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Expression of Feline Infectious Peritonitis Coronavirus Antigens on the Surface of Feline Macrophage-like Cells

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SUMMARY

Growth of feline infectious peritonitis virus in a continuous feline cell line is described and evidence for the macrophage-like character of these cells is presented. Under one-step growth conditions, cytopathic changes and giant cell formation were observed 12 h after infection; more than 99% of the virus remained cell-associated 15 h after infection. Viral proteins at the surface of infected cells were detected by immunofluorescence. The exposed antigens were localized on four proteins with molecular weights of 225.5K, 175K, 138K and 25K using radioiodination followed by immunoprecipitation. Another viral polypeptide of 44K (the nucleocapsid protein) was only labelled when the cell membranes had been disrupted. Expression of viral antigens on the cell surface may be a significant factor in the immune pathogenesis of feline infectious peritonitis.

INTRODUCTION

Feline infectious peritonitis virus (FIPV), a coronavirus, causes disease in cats and other *Felidae*. The pathogenesis of feline infectious peritonitis (FIP) is not understood; indications for an involvement of the immune system, however, have been given (Horzinek & Osterhaus, 1979). Actively acquired or transferred coronavirus antibodies have been found to make a cat more prone to a fatal course of FIP (Pedersen & Boyle, 1980; Weiss & Scott, 1981). A longitudinal study of the events during experimental FIP made us advance a hypothesis for the pathogenetic mechanism during FIPV infection (Jacobse-Geels *et al.*, 1982). A major role is attributed to the macrophage, which is the predominant if not the only target cell of FIPV *in vivo* (Pedersen, 1976), and an immune enhancement of macrophage infection (Halstead *et al.*, 1973; Peiris & Porterfield, 1979) has been postulated. Until recently, *in vitro* cultivation of FIPV could only be achieved in organ or peritoneal cell cultures (Pedersen, 1976; Hoshino & Scott, 1978). Meanwhile, however, several authors have also described growth of FIPV in continuous cell lines of feline origin (Black, 1980; Hitchcock *et al.*, 1981; Evermann *et al.*, 1981; Woods, 1982). In this study, we present evidence that the cells of the fcwf line (Pedersen *et al.*, 1981) used for propagating FIPV possess properties of macrophages; in addition, exposure of viral antigen on the surface of infected cells is demonstrated, which is considered relevant for the mechanism of FIP immune pathogenesis.

METHODS

Cells. Monolayers of fcwf (*Felis catus* whole foetus) cells (Pedersen *et al.*, 1981) were grown in Dulbecco's modification of Eagle's medium (DMEM) containing 10% foetal calf serum (FCS) and antibiotics.

Cytological studies. Fcwf cells were grown on 20 × 20 mm coverslips in 35 mm diam. wells (Costar). Three days after seeding, 0.1 ml of a suspension of latex spherules (1.1 μm diam., 10¹⁰ particles/ml) or carbon particles (10% Indian ink) in DMEM was added to the cultures. After 1 h (latex) or 2 h (carbon) incubation at 37 °C the cells were washed extensively with phosphate-buffered saline (PBS) and stained with 0.75% crystal violet in 10% formaldehyde or with a 10% Giemsa solution in PBS. The coverslips were mounted in 50% (v/v) glycerol in PBS and screened for phagocytosis.

Non-specific esterase staining was done according to Yam *et al.* (1971).

Fc receptors were detected by the ability of the fcwf cells to form rosettes with antibody-coated sheep red blood cells (SRBC). Briefly, subconfluent monolayers of fcwf cells were overlaid with 1 ml of a 1% (v/v) suspension of SRBC coated with rabbit anti-SRBC IgG (RIV, Bilthoven, The Netherlands) in DMEM and incubated for 15 h at 37 °C. The monolayers were washed twice with DMEM, fixed briefly with 1% glutaraldehyde in DMEM and stained according to Papanicolaou.

Plasminogen activator was assayed as described by De Weger *et al.* (1983) and lysozyme activity (in the culture supernatant) following Van Loveren *et al.* (1981).

Virus growth. Cell culture-adapted FIPV was obtained from Dr N. C. Pedersen (School of Veterinary Medicine, University of California, Davis, Ca., U.S.A.). In all infection experiments the cells were pre-washed with PBS, containing 50 µg/ml DEAE-dextran (PBS-DEAE; Pharmacia) and inoculated with FIPV diluted to the desired concentration in PBS containing 1% FCS. After adsorption (1 h at 37 °C), the inoculum was removed and DMEM containing 2% FCS and 0.001% trypsin (Difco) was added. When high virus yields were required, the infected monolayers were trypsinized on day 1 after infection in order to disperse infected cells amongst the remaining non-infected cells. Cultures were allowed to settle in the same flask and were incubated for another day. Virus was harvested by three freeze-thaw cycles at -70 °C of the infected monolayers in DMEM containing 20% FCS. The resulting suspension was clarified by centrifugation at 500 g for 10 min and the virus was stored at -70 °C in small aliquots.

Infectivity assay. Fcwf cell monolayers in Costar cluster dishes (24 wells, 16 mm diam.) were infected with serial tenfold dilutions of the virus suspension in PBS containing 1% FCS. After 5 to 7 days at 37 °C, the titration endpoints were read either directly (by cytopathology) or after immunoperoxidase staining. In the latter case, the monolayers were rinsed with PBS and fixed with ethanol containing 5% (v/v) acetic acid for 10 min at -20 °C. After another rinse the monolayers were incubated for 1 h at 37 °C with peroxidase-conjugated anti-FIPV IgG, which had been prepared from an ascitic fluid of a cat that had died of FIP. The conjugate was diluted 1/200 in PBS containing 1% (v/v) newborn calf serum. After three cycles of washing with PBS, peroxidase activity was visualized using 0.0025% *o*-dianisidine (Fluka, Buchs, Switzerland) in 0.01 M-Tris-HCl buffer, pH 7.4, containing 0.9% (w/v) NaCl and 0.01% (v/v) H₂O₂. Titration endpoints (TCID₅₀) were calculated using the Kärber formula.

Demonstration of viral antigen on cell membranes. Expression of viral antigen on the surface of host cells was monitored by immunofluorescence of unfixed cells. Infected monolayers (m.o.i. approximately 15) were incubated with an anti-FIPV serum for 30 min at 4 °C and washed repeatedly with ice-cold PBS. After incubation with fluorescein-labelled Protein A from *Staphylococcus aureus* (Pharmacia) the cells were examined using an epifluorescence microscope (Zeiss, Oberkochen, F.R.G.).

To identify the viral antigens exposed, surface proteins of cells 20 h post-infection were labelled with ¹²⁵I as described by Markwell & Fox (1978). Infected monolayers in Costar cluster wells (35 mm diam.) were washed three times with PBS and subsequently 1 ml PBS containing approximately 500 µCi Na[¹²⁵I] (Amersham International; sp. act. 16.3 mCi/µg) was added. An 18 × 18 mm coverslip coated with 10 µg of Iodogen (Pierce, Rockford, Ill., U.S.A.) was carefully placed on top of the fluid. After 10 min of incubation at room temperature with occasional shaking, the coverslip was removed and 100 µl of a 10 mM-tyrosine solution was added. The monolayers were washed twice with PBS containing 1 mM-tyrosine and were lysed with 0.5 ml TES buffer (20 mM-Tris-HCl pH 7.4, 1 mM-EDTA, 100 mM-NaCl) supplemented with 0.5% Triton X-100, 0.5% (w/v) 1,5-naphthalenedisulphonate-disodium salt and 2 mM-phenylmethylsulphonyl fluoride (lysis buffer). The lysates were centrifuged at 10000 g for 5 min and the cytoplasmic supernatant was stored at -70 °C. The intracellular proteins were detected after applying 1.0 ml of lysis buffer containing 500 µCi Na[¹²⁵I] to the infected cells and incubating for 10 min under an Iodogen-coated coverslip. The labelled lysates were then passed through a Sephadex G-25 column (Pharmacia; bed vol. 8 ml) and fractions containing labelled protein were pooled and stored at -70 °C.

Immunoprecipitation. After clarification, 100 µl volumes of the lysates prepared as described above were mixed with 5 µl quantities of anti-FIPV serum. After overnight incubation at 4 °C, 3 M-KCl was added to a final concentration of 0.5 M. For precipitation of the immune complexes, 40 µl of a 10% suspension of formaldehyde-fixed *S. aureus* cells in TES buffer containing 0.5% (v/v) Triton X-100 was added and incubated at room temperature for 45 min. Precipitates were washed three times with TES buffer containing 0.5% Triton X-100 and dissolved in 50 µl of electrophoresis sample buffer; analysis by polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously (Rottier *et al.*, 1981).

Virus growth curve. Monolayers of fcwf cells in 35 mm diameter wells were inoculated with FIPV at an m.o.i. of approximately 30 TCID₅₀ units per cell. After 1 h at 37 °C the inoculum was removed and the wells were washed with PBS. The wells were filled with 1 ml DMEM supplemented with 2% FCS and 0.001% trypsin; incubation was continued for different periods of time. The culture medium was harvested and centrifuged at 500 g for 5 min. The supernatants were used for titrating cell-free virus and the pelleted cells were resuspended in 1 ml DMEM containing 20% FCS and added to the original well. Intracellular virus was released by three freeze-thaw cycles and titrated subsequently.

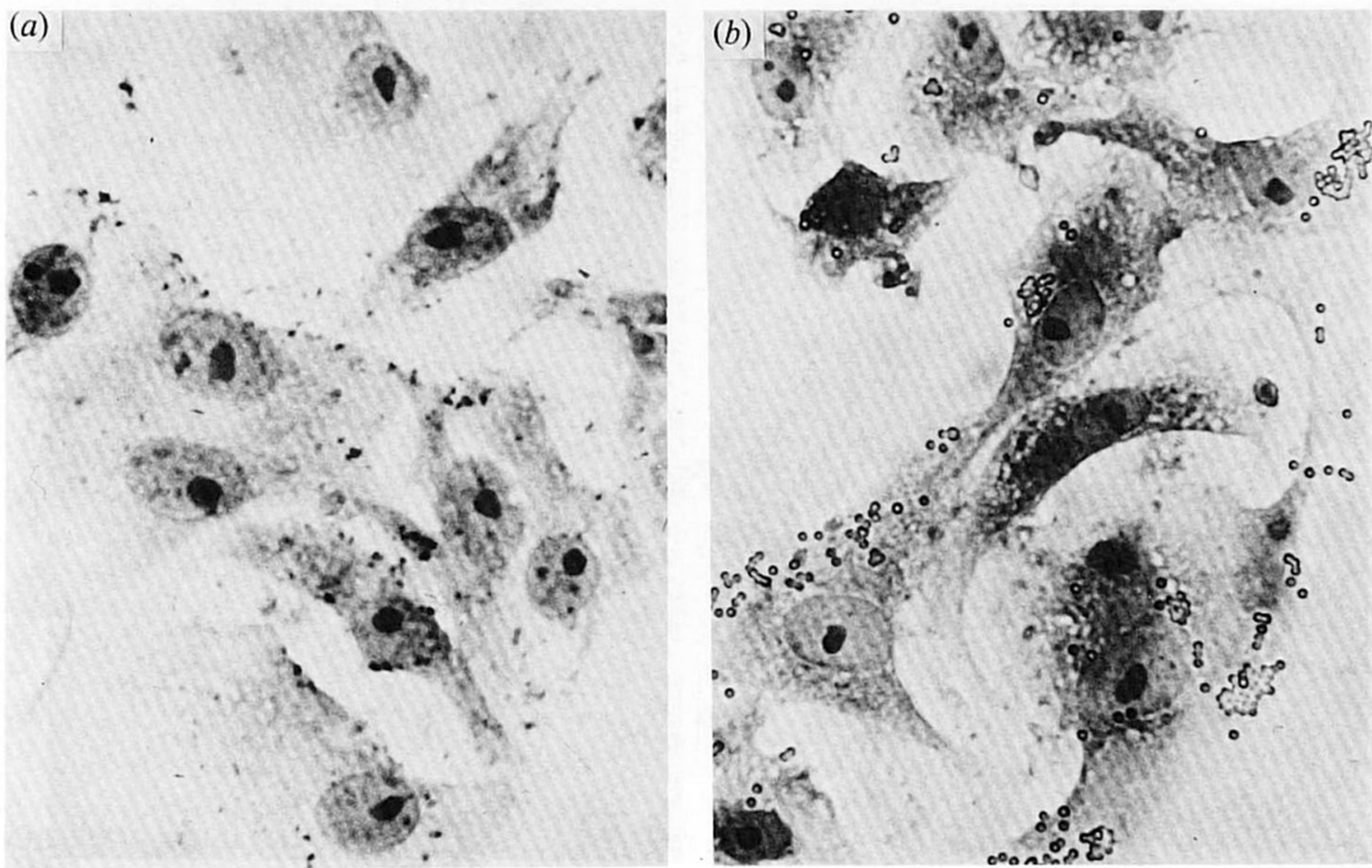


Fig. 1. Phagocytosis of (a) carbon and (b) latex particles by fcwf cells.

RESULTS

Characteristics of fcwf cells

Fcwf cells grow in monolayers on glass or plastic surfaces, and have a spindle to stellate morphology. The cells contain an oval nucleus with one or two nucleoli. Their growth ability is good in low passages (doubling time 24 h) but gradually decreases beyond passage number 25 (doubling time 48 h). Staining for non-specific esterases revealed a strong activity distributed evenly throughout the cytoplasm in most cells. Phagocytosis of carbon particles was found in about 60% of the cells and latex particles were taken up by more than 70% (Fig. 1). Fcwf cells are capable of binding antibody-coated SRBC as depicted in Fig. 2; approximately 1 to 15 SRBC were attached per cell. Neither plasminogen activator nor lysozyme activity was found in culture supernatants.

Growth of FIPV in fcwf cells

Optimal conditions for virus adsorption and replication in fcwf cells were determined in pilot experiments. One to 2 h of adsorption at 37 °C proved to produce maximal virus yields. Trypsin was added to the viral growth medium at a final concentration of 0.001% (w/v) since in its presence the number of fluorescent foci was doubled and the formation of syncytia enhanced, thereby facilitating the reading of cytopathic effect (c.p.e.) by light microscopy.

The growth curve of FIPV in fcwf cells was determined (Fig. 3). Cell-associated infectivity increased during the first 15 h post-infection followed by a decrease of approximately 2 log₁₀ units in the subsequent 9 h period. Increasing amounts of extracellular virus were found during single-cycle growth but more than 99% of the infectious particles were cell-associated at 15 h after infection. Cytopathic changes started 12 h post-infection, when small syncytia were seen; these increased in size and contained about 10 nuclei at 15 h. At 24 h, the c.p.e. was pronounced; when incubation was continued, cell rounding and detachment ensued.

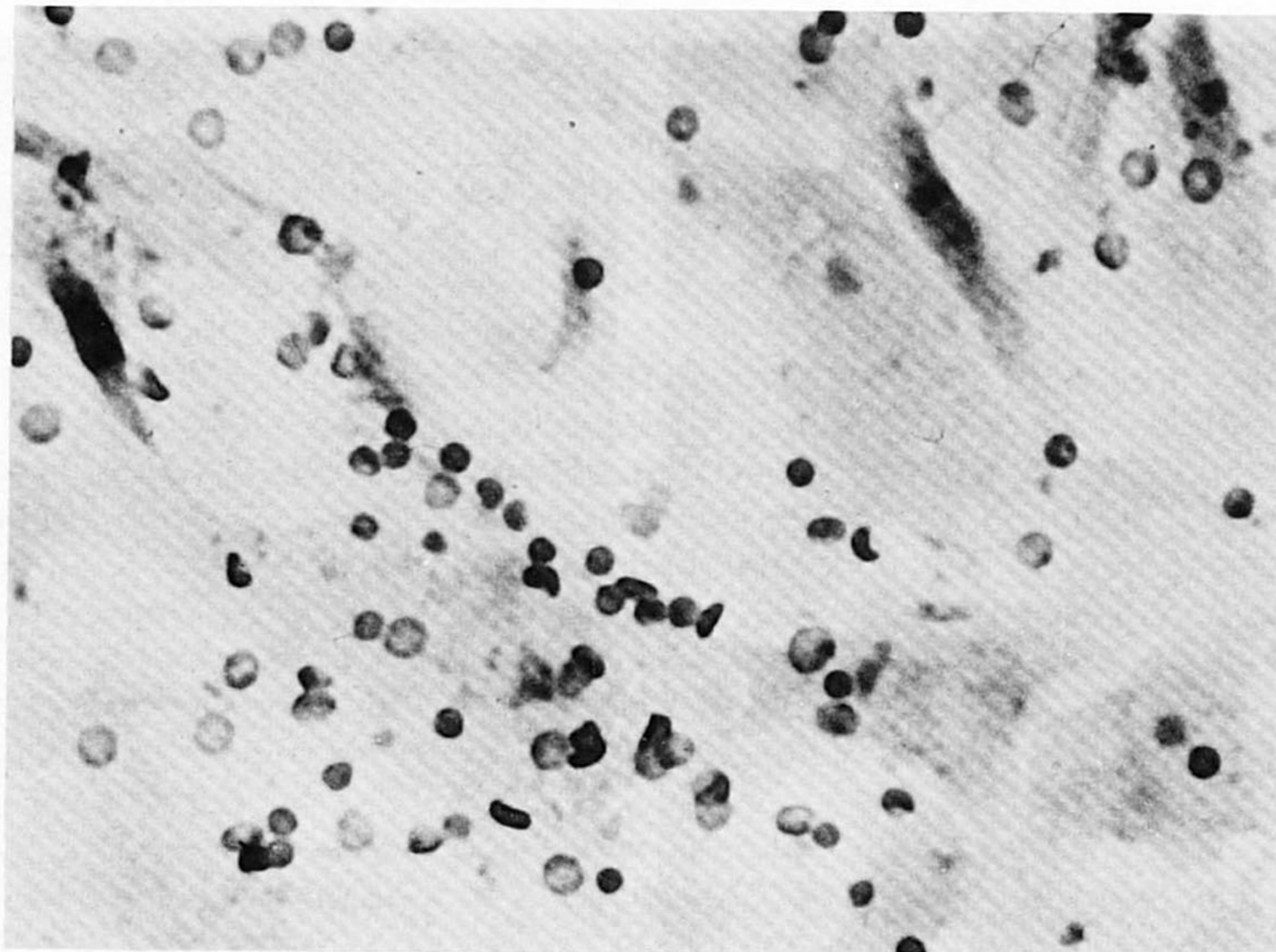


Fig. 2. Rosette formation by fcwf cells of sheep red blood cells opsonized with rabbit anti-SRBC antibodies.

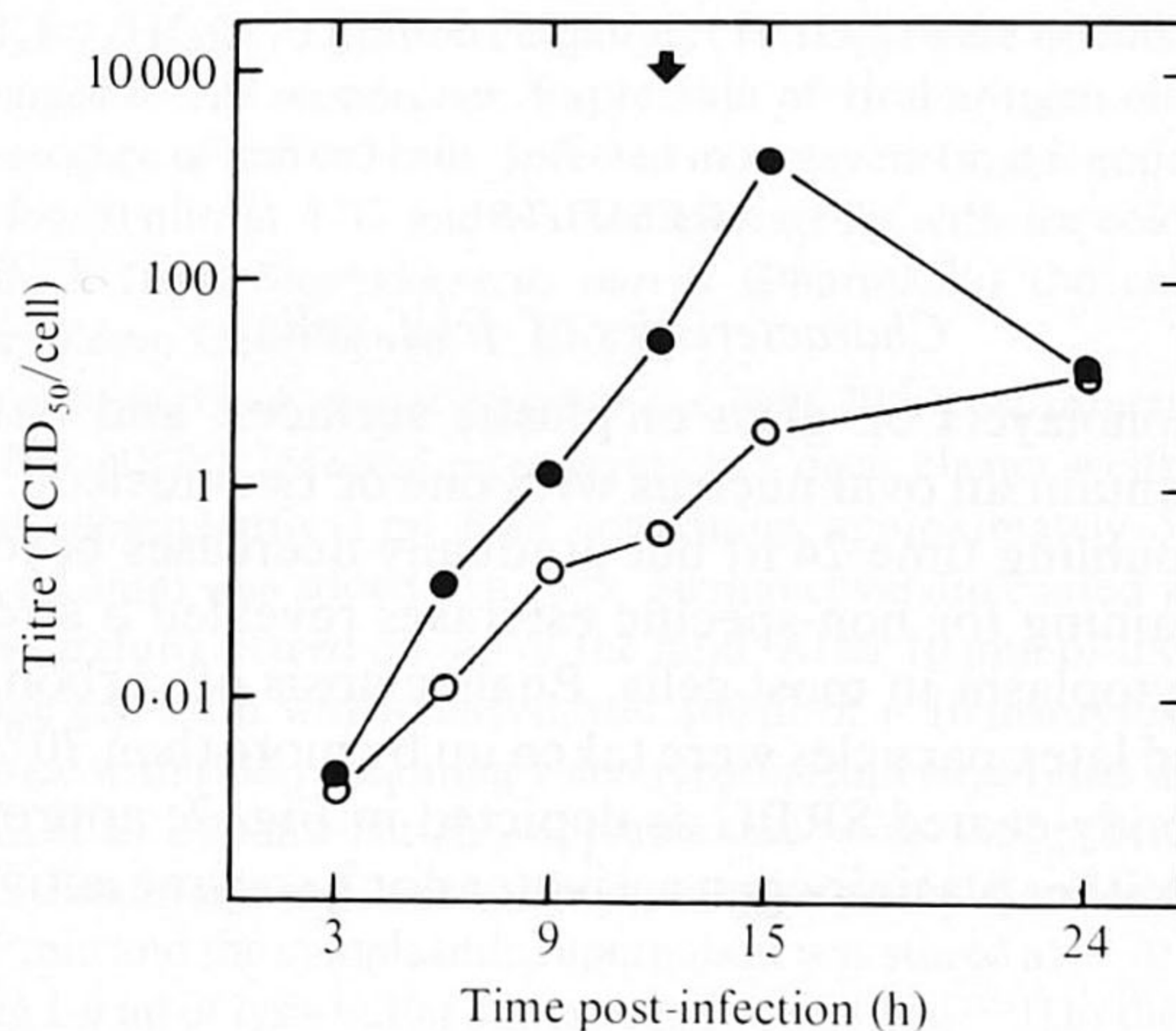


Fig. 3. Growth of feline infectious peritonitis virus in fcwf cells: ●, cell-bound infectivity; ○, extracellular infectivity. The arrow indicates the beginning of cytopathic effects.

Expression of viral antigen on the cell membrane

By immunofluorescence, granular accumulation of viral antigen could first be detected on cell membranes by 16 h post-infection and was very distinct at 24 h (Fig. 4). Surface labelling with ^{125}I followed by immunoprecipitation and SDS-PAGE revealed a protein pattern as shown in Fig. 5. Antiserum against FIPV recognized four proteins with apparent molecular weights of 225 500 (225.5K), 175K, 138K and 25K (Fig. 5, lane *a*). No corresponding proteins were recognized in mock-infected control cells (lane *c*) nor were they precipitated by normal cat serum in infected (lane *b*) or in mock-infected cells (lane *d*). When detergent-disrupted, FIPV-infected cells were labelled, the immune serum recognized an additional protein of 44K (Fig. 5, lane *e*) and a protein of 34K mol. wt. which is also seen in mock-infected cells (lane *g*).

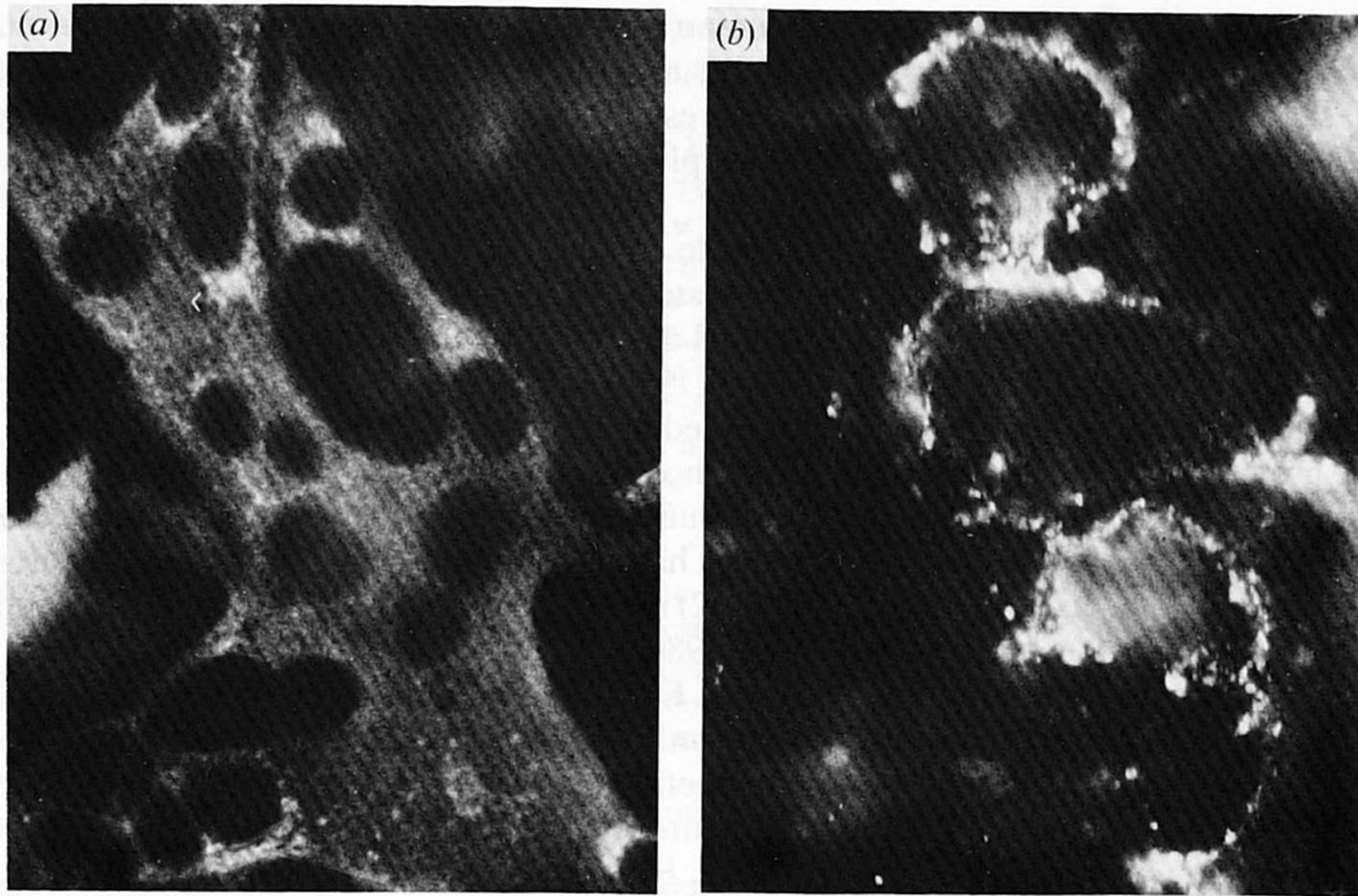


Fig. 4. Immunofluorescence of feline infectious peritonitis virus-infected fcwf cells 20 h post-infection. (a) Cytoplasmic fluorescence in acetone-fixed cells; (b) membrane fluorescence in unfixed cells.

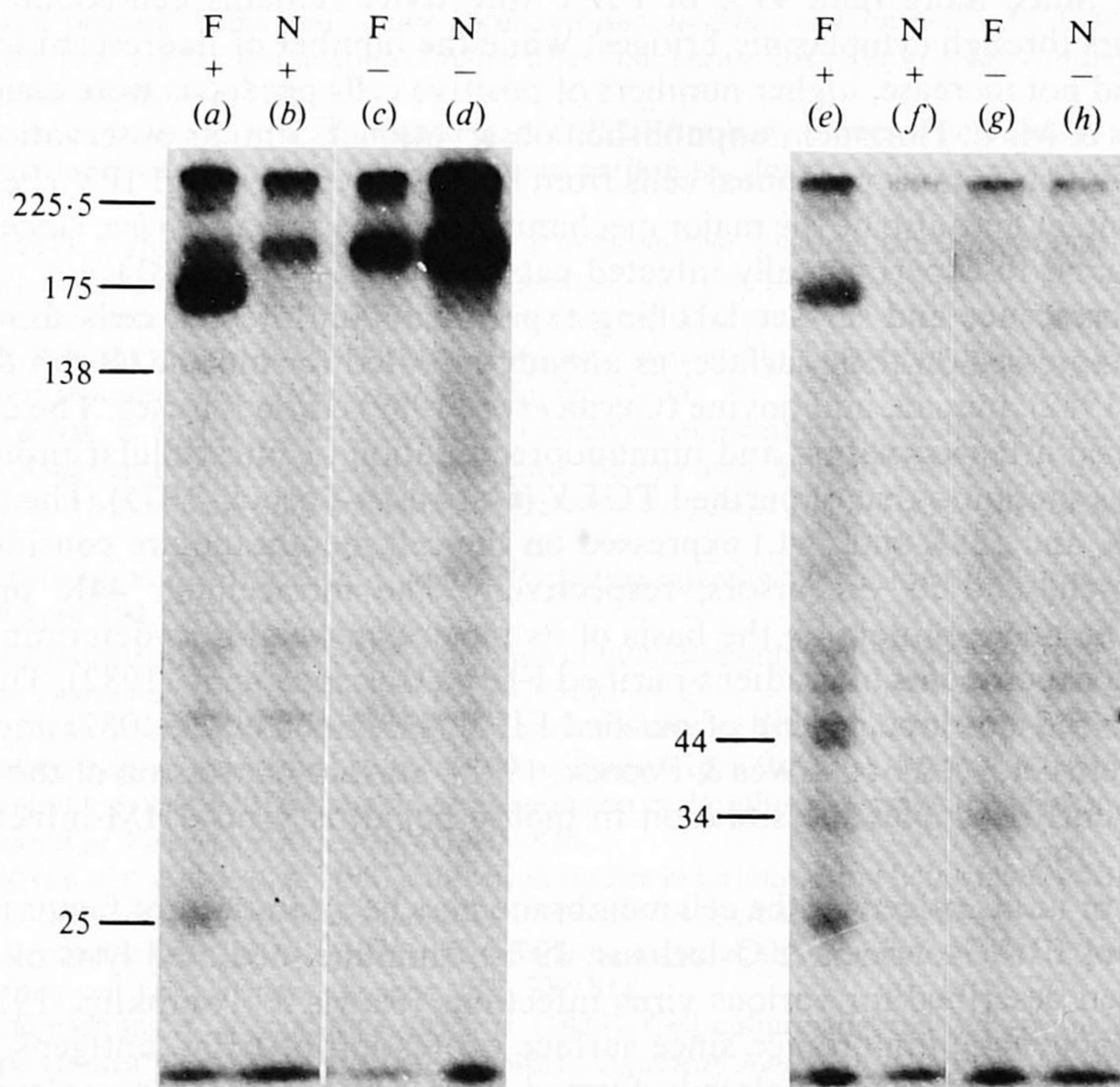


Fig. 5. Electrophoretic analysis of ¹²⁵I-labelled proteins of FIPV-infected (+) and mock-infected (-) fcwf cells after immunoprecipitation with FIP immune serum (F) or normal cat serum (N). (a to d) Surface proteins; (e to h) intracellular proteins. The numbers are mol. wt. × 10⁻³ of the virus proteins.

DISCUSSION

Distinct staining of non-specific esterase, the most reliable cytochemical marker for macrophage identification (Kaplow, 1981), was observed in fcwf cells. They expressed

phagocytic properties as described for human and mouse macrophage lines (Morahan, 1980) and Fc receptors could be detected on their surface. The absence of lysozyme or plasminogen activator activity does not invalidate these results; in a number of murine macrophage lines lysozyme activity was only marginal and plasminogen activator assays usually give very fluctuating results (Morahan, 1980).

Fcwf cells and a strain of FIPV adapted to this line of macrophage-like cells were studied since they constitute a reproducible *in vitro* system. FIPV has been propagated also in other cell lines, however, which have not been qualified as macrophage-like (O'Reilly *et al.*, 1979; Black, 1980; Hitchcock *et al.*, 1981; Evermann *et al.*, 1981), and some not even of feline origin (H. E. L. Jacobse-Geels & M. C. Horzinek, unpublished observations). Pedersen (1976) was the first to report growth of FIPV in explanted autochthonous peritoneal cells and Weiss & Scott (1981) demonstrated viral antigen in cultivated buffy coat cells of experimentally infected cats. Replication of coronaviruses in macrophages has also been described for a human coronavirus strain (229E; Patterson & Macnaughton, 1982) and mouse hepatitis virus (MHV-3; Virelizier & Allison, 1976; Macnaughton & Patterson, 1980).

Our studies have shown that replication of FIPV attains maximum yields in fcwf cells after 15 h; in feline embryonic lung cells, maximum amounts of cell-free virus were not found until 30 h (Beesley & Hitchcock, 1982). Growth kinetics similar to those we found have been reported for the closely related transmissible gastroenteritis virus (TGEV) of swine, when grown in primary pig kidney cells (Pensaert *et al.*, 1970). However, replication of FIPV is slower than that of MHV-A59 which is completed at 10 h post-infection; about 80 particles per cell are synthesized (Spaan *et al.*, 1981), whereas FIPV-infected cells contain more than 1000 infectious units at 15 h. Since more than 99% of FIPV infectivity remains cell-bound, virus spread probably occurs through cytoplasmic bridges; while the number of fluorescent foci in infected monolayers did not increase, higher numbers of positive cells per focus were counted (H. E. L. Jacobse-Geels & M. C. Horzinek, unpublished observations). Similar observations were made in explanted autochthonous peritoneal cells from kittens infected with FIPV (Pedersen, 1976). Cell-to-cell contact may also be the major mechanism of virus spread *in vivo*, since viraemia was strictly cell-bound in experimentally infected cats (Weiss & Scott, 1981).

Immunofluorescence and surface labelling experiments on infected cells showed that viral antigens are expressed on their surface, as already reported for murine (Robb & Bond, 1979; Collins *et al.*, 1982), human, and bovine (Gerna *et al.*, 1982) coronaviruses. The electrophoretic pattern obtained after iodination and immunoprecipitation of intracellular proteins of FIPV-infected cells is similar to that of purified TGEV (Garwes & Pocock, 1975). The larger proteins (225.5K, 175K and 138K mol. wt.) expressed on the cell membrane are considered to be the peplomer protein and its precursors, respectively. The intracellular 44K protein can be identified as the nucleoprotein on the basis of its molecular weight, as determined recently in electroblotting experiments of gradient-purified FIPV (Horzinek *et al.*, 1982). The 25K protein corresponds to the envelope protein of purified FIPV (Horzinek *et al.*, 1982) and to one of the envelope proteins of TGEV (Garwes & Pocock, 1975). Surface expression of the peplomer and envelope proteins resembles the situation in mouse hepatitis virus JHM-infected L241 cells (Collins *et al.*, 1982).

Expression of viral antigen on the cell membrane may be an important factor in the immune pathogenesis of FIP (Horzinek & Osterhaus, 1979). Immune-mediated lysis of infected cells, which has been described for various virus infections (Rawls & Tompkins, 1975), may be of particular pathogenetic importance since surface expression of FIPV antigens is seen late in infection when virus progeny is already formed. The activity of the complement system is reduced in terminal FIP cases, possibly as a result of its activation during antibody-mediated lysis of virus antigen-bearing cells. A pronounced increase in T lymphocytes is also seen (Horzinek *et al.*, 1979) which may be an indication of enhanced direct lymphocyte-mediated cytotoxicity. The exclusive infection *in vivo* of macrophage-like cells (Pedersen, 1976) makes them candidates for an immune attack.

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