MATURATION OF RESPIRATORY SYNCYTIAL VIRUS WITHIN HEP-2 CELL CYTOPLASM

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Received August 6, 1996

Summary. – Electron microscopy of HEp-2 cells infected with respiratory syncytial virus (RSV) strain Long revealed the maturation of RSV on an ultrastructural level. The results showed that the virus maturated by two different pathways. In one of them, the virus assembled and matured before reaching the plasma membrane on the internal vesicle membrane within cytoplasm. The mature virus was delivered to the plasma membrane and to the extracellular space most likely by the transport vesicles and exocytosis. In the other pathway, the virus matured on the plasma membrane as described with other members of the family *Paramyxoviridae*. Using monoclonal antibodies (MoAbs), we localized viral nucleoprotein (NP) and envelope proteins in cytoplasm by immunoelectron microscopy (IEM).

Key words: respiratory syncytial virus; HEp-2 cells; immunoelectron microscopy; virus maturation; internal vesicle membrane; monoclonal antibody

Introduction

RSV (family *Paramyxoviridae*, subfamily *Pneumoviridae*, genus *Pneumovirus*) is the major cause of a lower respiratory tract disease in infants and children all over the world. The mechanism of acquiring immunity to RSV infection is unknown and no effective vaccine is at present available (Belshe *et al.*, 1982; Kim *et al.*, 1969). RSV is a membrane-bound pleomorphic particle with closely spaced short projections. The bilayered membrane encloses RNA nucleocapsid containing a non-segmented negative strand RNA genome (Huang and Wertz, 1982). The projections on spikes consist of two different glycoproteins, G and F, which are the most important proteins in the process of infection and immunity (Olmsted *et al.*, 1986). Recent molecular biological and biochemical studies on RSV revealed the detailed chemical composition and functions of these envelope proteins (Ball *et al.*, 1986; Collins, 1990; Collins, 1991; Fernie *et al.*, 1985; Gruber and Levine, 1985); however, the complete assembly of RSV in host cells has not been clarified. Most of previous morphogenetic studies have disclosed that RSV matures by budding through the plasma membrane (Armstrong *et al.*, 1962; Berthiaume *et al.*, 1974; Kalica *et al.*, 1973), although Norrby *et al.* (1970) have reported that maturation of RSV in Vero cells is likely to take place on the internal vesicles membrane within cytoplasm. In the course of our recent IEM study aimed to find the virus-host cell interaction for new vaccination strategy, we observed many complete virus particles inside the RSV-infected HEp-2 cells.

In this report, we present two different pathways of maturation of RSV in HEp-2 cells and also the distribution of viral NP and envelope proteins F and G in the cytoplasm as observed by IEM using MoAbs.

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Abbreviations: IEM = immunoelectron microscopy; MoAb = monoclonal antibody; NP = nucleoprotein; PLP = periodate-lysine-paraformaldehyde; RSV = respiratory syncytial virus

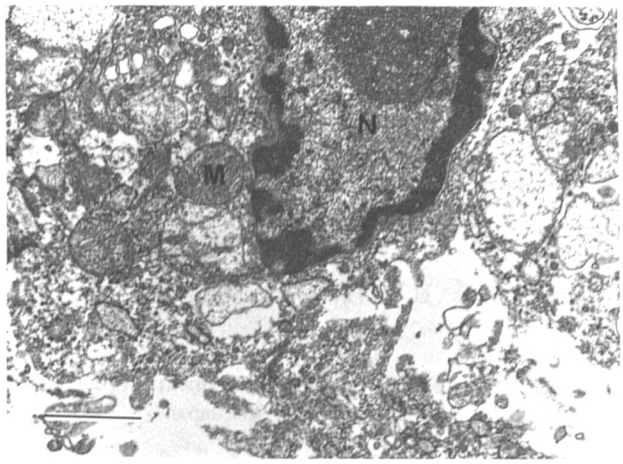

Fig. 1
IEM of uninfected HEp-2 cells
PLP fixation and saponin permeabilization. Anti-G MoAb and immunogold used. N = nucleus, M = mitochondria. Bar = 1000 nm.

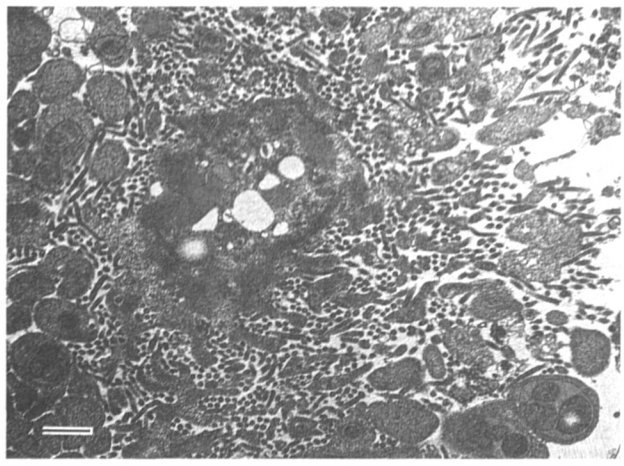

Fig. 2a
Ultrathin section through the surface of HEp-2 cell 3 days after infection with RSV
Many mature virus particles released into the extracellular space seen, suggesting no budding of RSV through the plasma membrane. Bar = 1000 nm.

Materials and Methods

Virus and cells. The long strain (type A) of RSV was grown in HEp-2 cells. Eagle's Minimal Essential Medium (Gibco) containing 7% foetal calf serum was used for cell propagation. The same medium with 2% serum was used for virus growth. Cells were infected at multiplicity of 0.05. The infected HEp-2 cells were subjected to IEM 3 days after virus inoculation.

MoAbs and colloidal gold. MoAbs against RSV proteins F and G and NP were used in IEM (Tsutsumi *et al.*, 1987; Tsutsumi *et al.*, 1988). A protein A-gold complex, 5 nm in diameter, was prepared as described earlier (Clot and Genzl, 1981; Tanaka *et al.*, 1984) and used for detection of deposited MoAbs.

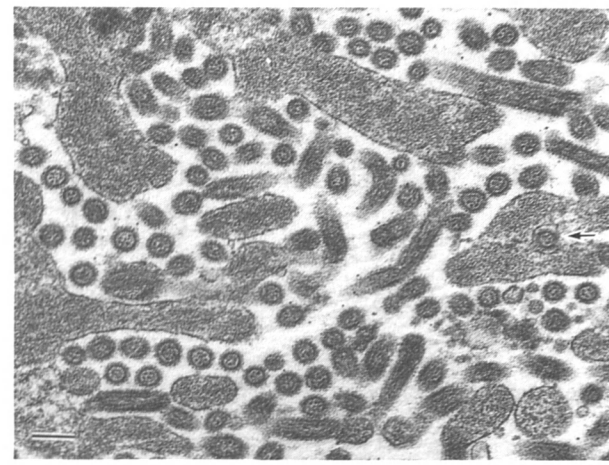

Fig. 2b
Magnification of Fig. 2a
An arrow shows a virus particle in a vesicle. Glutaraldehyde fixation. Bar = 200 nm.

IEM. Two pre-embedding methods with different fixatives were used. (1) The surface-labelling method: RSV-infected HEp-2 cells were collected by low-speed centrifugation and fixed with 1% glutaraldehyde in 0.1 mol/l cacodilate buffer at 4°C for 1 hr. The cells were washed in the same buffer, followed by 0.1 mol/l glycine pH 7.2 to block the excessive aldehyde residues (Hung *et al.*, 1987). (2) The penetrating-labelling method: the infected cells were fixed with periodate-lysine-paraformaldehyde (PLP) Bohn, 1978; McLean and Nakane, 1974) and treated with 0.03% saponine. The fixed cells were incubated with each MoAb diluted 10 times in 1% bovine serum albumin at 37°C for 1 hr. After extensive washing, the cells were incubated overnight in the protein A-colloidal gold complex at 4°C. To eliminate the unadsorbed colloidal gold, repeated washing with phosphate-buffered saline was done by low-speed centrifugation. The pelleted cells were fixed in 1% osmium-tetroxide and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and then with lead citrate. Hitachi H7000 electron microscope was used. The specificity of the labelling was proved using uninfected cells treated with MoAbs and colloidal gold (Fig. 1).

Results

Localization of mature RSV particles within HEp-2 cells

Ultrathin sections of a portion near the surface of HEp-2 cells revealed the release of a high number of virus particles outside the cells suggesting that this is a phenomenon

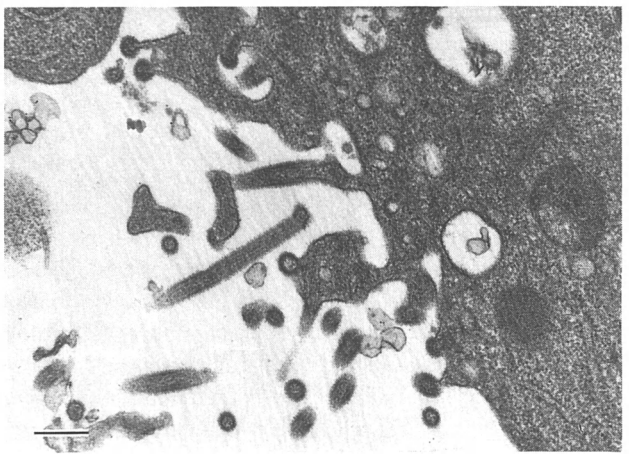

Fig. 3
RSV budding through the plasma membrane and the membrane of small vesicle close to the plasma membrane of HEp-2 cell
Glutaraldehyde fixation. Bar = 300 nm.

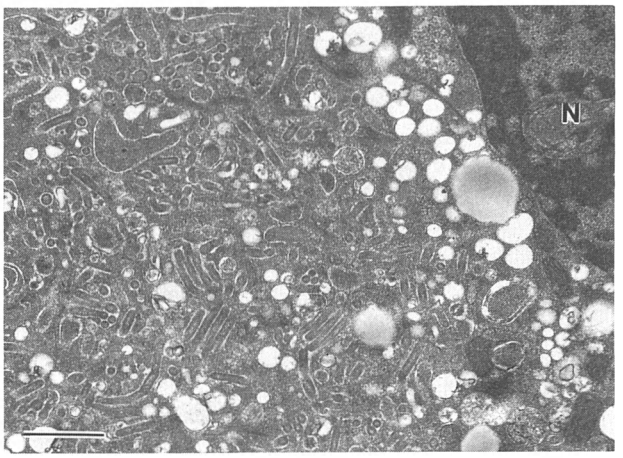

Fig. 4
Localization of mature RSV particles in the cytoplasm close to the nucleus (N) of HEp-2 cell
Glutaraldehyde fixation. Bar = 1000 nm.

caused by the lysis of the cells or by the burst of mature virus particles from the cells (Fig. 2a). A more enlarged picture (Fig. 2b) shows that the virus particles are mature complete virions with spikes on the surface and moreover, nucleocapsids are observed inside the bilayered membranes. Their size is approximately 100 – 300 nm and they are arranged in one direction like looking for the exit through the channels. On the other hand, one complete virus particle is seen in a vesicle (arrow). This phenomenon suggests another pathway for maturation of RSV besides budding from the plasma membrane, as reported for paramyxoviruses (Fig. 3) (Armstrong *et al.,* 1962; Ball *et al.,* 1986; Berthiaume *et al.,* 1974; Kalica *et al.,* 1973). On the basis of these observations we attempted to study the maturation process of RSV by IEM. In the merotomied samples of infected HEp-2 cells, mature virions were found in cytoplasmic vesicles. These particles were of filamentous shape, approximately 500 – 1000 nm in length and their cross-sections were round, 100 nm in diameter, containing a bilayered envelope with fully developed spikes (Fig. 4). Many single particles with diameters corresponding to those of the particles with spikes were seen in the intracytoplasmic vesicles.

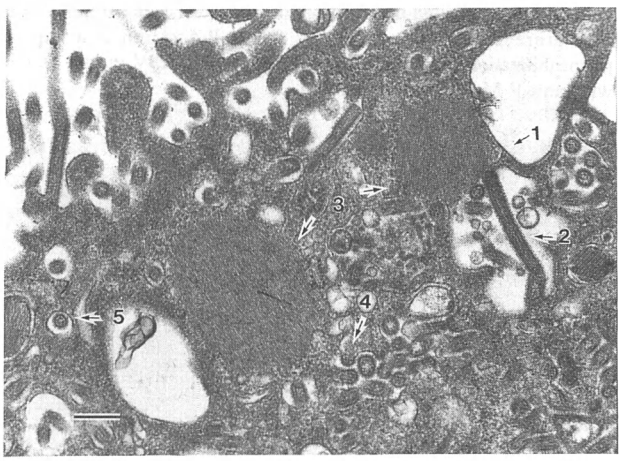

Fig. 5
The assembly of RSV within cytoplasm of HEP-2 cell
An intracytoplasmic membrane with visible spikes (arrow 1). The same spikes seen on the surface of RSV particles within an intracytoplasmic vesicle (arrow 2). NP accumulated in the vicinity of intracytoplasmic membrane carrying spikes (arrow 3). Virus particles having spikes released through the intracytoplasmic vesicle membrane by budding (arrow 4). Mature virus particles in small vesicle near the surface of the cell (arrow 5). Glutaraldehyde fixation. Bar = 300 nm.

Intracellular membranes (Fig. 5, arrow 1) as well as plasma membrane (Fig. 6a) were partially occupied by visible spikes, being morphologically the same as those seen on the surface of the complete virus particle (Fig. 5, arrow 2). Ribonucleoprotein units were accumulated in the vicinity of the spiked membranes (Fig. 5, arrow 3).

Concerning the localization of mature virus particles within cytoplasmic vesicles, we assume that after the arrangement of glycoprotein spikes on the intracytoplasmic membrane, ribonucleoprotein-like viral structural unit accumulated underneath the modified membrane (Fig. 5, arrow 4). Then mature virions were released into the intracytoplasmic vesicles by a budding process different from that shown in Fig. 3, in which mature virions were budding from the plasma membrane. Our observations suggest also that mature virions are transported outside of the infected cell by transport vesicles.

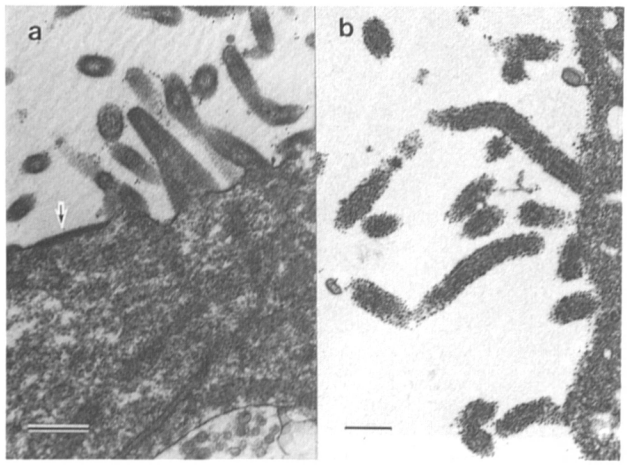

Fig. 6
Localization of F protein in the envelope of RSV
(a) Glutaraldehyde fixation, RSV budding through the plasma membrane of HEp-2 cell. Plasma membrane partially modified by spikes (arrow). The sample labelled with anti-F MoAb. Only a few gold particles revealed the F protein on the outer side of the envelope of mature RSV. Bar = 300 nm. (b) PLP-fixation and saponin permeabilization. Extracellular free and budding RSV virions labelled with anti-F MoAb. Bar = 300 nm.

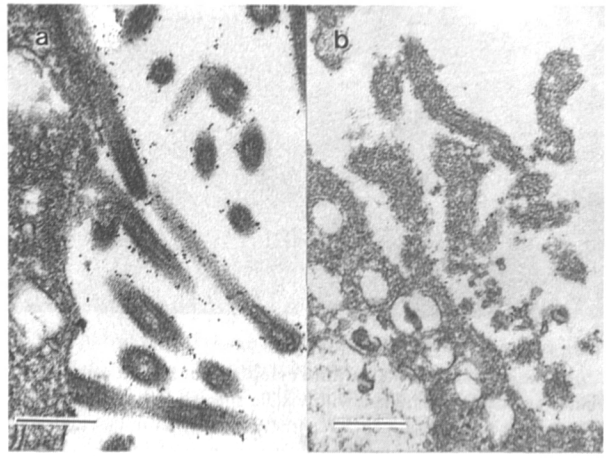

Fig. 7
Localization of G protein on the envelope of RSV
(a) Glutaraldehyde fixation localized G protein on the outer side of the envelope of virus particles. Bar = 300 nm. (b) PLP-fixation and saponin permeabilization. G protein detected on the outer side of the virus envelope. Bar = 300 nm.

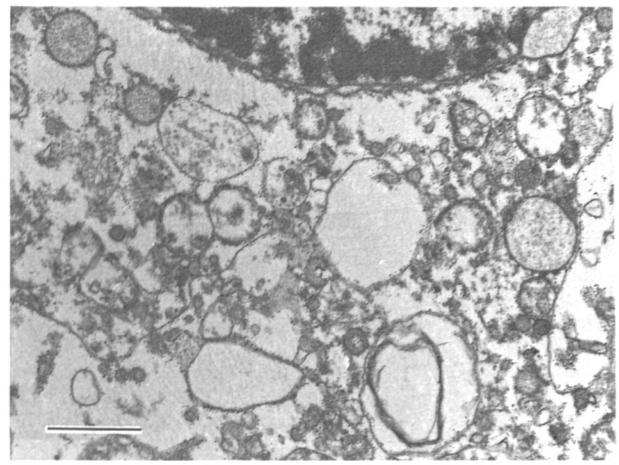

Fig. 8
Distribution of RSV F protein along the membranes of intracellular vacuoles
Gold particles deposited on the outer and inner surfaces of vesicles. PLP-fixation and saponin permeabilization. Bar = 1000 nm.

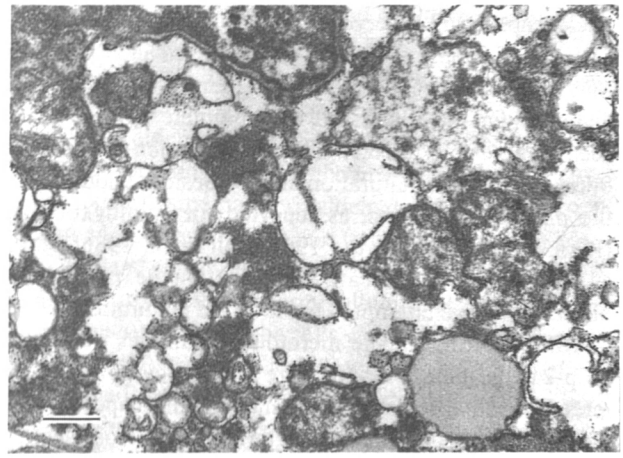

Fig. 9
Distribution of RSV G protein along the membranes of intracellular empty vacuoles
Gold particles deposited on the outer and inner surfaces of vesicles. NP inclusions observed in a close contact with the labelled membranes. PLP-fixation and saponin permeabilization. Bar = 300 nm.

Morphological identification of viral surface glycoproteins and NP by IEM with MoAbs

To confirm the maturation of virions in intracytoplasmic vaculoes, the distribution of viral glycoproteins F and G and NP in infected cells was studied. The infected cells were treated with MoAbs and colloidal gold A-complex and fixed with glutaraldehyde or PLP.

MoAbs against proteins F and G revealed a small amount of gold attached to the surface of released virions in the glutaraldehyd-fixed cells (Figs. 6a,7a), however, F protein was seen densely arranged around budding particles in PLP-fixed cells (Fig. 6b). Although proteins G and F were distributed in the plasma membrane (Figs. 6b, 7b), both were accumulated in the membranes of intracytoplasmic vacuoles. Many vesicles having different size were distributed

widely in the cytoplasm and most of them contained colloidal gold associated with MoAbs against protein F and G on the outer and inner surfaces of the PLP-fixed cells (Figs. 8,9); however, no colloidal gold was detected around NP (Fig. 9). NP inclusions were observed in the cytosol in a close contact with the labelled membranes, suggesting the site of RSV assembly (Fig. 9).

On the other hand, in the samples fixed with PLP, infected cells labelled with MoAbs against NP exhibited deposition of colloidal gold particles on the surface of inclusions within the cytoplasmic matrix (Fig. 10). However, the MoAbs and gold particles could not penetrate the intact plasma membrane in the glutaraldehyde-fixed cells; it thus appears that NP inclusions remained mostly unlabelled (data not shown).

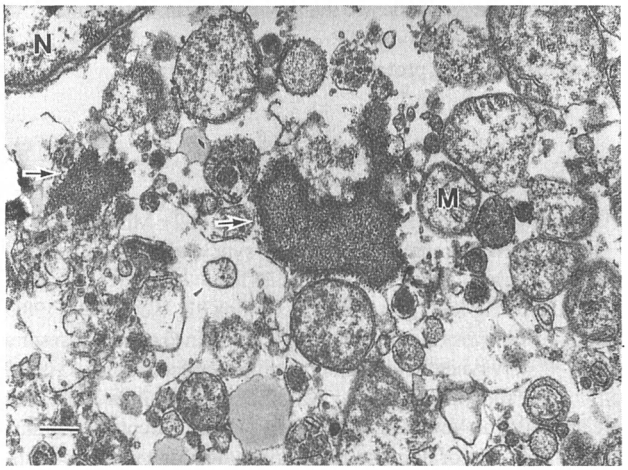

Fig. 10
Localization of the intracytoplasmic granular type NP of RSV in HEp-2 cell (arrows)
PLP-fixation and saponin permeabilization. Labelling with anti-NP MoAb. N = nucleus, M = mitochondria. Bar = 300 nm

Discussion

In this study, IEM was performed with MoAbs against RSV glycoproteins G and F, and NP with colloidal gold as a marker to confirm the maturation of RSV virions in the intracellular vesicles not only by the budding process through the plasma membranes.

Using the surface-labelling (glutaraldehyde fixation) and the penetrating-labelling (PLP fixation) methods, IEM exhibited a certain level of colloidal gold targeting. A rigid preservation of cell ultrastructure by glutaraldehyde fixation did not allow the intracellular localization of envelope proteins G and F or NP.

On the contrary, a poor preservation of cell ultrastructure by PLP fixation showed granular NP inclusions in cytoplasm. It is likely that an efficient labelling of intracellular mature virions by either fixation method could not be achieved without severe destruction of cell ultrastructure.

In this investigation, IEM with MoAbs and colloidal gold bound to protein A indicated that RSV NP is synthesized in the cytoplasmic matrix, and envelope proteins F and G along the intracellular membranes in RSV-infected HEp-2 cells. The proliferated intracellular lumen-side-labelled membranes and the presence of mature RSV particles within intracytoplasmic vesicles suggested that the assembly and maturation of RSV might occur by budding of NP through the intracytoplasmic vesicles.

It is widely accepted that the maturation of a RSV virion resembles that of other paramyxoviruses (Dubois-Dalq *et al.,* 1984). According to this theory, RSV virions assemble in the plasma membranes of infected cells and mature by budding through the plasma membrane.

Our results show clearly that the assembly and maturation of RSV in HEp-2 cells occurred not only on the plasma membrane but also on the inner cytoplasmic vesicle membranes, most likely proliferated from trans-Golgi compartment. Norrby *et al.* (1970) have already reported from their morphogenetic study of RSV in Vero cells that the maturation of RSV was likely to take place on the internal vesicle membrane within cytoplasm. However, the possibility that this intracytoplasmic structure represented the invagination of the plasma membrane cannot be excluded.

Our results indicate that RSV maturation proceeds by an alternative pathway on an intracytoplasmic membrane because the existence of many mature viral particles inside the cell cannot be explained by mere invanigation of the plasma membrane. Moreover, the numeorus small vesicles found on the surface of the infected HEp-2 cells might be transport vesicles that have not been described before on an ultrastructural level in any RSV-infected cells. One of the known hypotheses maintains that the transport vesicles are incorporated into the plasma membrane and that the RSV particles budding through the plasma membrane is just the final step of the exocytosis process. On the other hand, many investigators stress the importance of matrix (M) protein as a mediator of association between NP and envelope proteins in other paramyxoviruses (Brown *et al.,* 1987; Kristensson and Orvell, 1983; Portuer and Murti, 1986; Yoshida *et al.,* 1976). An interesting suggestion has been made by Yoshida *et al.* (1976) that M protein could have a role in NP folding after synthesis in cytoplasm (Yoshida *et al.* 1976). In our opinion, the preparation of a MoAb against protein M of RSV is a prerequisite for the localization of M protein in RSV-infected cells and for understanding its potential role in RSV assembly.

Acknowledgements. We thank Mrs J.Y. Zhow, Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, P.R. of China for providing protein A-colloidal gold complex.

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