Alterations of the Immunological Specificity of Plasma Membranes from Cells Infected with Marek’s Disease and Turkey Herpes Viruses

By O. R. KAADEN AND B. DIETZSCHOLD
Federal Research Institute for Animal Virus Diseases, 74 Tübingen
(Postfach 1149), BRD (West Germany)

(Accepted 23 May 1974)

SUMMARY
Highly purified plasma membranes were isolated from chicken embryo fibroblasts infected with Marek’s disease virus (MDV) or turkey herpes virus (HVT). The purification was monitored by the incorporation of fucose and marker enzymes specific for plasma membranes. Polyacrylamide gel electrophoresis showed that the membrane preparations contained two new virus-induced proteins. When reacted in the double immunodiffusion test solubilized plasma membranes from MDV-infected cells formed two specific precipitation bands with Marek’s disease immunoglobulins.

Antisera prepared against plasma membranes from MDV- or HVT-infected cells neutralized extracellular infectious HVT. After incubation of plasma membranes from MDV-infected cells with Marek’s disease antibodies the buoyant density of the membranes increased from 1.05 g/ml to 1.08 g/ml due to the binding of immunoglobulins to the virus-induced membrane proteins. The Marek’s disease mortality of chickens twice vaccinated with a plasma membrane preparation from HVT-infected cells was reduced by 94%.

From the results it was concluded that the plasma membranes acquire new immunologic specificities after infection with MDV or HVT.

INTRODUCTION
During the replication of several viruses containing DNA, new membrane or surface antigens are induced. By using the immunofluorescence techniques, such antigens were detected in cultured cells after infection with polyoma virus (Irlin, 1967), Shope papilloma virus (Ishimoto & Ito, 1969), Epstein-Barr virus (Klein et al. 1967), herpes simplex virus, strain HFE (O’dea & Dineen, 1957), and Marek’s disease virus (Chen & Purchase, 1970). Recently, the incidence of a cell surface antigen in quail cells infected with cell-free herpes virus of turkeys was reported by Ishikawa et al. (1972).

In the present study we report on the polypeptide composition and the alterations of the immunologic specificity of plasma membranes prepared from MDV and HVT infected cells.

METHODS
Cells. Chicken embryo fibroblast cells (CEF) were prepared from 11-day-old Rhode Island Red embryos (RIR, line Houghton Poultry Research Station). The conditions of cell cultivation and the media used have already been described (Kaaden & Dietzschold, 1972).
Labelling of cells. Cultured cells were labelled with 5 μCi/ml [35S]-L-methionine (372 mCi/mmol) or 20 μCi/ml [3H]-methyl-L-methionine (100 mCi/mmol) during 24 h incubation in the maintenance medium. The incubation of cells with 10 μCi/ml [3H]-L-fucose (500 mCi/mmol) was extended to 36 h. The maintenance medium used for radioactive-labelling contained a 1:10 mixture of TCM 199 and Hank's solution and 2% of dialysed calf serum. The radioactive precursors were purchased from Radiochemical Centre, Amersham, England.

Viruses. The attenuated MDV, strain HPRS-16 att, was used at the 15th culture passage level for the infection of cells. The experiments with the HVT, strain FC-126 (Witter et al. 1970), were performed with a virus stock prepared from the 25th CEF passage. Extracellular infectious HVT was prepared according to the method of Calnek, Hitchner & Adldinger (1970).

The viruses were propagated in roller bottles of CEF and infected cells stored in liquid nitrogen. The infectivity of MDV and HVT was assayed in secondary CEF (Kaaden & Dietzschold, 1972).

Membrane preparation. Membrane fractions from normal CEF, MDV- or HVT-infected CEF were isolated and fractionated following the method of Bingham & Burke (1972). The membrane band formed at a dextran density of 1.048 g/ml was removed and used throughout all experiments.

Electron microscopy. Isolated membranes were fixed overnight with 3% (v/v) glutaraldehyde, rinsed three times with phosphate buffer, pH 7.2 and fixed with 1% (v/v) OsO4 for 1 h. The preparations were dehydrated by sequential washings with absolute ethanol and finally embedded in Epon-Araldite. Thin sections were cut with a LKB Ultratome 4804A using glass knives, stained with uranyl acetate, and examined in a Siemens Elmiskop 101 at an instrumental magnification of ×10000.

Estimation of radioactivity. Samples were counted in a liquid scintillation counter (Tri-carb, model 3375, Packard, Frankfurt, Germany).

Protein estimations for determination of specific activities were performed by the method of Lowry et al. (1951) using bovine serum albumin as a protein standard.

Enzyme assays. The activity of NADPH: ferricytochrome c oxidoreductase (E.C. 1.6.2.3) was determined by the method of Ragnotti, Lawford & Campbell (1969). Succinate cytochrome c reductase (E.C. 1.3.99.1) and 5'-nucleotidase (E.C. 3.1.3.5) activities were both measured by the method of Avruch & Wallach (1971). NADP, cytochrome c and AMP were purchased from Boehringer, Mannheim, Germany. Tracer [3H]-AMP (27.5 Ci/mmol) was a product of Radiochemical Centre, Amersham, England.

Polyacrylamide gel electrophoresis (PAGE). Cellular membrane fractions were removed from the dextran T-40 gradients and precipitated by addition of ice-cold 10% (w/v) trichloroacetic acid (TCA). The precipitate formed was sedimented by centrifuging for 30 min at 35000 g in the Spinco ultracentrifuge (type 30 rotor). The final precipitate was washed with cold ethanol. Analytical PAGE in 10% (w/v) polyacrylamide gels was carried out as described by Bolognesi & Bauer (1970) with the following modifications. The protein samples were treated with SDS (BDH Chemical Ltd, Poole, England) and dithiothreitol (DTT) (Calbiochem, San Diego, U.S.A.) to give a final concentration of 1% (w/v) and 0.1 M, respectively, and heated for 1 min at 100 °C. Samples of 100 μl containing approx. 50 to 100 μg of protein were applied to the gels (6 × 90 mm). After extrusion from the tubes, the gels were sequentially sliced with a razor blade into sections of approx. 1.25 mm, and dissolved in 0.5 ml 30% H2O2 (Merck, Darmstadt, Germany) for 5 h at 80 °C. Radioactivities were measured in vials containing 10 ml of Insta-Gel® (Packard, Frankfurt, Germany).
Herpes virus infected cell membranes

In co-electrophoresis experiments with materials labelled with different isotopes, the $[^{3}H]$ values were corrected for $[^{35}S]$-overlap into the $[^{3}H]$-channel, whereas the extent of the $[^{3}H]$-cross-over into the $[^{35}S]$-channel was insignificant. The counting efficiencies were 17% for $[^{3}H]$- and 68% for $[^{35}S]$-labelled samples.

Protein staining with 0.25% Coomassie blue (Serva, Heidelberg, Germany) dissolved in 10% (w/v) acetic acid and 50% (v/v) methanol was carried out for 1 h at room temperature.

Serological tests. The agar gel precipitation test (AGP) of Chubb & Churchill (1968) was performed in 0.75% agarose gels as described elsewhere (Kaaden, Dietzschold & Ueberschaer, 1974). All tests were carried out with immunoglobulins prepared from sera of chickens which had been infected by natural exposure to MDV, strain HPRS-16. Immunoglobulins were precipitated from chicken sera by using sodium sulphate (Orleans, Rose & Marrack, 1961).

For the virus neutralization test (VNT), cell-free HVT was prepared from HVT-infected CEF according to the method of Calnek and the stock virus was diluted with SPGA solution (Calnek et al. 1970) containing about 100 p.f.u./0.1 ml and stored at -65 °C. Twofold serial dilutions of serum were prepared from an initial 1:5 dilution in phosphate-buffered saline (PBS). Equal parts of serum dilution were mixed with the virus dilution. For the HVT control, PBS was substituted for serum, and known positive and negative sera were included in each test. After a 60 min reaction time at 37 °C, 0.1 ml of the mixture was inoculated onto each of three 60 mm Petri dishes of secondary CEF. Five ml of maintenance medium were added after a 30 min adsorption period. Focal lesions were enumerated after 3 days of incubation at 37 °C. The titre of neutralizing antibodies was expressed as the reciprocal of the highest serum dilution causing a 50% reduction of the virus titre.

Isopycnic sedimentation of membrane-immunoglobulin complexes. Highly purified radioactively-labelled CEF membranes were mixed with increasing quantities of immunoglobulins and incubated overnight at 4 °C (Roizman & Spear, 1970). The mixture was then layered on the top of a preformed linear dextran T-40 gradient (10 to 25% w/v) and centrifuged for 16 h at 200000 g in the SW 50.1 rotor. After the sedimentation the gradients were fractionated and the TCA-insoluble radioactivity was measured. The density of the dextran fractions was determined by weighing 100 µl samples in calibrated pipettes.

Vaccination and evaluation procedures. The methods have been previously described (Kaaden et al. 1974).

Production of antisera. Anti-cellular membrane sera were prepared by immunization of rabbits with gradient-purified plasma membranes from virus-infected or uninfected CEF, respectively. Three doses of membranes (protein content approx. 200 µg/dose) emulsified with complete Freund’s adjuvant were applied during 3 weeks by intramuscular route. A booster inoculation was given 4 weeks later. Blood was obtained from the ear vein 14 days after the last injection and tested for specific activity in the neutralization test.

RESULTS

Characterization of the isolated membrane fraction

Electron microscopic examination

The electronmicrograph (Fig. 1) of the membrane fraction isolated from the dextran gradient revealed that it consisted predominantly of large sheets and fragments of membranes and microvesicles. As expected, neither enveloped herpes virus particles nor ‘naked’ nucleocapsids were found by the electron microscopic examination of the material banding
Enzymatic composition

In the crude cytoplasmic extract the specific enzyme activities expressed as units per mg protein were determined to be: 5'-nucleotidase 2.48, succinate dehydrogenase 10.5, NADPH:ferricytochrome c oxidoreductase 2.92. In the cellular membranes banding at a dextran density of 1.05 g/ml only an activity of 5'-nucleotidase (sp. act. 15.8 units per mg membrane protein) was obtained.

Moreover, the incorporation of [3H]-labelled fucose was estimated to be $48 \times 10^{-3}$ ct/min/mg protein for the cytoplasmic extract, whereas the corresponding value for the purified membrane fraction was $18.5 \times 10^{-3}$ ct/min/mg protein. Because of their morphologic characteristics, enzymic composition, and fucose content the isolated cellular membranes were classified as plasma membranes (PM).

Electrophoretic analysis

In co-electrophoresis experiments using [3H]- and [35S]-L-methionine the polypeptide pattern of the isolated PM from uninfected and MDV-infected CEF were analysed in the analytical SDS-PAGE. A typical pattern obtained after co-electrophoresis of PM from normal and MDV-infected CEF is shown in Fig. 2. Under the conditions used, at least nine polypeptides were seen in the membrane fraction from uninfected CEF. In the PM fraction from MDV-infected CEF at least two virus-induced polypeptides (VIP), designated VIP-1 and VIP-2, were detected by the SDS-PAGE. The peak fractions of VIP-1 and VIP-2 represented 1.9 and 5.0%, respectively, of the total radioactivity put on the gel.
Herpes virus infected cell membranes

Fig. 2. SDS polyacrylamide gel electrophoresis pattern of plasma membranes obtained from fractions banding at a dextran T-40 density of 1.048 g/ml. SDS-treated samples of PM from [³H]-methyl-L-methionine-labelled uninfected CEF (○●●○, protein content 28 μg, sp. act. 2890 [³H] d/min) and from [³S]-L-methionine-labelled MDV infected CEF (○○○○○, protein content 34 μg, sp. act. 2510 [³S] d/min) underwent electrophoresis on a 10% acrylamide gel column (90 × 6 mm) for 5 h at 2 mA.

The polypeptide pattern of PM from HVT-infected CEF obtained after the SDS-PAGE was identical to that of PM of MDV-infected CEF (results not shown).

Detection of antigenic H components in the isolated plasma membranes

Precipitating antigens

Isolated PM from uninfected or MDV-infected cells were disintegrated by treatment with pyridine (Blumenfeld, 1968), sodium deoxycholate (SDOC) (Ne'eman et al. 1972) or Triton X-100 and tested in the double immunodiffusion tests. SDOC or pyridine-treated PM from MDV-infected CEF formed two specific precipitation bands with MD-immunoglobulins (Fig. 3). No precipitation band was observed, if PM from infected CEF were allowed to react with immunoglobulins from normal chicken sera. MD-immunoglobulins and PM from uninfected CEF also gave negative results in the AGP tests. A third precipitation line was detected in the sonicated antigen preparation of HPRS-16-infected CEF (Fig. 3).

Virus neutralization with MDV- and HVT-PM-antisera

The virus-neutralizing antibody titres of rabbit antisera prepared against PM from HVT- or MDV-infected CEF were 1:80 or 1:30, respectively. In contrast, antiserum against PM from uninfected CEF exhibited a very weak virus neutralizing activity against extracellular HVT (titre ≤ 1:5). From these data it is clearly evident that extracellular infectious HVT was neutralized by both anti-MDV-PM and anti-HVT-PM antisera.

Sedimentation of the PM-immunoglobulin mixture

Membranes from MDV-infected cells obtained by the isopycnic sedimentation in the dextran gradient were mixed with immunoglobulins from MD-reconvalescent birds or from normal chicken sera (NCS) and recentrifuged in another dextran gradient. The
O. R. KAADEN AND B. DIETZSCHOLD

Precipitation test in 0.75% agarose gel of solubilized plasma membranes from MDV-infected CEF (MDV-PM) or BC-antigens from MDV-infected CEF with immunoglobulins from MD-infected chickens. (1) Immunoglobulins from MD-infected chickens (protein content 6.2 mg/ml) were allowed to react with an extract of MDV-infected CEF, sonicated for 30 s at 50 W in a Branson B12 sonifier; (2) SDOC-treated (16 mg/ml SDOC for 30 min at 20 °C) MDV-PM; (3) immunoglobulins from normal chicken sera (protein content 6.8 mg/ml); (4) pyridine-treated (10% v/v for 20 min at 20 °C) MDV-PM; (5) MDV-PM treated with 1% Triton X-100 (6) and PM from uninfected CEF, treated with SDOC (7).

Fig. 3. Precipitation test in 0.75% agarose gel of solubilized plasma membranes from MDV-infected CEF (MDV-PM) or BC-antigens from MDV-infected CEF with immunoglobulins from MD-infected chickens. (1) Immunoglobulins from MD-infected chickens (protein content 6.2 mg/ml) were allowed to react with an extract of MDV-infected CEF, sonicated for 30 s at 50 W in a Branson B12 sonifier; (2) SDOC-treated (16 mg/ml SDOC for 30 min at 20 °C) MDV-PM; (3) immunoglobulins from normal chicken sera (protein content 6.8 mg/ml); (4) pyridine-treated (10% v/v for 20 min at 20 °C) MDV-PM; (5) MDV-PM treated with 1% Triton X-100 (6) and PM from uninfected CEF, treated with SDOC (7).

radioactivity profiles of the gradients are shown in Fig. 4. The buoyant density of the radioactively-labelled membranes from MDV-infected cells after co-sedimentation with immunoglobulins from NCS did not change if compared with the density of the starting membrane material alone. A single peak of radioactivity was found at a density of 1.048 g/ml. After co-sedimentation of the PM-MD-immunoglobulin mixture, however, two peaks of radioactivity were detected. The buoyant density of the major fraction was estimated to be 1.076 g/ml. A minor component corresponding to a density of 1.048 g/ml gave a second peak (II) of radioactivity in the upper part of the dextran gradient. In control experiments using radioactively-labelled immunoglobulins or the immunodiffusion test with rabbit anti-chicken sera, chicken immunoglobulins were detected in the denser band (peak I) (results not shown). Peak II and the single peak of the radioactively-labelled PM incubated with NCS were free of serologically detectable amounts of chicken immunoglobulins.

The results of the reverse experiments showed, that the starting buoyant density of PM from uninfected CEF after co-sedimentation with MD-immunoglobulins remained unaltered. Therefore, it was concluded, that the increase of the buoyant density of PM from virus-infected cells is due to the binding of MD-immunoglobulins.

Protective effect of PM from HVT-infected CEF against MD

In order to study the immunizing effect of purified PM from uninfected and HVT-infected CEF, two groups of chickens were twice vaccinated at 14-day intervals. The
**Herpes virus infected cell membranes**

![Graph](image)

Fig. 4. Isopycnic sedimentation of [\(^{35}\)S]-methionine-labelled plasma membranes from MDV-infected CEF with immunoglobulins from normal chicken sera (a) or MDV-infected chickens (b). The PM-immunoglobulin mixtures were incubated overnight at 4 °C and centrifuged in a dextran T-40 gradient (Spinco rotor SW41 for 16 h at 200,000 g). The sedimentation is from right to left.

**Table 1. Immunization of Rhode Island Red chickens against Marek's disease with purified plasma membranes from HVT infected CEF**

<table>
<thead>
<tr>
<th>Vaccine prepared from</th>
<th>Protein content ((\mu)g/g)</th>
<th>Vaccination date*</th>
<th>Marek's disease dead/survivors (%)</th>
<th>Gross lesions</th>
<th>Reduction of the specific mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membranes from HVT infected CEF</td>
<td>40</td>
<td>1st day</td>
<td>1/20 (5)</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>Plasma membranes from uninfected CEF</td>
<td>50</td>
<td>15th day</td>
<td>14/16 (88)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* For the challenge infection with the pathogenic MDV, strain HPRS-16, the vaccinated chickens were exposed to natural contact with three chickens showing clinical symptoms of MD.

vaccine was prepared from a stock of PM which was free of infectious HVT or MDV as proven in tissue cultures.

The specific mortalities after the challenge infection with the pathogenic MDV during an observation period of 20 weeks are given in Table 1. After two doses of PM from HVT-infected CEF the specific MD mortality was reduced by 94%. PM from uninfected CEF
did not have a protective effect against MD in vaccinated control groups. In this group, 14 out of 16 died by MD or showed typical pathological gross lesions after the sacrifice.

**DISCUSSION**

The aim of the described experiments was to isolate cellular membranes from MDV and HVT-infected CEF and to look for virus-induced alterations of their protein composition and antigenicity. Using the method of Bingham & Burke (1972) highly purified membrane preparations were obtained. Because of their enzyme composition, morphologic characteristics, buoyant density and the ability to incorporate fucose, they were classified as plasma membranes (Ragnotti et al. 1969; Atkinson & Summers, 1971). The electrophoretic examination of the isolated PM from MDV-infected CEF in the SDS polyacrylamide gel electrophoresis compared with the PM of uninfected CEF revealed two new methionine-labelled polypeptides. These proteins designated VIP-1 and VIP-2 were found to be specific for the infections with MDV (Fig. 2). From the analysis of the radioactively-labelled PM in the PAGE it was calculated that the peak fraction of VIP-1 represents 1.9% of the total radioactivity recovered from the gel. However, the corresponding value of the VIP-2 was estimated to be 5.0%. The presence of one or two virus-induced polypeptides which may be suggested from the electrophoretic studies was clearly demonstrated by the serological experiments. Solubilized PM from MDV-infected CEF formed two specific precipitation lines with the homologous immunoglobulins (Fig. 3). The incidence of virus-induced membrane alterations in virus-infected cells was demonstrated by virus neutralization, by immunodiffusion as well as by polyacrylamide gel electrophoresis.

The presence of MDV- and HVT-induced proteins in the PM of infected cells could be further demonstrated by the virus neutralization test. At least one component in the PM from MDV-infected CEF assumed to be virus-induced stimulated the formation of cross-neutralizing antibodies against HVT as may be concluded from the results of the VNT. Rabbit antisera prepared against PM from virus-infected cells neutralized extracellular infectious turkey herpes virus. Although the homologous neutralizing serum titre was about 1.9-fold higher, HVT was also neutralized but to a lower extent by the MDV-PM-antiserum. The results of the neutralization experiments prompted us to prove the immunizing effect of the PM from HVT-infected cells against a natural MD infection in vaccinated chickens.

Surprisingly, the specific MD-mortality among chickens twice vaccinated by a PM preparation from HVT-infected CEF was reduced by 94%. Little is known about the mechanisms of immunization after the vaccination against MD with the HVT. A correlation seems to exist between the incidence of HVT neutralizing antibodies after the vaccination and the protection against MD (Calnek, 1972), although the role of the cell-mediated immune mechanisms has not been explored in detail.

The detection of MDV- and HVT-induced proteins in the PM from infected cells by the PAGE, equilibrium sedimentation of the PM-immunoglobulin mixtures, virus neutralization tests, immunodiffusion and vaccination experiments extend our knowledge about the membrane antigens in MDV-infected cells as described by Ahmed & Schidlovsky (1972) and Chen & Purchase (1970). Heine, Spear & Roizman (1972) concluded from the electrophoretic and serological examination of cellular membranes from herpes simplex type I-infected cells that the glycoproteins bound to the cellular membranes and those of the virus envelopes have a similar polypeptide composition and antigenicity. From the data presented in this study the question arises of whether the proteins detected in the PM preparations
Herpes virus infected cell membranes from MDV- and HVT-infected cells are identical in their antigenicity with structural proteins of the MD or turkey herpes virus particles, or whether they are only specific for the corresponding virus infection. Efforts are being undertaken to prepare highly purified MDV and HVT preparations and to compare the virus structural proteins with the polypeptides of the plasma membranes from virus-infected cells.

The authors are indebted to Dr Frank Weiland for performing thin sections of the plasma membranes and Dr J. Cox for his reading of the manuscript. The skilful technical assistance of Gisela Mayer, Jaroslava Maskova and D. Sautter is gratefully acknowledged.

REFERENCES

O. R. KAADEN AND B. DIETZSCHOLD


(Received 25 March 1974)