation indirect immunolabelling at 4° C. Incubation with anti-HTLV-I IgG+ patient serum diluted 1:100 in PBS for 15 min, mouse monoclonal-anti-human IgG diluted 1:200 in PBS for 15 min, goat-anti-mouse IgG-G5 diluted 1:10 in PBS for 15 min. Conventional double fixation, embedding and contrasting. Sequential steps of HTLV-I maturation at the cell membrane. **a.** HTLV-I envelope-antigen-positive area in the cell membrane. × 100,000. **b.** Envelope-antigen positive protrusion in the cell surface. × 100,000. **c.** Sectorial localization of the envelope-antigen in the coat of the mature virus. × 250,000

Fig. 8. Detection of the HTLV-I p19 (core) antigen in C91PI T-lymphoma cells. Postfixation indirect immunolabelling. Method: «minimal fixation». Permeabilization using 0.2% Triton-X-100 for 5 min. Incubation with mouse monoclonal anti-p19 diluted 1:100 in PBS for 30 min, goat anti-mouse IgG-G5 diluted 1:10 in PBS for 30 min. Conventional postfixation, embedding. **a.** Note the heavily labeled cytoplasmic matrix-areas. × 150,000. **b.** Surface labelling of HTLV-I-infected cells for p19. Note the weak reaction on the plasma membrane. × 100,000. **c.** The permeabilized mature virus is strongly positive for p19. × 80,000

Fig. 9. Detection of beta2-microglobulin in the envelope of HTLV-I. Prefixation indirect immunolabelling. Method: Incubation with anti-human beta2-microglobulin (polyclonal) diluted 1:20 in PBS for 15 min at 4° C, goat-anti-rabbit IgG-ferritin diluted 1:20 in PBS for 15 min. Conventional fixation and embedding. **a.** on the surface of beta2+ C91PI cells the mature viruses are heavily labeled. × 70,000. **b.** In a control experiment another surface antigen of the C91PI cells, the Fib-75 is not present on the virus. × 70,000

Fig. 10. HIV-I particles on the surface of H9-T4+ cells by scanning electron microscopy. Note the presence of numerous round profiles closely associated with the plasma membrane. × 60,000

Fig. 11. Transmission electron microscopy of an HIV-I-producing cell. Note the presence of typical asymmetric D-type particles at the cell surface. (Rod-shaped and C-like profiles are evident). × 80,000

Fig. 12. Detection of HIV-I envelope-antigen (gp120) during the budding process in H9 cells. Prefixation indirect immunolabelling. Method: Incubation at 4° C with HIV-I (HTLV-III)-antibody-positive pre-AIDS patient serum diluted 1:100 in PBS for 15 min, with a second layer of mouse anti-human monoclonal IgG diluted 1:100 in PBS for 15 min and with a third Layer of goat anti-mouse IgG-G5 diluted 1:10 in PBS for 15 min. Conventional fixation, embedding and contrasting. **a.** Membrane segment in H9 cells containing the gp120. × 10,000. **b.** Protrusion of a cell membrane segment carrying gp120. × 100,000. **c.** Mature HIV-I particle. Gp120 is localized into well-defined sectors of the envelope. × 100,000

Fig. 13. Demonstration of HLA antigens in the envelope of HIV-I particles. Prefixation indirect immunolabelling. **a.** Detection of beta2-microglobulin in the envelope. Method: Incubation with mouse monoclonal anti-human beta2-microglobulin diluted 1:100 in PBS for 15 min at 4° C, second incubation with goat-anti-mouse IgG-G5 diluted 1:10 in PBS for 15 min. Conventional fixation and embedding. Note the presence of beta2-microglobulin on the surface of H9 cells as well as in the envelope of the virus. × 20,000. **b.** Visualization of HLA-DR in the envelope. Method: Incubation with mouse monoclonal anti-HLA-DR diluted 1:100 in PBS for 15 min at 4° C, second incubation with goat-anti-mouse IgG-G5 diluted 1:10 in PBS. Conventional fixation and embedding. × 30,000. **c.** Absence of Fib-75 HLA-related epitope from the envelope. Method: Incubation with Fib-75 mouse-monoclonal IgG diluted 1:100 in PBS at 4° C for 15 min, second incubation in goat-anti-mouse IgG-G5 diluted 1:10 in PBS. Conventional fixation and embedding. Note the absence of the epitope from the cell surface and from the envelope of the mature particles. × 70,000.

All bars on figures represent 0.1 micron.

3. Immunoelectron microscopy of HIV-1

The acquired immunodeficiency syndrome (AIDS) is caused by another member of human T-cell-lymphotropic virus family, by HIV. It was first isolated in France (Barre-Sinoussi et al., 1983) and was called lymphadenopathy associated virus (LAV), then it was isolated in the USA and was designated as HTLV-III (Gallo et al., 1984). Another name of it is adenopathy-related virus (ARV) (Levy et al., 1984). The International Committee for Taxation of Viruses (ICTV) termed this virus as HIV and put it into the Lentivirus genus, based

on morphologic and genetic relationships with these viruses (Gonda et al., 1985). Some homology has been found with HTLV-I in the gag and pol genes (Ratner et al., 1985). Interestingly, there is also a limited serological cross-reactivity between HIV and HTLV-I. Beside the gag and pol genes, the HIV genom contains a 2.5 kbase open reading frame which corresponds to the env and lor genes of HTLV-I (Gelderblom et al., 1988). The major antigen protein of HIV is gp120, the precursor of which is gp160 (Allan et al., 1985). The cellular receptor of HIV is the CD4 protein of the T-helper lymphocytes which specifically interacts with the gp120 of HIV (Sattentau and Weiss, 1988). In some isolates it was possible to identify gp41, the putative transmembrane-protein of the HIV (Shangadharan et al., 1984). Beside the env, gag, and pol there are few open reading frames in HIV genom coding 4 proteins (Palmer and Goldsmith, 1988). The morphological substrate of gp120 and gp41 is a knob-like structure in the envelope (Gelderblom et al., 1987). The loose association between the two gps is casused by the lack of S-S bridges between them and this is the cause of the shedding of gp120 from the virus, resulting in a series of events associated with the HIV infection (binding to and depletion of protective antibodies, formation of antibody-antigen complexes and masking of CD4 epitopes on infected T-cells (Klatzmann and Gluckmann, 1986).

LAV-II (HIV-II) was isolated in Africa where HIV-I is not endemic (Clavel et al., 1986). The differences between HIV-I, and HIV-II is in the envelope gp (140/120). HIV-I virus has an immunological crossreactivity with STLV-III the causative agent of simian AIDS (Palmer and Goldsmith, 1988). HTLV-IV, isolated in West-Africa, does not cause AIDS; it is not cytopathic for CD4 cells (Kanki et al., 1986). The HIV-i has a diameter of 100nm with a cone-shaped core (100nm × 45nm) (Gallo et al., 1984; Gelderblom et al., 1988; Palmer and Goldsmith 1988) (Fig. 10) covered by a core-shell of 5 nm. The p17 could be a stabilizer protein underneath the viral envelop. The env projections are the gp120-trimers and gp41 is also accessible to the surface with gp17 (Gelderblom et al., 1987). In AIDS patient's serum antibodies against gp120, gp41 and p17 could be detected (Ranki et al., 1987). The core-shell protein p24 and the RNA-associated p15 are also structural components of HIV-I (Gelderblom et al., 1988). During the budding process the cell membrane gp120 is incorporated into the mature particle (Fig. 11). The sectorial localization of this gp is very similar to the one observed in the case of HTLV-I. Avian and murine oncoviruses incorporate host antigens into their envelope (Bubbers and Lilly, 1977; Azocar and Essex, 1978). It was demonstrated that HLA-DR is specifically picked up by the budding HIV-I (Timár et al., 1987b; Gelderblom et al., 1988) (Fig. 12), but beta2-microblobulin could also be detected in the envelope (Timár et al., 1987b) (Fig. 13). This fact could explain some pathological events in AIDS patients and some characteristics of anti-HIV immune reactions. It is important to note that HLA-DR is specifically recognized by CD4 epitopes, so these interactions could further support the HIV-gp120/CD4 connections. The presence of HLA-class I epitopes in the envelope of HIV could result in altered MHC-restricted anti-HIV immune reactions. This fact could also substantiate a well known clinical observation: that in HIV-infected patients the beta2-microglobulin level in the serum is frequently increased (Lecey et al., 1987).

Concluding remarks

It is now evident from the recent ultrastructural and immunocytochemical investigations on human retroviruses, that electron microscopy has significantly contributed to the identification of these viruses in the pioneering period of the newly discovered diseases: ATL and AIDS. After a few years of systematic studies revealing and identifying the fine structure of these viruses it is now obvious that immunoelectron microscopy has provided necessary proofs to substantiate the biochemical data on viral proteins and has helped to construct the model of these viruses. With the development of powerful new electron microscopical techniques, such as low temperature embedding and cryosubstitution, it is now possible to apply them in everyday diagnosis of human retrovirus infections.

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