Ultrastructural demonstration of retrovirus antigens with immuno-gold staining in prodromal acquired immune deficiency syndrome

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SUMMARY The peripheral lymphocytes of a patient with prodromal acquired immune deficiency syndrome contained giant multivesicular bodies. These were specifically stained by immuno-gold labelled polyclonal antibodies against the major core protein p24 of bovine leukaemia virus and human T cell leukaemia virus I. Moreover, the patient's serum was positive for bovine leukaemia virus by the ELISA method.

It is increasingly likely that the transmissible causative agent of acquired immune deficiency syndrome (AIDS) is a variety of T lymphotropic retrovirus. Vilmer *et al*¹ have isolated a lymphadenopathic virus from two siblings with haemophilia, one of whom had AIDS. Popovic *et al*² have developed a cell system for the reproducible detection of human T cell leukaemia virus III from patients with AIDS and pre-AIDS.

Ultrastructural immunological staining has recently progressed with the use of colloidal gold-antibody probes.³ We report the observations made with this technique on the peripheral lymphocytes from a prodromal case of AIDS using a panel of polyclonal and monoclonal antibodies directed against different retrovirus antigens.

Case report

A 56 year old Zairean woman was admitted for severe weight loss, diffuse lymph node enlargement, and slight fever. She denied any sexual contact for four years and had not received any transfusions or immunosuppressive treatment. She had not been outside Europe for 20 years. Haematological data showed moderate lymphopenia ($2.5 \times 10^9/l$) and a loss of lymphocytes bearing the helper-inducer phenotype (3%) as defined by the monoclonal OKT4 antibody (OKT4:OKT8 ratio 0.04). Cu-

taneous anergy for a panel of five antigens was noted. All in vitro measurements of T lymphocyte function were decreased, including mitogenic responses to phytohaemagglutinin, concanavalin A, candidin, γ -interferon production, and interleukin 2 production. Natural killer cell activity using K562 cells as targets was considerably decreased. Immunoglobulin concentrations were raised: IgG 73 g/l, IgA 3.15 g/l, Ig M 4.63 g/l. Extensive investigation showed no lymphoproliferative neoplasia or other immunosuppressive diseases. Lymph node biopsy showed follicular hyperplasia similar to that reported in AIDS. IgG antibodies against herpes virus, cytomegalovirus, Epstein-Barr virus, hepatitis B virus, and toxoplasma were present. There was no evidence of opportunistic infection at the time of the study but the patient is being investigated for interstitial pneumonitis and lymphocytic meningitis.

Methods

ULTRASTRUCTURAL IMMUNO-GOLD STAINING *Postembedding method*

Peripheral mononuclear cells isolated by Ficoll gradient were directly fixed by 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 2 h at room temperature. No postfixation was performed. The material was embedded in Epon as previously described.⁴ Ultrathin sections on nickel grids were first incubated with normal goat serum diluted 1/10 with Tris NaCl bovine serum albumin buffer, pH

Table 1 Immuno-gold reactions of patient's lymphocytes

Anti-bovine leukaemia virus antibodies		Anti-human T cell leukaemia virus 1 antibodies	
Polyclonal anti p-24	+	Polyclonal anti-p-24	+
Polyclonal anti gp51	+	Monoclonal anti-p-24	-
Monoclonal anti-p-24 (4'F5)	-	Monoclonal anti-p-19	-
Other anti-retrovirus antibodies		Control antibodies	
Polyclonal anti-p-30 (Rauscher)	±	Normal R S	-
Polyclonal anti-gp70 (Rauscher)	-	Rabbit anti-legumin	-
Polyclonal anti-avian myeloblastosis virus	-	Monoclonal (control)	-
		Goat antirabbit gold alone	-
		Anti-p 24 (BLV) depleted by the corresponding antigen	-

+ = positive staining, - = negative staining.

*A storage protein of plant origin. Serum of a rabbit hyperimmunised against this antigen did not show any reaction.

8.2; then with antiretrovirus antiserum (Table 1) diluted 1/1000 in the same buffer; and finally with the corresponding immuno-gold conjugate: goat antirabbit gold 5 nm, goat antimouse gold 5 nm, or rabbit antigoat gold 5 nm (obtained from Janssen Life Sciences Products, Beerse, Belgium).

Incubation of living cells

The mononuclear cells collected in RPMI were first incubated with the antiretrovirus serum diluted in phosphate buffered saline, 1% human AB serum, 0.2% azide sucrose free medium, and then incubated in the immuno-gold conjugate.

Finally, the material was fixed in 4% glutaraldehyde in Millonig buffer and postfixed with 2% OSO₄ before embedding in Epon.

Controls

Lymphocytes from the patient were tested with normal rabbit serum, polyclonal antilegumin rabbit serum, control monoclonal antiserum, and goat anti-rabbit gold alone. The specificity of the rabbit polyclonal anti-p24 bovine leukaemia virus antiserum was evaluated after depletion with lysed bovine leukaemia virus particles. Immuno-gold staining was also performed on the peripheral lymphocytes from a normal woman and from a man with active hepatitis B virus infection.

Bovine leukaemia virus antibodies were tested with the same postembedding technique on fetal lamb kidney cells producing bovine leukaemia virus and on centrifuged bovine leukaemia virus particles from the Laboratory of Virology, Institute of Molecular Biology, (A Burny). On the other hand, human T cell leukaemia virus antibodies were tested on virus producing cells issued from the laboratory of RC Gallo.

SEPARATION OF THE DIFFERENT POPULATIONS OF MONONUCLEAR CELLS

Fresh peripheral blood mononuclear cells were iso-

lated by Ficoll-Hypaque gradient. These cells were further fractionated by the formation of sheep red blood cell rosettes. The resulting T cell population was depleted in OKT4⁺ cells by treatment with the corresponding cytotoxic monoclonal antibody in the presence of complement.⁵ The isolation of OKT4⁺ cells for ultrastructural study was not possible because of their reduced number.

BOVINE LEUKAEMIA VIRUS SEROLOGY

Detection of bovine leukaemia virus p-24 cross reacting antibodies was performed by the ELISA method. In this assay, Microtest plates (Gibco 2-69620) were coated with 100 ng of purified bovine leukaemia virus p-24 protein dissolved in 0.01 M NaHCO₃-Na₂CO₃, pH 9.6, buffer and then saturated with bovine serum albumin. They were then incubated with dilutions of serum samples. After washing, peroxidase conjugated protein A (10 ng) was added and its binding was revealed by O-phenylene diamine. Optical density readings obtained in the absence of serum dilutions were zero. A detailed description of the ELISA technique has been given elsewhere.⁶

SPECIFICITY OF ANTI-BOVINE LEUKAEMIA VIRUS SERA

Purification of bovine leukaemia virus gp-51 and p-24 has been described by Bex *et al.*⁷ The purified proteins were injected into rabbits in order to obtain monospecific polyclonal antisera. The specificities of these sera were shown by radioimmune precipitation and polyacrylamide gel analysis of the precipitated antigen.^{8,9}

Results

ULTRASTRUCTURAL OBSERVATIONS

Tubuloreticular structures and numerous multivesicular bodies but no test tube or ring shaped forms were encountered in a few lymphocytes and

Fig. 1 Patient's lymphocyte with giant multivesicular bodies (M) and reticular inclusions (arrow). Original magnification $\times 60\ 000$.

Fig. 3 Postembedding immuno-gold staining with rabbit anti-bovine leukaemia virus p-24 goat antirabbit gold 5 nm on fetal lamb kidney cells producing bovine leukaemia virus. Original magnification $\times 60\ 000$.

Fig. 2 Postembedding immuno-gold staining with rabbit anti-bovine leukaemia virus p-24 goat antirabbit gold 5 nm on patient's lymphocyte. Original magnification $\times 60\ 000$.

Fig. 4 Immuno-gold staining (arrow) after incubation with rabbit anti-bovine leukaemia virus p-24 goat antirabbit gold 5 nm on living patient's lymphocyte. Original magnification $\times 60\ 000$.

Table 2 Anti-bovine leukaemia virus reactivity in patient's serum

Serum sample*	Optical density (492 nm)†
Normal human serum†	0.016‡
Patient's serum	0.205
Anti-bovine leukaemia virus p-24 rabbit serum	>1.500

*At 1/180 dilution.

†Normal human serum is a pool of 32 healthy blood donors.

‡This optical density reading is an absolute value. The background of the reaction is zero in the absence of serum dilutions.

monocytes of the patient. Neither tubuloreticular structures nor test tube or ring shaped forms were found in normal and chronic hepatitis controls.

A striking feature was the presence of great cytoplasmic vacuoles resembling giant multivesicular bodies in about 1% of the mononuclear cells (Fig. 1). They contained a granular material, some double outlined vesicles, and were often adjacent to "reticular" inclusions. In our experience, giant multivesicular bodies have been encountered in four prodromal cases of AIDS in black Africans. In the present case, such giant multivesicular bodies were not present either in the separate populations of suppressor cells (OKT8⁺ cells) nor in B cells and monocytes. Post-embedding immuno-gold staining performed with rabbit anti-bovine leukaemia virus p-24, rabbit anti-bovine leukaemia virus gp-51 polyclonal antibodies, and goat anti-human T cell leukaemia virus I p-24 polyclonal antibody was strongly positive in the giant multivesicular bodies (Fig. 2). No staining was observed with antiavian myeloblastosis virus polyclonal antibody and monoclonal antibodies against bovine leukaemia virus and human T cell leukaemia virus I. Anti-Rauscher virus p-30 polyclonal antibody gave a weak staining reaction (Table 1). There was no staining at the level of the tubuloreticular structures. In the OKT8⁺ cells, B lymphocytes, and monocytes, no staining was observed. No staining was detected with control rabbit, mouse, or goat-antirabbit gold antibodies. Moreover, the staining with anti-bovine leukaemia virus p-24 antibody was suppressed after depletion with the corresponding antigen. The staining of the fetal lamb kidney cells producing bovine leukaemia virus with anti-bovine leukaemia virus antibodies (Fig. 3) was positive in the cytoplasmic inclusions. A specific staining with a minimal background of the human T cell leukaemia virus producing cells with anti-human T cell leukaemia virus I antibodies was also obtained.

Finally, staining was also observed at the level of the giant multivesicular bodies of living cells with the rabbit anti-p-24 bovine leukaemia virus polyclonal antibody (Fig. 4). This reaction was still present after incubation with sodium azide and in

sucrose free buffers. During the development of the disease intracytoplasmic stained inclusions disappeared.

SEROLOGICAL OBSERVATIONS

As shown in Table 2, the patient's serum contained anti-bovine leukaemia virus p-24 reactivity.

Discussion

There is strong epidemiological, biological, and biochemical evidence that one or more retroviruses—lymphadenopathic virus,¹ human T cell leukaemia virus III²—are associated with human AIDS. Moreover, a similar syndrome has been induced in primates by injection of a type D retrovirus isolate.¹⁰ Electron microscopical studies of AIDS lymphocytes have defined different ultrastructural features of the disease.¹¹⁻¹⁷ Some of these structures are similar to those seen in virus infected cells. In the present case, we found, in addition to tubuloreticular structures and multivesicular bodies, cytoplasmic changes reminiscent of those encountered in fetal lamb kidney-bovine leukaemia virus infected cells. We were therefore interested in investigating whether bovine leukaemia virus related antigens could be shown at the ultrastructural level. For this purpose, we used an immuno-gold staining technique that has already been applied to the intracellular identification of viral antigens.¹⁸ The validity of this staining method for the detection of human T cell leukaemia virus I and bovine leukaemia virus antigens was confirmed in preliminary experiments involving virus producing cells. The use of glutaraldehyde alone as a fixative allows a satisfactory conservation of the ultrastructure without altering the specificity of antigen-antibody reactions.

In our patient we noted a specific reactivity with polyclonal antibodies directed against the major core protein of bovine leukaemia virus and human T cell leukaemia virus I. This cross reactivity is not surprising since bovine leukaemia virus and human T cell leukaemia virus I p-24s share common amino acid sequences. The lack of staining with the tested monoclonal antibodies is consistent with the hypothesis that the detected antigen belongs to a member of the bovine leukaemia virus-human T cell leukaemia virus family. A positive immuno-gold reaction was also present in unfixed cells incubated under conditions ensuring inhibition of pinocytosis of the 5 nm gold particles. The transient immunoreactivity of patient's cells is consistent with the findings of Popovic *et al.*,² who reported transient expression of cytopathic variants of human T cell leukaemia virus III in cells of patients with AIDS.

The presence of a positive bovine leukaemia virus serology further suggests that our patient has been in contact with a related virus.

Obviously, the importance of our findings obtained in a single case is difficult to assess and similar studies on other patients at different stages of the syndrome are needed to investigate the possible relation between the antigens found in the present case and the putative agent(s) of AIDS.

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