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Rapid preparation of plasma membranes from avian lymphoid cells and fibroblasts for virus binding studies

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Abstract

A simple and rapid protocol for the preparation of plasma membranes from chicken embryo fibroblasts and chicken lymphoid cells was developed. Characterization of the preparations by morphological, biochemical and serological methods indicated the specific enrichment of the plasma membranes as well as cell surface proteins. Binding of infectious bursal disease virus (IBDV) particles was demonstrated after immobilization of the plasma membranes, and cell type-specific differences were observed. Although the results of these studies reflect the interaction between IBDV and isolated cells only partially, the advantages of these plasma membrane preparations, the specific enrichment of cell surface proteins, their constant quality and the possibility to store aliquots over several months, make them a useful tool for virus binding studies with avian cells. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Plasma membrane preparation; Virus binding studies; Infectious bursal disease virus

1. Introduction

Cellular plasma membranes form the first barrier for viruses in the infection cycle. Investigations of early interactions of virus particles with their receptor sites on the cell surface may provide important insight into host cell tropism and therefore, pathogenicity, and antiviral strategies can be evaluated on the basis of these results. Permanent cell lines are used most often for the investigation of the early steps in the viral life cycle in order to applicate a defined counterpart. However, to answer specific questions, especially with regard to host cell tropism, it will sometimes be necessary to use primary cells in order to mimic the in vivo conditions. Preparation of primary cells, for example lymphocytes, is time-consuming and may often be unsatisfactory due to variable results associated with the quantity and quality of the

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preparations. Furthermore, rapid cell degeneration can produce misleading results during the course of binding studies.

The preparation of plasma membranes of primary cells can help overcome these problems. Starting with a pool of cells, plasma membranes with a constant quality can be produced and stored for several months. During the binding studies, misleading results due to contamination with intracellular compartments can also be avoided. A simple and rapid method is described now for the preparation of plasma membranes derived from chicken lymphoid cells and chicken primary fibroblasts. The purity of these preparations was confirmed by morphological, biochemical and serological investigations. Their binding activity was tested with infectious bursal disease virus (IBDV) particles and the results were compared to published binding studies with IBDV particles and primary cells (Nieper and Müller, 1996).

2. Materials and methods

2.1. Cells

Primary chicken embryo fibroblasts (CEF) were prepared according to standard procedures (Dulbecco and Vogt, 1954; Youngner, 1954); erythrocytes in the cell suspensions were removed by treatment with the ACK-buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA; pH 7.2) described by Coligan et al. (1992). Lymphoid cells from the bursa of Fabricius, spleen or thymus of 3- to 12-week-old specific pathogen free chicken were prepared by mincing these organs with scissors and centrifugation of the cell suspension in Ficoll-Paque (Pharmacia) as described elsewhere in detail (Müller and Becht, 1982). All cells were suspended in washing buffer consisting of 25.0 mM HEPES, 154 mM NaCl, 0.5 mM $MgCl_2$; pH 7.4 (Ferber et al., 1972) and were kept on ice during further manipulations whenever possible.

2.2. Plasma membrane preparation

Plasma membranes of CEF and lymphoid cells derived from bursa of Fabricius, spleen, or thymus were prepared following a protocol described by Maeda et al. (1983) with several modifications (see Fig. 1). All plastic and cellulose nitrate tubes were soaked in 1 mM EDTA pH 7.0 overnight and rinsed ten times with doubly distilled water before use. The cells, washed three times in the washing buffer described above, were resuspended in a 3-fold (w/v) excess of homogenization buffer (25.0 mM HEPES, 30 mM NaCl, 0.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 μM pepstatin A; pH 8.0) and kept on ice for 10 min. Thereafter, the cells were disrupted by 30–40 strokes in a 'tight fitting' (lymphoid cells) or a 'loose fitting' (CEF) Dounce homogenizer, respectively. Immediately after homogenization restoration buffer was added (25.0 mM HEPES, 0.6 M NaCl, 0.5 mM $MgCl_2$; pH 8.0) in order to achieve a final concentration of NaCl of 154 mM. EDTA was added to a final concentration of 1 mM. After centrifugation (5000 × g, 10 min, 4°C, SS34 rotor, Sorvall), a 25

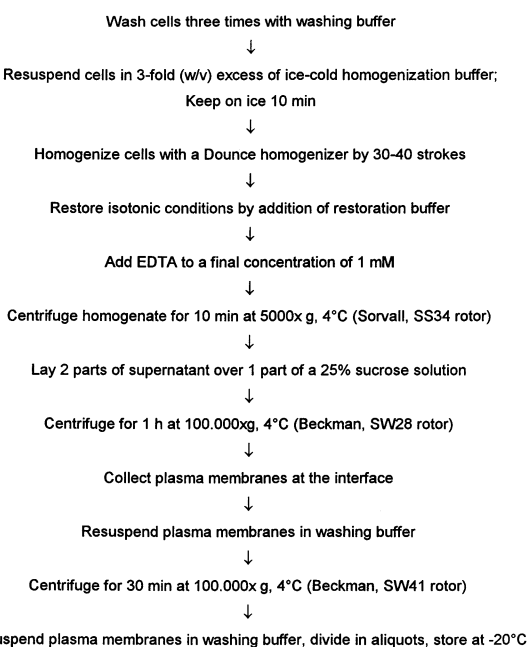

Fig. 1. Outline of protocol for preparation of plasma membranes from chicken embryo fibroblasts and lymphoid cells. See text for details.

ml volume of the resulting supernatant was layered over 12 ml of a 25% sucrose cushion (sucrose was dissolved in isotonic homogenization buffer). After centrifugation (100000 × *g*, 1 h, 4°C, SW28 rotor, Beckman L8-55), plasma membranes forming a white band at the interface were collected, diluted with washing buffer and sedimented by centrifugation (100000 × *g*, 30 min, 4°C, SW41 rotor, Beckman L8-55). They were resuspended in washing buffer and the protein concentration was determined in a microtiter plate assay using BCA protein reagent (Pierce). Aliquots of the membranes were stored frozen at −20°C up to three months.

2.3. Marker enzyme analysis

5′-nucleotidase is a marker enzyme of plasma membranes; this activity was determined using a diagnostic kit provided by Sigma. The activity of the plasma membrane-specific $(Na^+ + K^+)$-ATPase was determined according to Kinne et al. (1971). The activity of the glucose-6-phosphatase, a marker enzyme of the endoplasmic reticulum, was measured according to Harper (1962); 0.1 M imidazole buffer was used instead of a Na-citrate buffer as described by Nordlie and Lygre (1966). Alkaline and acid phosphatases were inhibited by EDTA and Glucose (Hübscher and West, 1965). Released inorganic phosphate was determined according to Chen et al. (1956).

2.4. Electron microscopy

Plasma membranes were fixed with 2.5% glutaraldehyde, washed with PBS and post fixed with 1% OsO_4. After dehydration in ethanol and propylene acid samples were embedded in epoxy resin (Luft, 1961). Ultra thin sections (0.1 μm) were stained with 2% uranyl acetate and examined with an electron microscope EM 902 (Zeiss).

2.5. SDS-PAGE, Coomassie staining, immunoblot

Proteins from cells and plasma membranes were dissociated in sample puffer (Laemmli, 1970); polypeptides (10 μg per slot) were separated in discontinuous SDS-polyacrylamide gels (15%) with or without reducing agents (Mini-Protean II, Bio-Rad). Gels were fixed (25% isopropanol, 10% acetic acid; 15 min), stained with 0.1% Coomassie brilliant blue R250 suspended in fixation solution for 15 min and destained (45% methanol, 10% acetic acid). For immunologic detection of plasma membrane specific proteins, separated polypeptides were transferred to nitrocellulose membranes (Millipore) by the semi-dry blotting procedure. After blocking (5% non fat dry milk in PBS, 0.05% Tween 20) membranes were incubated with a monoclonal antibody directed against chicken Ia antigen (generous gift from Thomas Graf, Heidelberg). Antibody binding was visualized by biotinylated antiglobulin, streptavidin peroxidase and chloronaphthol as substrate.

2.6. Virus binding studies

For binding studies in an ELISA-like procedure, plasma membranes were resuspended in coating buffer pH 9.6 (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 per 1000 ml) and coated to U-shaped wells of microtiter plates (Greiner) at 4°C overnight. After incubation with blocking buffer (1% BSA, 0.5% gelatine, 0.05% Tween 20, 0.1% thimerosal in PBS) for 3 h at 37°C, plasma membranes were incubated with purified IBDV particles for 2.5 h at 37°C. Binding of viral antigen was detected with virus-specific polyclonal antibodies from rabbits (1 h at 37°C) and the biotin-streptavidin-system. An amount of 1.2 phenylendiamine was used as substrate; extinction coefficients were measured at 492 nm. The plates were washed five times with PBS, 0.05% Tween 20 with an ELISA washer (Nunc) after each step of incubation.

3. Results

3.1. Preparation of plasma membranes

Using a single method for the preparation of plasma membranes from cells of different origin

and morphology may be critical, since buffers, mechanical forces during homogenization and the conditions during centrifugation have to be adapted to each cell type. Maeda et al. (1983) have established a simple and rapid method for the preparation of plasma membranes applicable to a broad range of cell types, including fibroblasts and lymphocytes. To increase the purity and yield of plasma membranes obtained from various types of chicken cells, this method was modified considerably. Further modifications were introduced with regard to the consecutive methods of characterization. An abbreviated protocol is shown in Fig. 1.

The first series of experiments was performed to optimize the washing, homogenization and restoration buffers described above (data not shown). Essentially, a phosphate-free buffer system was chosen to be able to characterize the membrane preparations biochemically by marker enzymes most of which release inorganic phosphate. When Tris buffers were used to stabilize the plasma membranes as recommended by Warren (1974), the enzymatic activities were very low in the preparations. Therefore, a HEPES-based buffer (Ferber et al., 1972), pH 8.0 to stabilize the cell membranes (Evans, 1987), was used. An 0.5 mM concentration of $MgCl_2$ was employed in order to stabilize the nuclear membranes during incubation in hypotonic homogenization buffer (Allan and Crumpton, 1970; Ferber et al., 1972) since $MgCl_2$ at higher concentrations led to the aggregation of lymphoid cells. Homogenization with Dounce homogenizers was given preference to the Polytron homogenizers used by Maeda et al. (1983) because their mechanical forces proved to be more gentle for cell disruption. To prevent osmolytic damage of the cell compartments and to conserve the structures and the enzymatic activities in the plasma membranes, isotonic conditions were re-established immediately after homogenization by the addition of restoration buffer. EDTA was added to complex divalent cations as these can lead to aggregations of plasma membranes with the nucleus (Ferber et al., 1972) and also may activate metalloproteases. As a further modification, an additional step of differential centrifugation was undertaken prior to density gradient centrifugation in order to remove cell debris, mitochondria and nuclei from the homogenates. Centrifugation of the membrane preparations in discontinuous sucrose gradients (40, 30, 20 and 10%) showed an increase of plasma membrane-specific enzymes and a decrease of the marker enzyme for the endoplasmic reticulum at lower sucrose concentrations; yields, however, at 10% sucrose concentration was only low. Satisfactory results with regard to purity and yield were obtained by centrifugation of the plasma membranes on a 25% sucrose cushion, in accordance to a protocol described by Perdue (1974).

3.2. Polypeptide patterns of avian cells and plasma membrane preparations

The patterns of the cellular proteins and those of the plasma membrane preparations obtained following the protocol outlined in Fig. 1 were determined by SDS-PAGE and Coomassie brilliant blue R250 staining (Fig. 2). In all of the plasma membrane preparations several polypeptides, present in the cell lysates and presumably representing histone proteins, were missing: A group of three proteins with apparent molecular weights between 14 and 16 kDa and one protein of 32 kDa. There was, however, a significant enrichment of two proteins of approximately 40 and 43 kDa and one high molecular weight protein in all plasma membrane preparations. Plasma membrane preparations of different lymphoid cell types had similar polypeptide patterns; however, the polypeptide pattern of plasma membranes prepared from CEF proved to be different, especially in the high molecular weight region.

3.3. Biochemical analysis of marker enzymes

The activities of marker enzymes for plasma membranes ($(Na^+ + K^+)$-ATPase, 5′-nucleotidase) or the endoplasmic reticulum (glucose-6-phosphatase) as the major contaminant indicate the purity of plasma membrane preparations. These enzyme activities, determined in the cell homogenates and the plasma membrane prepara-

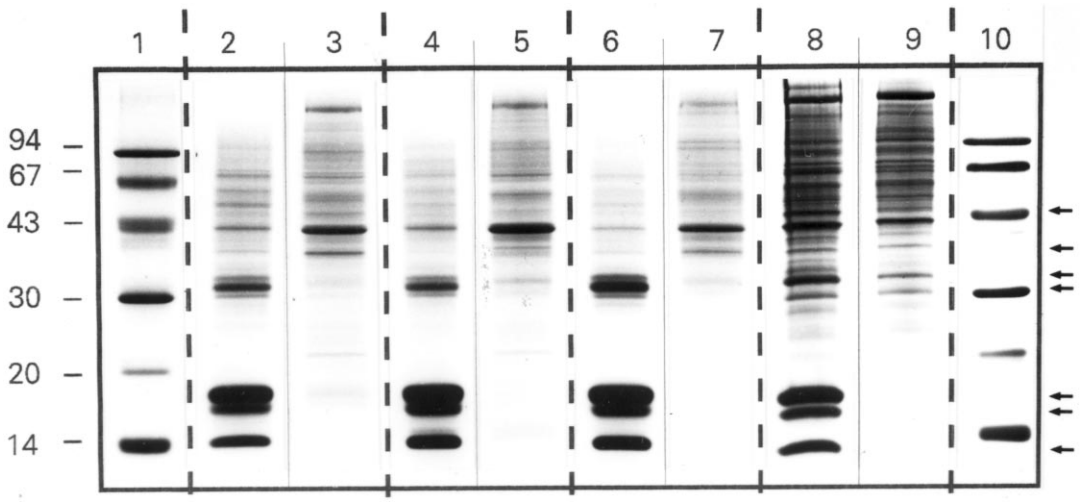

Fig. 2. Polypeptide patterns of cell homogenates and plasma membrane preparations. Proteins (10 μg per lane) were separated by SDS-PAGE and stained with Coomassie brilliant blue. Lanes 1 and 10, molecular weight marker proteins (kDa, numbers shown at the left). Cell homogenates and plasma membrane preparations from lymphoid cells isolated from bursa of Fabricius in lanes 2 and 3, spleen in lanes 4 and 5, thymus in lanes 6 and 7 and from CEF in lanes 8 and 9, respectively. Arrows indicate polypeptides mentioned in the text.

tions, are summarized in Table 1. The specific activities of marker enzymes for plasma membranes were significantly increased in the plasma membrane preparations: As compared to the cell homogenates, the activity of the $(Na^+ + K^+)$-ATPase was increased 6–12-fold and the 5′-nucleotidase activity was increased 4–10-fold. The highest increase in specific activity of both enzymes was observed in plasma membranes derived from CEF. However, an increase in the activity of glucose-6-phosphatase (2–3-fold) was also observed and all attempts to reduce this activity by modifying the isolation protocol were unsuccessful.

3.4. Morphological examination by electron microscopy

The morphological examination of the plasma membrane preparations by electron microscopy revealed vesicles of various size and shape. No nuclei or mitochondria could be detected, but at the inner side of some of the vesicles, small cytoplasmic hems could be observed (Fig. 3). These results are in accordance with those of the biochemical characterization.

3.5. Analysis of the plasma membrane preparations by serological methods

Chicken Ia antigen, a cell surface molecule present on chicken lymphoid cells, corresponds to the human MHC II antigen (Guillemot et al., 1984); CEF do not express this antigen. To benefit from the high resolution of serological methods, this protein was investigated by Western blotting in order to demonstrate the enrichment of plasma membrane-specific proteins by the established protocol. In addition, this test could indicate alterations of the plasma membrane proteins by this isolation procedure. Proteins of cell homogenates and plasma membrane preparations were separated by SDS-PAGE under non-reducing conditions, transferred onto nitrocellulose membranes and reacted with a monoclonal antibody directed against the chicken Ia antigen (Fig. 4). The antibody bound to the non-reduced molecule with an apparent molecular weight of 55 kDa. A constant amount of protein had been loaded on each slot; therefore, the strong increase of chicken Ia antigen in the plasma membranes of lymphoid cells indicates the efficiency of the applied protocol.

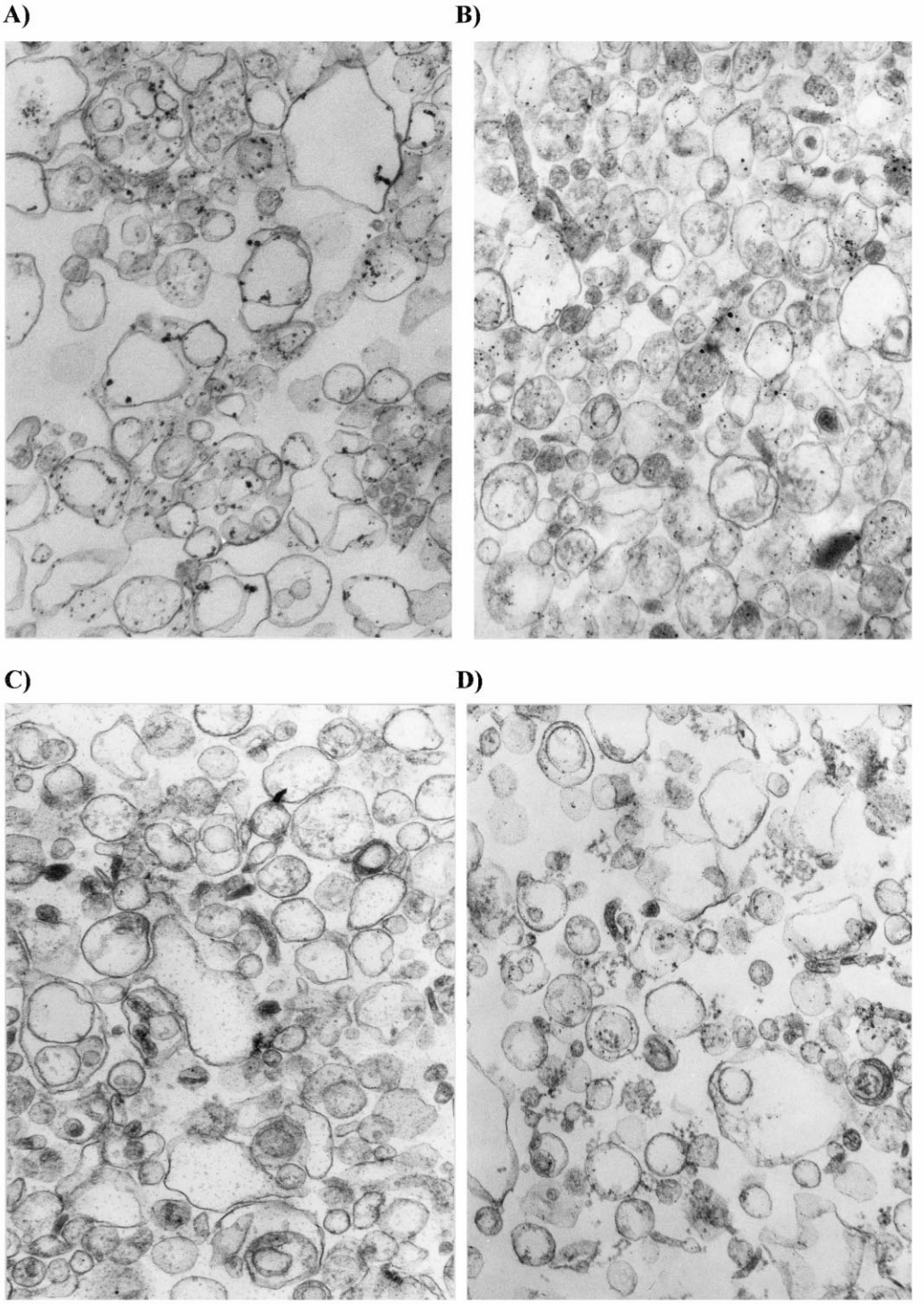

Fig. 3. Electron microscopic analysis of plasma membrane preparations from lymphoid cells of bursa of Fabricius (A), spleen (B), thymus (C) and chicken embryo fibroblasts (D).

Table 1
Marker enzyme analysis

	$(Na^+ + K^+)$-ATPase		5′-nucleotidase		Glucose-6-phophatase	
	Specific activity[a]	Enrichment[b]	Specific activity[a]	Enrichment[b]	Specific activity[a]	Enrichment[b]
Bursa						
ch	0.25 ± 0.1	9.2	1.0 ± 0.1	4.0	1.6 ± 0.5	2.1
pm	2.30 ± 0.8		4.0 ± 1.0		3.3 ± 1.0	
Spleen						
ch	0.35 ± 0.2	6.3	1.2 ± 0.4	7.9	7.7 ± 0.1	1.0
pm	2.20 ± 0.7		9.5 ± 0.5		7.6 ± 0.1	
Thymus						
ch	0.38 ± 0.2	10.0	0.7 ± 0.3	5.8	14.1 ± 1	1.8
pm	3.79 ± 1.3		4.1 ± 2.0		3.3 ± 0.3	
CEF						
ch	0.22 ± 0.1	12.0	0.5 ± 0.2	10.2	1.0 ± 0.5	2.9
pm	2.51 ± 1.0		5.1 ± 1.0		2.9 ± 0.4	

Specific activity of marker enzymes of the plasma membrane ($(Na^+ + K^+)$-ATPase, 5′-nucleotidase) and endoplasmic reticulum (glucose-6-phosphatase) in the cell homogenates (ch) and plasma membrane preparations (pm), preparations of lymphoid cells from bursa of Fabricus, spleen, thymus and chicken embryo fibroblasts (CEF). Results presented as mean ± S.E. of three different preparations.

[a] Specific activity = μmol/mg × h.

[b] Enrichment = Specific activity in pm/Specific activity in ch.

3.6. Binding of IBDV to immobilized plasma membranes

In order to test binding of IBDV particles to the plasma membranes, an ELISA-like solid-phase virus binding assay was developed. Increasing amounts of protein (up to 10 μg/well) were coated onto plastic wells and incubated with a constant amount of IBDV particles. Viral antigens bound to the membranes were detected with an IBDV-specific polyclonal antiserum and the biotin-streptavidin system. As shown in Fig. 5, the plasma membrane preparations obtained from all types of chicken cells bound IBDV particles. Virus binding to the plasma membranes was related directly to the amount of the membrane proteins added, indicating that these were not severely affected by the isolation procedure. The same efficiency of binding was observed with both IBDV serotypes. Plasma membranes derived from CEF bound IBDV particles more efficiently than those derived from lymphoid cells. Binding of the serum proteins was less than 5% of total binding when virus was omitted. Similar results were obtained when a pre-immune serum was used. No binding of IBDV specific antibodies could be detected, when the membranes were omitted. The assay was equally sensitive and specific when culture supernatants from IBDV infected CEF were used in place of gradient-purified virus particles.

4. Discussion

Several methods for the preparation of plasma membranes from different types of tissues or isolated cells have been described. Most of these protocols were established for specific cell types or applications and cannot be used with other cell or tissue types without considerable loss of purity or yield. For chicken cells, only protocols for the preparation of plasma membranes from CEF have been published (Perdue and Sneider, 1970; Perdue, 1974). These, however, caused denaturation of marker enzymes and the selective loss of some membrane components (Perdue, 1974). Therefore, a protocol was established for chicken cells, based on a method applicable to a wide

range of different cell types and tissues, including human fibroblasts and lymphocytes (Maeda et al., 1983). Modifications of the buffer system, concentration of Mg^{2+} and sucrose, as well as the procedures of homogenization and centrifugation, increased purity and yield of the plasma membranes prepared from chicken embryo fibroblasts and lymphoid cells considerably.

The plasma membranes were examined by biochemical and serological methods and visualized by electron microscopy. The biochemical examination indicated the enrichment of plasma membrane markers as opposed to endoplasmic reticulum markers. Contamination with membranes from other organelles, notably from Golgi, have been neglected, considering that these might not be essential in virus binding studies. Using this simple and rapid method a level of purity was achieved which, as indicated by $(Na^+ + K^+)$-ATPase activity, was considerably higher than described for chicken cells before (Perdue and Sneider, 1970). Glucose-6-phosphatase was also enriched, however to a significantly lower extent, indicating traces of endoplasmic reticulum. Allan and Crumpton (1970), and Johnstone and Crumpton (1980) achieved a 9–11-fold enrichment of 5′-nucleotidase activity in plasma membrane preparations from pig lymphocytes, whereas only a 4–8-fold enrichment was achieved with chicken lymphoid cells in the experiments described above. Glucose-6-phosphatase activity, however, was enriched to the same extent. Our efforts to remove endoplasmic reticulum, e.g. by rising pH values during homogenization and centrifugation or by additional washes of the plasma membranes in hypotonic buffers, remained without success.

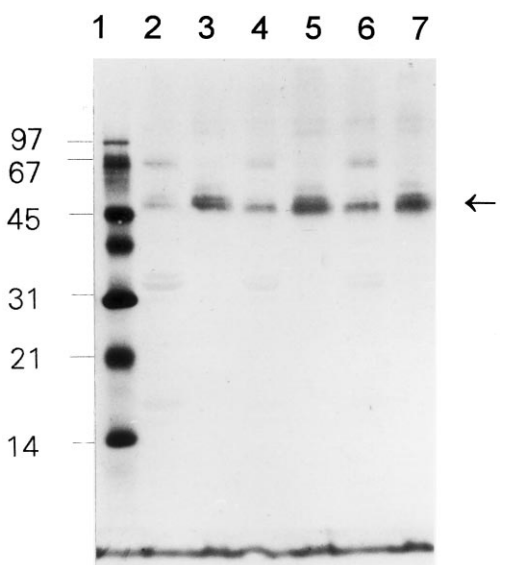

Fig. 4. Demonstration of chicken Ia antigen in cell homogenates and plasma membrane preparations from lymphoid cells. Lane 1, molecular weight marker proteins (kDa, numbers shown at the left). Cell homogenates and plasma membrane preparations from lymphoid cells isolated from bursa of Fabricius in lanes 2 and 3, spleen in lanes 4 and 5, thymus in lanes 6 and 7.

Immobilized isolated plasma membranes have been used in binding studies, among others, with mouse hepatitis virus (Boyle et al., 1987), human coronavirus 229E (Yeager et al., 1992) and hepadnavirus (Pontisso et al., 1989a,b; Qiao et al., 1992). The enrichment of the chicken Ia antigen in the plasma membrane preparations from lymphoid cells indicates that cell surface proteins were not severely altered by the preparation procedure. Binding of IBDV particles of both serotypes to the plasma membranes could be demonstrated in an ELISA-like procedure. By a modified blotting technique (VOPBA), described elsewhere in detail (Nieper and Müller, 1996), the specificity of binding of IBDV particles to these plasma membrane preparations was demonstrated in competition experiments.

Differences in binding of virus particles of different serotypes to cells or plasma membrane preparations have been described for reovirus and Theiler virus: In vitro binding studies with reovirus serotype 1 and 3 demonstrated a common and a serotype 3-specific receptor on L cells (Ambler and Mackay, 1991) and endothelial cells (Verdin et al., 1989), whereas in VOPBA studies with plasma membranes derived from L cells (Choi et al., 1990) or endothelial cells (Verdin et al., 1989) no differences in virus binding could be observed. Similar results were obtained with two serotypes of Theiler virus and BHK cells and plasma membranes (Fotiadis et al., 1991; Kilpatrick and Lipton, 1991). In the ELISA-like procedure, IBDV particles of both serotypes

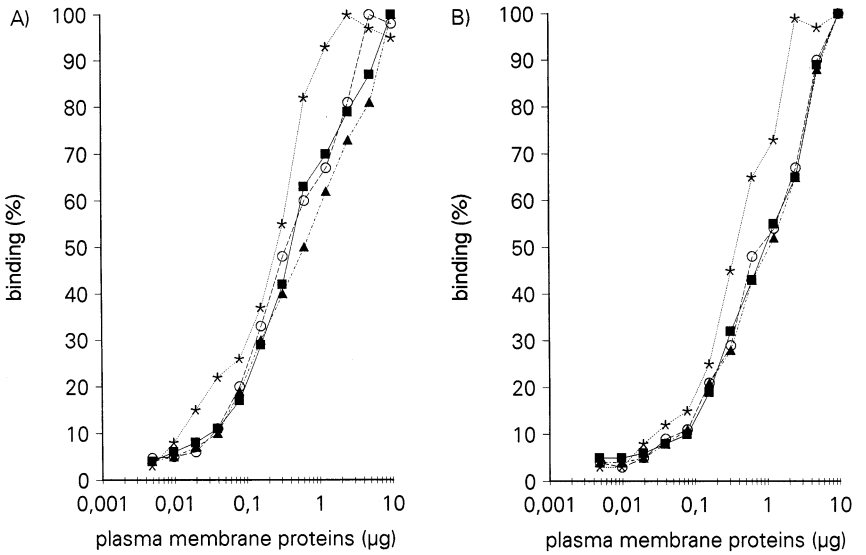

Fig. 5. Binding of a constant amount of purified IBDV particles (A, serotype 1 strain Cu-1; B, serotype 2 strain 23/82) to various amounts of immobilized plasma membrane proteins (0.04–10 μg). The percental adsorption is shown to compare the binding of both strains to the plasma membranes. OD 2.0 was taken as 100%. Specific binding was determined by substracting unspecific binding of serum and virus particles to the plate from total binding.

bound to plasma membranes derived from CEF more efficiently than to those derived from lymphoid cells. These differences were more prominent in in vitro binding studies performed with IBDV particles and avian cells (Nieper and Müller, 1996). Furthermore, cell and serotype specific differences observed in the in vitro binding studies with isolated lymphoid cells could not be shown with the plasma membrane preparations. In the in vitro studies virus particles of serotype 2 bound to a higher extent than serotype 1 particles. In addition it had been observed that the efficiency of binding of serotype 1 particles to isolated lymphoid cells was in the order bursa of Fabricius, spleen and thymus. These differences can be due to several reasons. (1) The in vitro studies were based on cell numbers. CEF are larger than lymphoid cells; lymphoid cells isolated from bursa of Fabricius and spleen are larger than those isolated from thymus. Cells with a larger surface might express more virus binding sites and higher amounts of protein were present in the test. It might be speculated, therefore, that a larger number of virus particles can be bound. In contrast, the same amount of protein had been used for each cell type in the ELISA study. (2) Immobilization of plasma membranes can lead to alterations of their binding activity (Yic and Lingwood, 1992). (3) The preparation procedure, e.g. the disruption of the native membranes, might result in alterations of virus binding structures. Similar differences have been described with human rhinovirus (Staunton et al., 1989) and murine Moloney leukemia virus (Johnson and Rosner, 1986).

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