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Susceptibility of chicken lymphoid cells to infectious bursal disease virus does not correlate with the presence of specific binding sites

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Pathogenic serotype 1 strains of infectious bursal disease virus (IBDV) replicate efficiently in lymphoid cells of the bursa of Fabricius of chicken. Lymphoid cells in other organs are not susceptible. Apathogenic serotype 2 strains do not replicate in lymphoid bursa cells or in other lymphoid cells. Chicken embryo fibroblasts (CEF), however, efficiently replicate strains of either serotype. Binding studies showed that strains of both IBDV serotypes bind to lymphoid cells isolated from the bursa, thymus or spleen, indicating that restriction of IBDV replication to lymphoid B cells is not determined by the presence of specific receptor sites. The specificity of binding was demonstrated by saturation and competition experiments. These revealed the presence of different receptors: CEF had receptors common to both serotypes and specific ones for each serotype. Receptor sites common to both serotypes were also present on lymphoid cells; however, additional serotype-specific sites were only demonstrated for the apathogenic serotype 2 strain. Strains of both serotypes specificially bound to proteins with molecular masses of 40 kDa and 46 kDa, exposed on the surface of CEF and lymphoid cells. Competition experiments indicated that these proteins might represent the common receptor sites of IBDV.

Introduction

Infectious bursal disease virus (IBDV), a member of the family *Birnaviridae*, is the aetiological agent of Gumboro disease and causes severe economic losses to the poultry industry. The target organ for pathogenic serotype 1 strains is the bursa of Fabricius (BF), as demonstrated by experiments in which bursectomized chicken survived IBDV infections lethal for normal chicken (Käufer & Weiss, 1980). Between 3 and 6 weeks after hatching, when the BF reaches maximum development, chicken are highly susceptible to the virus. Infections result in high mortality during the acute course of the disease or in B cell deficiency after recovery from infection (for review see Becht, 1980; Kibenge *et al.*, 1988). Chicken infected immediately after hatching develop a chronic infection with atrophia of the BF and B cell depletion (Winterfield *et al.*, 1972; Hudson *et al.*, 1975). Chicken infected with IBDV when older than 12 weeks do not show clinical signs (Becht, 1980).

High concentrations of virulent IBDV serotype 1 strain Cu-1 (Nick *et al.*, 1976) antigens and high infectivity titres have been demonstrated in the BF of infected chicken, whereas only traces of antigen and low virus titres were detected in the thymus and spleen (Cursiefen, 1980; Käufer & Weiss, 1980). Analogous results were obtained with lymphoid cells isolated from these organs and from peripheral blood (Müller, 1986; Burkhardt & Müller, 1987). *In vitro* infection studies showed that IBDV replicates in a population of proliferating B cells (Müller, 1986) but not in very immature lymphoblasts (Beug *et al.*, 1981) or competent B cells (Becht, 1980). A second serotype has been isolated predominantly from turkeys (McNulty *et al.*, 1979). Serotype 2 strains, e.g. strain 23/82 (Chettle *et al.*, 1985), do not replicate in lymphoid cells and are apathogenic for both turkeys and chicken (Jackwood *et al.*, 1982, 1984, 1985; Ismail *et al.*, 1988). Strains Cu-1 (Nick *et al.*, 1976) and 23/82 (Becht *et al.*, 1988) can be propagated in chicken embryo fibroblasts (CEF). The reasons for these differences in cell tropism are still unknown. The results of experiments presented here show that early stages of virus–cell interaction are not responsible for the differences observed in susceptibility of lymphoid cells and therefore pathogenicity.

Methods

Virus and cell culture. Serotype 1 strain Cu-1 of IBDV (Nick *et al.*, 1976) was adapted to CEF and remained pathogenic for chicken (Lange *et al.*, 1987). Strain 23/82, isolated from healthy turkeys, is

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serotype 2 and was a generous gift from N. E. Reed (New Haw, Weybridge, UK). Viruses were propagated in CEF as described (Cursiefen *et al.*, 1979). Virus particles were radiolabelled and purified by caesium chloride density centrifugations as described previously (Müller & Becht, 1982). [^{35}S]Methionine-labelling was at approximately 5000 c.p.m. per 1–10 $\times$ 10^6 virus particles.

Cells and plasma membrane preparations. Erythrocytes were removed from freshly prepared CEF by 10 min incubation in lysis buffer (0·15 M-NH_4Cl, 1·0 mM-$KHCO_3$, 0·1 mM-EDTA pH 7·2) and subsequent centrifugation at 300 *g* for 5 min (Coligan *et al.*, 1992). Lymphoid cells from BF, spleen and thymus of 5- to 6-week-old SPF chicken were isolated by Ficoll-Paque (Pharmacia) purification as described (Müller & Becht, 1982). In binding studies, cells were incubated in Iscove's modified Dulbecco's medium (IMDM) supplemented with 25 mM-HEPES and 0·5% BSA (binding medium). Plasma membranes of CEF and lymphoid cells were prepared according to Maeda *et al.* (1983) with modifications and characterized by morphological, biochemical and immunological methods (H. Nieper & H. Müller, unpublished results).

Cell surface labelling with biotin. Isolated CEF and lymphoid cells were washed three times with PBS or Hanks' buffered salt solution (HBSS) and labelled with water-soluble biotin with an extended spacer arm (Immunopure NHS-LC-Biotin; Pierce) according to Meier *et al.* (1992). Briefly, 1 $\times$ 10^7 cells per ml were incubated with 0·5 mg/ml NHS-LC-Biotin (CEF, 20 min; lymphoid cells, 5 min) at 4 °C. Unbound biotin was removed by washing the cells three times with PBS or HBSS supplemented with 10 mM-glycine. CEF were used immediately in further studies. Lymphoid cells were subsequently lysed with 0·5% Triton X-100 in HBSS (Coligan *et al.*, 1992) and unsoluble material was removed by centrifugation (15000 *g*, 15 min). Supernatants were used in further experiments.

Binding of virus to isolated cells. In order to screen the binding of representatives of the two serotypes to avian cells, 1 $\times$ 10^8 cells were incubated in 500 µl binding medium containing highly purified complete IBDV particles (Müller & Becht, 1982) at a particle to plaque ratio of about 1:10, as determined previously (Müller *et al.*, 1986). After incubation for 4 h at 4 °C to avoid virus uptake, cells were washed three times with IMDM. Washing solutions were collected and the infectivity was titrated in a plaque assay (Nick *et al.*, 1976).

Binding studies with radiolabelled virus particles. CEF and lymphoid cells isolated from bursa, spleen or thymus were incubated with [^{35}S]methionine-labelled virus particles (approximately 5000 c.p.m.). After incubation at 4 °C with intermittent shaking, cells were washed three times with IMDM and lysed with PBS containing 1% Triton X-100. Radioactivity in the lysates and the collected washing solutions was determined by liquid scintillation counting. Samples were analysed in duplicates and each experiment was repeated at least three times. Binding in the presence of high excess of unlabelled virus particles revealed about 40% non-specific binding in the case of CEF and about 50 to 70% in the case of lymphoid cells.

Virus overlay protein-binding assay (VOPBA). Proteins in cell homogenates or plasma membrane preparations (20 µg protein per slot, BCA* protein assay; Pierce) were separated by SDS–PAGE (MiniProtean II; Bio-Rad) under reducing conditions and transblotted to nitrocellulose paper (Millipore). Sheets were incubated overnight in 5% non-fat dried milk in PBS-T (PBS containing 0·05% Tween 20), cut into strips and incubated with purified virus particles for 2 h at room temperature. The strips were washed extensively with PBS-T and binding of virus particles was detected with virus-specific MAbs, kindly provided by H. Becht (Giessen, Germany), the biotin–streptavidin system (Amersham) and chloronaphthol as substrate (Sigma). Strips incubated with radiolabelled virus particles were exposed to X-ray film (Fuji). Molecular mass determinations were made using biotinylated reference proteins (Pharmacia).

Results

Representatives of both IBDV serotypes bind to avian cells

In a first series of experiments, binding of IBDV to avian cells was determined by incubation of 1 $\times$ 10^8 cells in medium containing 500 p.f.u. of highly purified complete IBDV particles (particle:p.f.u. ratio approximately 10). After 4 h at 4 °C the cells were removed by centrifugation and the infectivity remaining in the supernatant was determined by plaque assay. The highest reduction of input infectivity was observed after the incubation of CEF with the representatives of both serotypes: Cu-1 (serotype 1), 87%; 23/82 (serotype 2), 85%. A similar reduction of input infectivity was observed when lymphoid cells were incubated with strain 23/82 (bursa, 70%; spleen, 80%; thymus, 65%), indicating that the apathogenic serotype 2 strain efficiently bound to these cells. Surprisingly, the infectivity of medium containing the pathogenic strain Cu-1 was only slightly reduced by lymphoid cells (bursa, 15%; spleen, 12%; thymus, 6%) in numerous repeated experiments. This observation was substantiated by increasing the cell numbers, e.g. tripling of the number of thymus cells resulted in values similar to those obtained with bursa or spleen cells. When less than 1 $\times$ 10^7 lymphoid cells were incubated with strain Cu-1, no reduction was observed. To test for cell-associated infectivity, the cells were inoculated onto CEF monolayers in an infectious centre assay. As expected, these experiments (data not shown) confirmed the results described before: CEF showed the highest virus titres, lymphoid cells incubated with 23/82 had slightly lower titres, but only low titres were observed with lymphoid cells incubated with Cu-1. In order to determine age-dependent effects on binding, lymphoid cells isolated from chickens at various times after hatching (3 to 15 weeks) were tested; analogous results were obtained in each case. In further experiments, various numbers of cells were incubated with suspensions of purified virus particles, grown in the presence of [^{35}S]methionine. The results of these experiments (Fig. 1) confirmed those obtained in the previous study and showed that significantly higher amounts of serotype 2 virus particles bound to CEF than to lymphoid cells.

IBDV binding kinetics

The differential binding of the two serotypes to lymphoid cells was unexpected. To investigate these differences, constant amounts of radiolabelled virus particles were

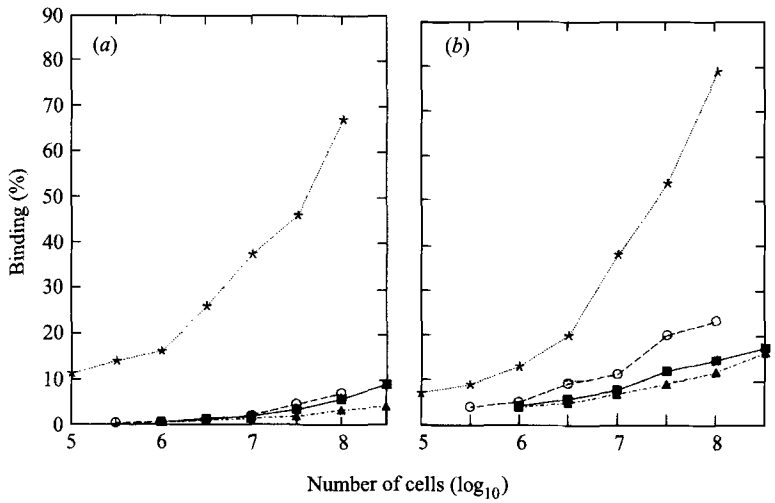

Fig. 1. Binding of [^{35}S]methionine-labelled IBDV particles (5000 c.p.m.) to increasing numbers of avian cells after incubation at 4 °C for 4 h. (*a*) IBDV Cu-1, serotype 1; (*b*) 23/82, serotype 2. Cell types: CEF (*), bursa (■), spleen (○), thymus (▲).

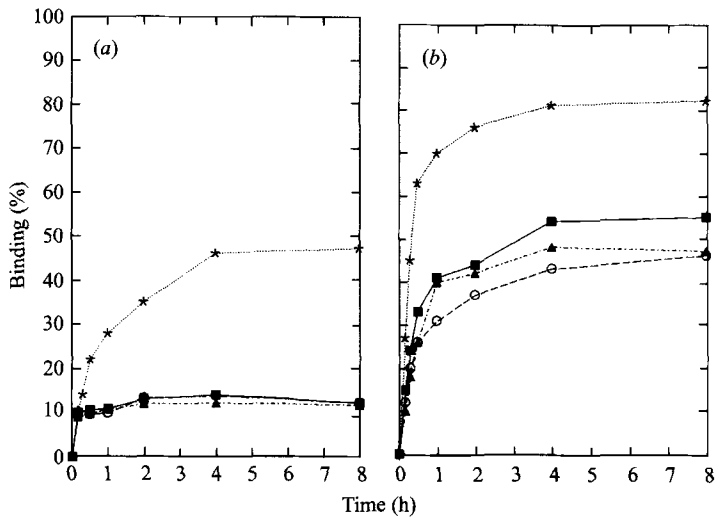

Fig. 2. Binding of [^{35}S]methionine-labelled IBDV particles to CEF (5×10^7) and lymphoid cells (5×10^7; $1{\cdot}5 \times 10^8$ in the case of thymus cells and Cu-1). At the times indicated, cells incubated at 4 °C were washed twice and solubilized. Radioactivity in the cell lysates and the combined supernatants and washing solutions was determined by liquid scintillation counting. Percentage of cell-bound radioactivity is shown. (*a*) Cu-1 serotype 1, (*b*) 23/82 serotype 2. Cell types: CEF (*), bursa (■), spleen (○), thymus (▲).

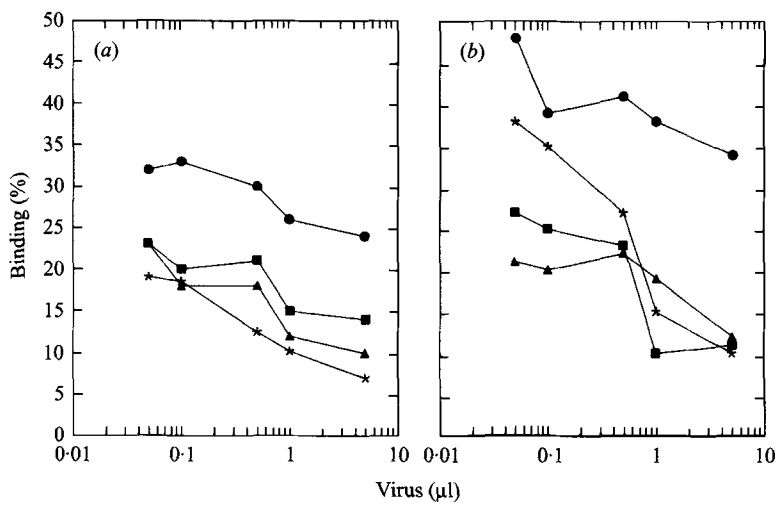

Fig. 3. Saturation of IBDV binding to avian cells (see legend to Fig. 2). Cells were incubated in the presence of various amounts of [^{35}S]methionine-labelled IBDV particles for 4 h at 4 °C and processed further as described in the legend to Fig. 2. (*a*) Serotype 1, (*b*) serotype 2. Different preparations of IBDV were used with CEF and lymphoid cells. Cell types: CEF (*), bursa (■), spleen (●), thymus (▲).

incubated for various times with the different cell types. The time course of binding depicted two different reaction patterns (Fig. 2). With CEF, both strains reached half maximum binding within 30 min. The same values were obtained when the serotype 2 strain 23/82 was incubated with the lymphoid cells. In all these cases

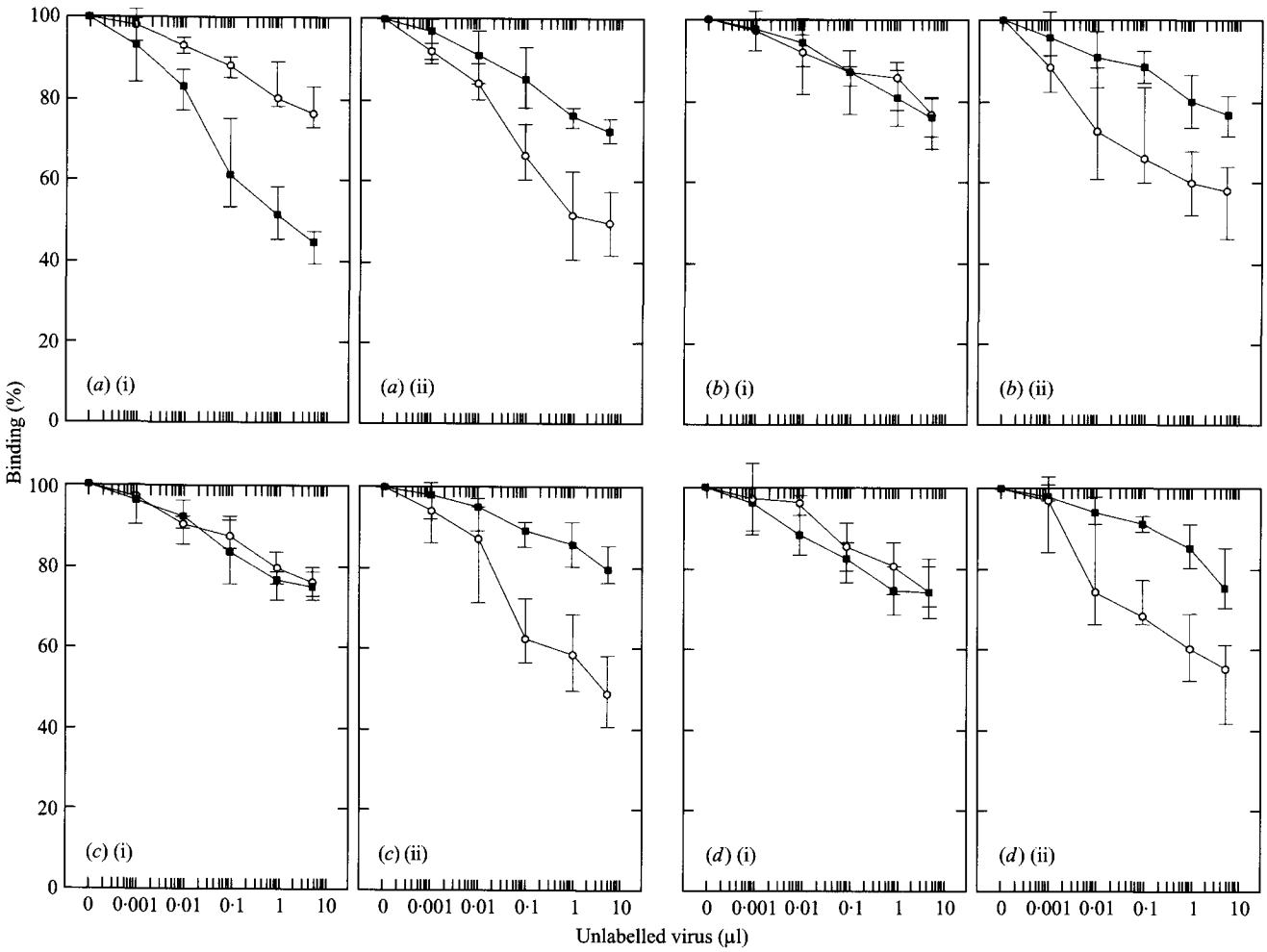

Fig. 4. Competition of binding of IBDV to avian cells. (*a*) CEF; (*b*) bursa; (*c*) spleen; (*d*) thymus. Cells were incubated with a constant amount of [^{35}S]methionine-labelled Cu-1 (i) or 23/82 (ii) virus particles (about 5000 c.p.m.) in the presence of increasing concentrations of unlabelled IBDV particles for 4 h at 4 °C and processed further as described in legend to Fig. 2. Binding of [^{35}S]methionine-labelled virus particles in the absence of unlabelled virus was taken as 100%. (■) Competition by the presence of unlabelled Cu-1; (○) competition by the presence of unlabelled 23/82.

a plateau was reached after incubation for about 4 h. In contrast, binding of the serotype 1 strain Cu-1 to lymphoid cells was significantly more rapid. About 80% of maximum binding was observed after an incubation period as short as 10 min; in this case a plateau was reached after about 2 h. When the plateau was reached, the numbers of virus particles which bound to the various cell types were in the same ratios to each other as those determined in the previous section. This indicates that even after a prolonged incubation the number of Cu-1 particles bound to lymphoid cells did not increase.

Binding of both serotypes of IBDV is specific

The differences observed in binding could be due to unspecific binding or to additional receptor sites. Therefore, saturation and competition experiments were performed to demonstrate specificity of binding. Saturation experiments were performed according to Verdin *et al.* (1989). Assuming that binding of virus particles to the cell surface is mediated by specific receptor sites, large amounts of virus particles should lead to saturation of these sites. When all the binding sites are occupied, a further increase in virus particle number will lead to a relative decrease of the binding rate. The results of such experiments are shown in Fig. 3: binding of IBDV to avian cells proved to be specific, since both strains showed a decrease of the percentage binding when the particle numbers were increased, regardless of the cell type. The highest proportion of specific binding sites was present on CEF, as indicated by a strong decline of the percentage binding rates. In the case of lymphoid cells, however, the decline was less obvious, particularly in the case of the serotype 1 strain Cu-1. This may be indicative of a low number of specific binding sites.

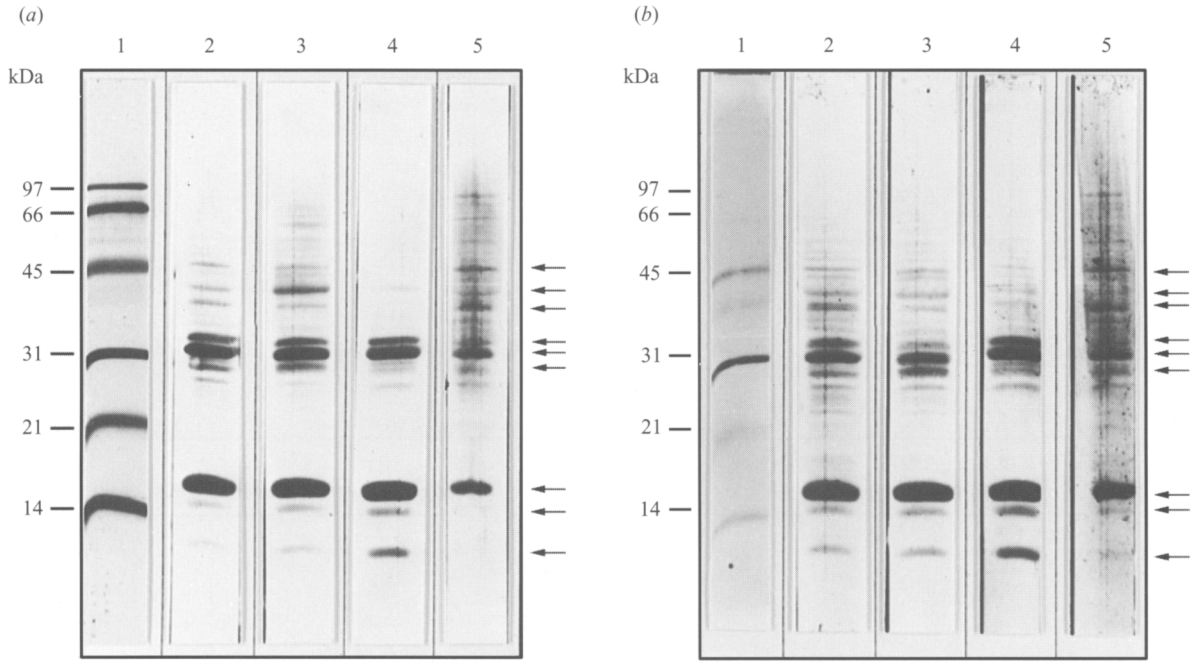

Fig. 5. VOPBA studies with IBDV and avian cells. Proteins in total cell lysates (20 μg per lane) separated by SDS–PAGE and transferred to nitrocellulose membrane were incubated with purified IBDV particles. Binding of virus was detected with IBDV-specific MAbs and the biotin–streptavidin system. Lanes 1, molecular mass marker proteins. Lymphoid cells isolated from bursa (lanes 2), spleen (lanes 3) and thymus (lanes 4); CEF in lanes 5. Arrows indicate prominent virus-binding proteins. (*a*) serotype 1; (*b*) serotype 2.

Competition experiments with radiolabelled virus particles and unlabelled virus particles of the same serotype demonstrated the specificity of the binding sites. Incubation of cells with radiolabelled virus particles in the presence of unlabelled virus particles of the heterologous serotype should indicate whether strains of both serotypes use the same receptor sites. In these experiments, two reaction patterns were again observed (Fig. 4). In the case of CEF, binding of both strains was competed more efficiently by the homologous virus strain (up to 55%) than by the heterologous strain (32%; Fig. 4*a*); similar results were obtained after incubation of serotype 2 strain 23/82 with the lymphoid cells (Fig. 4*b–d*, ii). In contrast, when lymphoid cells were incubated with radiolabelled serotype 1 virus particles, the maximum inhibition was only about 30%, irrespective of the serotype used for competition (Fig. 4*b–d*, ii). This reaction pattern is similar to that observed when lymphoid cells were incubated with radiolabelled serotype 2 virus particles in the presence of the heterologous virus strain (Fig. 4*a*) and to the competition of the binding of either serotype to CEF by the heterologous virus strain (Fig. 4*b–d*, ii). Binding in the presence of a 500-fold excess of unlabelled virus particles (equivalent to 5 μl in Fig. 4) of the homologous strain revealed about 40% non-specific binding in the case of CEF and about 50 to 70% in the case of lymphoid cells.

Demonstration of virus-binding proteins

In VOPBA experiments, a modified Western blotting technique (Boyle *et al.*, 1987), attempts were made to determine the size and numbers of virus-binding proteins. In assays using cell homogenates (Fig. 5) prepared from CEF or the various lymphoid cells, virus particles of both serotypes bound to proteins with molecular masses of 16 kDa, 14 kDa and 12 kDa, and a second group of proteins of about 32 kDa; virus binding was also observed in a third group of proteins of 40 kDa and 46 kDa, but to a considerably lower extent. When plasma membrane preparations prepared from either cell type were used, virus particles did not bind to proteins corresponding to the first group and binding to proteins in the second group was significantly reduced. Binding to the proteins in the third group remained virtually unaltered (Fig. 6*a–d*, lanes 3–8). In this case, a fourth group of virus-binding proteins with molecular masses of about 25 kDa became more prominent, particularly in plasma membrane preparations of lymphoid cells. In both series of experiments, no differences were observed in this pattern of virus-binding proteins, regardless of the cell type or the virus strain. In cell surface labelling experiments with NHS-LC-Biotin, the protein bands in the 40 to 46 kDa group were clearly visible (Fig. 6*a–d*, lanes 1 and 2), whereas the protein bands in the first and

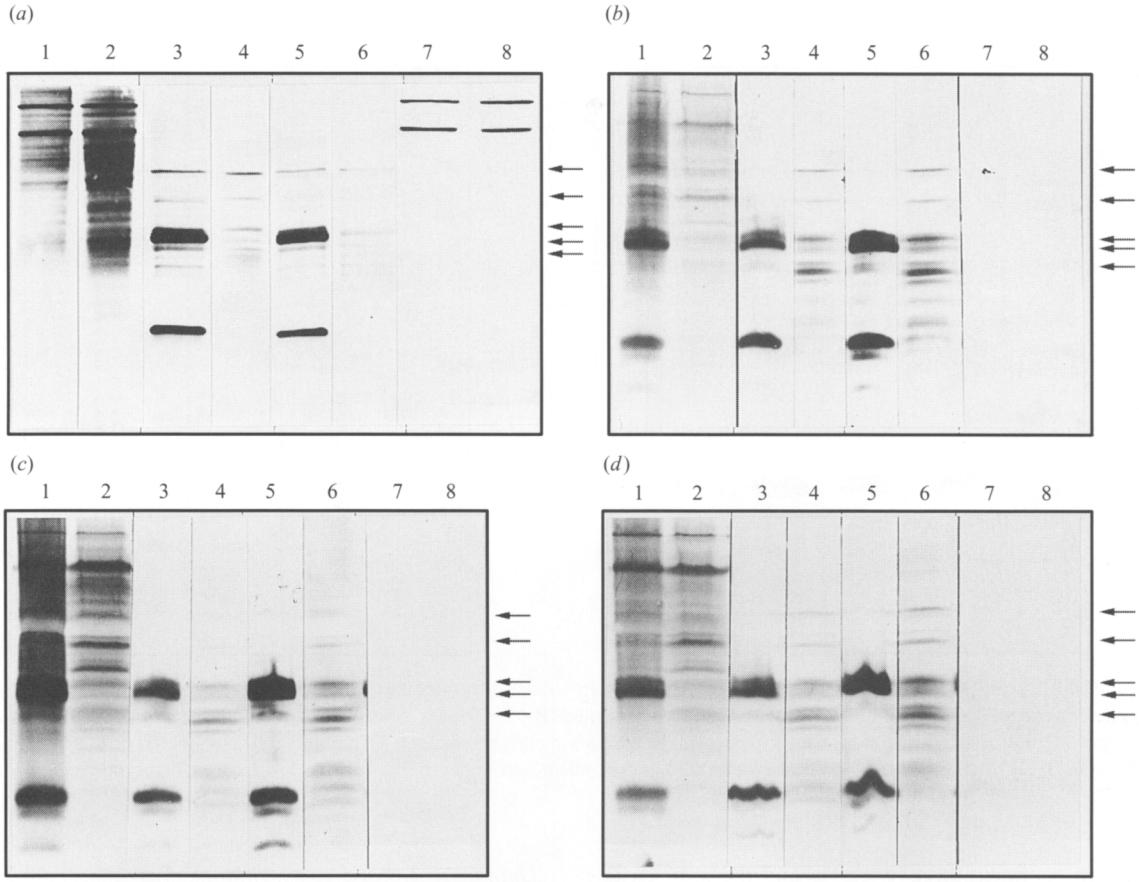

Fig. 6. Binding of IBDV to cell surface proteins of avian cells. Proteins (20 μg per lane) were separated by SDS–PAGE and transferred to nitrocellulose membranes. Lanes 1 and 2, cellular proteins after surface labelling with biotin; total cell lysate (lanes 1) and plasma membrane-enriched fraction (lanes 2). Lanes 3 to 8, VOPBA studies. Total cell lysates were separated in lanes 3, 5 and 7; plasma membranes in lanes 4, 6 and 8. Binding of virus (Cu-1, lanes 3 and 4; 23/82, lanes 5 and 6) was detected with MAbs as described in Fig. 5. Lanes 7 and 8, no virus added. Arrows indicate virus binding proteins. (*a*) CEF; lymphoid cells isolated from (*b*) bursa, (*c*) spleen and (*d*) thymus.

second group were not. This shows that only the former proteins are present on the cell surface.

Competition experiments proved the specificity of virus binding (Fig. 7). Binding of radiolabelled virus particles to the group of proteins with molecular masses of 32 kDa and the 25 kDa group detected in plasma membrane preparations was not inhibited by an excess of unlabelled homologous or heterologous virus particles. In contrast, binding to the 46 kDa proteins was efficiently inhibited by homologous, as well as by heterologous unlabelled virus particles. In some of these experiments, it proved to be difficult to demonstrate the binding of labelled virus particles to the 40 kDa protein.

Discussion

In the binding studies described here, detection of receptors is at the lower limits of its sensitivity, and the use of freshly isolated cells may result in considerable variation of the starting material. The results of these studies show that replication of serotype 1 strains in lymphoid bursa cells is not determined by cell type-specific receptor sites since sites for both serotypes were demonstrated on all types of lymphoid cells, as well as CEF. However, cell- and serotype-specific differences were observed which indicate two characteristic binding patterns: (i) in experiments with CEF, large amounts of virus particles of both serotypes bound within 4 h; the half maximum binding was reached within 30 min. The specificity of the binding sites was demonstrated in saturation and competition experiments using virus particles of the homologous serotype. Competition was less efficient when virus particles of the heterologous serotype were used. Using lymphoid cells similar results were obtained in binding studies with the serotype 2 strain, although total binding was lower and saturation was therefore less obvious. (ii) In contrast, the serotype 1 strain hardly bound to lymphoid cells. Half maximum binding was reached in less than 10 min; maximum binding was observed after 2 h. In competition experi-

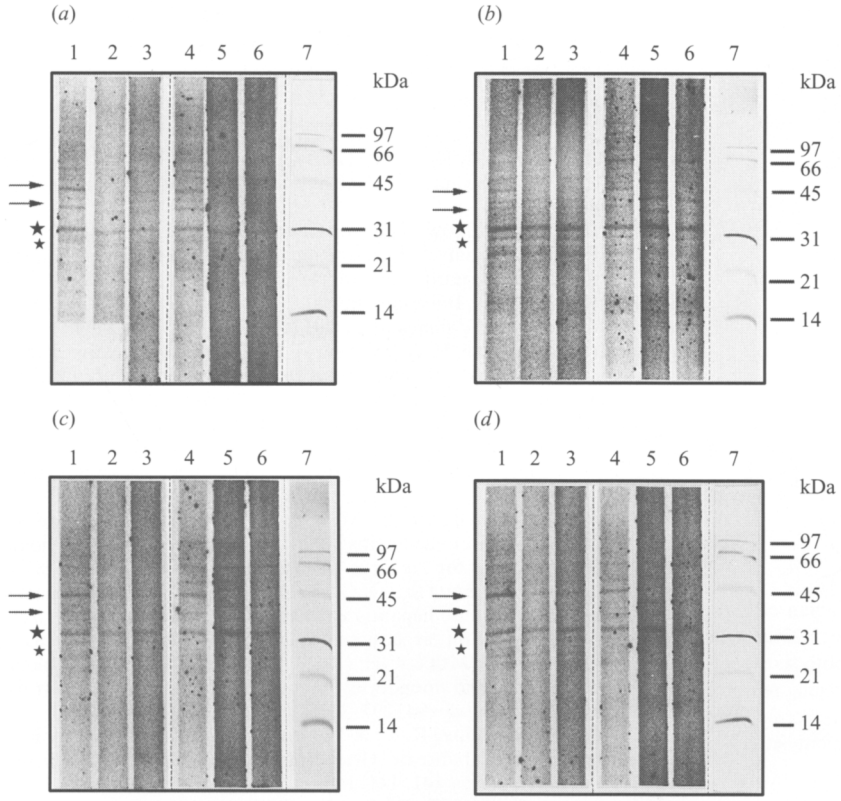

Fig. 7. VOPBA studies with [^{35}S]methionine-labelled IBDV particles (lanes 1–3, Cu-1; lanes 4–6, 23/82) either alone (lanes 1 and 4) or in the presence of unlabelled particles of the homologous (lanes 2 and 5) or heterologous (lanes 3 and 6) serotype. Lane 7, molecular mass marker proteins. Specific binding is indicated by arrows, unspecific binding is indicated by asterisks. (*a*) CEF; lymphoid cells isolated from (*b*) bursa, (*c*) spleen and (*d*) thymus.

ments, virus particles of either serotype inhibited binding of serotype 1 to the same extent (Fig. 4*b–d*, i). Competition was low and similar to that observed when either serotype and CEF were incubated in the presence of the heterologous serotype (Fig. 4*a*), or when binding of the serotype 2 strain to lymphoid cells was tested in the presence of the heterologous serotype (Fig. 4*b–d*, ii).

Taking into account these binding characteristics, a model based on the presence of receptor sites common to both IBDV serotypes and serotype-specific receptor sites can be proposed (Fig. 8). CEF have common receptor sites as well as receptor sites specific for each of the two serotypes (Fig. 8*a*). Lymphoid cells also have common receptor sites and receptor sites specific for serotype 2 strains (Fig. 8*b*); a serotype 1-specific receptor site is missing (Fig. 8*c*). When both types of receptor sites present on the cell surface are used for virus binding, large amounts of virus particles bind, and it takes a long time to reach half maximum binding (Fig. 2*b*). In this case, competition is considerably more efficient by the homologous than by the heterologous strain. In contrast, when only the common receptor site is used for binding, as in the case of the serotype 1 strain and lymphoid cells, only small amounts of virus particles bind, and half maximum binding is reached within a short time (Fig. 2*a*); both the homologous and the heterologous serotypes are able to compete for binding with the same efficiency (Fig. 4). Serotype-specific differences in binding have also been described for the two Theiler's murine encephalomyelitis virus types (TMEV; Fotiadis *et al.*, 1991) as well as for reovirus 1 and 3 (Ambler & McKay, 1991; Verdin *et al.*, 1989; El-Ghorr *et al.*, 1992).

VOPBA studies have been used to identify the cellular receptors for murine hepatitis virus (Boyle *et al.*, 1987), reovirus (Verdin *et al.*, 1989; Choi *et al.*, 1990), murine polyomavirus (Marriott *et al.*, 1987), the minor receptor for human rhinovirus (Mischak *et al.*, 1988), rotavirus (Bass *et al.*, 1991), hepatitis B virus (Mehdi *et al.*, 1994), lymphocytic choriomeningitis virus (Borrow & Oldstone, 1992), human T cell lymphotropic virus type 1 (Gavalchin *et al.*, 1993), visna virus (Dalziel *et al.*, 1991; Crane *et al.*, 1991) and TMEV (Kilpatrick & Lipton, 1991). It is a prerequisite that the virus-binding structure is a single polypeptide chain which is able to retain binding activity in the presence of detergents and without the involvement of other membrane components (Haywood, 1994). The IBDV-binding sites demonstrated on the surface of CEF and lymphoid cells meet these requirements. In VOPBA studies with total cell lysates several polypeptides were demonstrated as binding sites. The specificity of two virus-binding proteins with molecular masses of about 46 kDa and 40 kDa present in plasma membrane preparations could be proven by competition experiments. It may be speculated that these proteins

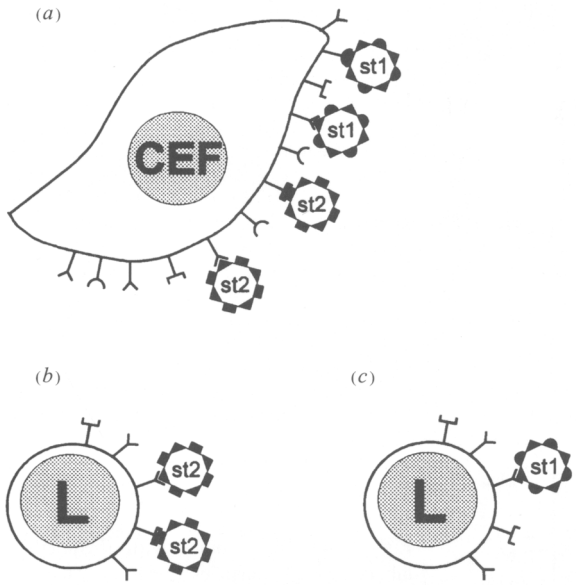

Fig. 8. Schematic representation of IBDV binding to avian cells. L, avian lymphoid cell; st1, serotype 1-specific virus particle; st2, serotype 2-specific virus particle; (Y) common receptor site for both serotypes; (Y) serotype 1-specific receptor site; (Y) serotype 2-specific receptor site; (▲) common virus attachment site; (◗) serotype 1-specific virus attachment site; (■) serotype 2-specific virus attachment site. For details, see text.

represent the postulated common receptor site, because binding of both IBDV serotypes to these proteins could be demonstrated for all of the cell types under investigation. In each case, obviously similar amounts of virus particles were bound. This is in contrast to the results of binding studies with isolated cells. However, this discrepancy may be explained by the fact that identical amounts of protein were investigated instead of similar cell numbers. Until now, it remains unclear if there exists any relationship between the two proteins regarded as specific for binding of IBDV, for example, a dimeric structure or degradation of high molecular mass protein(s). Identical patterns were observed when samples were loaded on the gels without using 2-mercaptoethanol and without prior boiling (data not shown). Binding of IBDV to proteins with molecular masses of 32 kDa and 25 kDa has to be regarded as unspecific since even a high excess of virus particles of the homologous or the heterologous serotype failed to inhibit binding. Serotype-specific binding sites could not be demonstrated by this technique, probably because these structures were destroyed under the denaturating conditions used in VOPBA. Further studies will have to establish the nature of the 46 kDa and 40 kDa cell surface proteins and to confirm their function in the early steps of IBDV infection.

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