

Production and characterization of monoclonal antibodies to four Egtved virus structural proteins

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ABSTRACT: With the aim of studying molecular mechanisms of virulence and immunogenesis of Egtved virus, monoclonal antibodies (MAbs) were produced against 4 dominant virus proteins (G, N, M₁ and M₂). The reactivity of each MAb was determined by ELISA, immunoblotting, immunofluorescence and plaque neutralization. Antibodies specific for each of the 4 proteins, as demonstrated by immunoblotting, gave characteristic reactions in ELISA as well as immunofluorescence. None of the MAbs were able to neutralize virus in vitro. When analysed in immunofluorescence using cell cultures fixed at different times after inoculation with live virus, the N-protein was first to be detected followed by M₁, G and M₂. G-specific MAbs reacted with either a 'reticular', or a 'Golgi'-form of the G-protein. Results are consistent with published information on the protein composition and cellular appearance pattern of other rhabdoviruses studied in vitro.

INTRODUCTION

Egtved virus, the causative agent of viral haemorrhagic septicaemia (VHS) of rainbow trout *Salmo gairdneri*, is one of the most severe pathogens encountered in European trout farms. The virus apparently belongs to the lyssa virus group in the rhabdovirus family (Lenoir & de Kinkelin 1975, McAllister & Wagner 1975). Attempts to develop vaccines on the basis of inactivated or attenuated virus have not so far led to products inducing sufficient protection against VHS (de Kinkelin et al. 1984).

It remains to be seen, however, whether improved VHS-vaccine potency might be obtained in artificial vaccines containing primarily or exclusively the virus antigens which induce protective immunity in fish. Such vaccines would have to be based on a detailed study of the molecular mechanisms of virulence and immunogenesis. Such a study requires separation and purification of the 5 polypeptides of Egtved virus which have been designated L (RNA-dependent RNA polymerase), G (envelope glycoprotein), N (nucleocapsid related protein), and M₁ and M₂ (matrix proteins 1 and 2) according to the accepted nomenclature of Rabies virus proposed by Wagner et al. (1972). A possible means of obtaining the virus polypeptides in pure form is affinity chromatography based on specific monoclonal antibodies (MAbs).

The present work deals with the production and preliminary characterization of monoclonal antibodies against 4 different polypeptides of Egtved virus strain F1 (Jensen 1965). The methods applied in the characterization of the antibodies were: enzyme-linked immunosorbent assay (ELISA), immunoblotting, immunofluorescence (IFAT), double-diffusion and plaque neutralization (50 % PNT).

MATERIALS AND METHODS

Fish cell lines. For production of virus Bluegill fry (BF-2) cells (Wolf & Quimby 1962) were used. Epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983) were utilized for preparation of virus-infected cell cultures for IFAT and 50 % PNT. Both cell lines were cultivated in stationary bottles and trays in Eagle's MEM supplemented with 2 % or 10 % foetal bovine serum and antibiotics in standard concentrations. Buffers used were bicarbonate when cultivation was in bottles, and Tris when cultivation was in open vessels. Cultivation temperature was 20 and 25 °C for BF-2 and EPC cells, respectively. After inoculation of cell cultures with virus, incubation was performed at 15 °C.

Viruses. Egtved virus, reference strain F1 (Jensen 1965) at passage level 254, was concentrated and gradient-purified as described by de Kinkelin (1972) for

immunization of mice as well as for use in ELISA and SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Rhabdovirus anguilla (EVX) (Hill et al. 1980), purified following the same procedure, was used as control antigen when the specificity of antibodies was tested in ELISA and by immunoblotting. For preparation of virus-infected cultures for immunofluorescence the following virus strains were used: Egtved virus field strains Gelsbro (serologically related to reference strain F1), EVX and spring viraemia of carp (SVC) virus (Fijan et al. 1971).

Immunization of mice. Six-wk-old female mice (BALB/c) were given 5 intraperitoneal injections of 10^8 to 10^9 50 % tissue culture infective doses (TCID₅₀) over a period of 2 mo. For the first 3 injections virus was mixed with equal parts of Freund's incomplete adjuvant. Two mice were given an intravenous booster injection of 10^9 TCID₅₀ in Week 9 when ELISA and IFAT had documented the presence in the mice of Egtved-virus-specific antibodies. Three d after the booster the mice were killed and the spleens removed for fusion.

Cell fusion. Spleen cells from the immunized mice were fused with cells of the non-immunoglobulin-secreting myeloma cell line P3-X63-Ag8.653 (Kearney et al. 1979) according to the procedure described by Astaldi et al. (1980). About 2×10^7 myeloma cells and 10^8 mouse spleen cells were mixed in cell culture medium RPMI 1640 (Gibco). The cells were spun down at $400 \times g$ for 10 min at 6 °C and subsequently resuspended in 0.5 ml RPMI 1640 medium with 50 % polyethylene glycol (PEG) 4000 (Merck). After resuspension the cells were diluted over 5 min with 10 ml RPMI 1640, pelleted and washed once in medium supplemented with 15 % bovine serum, 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine (HAT-medium). The cells were then plated on five 96-well tissue culture trays (Costar) in the above HAT medium supplemented with 4 % human endothelial cell supernatant (HECS) from Costar.

Cultivation of hybridoma cells. The hybridoma cells were grown at 37 °C in an atmosphere containing 6 % CO₂ and 80 % humidity. Ten d after fusion the HAT medium was replaced by HT medium without HECS. From this point on it was attempted to maintain a 50 to 90 % cell confluency by changing the medium at 2 to 4 d intervals and by varying the serum concentration in the medium between 5 and 15 %.

Cloning. Hybridoma cell lines were cloned at least twice by limiting dilution, in order to obtain stable clones of immunoglobulin(Ig)-secreting cell populations.

ELISA. The assay used for screening of hybridoma supernatants (HSNs) was designed according to the principles of indirect ELISA described by Voller et al.

(1979). Microtiter plates were coated overnight at 4 °C with 50 µl per well of either purified Egtved virus diluted 1:1000 in phosphate-buffered saline (PBS), pH 7.2, or of PBS alone. After temperature alignment for 1 h at room temperature (the following steps were all performed at room temperature) the plates were washed once in PBS with 0.05 % Tween 20 (PBS-T) followed by blocking with PBS-T with 1 % bovine serum albumin (BSA) (PBS-T-BSA), 200 µl per well, for 1 h. After 3 additional washings the microtitre plates were incubated for 1 h with 50 µl per well of the HSNs to be examined. A serum pool from mice immunized with Egtved virus strain F1 was used in dilution 1:1000 (later bleedings 1:10 000) as a positive standard. The washing procedure was repeated and followed by a 1 h incubation with horse radish peroxidase (HRP)-conjugated rabbit Ig to mouse Ig (Dakopatts, Copenhagen) diluted 1:1000 in PBS-T-BSA, 50 µl per well.

Finally, after renewed washing, the bound enzyme was visualized using H₂O₂-OPD substrate (50 µl well⁻¹). The colour reaction was stopped with 2M H₂SO₄ (100 µl well⁻¹) when the standard test serum reached maximum absorbance (about A₄₈₆ = 3) in wells coated with virus (usually after 10 to 20 min). Spectrophotometric measurements were done in an automatic spectrophotometer (Kontron SLT 210). In the initial screening a HSN was regarded as ELISA-positive when its A₄₈₆ was above 0.3 in the virus-coated well, provided that the A₄₈₆ of the corresponding well coated with PBS did not exceed 50 % of the value with virus antigen.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (1970) in 0.7 or 1.5 mm thick slab gels, using a vertical slab gel system from Pharmacia. The stacking gel contained 3.5 % acrylamide/0.11 % bisacrylamide, and the separating gel 10%/0.31 %. Wells in the gels were loaded with about 8 µg virus protein per cm² cross-section area. Molecular weight standards from Kem-En Tec (Hellerup, Denmark) were included in each run. After electrophoresis the gels were either used for immunoblotting or stained by the silver staining method described by Morrissey (1981).

Immunoblotting. The protein bands were transferred from polyacrylamide gels to nitrocellulose membranes (Whatman) in a semi-dry electroblotter (Ancos, Ølstykke, Denmark) according to the principles described by Kyhse-Andersen (1984). Free binding sites were blocked by incubation in PBS with 2 % Tween 20 for 3 min. After washing in PBS-T the membranes were cut into 2 to 4 mm wide strips and incubated overnight at 4 °C (or occasionally 2 h at room temperature) with the individual HSNs diluted in PBS-T-BSA. As a positive control reagent, diluted mouse antiserum to strain F1 of Egtved virus was used. HRP-

conjugated antiserum was the same as in ELISA. Visualization of bound conjugate was performed using 3-amino-9-ethyl carbazole (Sigma) (1 mg in 100 ml acetone) of which solution 2 ml were added to 50 ml 50 mM sodium acetate buffer pH 5.5, containing 0.015 % H₂O₂. The reaction was stopped after 10 to 30 min by removal of substrate. As a positive control reagent, diluted mouse antiserum to strain F1 of Egtved virus was used. The reaction was stopped when the 4 dominating virus protein bands were clearly stained by the control serum (usually 10 to 30 min).

To check the efficiency of the blotting procedure a few strips of the nitrocellulose membranes were routinely stained for total protein with colloidal gold (Moremans et al. 1985).

Plaque neutralization test (PNT). 50 % Plaque neutralization tests were used for screening of HSNs for neutralizing activity. The technique used was basically as the micro-method previously described (Olesen & Jørgensen 1986) except that complement was omitted.

Indirect fluorescent antibody technique (IFAT). Coverglass cultures, 9 × 22 mm, of EPC cells were infected with 2 × 10⁸ TCID₅₀ per ml of Egtved virus strain F1 or with 1 × 10⁷ TCID₅₀ per ml of strain Gelsbro. Control coverglass cultures were infected with EVX or SVC virus. Following 1 rinse in medium without serum the cultures were fixed for 10 min in acetone, either at 1 h intervals between 1 and 12 h post-infection (p. i.) (F1) or at 24 h p. i. (Gelsbro, EVX and SVC). The fixed cell cultures were stored at -20 °C until used. In IFAT, hybridoma supernatants, undiluted or diluted in PBS, or mouse antiserum to Egtved virus strain F1 diluted 1:100 in PBS, were applied as the first antibody layer and fluorescein isothiocyanate (FITC)-conjugated rabbit antiserum to mouse Ig (Dakopatts, Copenhagen), 1:40, as the second antibody layer. Incubation times and temperatures as well as rinsing and mounting procedures were essentially as already described (Jørgensen 1974). Examination was carried out in an Olympus Vanox epifluorescence microscope supplied with a 200 W mercury lamp, interference filters (Rygaard & Olsen 1969) and fluorescence oil-objectives × 40 and × 100.

Protein determination. The protein content in suspensions of purified virus was estimated by the dye (Coomassie blue G250, Merck) binding microassay described by Rylatt & Parish (1982), slightly modified as proposed by Macart & Gerbaut (1982), by adding 30 mg SDS per l colour reagent. BSA (Sigma) was used as standard protein.

Ig class determination. The Ig class and subclass of the MAbs was determined by means of the Ouchterlony double-diffusion technique applying reagents from Serotec (UK).

Ig quantification in hybridoma supernatants. The

concentration of murine antibody in HSNs was estimated by ELISA. Instructions about the procedure as well as a standard reagent (an HSN containing 20 µg of mouse Ig per ml) were kindly provided by Dr Claus Koch, the State Serum Institute, Copenhagen. In brief, rabbit Ig against mouse Ig (Dako, Denmark) was applied to the wells of microtitre plates as a catching antibody layer. Second layer was 2-fold dilutions of unknown or standard HSN, third layer was HRP-conjugated rabbit antiserum to mouse Ig. The Ig concentration of unknown HSNs were estimated from the correlation between A₄₈₆ and dilution factor for the 20 µg ml⁻¹ standard HSN.

RESULTS

SDS-PAGE of purified viruses

Purified Egtved virus, used for immunization, immunoblotting and ELISA, contained 3 × 10¹⁰ TCID₅₀ ml⁻¹. The protein content was approximately 50 to 60 µg ml⁻¹. Examination in SDS-PAGE revealed 5 major bands in silver-stained gels as well as in gold-stained blotting strips (Fig. 1, Strip 15). According to the molecular weight standards used the molecular weight of the respective 5 polypeptides were about 190, 70, 41, 28 and 24 kd. Based on the nomenclature of rhabdovirus proteins proposed by Wagner et al. (1972) the major protein bands are called L, G, N, M₁ and M₂.

Purified EVX contained 2 × 10⁹ TCID₅₀ and the protein concentration was estimated to 15 to 20 µg ml⁻¹. Four bands were visible on the gold-stained blotting strips (3 major and 1 minor one). The expected high molecular weight L-band was not visible, probably due to low concentration and/or slow migration out of the gel during blotting (Fig. 1, Strip 1).

Hybridoma cell line generation and initial screening

Ten d after fusion, cell growth was observed in about 95 % of the inoculated 960 wells in the fusion trays. Each well contained 1 to 5 cell clones. About 25 % of the cell cultures produced antibodies which were detectable during the initial ELISA screening 13 d after the fusion. Subcultivation followed by repeated testing reduced this to about 10 % (92 cell cultures). None of the supernatants from these cultures appeared positive when examined in plaque neutralization test.

In immunoblotting 53 of the 92 tested HSNs gave a detectable reaction with 1 or more of the 4 protein bands G, N, M₁ and M₂, the remaining 39 being negative. The 29 cell cultures producing the most strongly

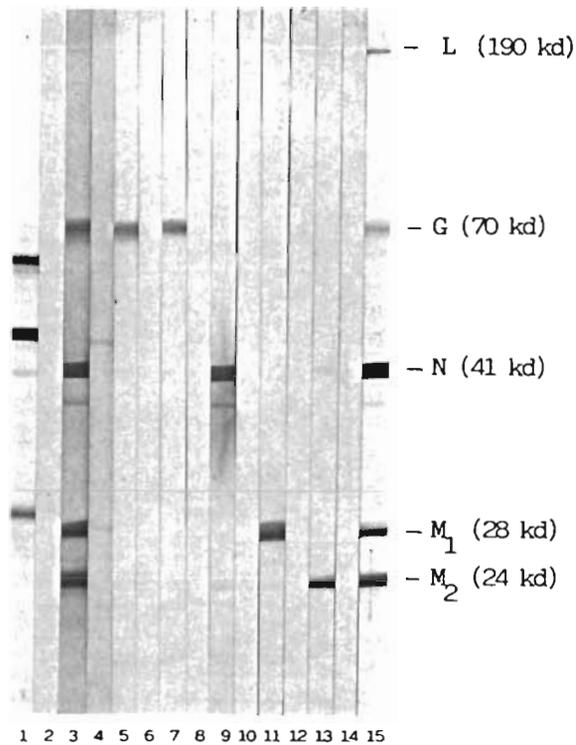


Fig. 1. Immunoblotting with MABs and mouse serum. After transfer the nitrocellulose membrane was cut into strips which were either stained with colloidal gold or immunostained. Antigens were Egtved virus (VHSV) and Rhabdovirus anguilla (EVX). Strips: 1, EVX with gold; 2, VHSV with serum from non-immunized mouse; 3, VHSV and 4, EVX both with serum from mice immunized with VHSV; 5, VHSV and 6, EVX both with G-specific MAB G-gol type (IP1D11); 7, VHSV and 8, EVX both with G-specific MAB G-ret type (IP1H3); 9, VHSV and 10, EVX both with N-specific MAB (IP5B11); 11, VHSV and 12, EVX both with M₁-specific MAB (IP1C6); 13, VHSV and 14, EVX both with M₂-specific MAB (IP1C3); 15, VHSV with gold. Mouse sera were diluted 1000 times in PBS-T-BSA. HSNs were diluted to about 30 ng mouse Ig ml⁻¹. Strips were incubated 2 h at room temperature with the primary antibody. Other parameters as described in the text

and most monospecifically reacting HSNs were cloned. After cloning monospecifically reacting HSNs were analysed in IFAT. Five different staining patterns of virus-infected cells were observed, correlating with the different protein specificities seen by immunoblotting. Cell clones representing each of the 5 patterns were selected for further propagation.

Immunoblotting

Immunoblotting results with the 5 finally selected cell lines are shown in Fig. 1. None of the HSNs from these cells reacted with a cellular control antigen (not illustrated) or with EVX virus proteins. It appeared that anti-M₂ MAB cross-reacted slightly with the N protein and anti-N MAB possibly slightly with the M₂-protein.

However, monospecificity in immunoblotting turned out to be a matter of dilution factors since all the antibodies showed some faint shadow-like staining of protein bands other than the intensively stained main band, if a high concentration of MAB was used.

ELISA

As shown in Fig. 2 the 5 selected MABs of different specificity reacted with different intensities in ELISA. The intensity of the reactions decreased in the following order: anti-M₂, anti-N, anti-M₁ and anti-G.

None of the HSNs reacted with EVX virus in ELISA (not shown).

IFAT

The IFAT reaction of HSNs, previously characterized by immunoblotting, with Egtved virus-infected cell cultures, fixed 24 h p. i., are shown in Fig. 3. Antibody against the N-protein (Fig. 3d) gave a strong, coarsely granular staining primarily of the peripheral part of the cytoplasm in the majority of the infected cells. Antibody against M₁ induced a staining which resembled that of anti-N but less intensive. Anti-M₁ additionally stained membranes or membrane-associated material (Fig. 3e).

Antibody reacting with M₂ caused a fine granular staining throughout the cytoplasm. Furthermore, the cell membranes including membrane protusions were stained (Fig. 3f).

The reaction of antibodies specific for the G-protein showed 2 different patterns varying from one HSN to another. One of the patterns involved staining of reticular structures, in some cells more evidently than in others, evenly distributed throughout the cytoplasm. In most of the infected cells cisternae-like structures in a juxtannuclear position were stained as well. The specificity of the antibody in question is referred to as anti-G-reticular (anti-G-ret) (Fig. 3a, b).

The other pattern involved staining of the cisternae-like structures close to the nucleus (probably some Golgi-associated structures) mentioned above and in addition a faint staining of cell membranes. The specificity of this antibody is referred to as anti-G-gol (Fig. 3c).

None of the HSNs examined reacted with EVX or SVC (not shown).

In order to obtain information about the earliest possible detection of the respective proteins in the infected cells, the 5 HSNs were applied on Egtved virus-infected EPC-cell cultures fixed at 1 h intervals p. i. The results appear in Table 1. The proteins became detectable in the following order: N (1 to 2 h p. i.), M₁ (6 h p. i.), G-gol and G-ret (7 to 8 h p. i.) and M₂ (8 h p. i.).

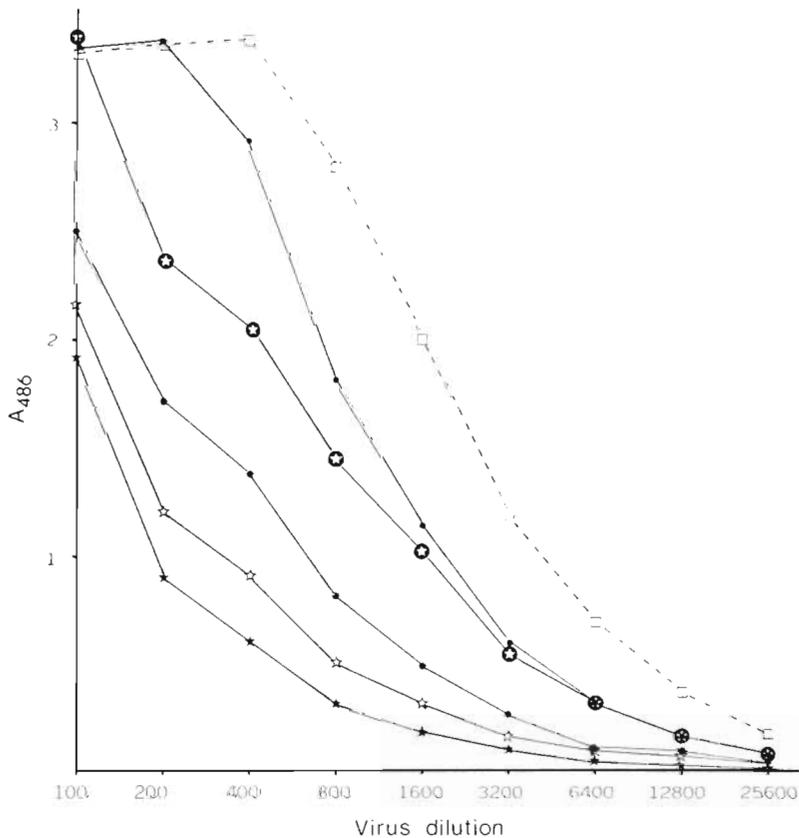


Fig. 2. Relation between A_{486} and dilution of gradient-purified Egtved virus in ELISA with the 5 finally selected MAbs and mouse serum as primary antibodies. HSNs were diluted to about $1 \mu\text{g mouse Ig ml}^{-1}$; sera from VHSV-immunized mice were diluted 10000 times. Other parameters as in 'Materials and Methods'. The reaction was stopped after 15 min. (★) G-specific MAb (G-gol) IP1D11; (△) G-specific MAb (G-ret) IP1H3; (●) N-specific MAb IP5B11; (▲) M₁-specific MAb IP1C6; (◆) M₂-specific MAb IP1C3; (□) mouse serum (10000^{-1})

DISCUSSION

An important prerequisite for the success of the present work was the initial development of the ELISA technique as a simple specific way of detecting Egtved virus-specific antibodies in HSNs. So far other workers have had little success in applying ELISA techniques in the study of Egtved virus (Dixon & Hill 1984). The use of gradient-purified virus in the coating layer is probably an important step.

It would have been possible to use IFAT instead of ELISA during the initial screening but this would have been very time-consuming.

Immunoblotting results (Fig. 1) revealed that the initial ELISA screening was sensitive enough to detect antibodies reacting with 4 of the 5 known polypeptides in the virion. However, antibodies specific for the G-protein in general gave ELISA reactions near the detection limit (Fig. 2). This means that some G-reactive MAbs of moderate or low affinity, or perhaps directed against less exposed determinants, might remain undetected. Bernard et al. (1985) have estimated that the G-protein makes up only about 7% of total virion protein mass. The percentage of L-protein is even smaller and furthermore the L-protein is a high-molecular-weight structure (about 190 kd). It thus

appears that a MAb directed against an unrepeatable determinant on L might very easily remain undetected.

Detection of L-specific MAbs in HSNs would probably require use of immunoblotting in the screening step. Even then difficulties might be encountered due to incomplete transfer of the L-band to the nitrocellulose membrane. Alternatively an ELISA with a heavy protein load in the coating step might prove usable, the main problem being an often high background level in the initial screening. Concerning the G-, N-, M₁- and M₂-proteins, immunoblotting seems to be quite a sensitive procedure in detecting low concentrations of specific MAbs. In fact HSNs from well-established cell lines could often be diluted about 8000 times and still give strong staining reactions. The observed cross-reaction of M₂-specific MAbs with the N-band was not seen in high dilutions of HSN. However, the cross-reaction pattern appeared with all of 4 M₂-reactive HSNs after cloning and therefore occurrence of some partially identical epitopes in the 2 protein bands cannot be excluded.

Several authors, however, have lately demonstrated the occurrence of different kinds of cross-reacting MAbs, which consequently may be more common than previously expected (Fox & Siraganian 1986, Ghosh & Cambell 1986).

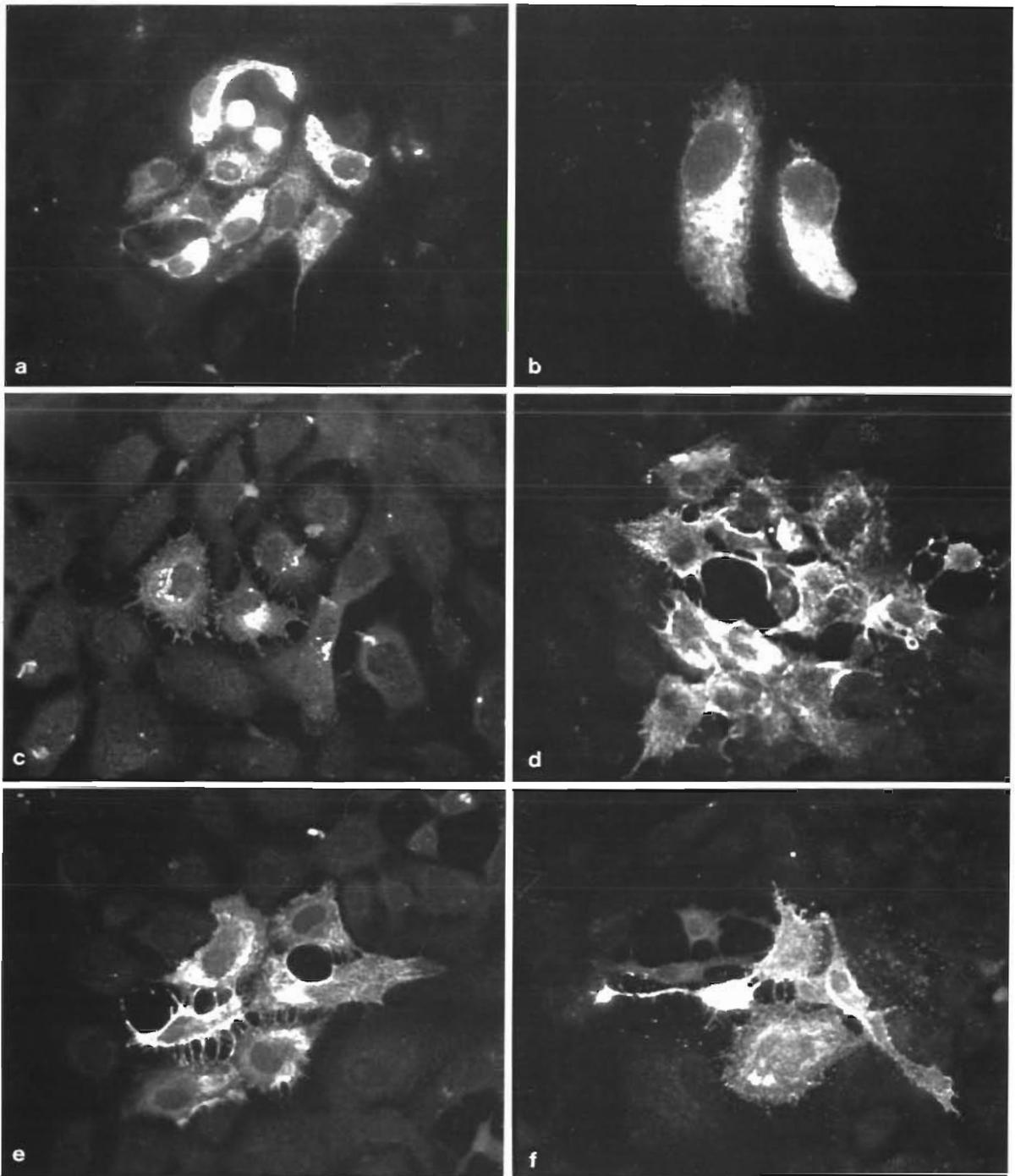


Fig. 3. Immunofluorescence micrographs illustrating the cellular staining patterns seen in Egtved virus-infected EPC cells fixed 24 h p. i. and examined with protein-specific MAbs as primary antibodies. (a) G-specific MAb, reticular type, $\times 370$. (b) Same, $\times 880$. (c) G-specific MAb, Golgi type, $\times 370$. (d) N-specific MAb, $\times 370$. (e) M_1 -specific MAb, $\times 370$. (f) M_2 -specific MAb, $\times 370$

In IFAT each antibody gave a characteristic staining pattern depending on the specificity. Similar results have been reported for vesicular stomatitis virus (VSV) which belongs to the vesiculo group in the rhabdovirus family (for review see Dubois-Dalcq et al. 1984). The

findings in this study concerning Egtved virus protein N and M_1 correspond well to the intracellular distribution of the VSV proteins N and NS respectively. The staining of peripheral cytoplasmic material probably concerns nucleocapsids immediately before the bud-

ding process. The staining of Golgi-like structures as well as membrane components by G-specific antibodies is a common phenomenon (Wehland et al. 1981, Bergmann et al. 1982). Conversely, the characteristic reticular pattern seen with the G-ret-specific antibodies does not seem to be a common observation in IFAT studies. Electron microscopical analysis, however, has proved the presence of viral glycoprotein in the endoplasmic reticulum of VSV-infected cells (Wehland et al. 1981, Bergmann et al. 1982). Presumably the reticular pattern in IFAT is due to selection of a MAb directed against a glycoprotein determinant which is well exposed in the endoplasmic reticulum.

The G-gol-specific antibodies probably react only with determinants added to (or demasked on) the G-molecule during its passage through the Golgi apparatus of the host cell.

In the study of the time of intracellular appearance for the different virion proteins, N was first to be detected followed by M₁, G and M₂ (Table 1). A nearly similar order of detection by means of radiochemical methods has been reported for IHNV, another fish rhabdovirus (Hsu et al. 1985).

Varying degrees of background staining with the different MAbs in IFAT made early detection of some proteins easier than others. The very early appearance (1 to 2 h) of N, however, is very significant and may reflect the function of the N-protein in genome folding and protection (Dubois-Dalcq et al. 1984).

With regard to rapid detection of Egtved virus by means of IFAT, our results have pointed out anti-N MAbs as potentially valuable specific reagents.

In ELISA M₂-specific MAb gave a very strong reaction with the F1 antigen and a reaction with the control antigens near background level, indicating easy and specific detection of the M₂ protein in this kind of assay. In concordance with this, Bernard et al. (1985) have found M₂ to be the dominating protein in the virion.

Table 1. Characteristics of 5 selected MAbs

Hybridoma cell line	Protein specificity	Immuno-globulin class/subclass	Time lapse p.i. until positive IFAT reaction ^a (h)
IP1D11	G (G-gd)	IgG ₁	7-8
IP1H3	G (G-ret)	IgG ₁	7-8
IP5B11	N	IgG ₁	1-2
IP1C6	M ₁	IgG _{2b}	6
IP1C3	M ₂	IgG ₁	8

^a HSNs were diluted to ca 2 µg mouse Ig ml⁻¹

None of the 92 HSNs tested in 50 % PNT contained detectable amounts of neutralizing antibody. Neither could neutralizing antibodies be detected in serum samples from the immunized mice at the time when the spleens were removed for fusion. After 6 mo of continued immunization of other mice low titres of neutralizing antibodies were synthesized. It remains to be seen whether the use of spleen cells from such a mouse for fusion might lead to hybridomas producing Egtved virus neutralizing antibodies.

The present MAbs appear to represent potentially valuable tools for future VHS studies. We intend to use them in immunosorbent work for preparation of pure Egtved virus polypeptides which will be essential in connection with immunization- and pathogenesis studies. In addition to being useful in basic VHS studies the MAbs seem to be potentially powerful diagnostic tools.

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