ULTRASTRUCTURAL LECTINOCYTOCHEMISTRY OF FOWL PLAGUE VIRUS-INFECTED AND UNINFECTED MDCK CELLS

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Summary. – Using horseradish peroxidase (HRP)-conjugated lectins for pre-embedding labelling we have shown differences in ultrastructural localization of saccharides in cell compartments of fowl plague (FP) virus-infected and uninfected MDCK cells. Lectinochemical staining of the cell compartments in the case of FP virus-infected MDCK cells was less intensive as compared with uninfected cells. Also certain differences in the staining of subcompartments of cell organells were seen. Staining of uninfected cells with *Pisum sativum* agglutinin (PSA)-HRP revealed an extensive visualization of Golgi complex, mainly its cis-part, TGN vesicles and lysosomes. Staining of FP virus-infected cells with the same lectin marked very lightly rough endoplasmic reticulum and not at all the Golgi complex. Staining with *Erythrina cristagalli* agglutinin (ECA)-HRP revealed a picture very similar to PSA-HRP staining of uninfected and FP virus-infected cells. The differences in the lectinochemical staining of cell organelles of FP virus-infected and uninfected cells may be connected with the inhibition of cell protein synthesis during FP virus morphogenesis.

Key words: lectinocytochemistry; electron microscopy; fowl plague virus; Golgi complex

Introduction

Exocytosed cellular or viral proteins undergo a series of structural changes on their pathway from the rough endoplasmic reticulum (ER) through the Golgi complex to the cell surface. These changes involve oligomerization, folding, sequential glycosylation at the Golgi complex and proteolytic cleavage which take place at trans-Golgi network (TGN) vesicles (Farquhar and Palade, 1981; Bennet *et al.*, 1988; Wilschut, 1989; Nayak and Jabbar, 1989, Hobman, 1993).

The Golgi complex is engaged in the biosynthesis of glycolipids and oligosaccharide chains of glycoproteins. Terminally glycosylated proteins are at the level of TGN sorted according to their destinations (Mellman and Simons, 1992).

The influenza haemagglutinin (HA) and neuraminidase (NA) belong to those glycoproteins that follow the general pathway of addition of N-linked sugars by the cellular glycosylation machinery during the transport of glycoprotein in the cell (Markoff *et al.*, 1983; Keil *et al.*, 1985). The HA and NA are integral parts of viral envelope glycoproteins. The HA is synthesized as a single polypeptid, folded and trimerized between ER and cis-part of Golgi complex (Gething *et al.*, 1986; Copeland *et al.*, 1986). The NA forms tetramers consisting from disulfide-linked subunits (Varghese *et al.*, 1983).

During the transport of influenza virus HA through the Golgi complex, most of oligosaccharide side chains are

Abbreviations: AlloA = *Allomyrina dichotoma* agglutinin; BSA = bovine serum albumin; DAB = diaminobenzidine; ER = endoplasmic reticulum; ECA = *Erythrina cristagalli* agglutinin; FP = fowl plague; Fuc = fucose; Gal = galactose; GalNAc = N-acetylgalactosamine; Glc = glucose; GlcNAc = N-acetylglucosamine; HA = haemagglutinin; HPA = *Helix pomatia* agglutinin; HRP = horseradish peroxidase; Man = mannose; MDCK = Madin-Darby canine kidney; MEM = Eagle's Minimal Essential Medium; MoAb = monoclonal antibody; NA = neuraminidase; PSA = *Pisum sativum* agglutinin; TGN = trans-Golgi network.

trimmed. The mannose-rich oligosaccharide side chains are first trimmed to the Man5GlcNAc2 structure, and N-acetylglucosamine, fucose, galactose and sialic acid residues are then added (Roth *et al.*, 1989).

The HA of FP virus (influenza A/FPV/Rostock/34 (H7)) contains up to seven oligosaccharide chains attached to asparagine. Five of them are processed complex oligosaccharides consisting of galactose, mannose, fucose and N-acetylglucosamine, and two are oligomannosides (Keil *et al.*, 1985). The NA of influenza virus A/Tokyo/3/67 (N2) contains two oligomannosidic side chains and two N-acetylglucosamine side chains (Ward *et al.*, 1983).

Lectinocytochemistry provides an useful method for localization of the Golgi complex subcompartments and is based on interactions of lectins with glycoconjugates present in the membranes or lumens of Golgi complex cisternae (Pavelka and Ellinger, 1991).

The present paper concentrates on affinity-cytochemical studies of FP virus-infected and uninfected Madin-Darby canine kidney (MDCK) epithelial cells using HRP-conjugated lectins. The HA of influenza virus was localized by colloidal gold-conjugated antibodies. The presented ultrastructural studies are an attempt to determine whether carbohydrate-binding reactions of lectins allow to localize differences that occur in the virus-infected and uninfected MDCK cell compartments with the aim to distinguish the ultrastructural pattern of glycosylation in normal and infected cells.

Materials and Methods

Lectins used in this study as well as their sugar specificities are listed in Table 1. The HRP-conjugates of lectins HPA, PSA, and ECA were purchased from Sigma Chem. Co. (St. Louis, MO), and that of AlloA was obtained from E-Y Laboratories (San Mateo, CA).

Cells. MDCK cell monolayers were grown in Eagle's Minimal Essential Medium (MEM) with 10% bovine serum on glass coverslips.

Table 1. Lectins used for pre-embedding labelling

Lectin	Specificity (Goldstein and Poretz, 1986)
Helix pomatia, HPA[a]	αGalNAc > αGlcNAc >> Gal
Pisum sativum, PSA[b]	αMan > αGlc = GlcNAc
Erythrina cristagalli, ECA[b]	Galß1,4GlcNAc > αGalNAc
Allomyrina dichotoma, AlloA[b]	αGal

[a]Used for labelling of uninfected cells. [b]Used for labelling of both uninfected and infected cells.

Virus. FP virus influenza (A/FPV/Rostock/34) was used.

Infection of cells. The MDCK cells were infected with FP virus at a multiplicity of infection of 50 PFU/cell and kept at room temperature for 1 hr. Cells were then rinsed and maintained in Eagle's Minimal Essential Medium (MEM) at 37 °C. At 5.5 hrs p.i. infected and uninfected cells were rinsed in MEM and phosphate-buffered saline (PBS), and processed for electron microscopy.

Lectinocytochemistry. Cells were fixed in a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/l cacodylate buffer pH 7.2 for 30 mins at 4 °C and then kept in cacodylate buffer for 2 – 3 days. After rinsing in PBS, the uninfected cells were incubated with lectin-HRP conjugates in PBS (concentrations 100 – 500 μg/ml) for 4 hrs at room temperature. The infected cells were incubated with two different concentrations of lectin-HRP conjugates, 20 and 100 μg/ml. All incubations media contained 0.1 mg/ml saponin and 1 mg/ml bovine serum albumin (BSA). The HRP was stained by diaminobenzidine (DAB) (0.5 mg/ml) in 0.02% H_2O_2 and 0.05 mol/l Tris-HCl pH 7.6 at room temperature for 15 mins (Pavelka and Ellinger, 1991). After rinsing in double-distilled water, the specimens were post-fixed in 1% osmium ferrocyanide for 30 mins, treated with veronal acetate-buffered osmium tetroxide (1%) for 4 hrs at 4 °C, dehydrated in ethanol and embedded in Epon 812. Ultrathin sections were examined unstained in Philips EM 300 or EM 400 electron microscopes at 80 kV.

Immuno-gold electron microscopy. HA of FP virus was localized using monoclonal antibody (MoAb) HC2 recognizing all conformations of HA and goat anti-mouse IgG conjugated with 10 nm gold (GAM 10; BioCell, Cardiff, UK) according to the procedure described by Čiampor *et al.* (1992).

Results

The pictures presented in this study compare the lectin-treated uninfected and virus-infected cells, as well as the lectin-untreated infected cells as the controls of specificity.

Localization of HA in FP virus-infected MDCK cells

MoAb HC2 which recognizes all conformations of FP virus HA, localized HA molecules 5.5 hrs p.i. in rough ER, cis-part of Golgi complex (Figs. 1A and 1B), on the surface of plasma membrane, and on the surface of budding or mature viral particles (Fig. 1C and 1D). Using pre-embedding labelling and saponin-permeabilized cells, the most intensive labelling was found on the cell surface and on the viral particles. Because of poor penetration of antibodies and conjugated complexes, the intracellular labelling revealed only a few gold particles localized on ER or on Golgi complex. These results confirm our previously published data on localization of HA during its exocytosis (Čiampor *et al.*, 1992). We used the above mentioned technique only in lectinochemical studies of transport of HA in the virus-infected cell.

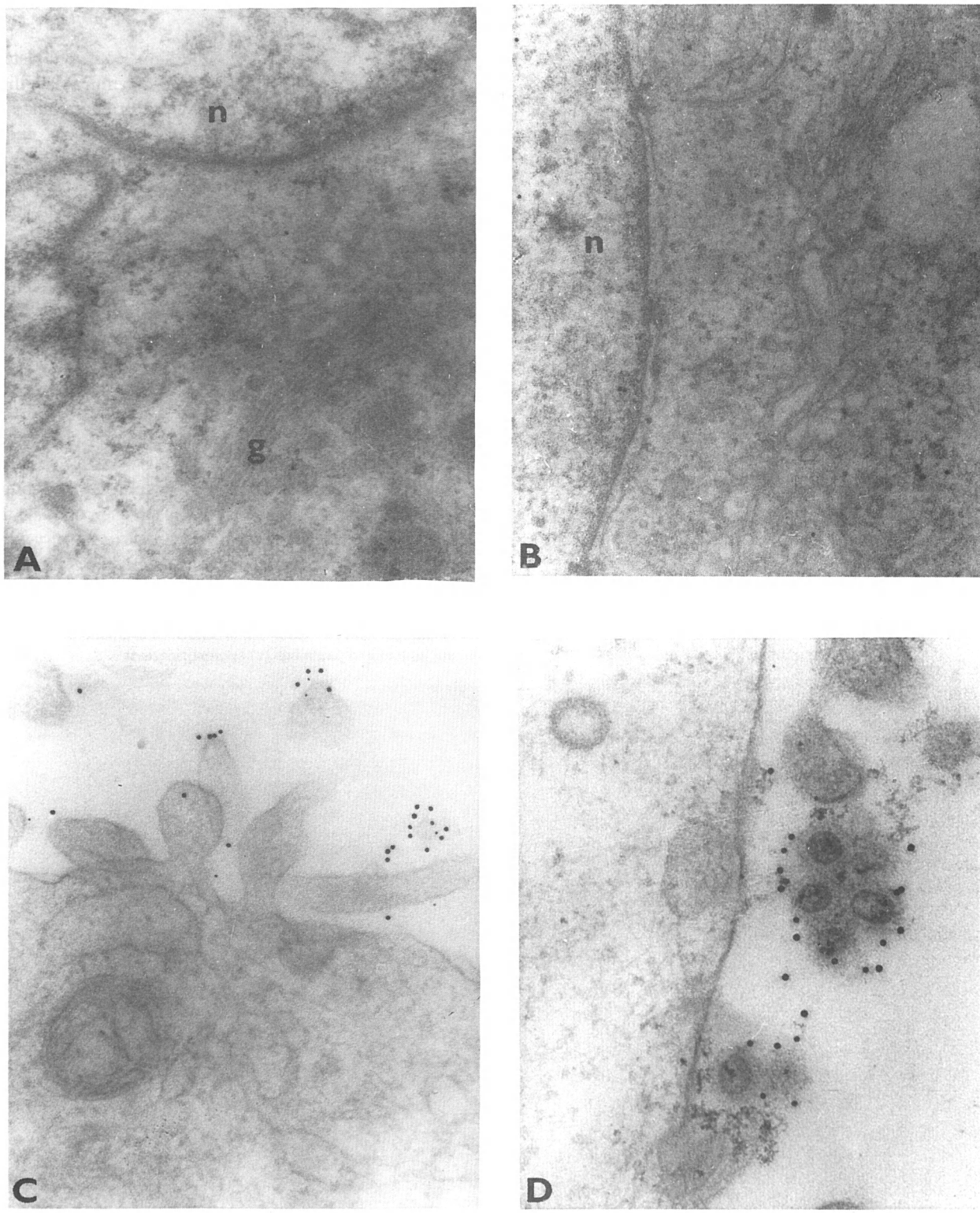

Fig. 1

Morphology and colloidal gold localization of HA in virus-infected MDCK cells without lectin treatment

All cell compartments are free of dense stain. No significant post-embedding labelling of HA with MoAb and colloidal gold can be observed (C, D). Using pre-embedding labelling with MoAb and colloidal gold, HA is found on the viral membrane. Golgi apparatus (g), nucleus (n), transport vesicle (v). Magnification 54,000 x (A), 68,000 x (B), 82,500 x (C), 110,000 x (D).

HPA

HPA binds GalNAc-residues with high affinity (Goldstein and Poretz, 1986). In uninfected MDCK cells, HPA induced intensive labelling of surface glycocalyx and lysosomes only. The Golgi coomplex and TGN vesicles were weakly reactive (Fig. 2).

PSA

PSA is Man-, Glc-, and GlcNAc-recognizing lectin (Goldstein and Poretz, 1986). In uninfected MDCK cells, the positive lectinochemical reaction was localized on perinuclear membrane, cis-part of Golgi complex, lysosomes and endosomes. Some of TGN vesicles exhibited a positive reaction. The medial-part of Golgi complex was free of reactive product (Fig. 3). In virus-infected cells the Golgi complex was labelled wery weakly without the intensive labelling of cis-part of Golgi complex visible in uninfected cells. A weak positive reaction was found on the rough ER and apparent positive reaction on lysosomes and endosomes. The labelling of budding virus particles on plasma membrane was also visible (Fig. 4A, B, C).

ECA

ECA is N-acetyllactosamine binding lectin (Goldstein and Poretz, 1986). The ECA-lectinocytochemical labelling of uninfected MDCK cells revealed a predominance of intensive positive reaction on secretory vesicles, endolysosomes and cell surface. Cis-part of Golgi complex and perinuclear membrane also exhibited a strong positive reaction (Fig. 5A, B). ECA-reaction product in virus-infected cells was distributed on endolysosomes and ER while the Golgi complex was almost unreactive. Cell surface membrane and budding or mature viral particles exhibited also a positive reaction product (Fig. 6A, B).

AlloA

AlloA has a high affinity to α-Gal residues (Goldstein and Poretz, 1986). Lectinochemical staining of uninfected cells showed a very intensive labelling of TGN vesicles, secretory vacuoles, perinuclear membrane, endolysosomes and surface cell membrane (Fig. 7). On the contrary, the virus-infected cells labelled with AlloA revealed a very weak labelling of all cytoplasmic structures. Only the cell surface

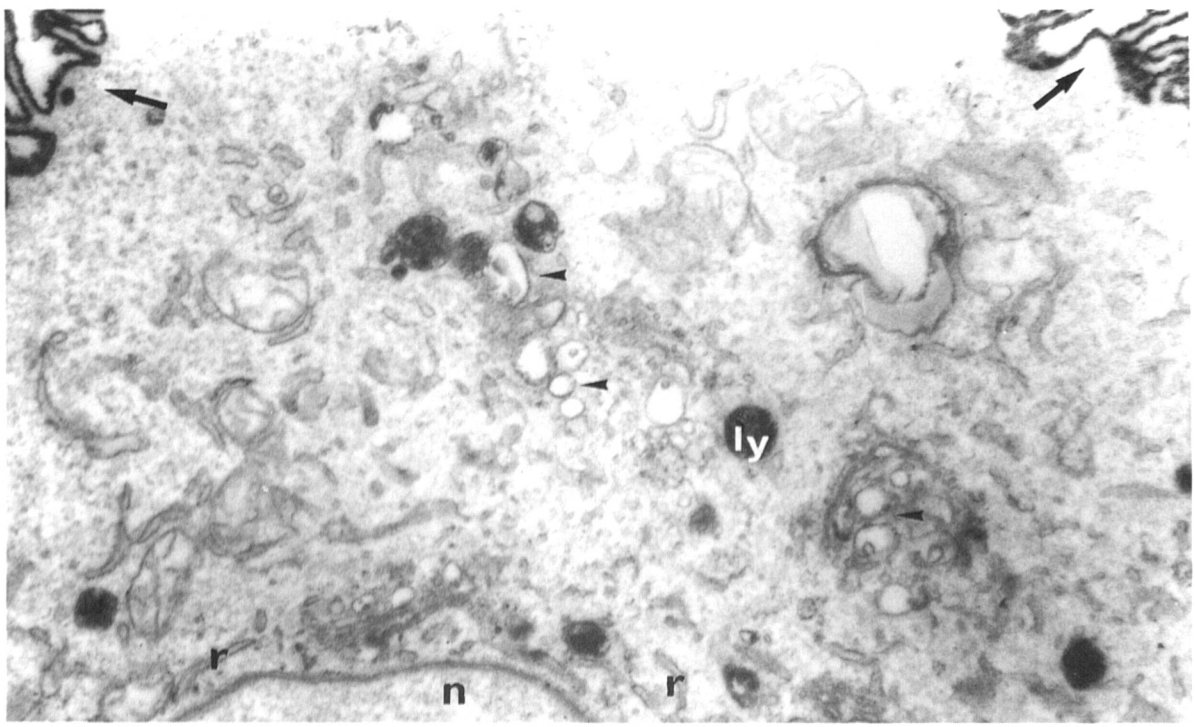

Fig. 2
Uninfected MDCK cells labelled with HPA-HRP
At the Golgi apparatus (g), only the transmost Golgi cisternae (arrow heads) show a weak label, whereas the other regions are negative. This lectin induces intensive labelling of lysosomes (ly) and surface glycocalyx (arrows). Nucleus (n), endoplasmic reticulum (r). Magnification 18,670 x.

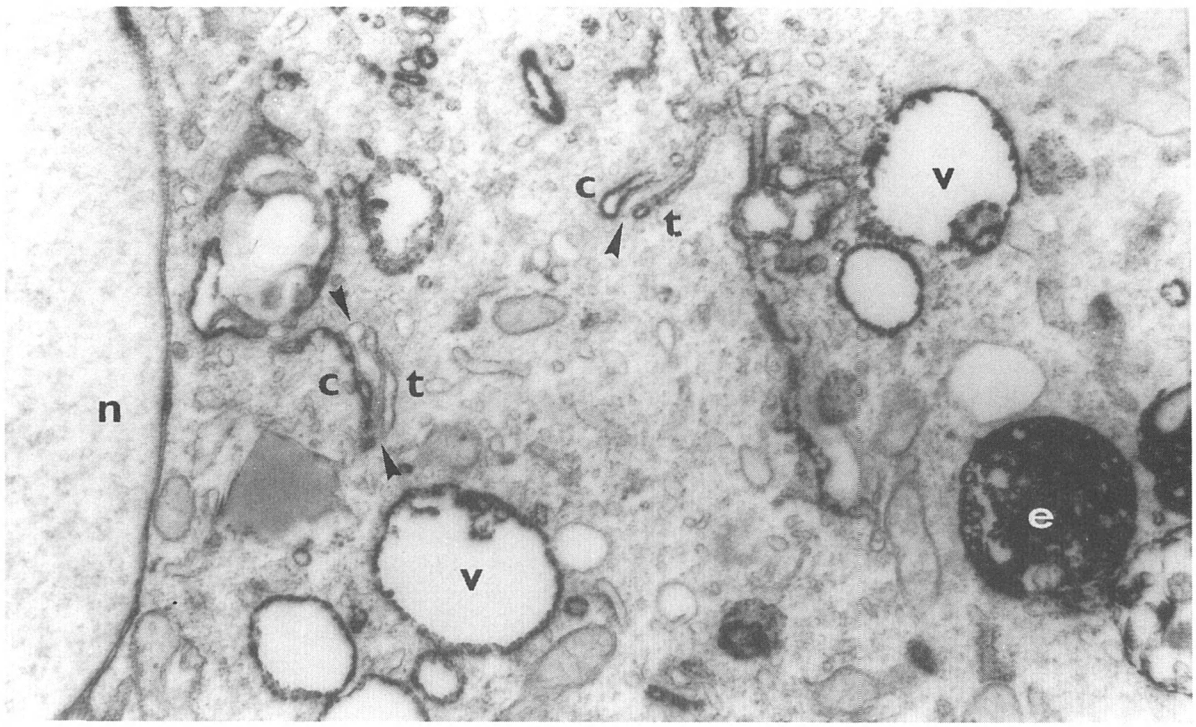

Fig. 3
Uninfected MDCK cells labelled with PSA-HRP
Label is concentrated in cis-Golgi (c) and partly in trans-Golgi cisternae (t), whereas none is in medial-Golgi region (arrow head). The membrane of transport vesicles (v) and endolysosomes (e) are intensively stained. Nucleus (n). Magnification 31,500 x.

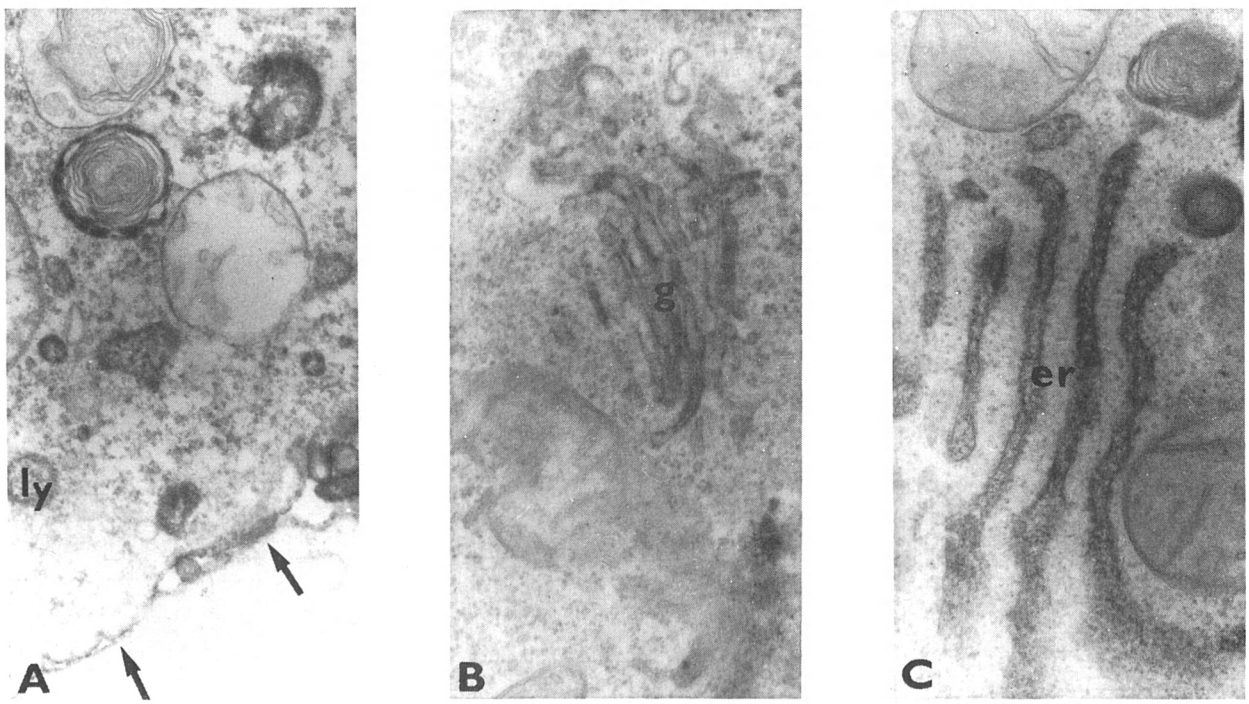

Fig. 4
Virus-infected MDCK cells labelled with PSA-HRP
A weak label is apparent in all cisternae of the Golgi apparatus (g) and unique labelling can be seen in the endoplasmic reticulum (er) and lysosomes (ly). Cell surface (arrows). Magnification 30,000 x (A), 32,000 x (B), 58,000 x (C).

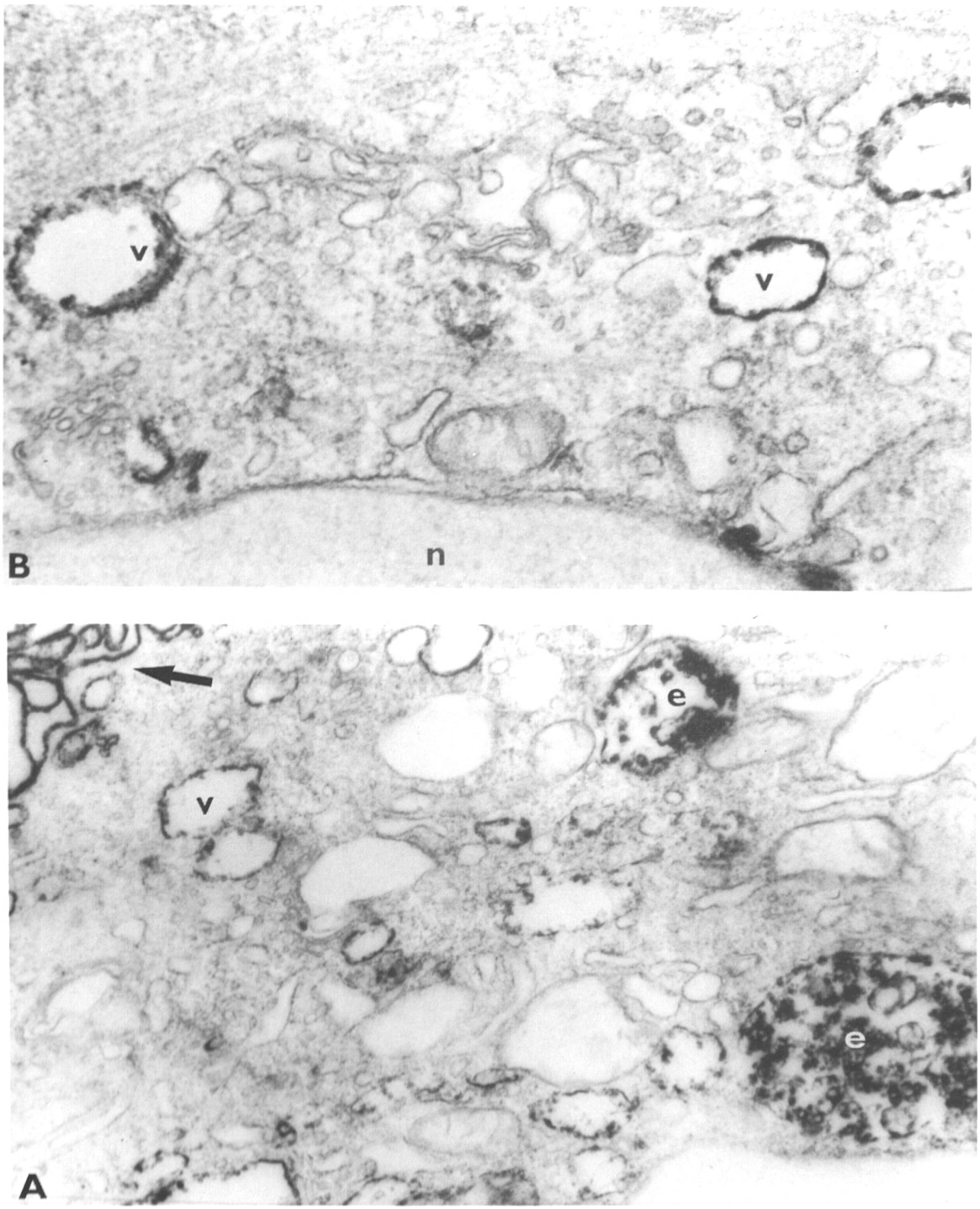

Fig. 5
Uninfected MDCK cells labelled with ECA-HRP
Label is apparent in secretory vesicles (v), endolysosomes (e), and on the cell surface (arrow). The Golgi apparatus is labelled irregularly or devoid of label. Nucleus (n). Magnification 38,640 x (A), 47,000 x (B).

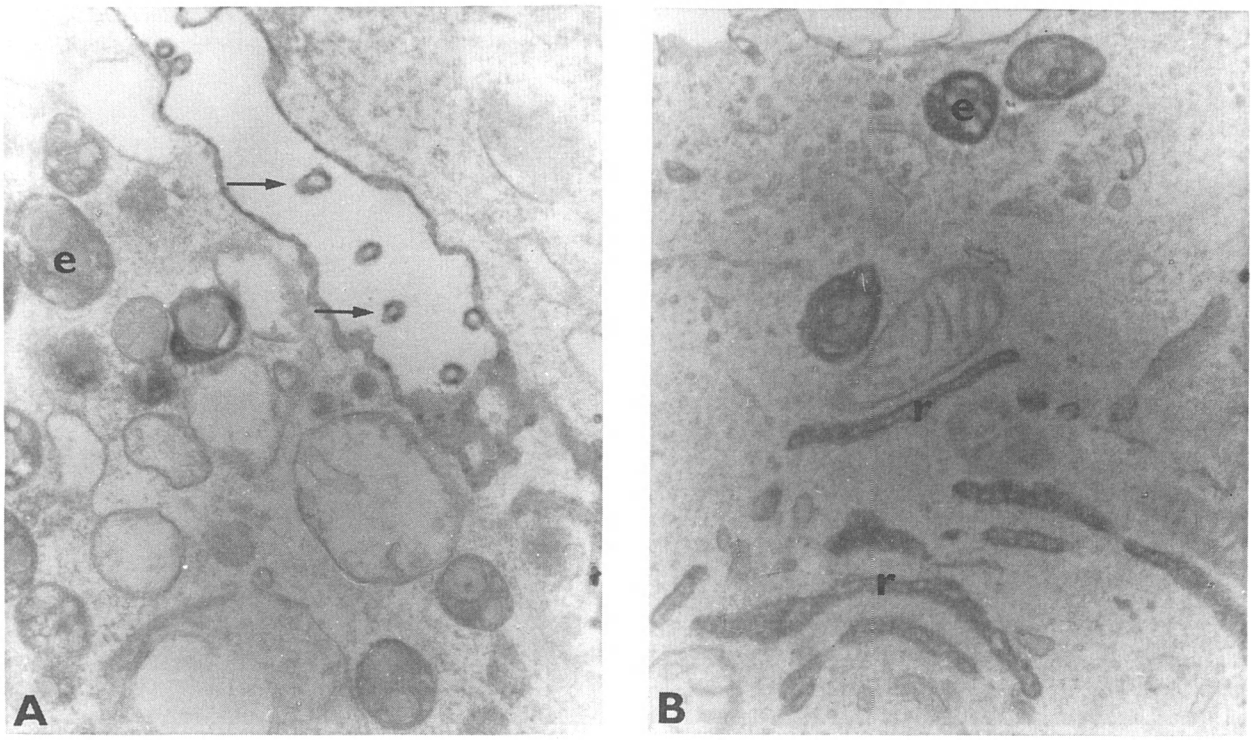

Fig. 6
Virus-infected MDCK cells labelled with ECA-HRP
Label is found in endolysosomes (e) and in the endoplasmic reticulum (r), whereas almost none is in the Golgi apparatus. Labelled virus particles (arrows). Magnification 18,750 x (A), 24,000 x (B).

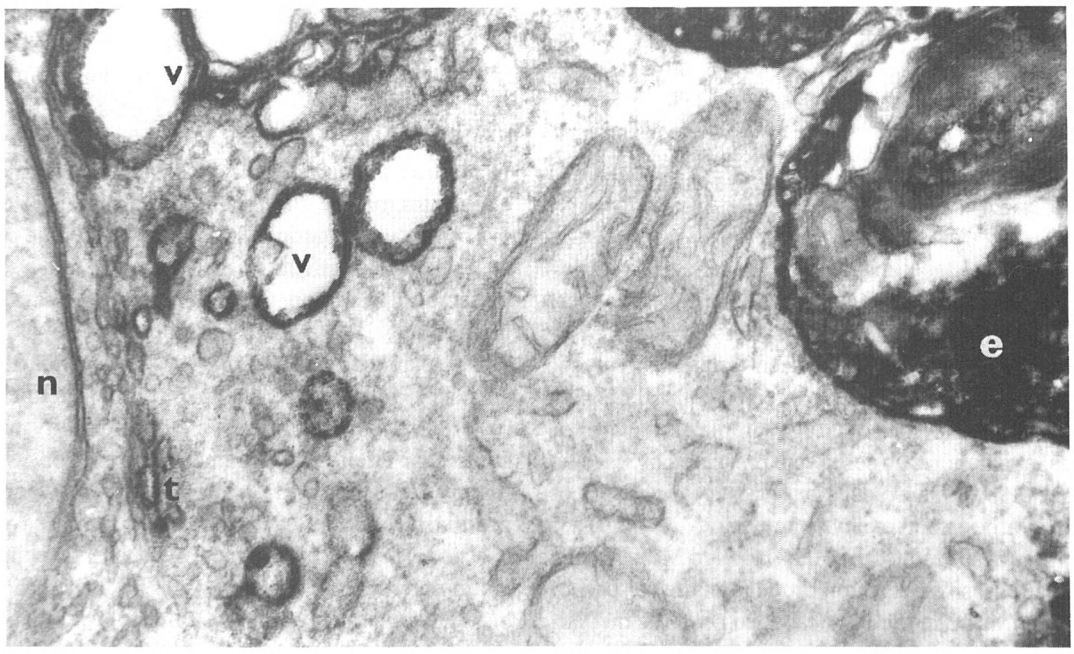

Fig. 7
Uninfected MDCK cells labelled with AlloA-HRP
Label is concentrated in the trans-Golgi network (t), whereas the other elements are free of label. The secretory vesicle membranes (v) and endolysosomes (e) are well visualized. Nucleus (n). Magnification 60,000 x.

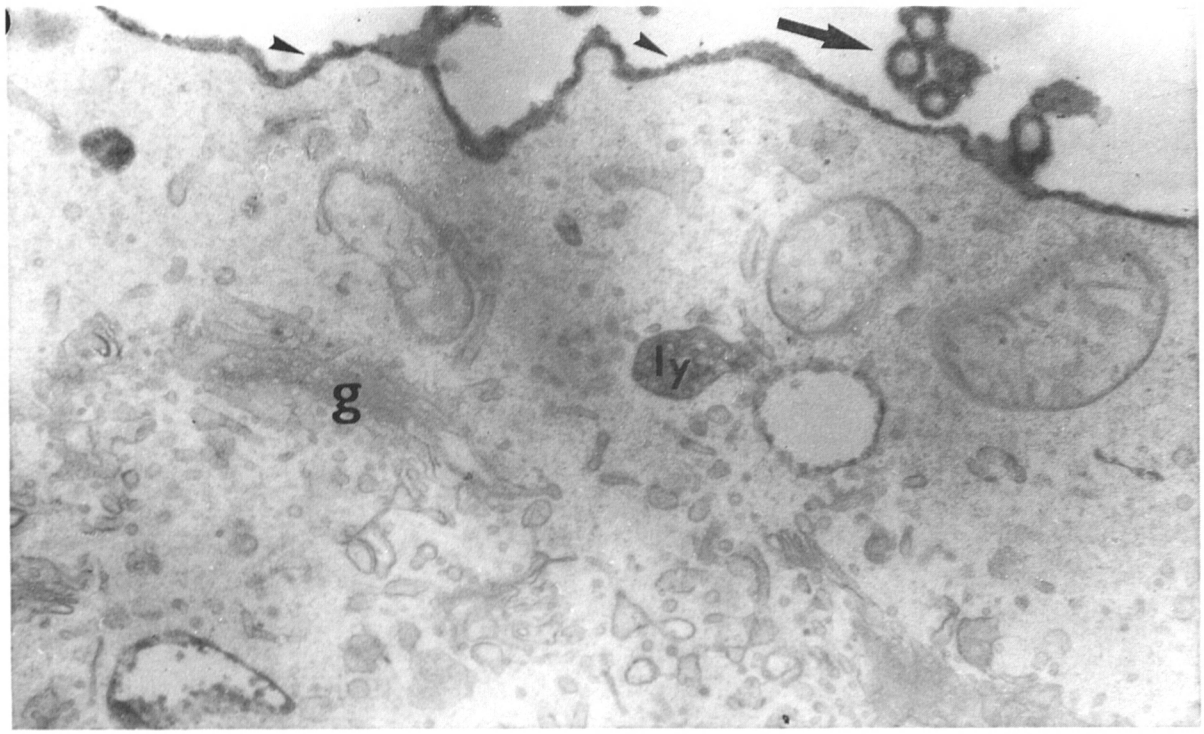

Fig. 8
Virus-infected MDCK cells labelled with AlloA-HRP
Label is clearly visible only in lysosomes (ly) and surface glycocalyx (arrow heads) with the virus particles (arrow). The Golgi apparatus (g) is devoid of label. Magnification 27,000 x.

and budding or mature viral particles revealed a strong positive product; weak reaction product was localized on lysosomes, but Golgi complex, TGN and ER were free of reactive product (Fig. 8).

Discussion

Lectinochemical labelling of MDCK cells showed differences between uninfected and virus-infected cells, particularly in Golgi complex vesicles and cisternae. The differences were probably due to diffrent amount of glycosylated proteins in infected and uninfected cells. Extensive lectinocytochemical studies of Golgi complex structures of rat intestinal and pancreatic cells were presented by Pavelka and Ellinger (1991). These cells have a very high level of cell protein synthesis and exocytic activity.

Comparing these results, the HPA-labelled MDCK cells did not reveal a positive reaction product on the cis-part of Golgi complex while in the PSA-labelled cells the medial-part of Golgi complex was non-reactive but the cis-part and TGN regions were reactive.

The infection of MDCK cells with FP virus resulted at 5.5 hrs p.i.in the following features of lectinochemical reactions.

(a) Altogether, the lectinochemical staining of infected cells was less intensive than that of uninfected cells. Cells infected with FP virus synthesize predominantly viral proteins (Griffiths *et al.*, 1982). It seems that the amount of viral glycosylated proteins is smaller than that of cellular ones. In addition, the permeability of the cell membrane for lectin-conjugates may be different in the case of infected cells.

(b) The virus particles were labelled on the cell surface. After 5.5 hrs of infection, the assembled and budding virus particles appear on the cell surface (Čiampor, 1972; Kingsbury, 1990). The amount of about 500 HA and 100 NA spikes (Taylor *et al.*, 1987) of each influenza virus particle is sufficient for positive lectino-chemical reaction.

(c) All lectins exibited very poor affinity to the Golgi apparatus. The lectinocytochemical reaction was apparent mainly in the transport vesicles. The viral glycoproteins are gradually concentrated in carrier vesicles during the exocytosis and are fully glycosylated after reaching the trans-Golgi region (Griffiths *et al.*, 1989; Klenk, 1990; Singh *et al.*, 1990; Čiampor *et al.*, 1992). It follows that the highest concentration of transported viral glycoproteins is present in the transport vesicles of TGN. Since the HA and NA are transmembrane proteins, the most intensive positive reaction was observed inside the vesicles because of HA and NA orientation.

(d) The ECA and PSA reaction products were also present in the ER. The possitive reactions indicate that the ER in infected MDCK cells is rich in mannose that corresponds to the results of Hirschberg and Snider (1987), and in N-acetyllactosamine residues probably attached to the viral proteins.

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