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Susceptibility to Duck Hepatitis B Virus Infection Is Associated with the Presence of Cell Surface Receptor Sites That Efficiently Bind Viral Particles

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To test the hypothesis that susceptibility of hepatocytes to duck hepatitis B virus (DHBV) infection requires cell surface receptors that bind the virus in a specific manner, we developed an assay for the binding of DHBV particles to monolayers of intact cells, using radiolabeled immunoglobulin G specific for DHBV envelope protein. Both noninfectious DHBV surface antigen particles and infectious virions bound to a susceptible fraction (approximately 60%) of Pekin duck hepatocytes. In contrast, binding did not occur to cells that were not susceptible to DHBV infection, including Pekin duck fibroblasts and chicken hepatocytes, and binding to Muscovy duck hepatocytes, which are only weakly susceptible (approximately 1% of cells) to DHBV infection, was virtually undetectable. Within a monolayer, individual Pekin duck hepatocytes appeared to differ markedly in the capacity to bind DHBV, which may explain difficulties that have been encountered in infecting 100% of cells in culture. We have also found that the loss of susceptibility to infection with DHBV that occurs when Pekin duck hepatocytes are maintained for more than a few days in culture correlates with a decline in the number of cells that bind virus particles efficiently. All of these results support the interpretation that the binding event detected by our assay is associated with the interaction between DHBV and specific cell surface receptors that are required for initiation of infection. Our assay may facilitate isolation and identification of hepatocyte receptors for this virus.

Hepadnaviruses replicate almost exclusively in the liver, where they produce both acute and persistent infections. This marked hepatotropism is believed to be a consequence both of tissue-specific transcription of virus genes and of the presence of molecules on the surface of hepatocytes that bind virus in order to facilitate virus uptake. Several reports have provided support, albeit indirect, for the existence of hepadnavirus receptors. For example, cell lines that are competent for human hepatitis B virus (HBV) DNA replication and assembly have been derived from human liver, but none are susceptible to infection by the virus, suggesting that the block to infection in these cells is at some early step (16, 17). Previous reports have identified binding sites for HBV on isolated human liver plasma membranes (11) and on a wide variety of cell types (9, 10), but in no case has susceptibility to infection with HBV been unequivocally demonstrated. Consequently, the biological relevance of such binding events remains unclear. More recently, Kuroki et al. (6) have used the ability of duck hepatitis B virus (DHBV) to bind to radiolabeled hepatocyte proteins in order to purify and clone a candidate cell surface receptor. However, this 180-kDa protein is found on both susceptible duck hepatocytes and resistant duck embryo fibroblasts, as well as a variety of other duck tissues, and the identity of this protein as a DHBV receptor remains to be demonstrated. In summary, cell surface receptors have not been identified for any of the hepadnaviruses, and their relative importance in determining tissue tropism and host range is therefore largely inferred.

It had been previously found that DHBV infects Pekin ducks and geese but not the closely related Muscovy duck or chickens (7). This finding, plus the fact that Pekin duck hepatocyte cultures are highly susceptible to DHBV infection (14, 18), provided a convenient model with which to examine the infectious pathway of hepadnaviruses and to further test the idea that a specific cell surface receptor actually exists, an important prelude to attempts to identify such a molecule. We now report evidence in support of the hypothesis that the degree of virus susceptibility in hepatocytes of various bird species (12) correlates with the efficiency with which these cells bind virus particles. Our previous observation that DHBV surface antigen (DHBsAg) particles inhibited infection in culture (14) is also consistent with this model. Thus, DHBV infection appears to be dependent on initial binding to a hepatocyte surface receptor that is species specific.

MATERIALS AND METHODS

Generation of MAbs to DHBV envelope proteins. BALB/c mice were immunized with a mixture of DHBV and DHBsAg particles that were purified from serum of congenitally infected Pekin ducks by pelleting followed by density gradient centrifugation on cesium chloride (13). In these preparations, DHBsAg is the predominant species (8). Splenocytes were fused to SP2 myeloma cells by standard procedures. Hybridoma supernatants were screened, by using an enzyme-linked immunosorbent assay (ELISA), for the presence of antibodies that bound to virus particles immobilized on nitrocellulose. Positive hybridomas were expanded and rescreened, and immunoglobulins from stable hybridomas were tested by Western blotting (immunoblotting) to determine whether specificity was directed toward the pre-S or S domains of the DHBV envelope proteins. Of the approximately 100 hybridomas detected that were producing anti-DHBV immunoglobulin G (IgG), only one was specific for the 17-kDa major S protein. One of the anti-pre-S antibodies, 1H.1 (IgG2a subtype), and the anti-S monoclonal antibody (MAb), 7C.12 (IgG2b subtype), were selected for expansion because they performed well in Western blots, in ELISAs, and for staining of fixed cells by immunofluorescence microscopy.

Purification and iodination of immunoglobulin. Ascites were generated in BALB/c mice following injection of the 7C.12 and 1.H1 hybridomas. IgG was purified from mouse ascites by affinity chromatography on Bio-Rad Affi-Gel–protein A, using the procedure recommended by the manufacturer. Isotypes were determined with an Isotype Ab-Stat kit (SangStat Medical, Menlo Park, Calif.). IgG was radiolabeled with ^{125}I NaI (NEN) by using Iodobeads (Pierce) to a specific activity of 6 to 10 μCi/μg of protein. Radiolabeled IgG was separated

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from unbound ^{125}I on a Sephadex G-50 column equilibrated with phosphate-buffered saline (PBS) plus 0.1% (wt/vol) bovine serum albumin and 0.05% (vol/vol) Tween 20. Fractions containing IgG were pooled, and fetal bovine serum was added to 5% (vol/vol) to stabilize the antibody preparation. Radiolabeled IgG was aliquoted and stored at −70°C.

Primary hepatocyte preparation and in vitro infection with DHBV. Primary hepatocytes were prepared from 1- to 2-week-old Pekin ducklings by collagenase perfusion and maintained in Liebowitz-15 (L15) medium (Gibco-BRL) as described previously (14) but lacking dimethyl sulfoxide, sodium bicarbonate, and glucose supplements. Fetal bovine serum was omitted from the medium unless noted otherwise.

Analysis of DHBV nucleic acids. Total intracellular DNA and nucleic acids enriched for DHBV covalently closed circular (CCC) DNA were prepared from cultured cells as described previously (12, 18). Nucleic acids were resolved on a 1.5% (wt/vol) agarose gel, then transferred to a nylon membrane (Amersham Hybond-N), and immobilized by UV cross-linking in a Stratagene Stratalinker. DHBV DNA was detected by using a full-length minus-strand-specific, ^{32}P-labeled DHBV RNA that was prepared by in vitro transcription of plasmid pSP65.DHBV5.1 with SP6 RNA polymerase as previously described (18). To prepare mRNA, cultured cells were lysed in 0.2 M Tris-HCl (pH 7.5)–0.2 M NaCl–25 mM EDTA–2% (wt/vol) sodium dodecyl sulfate (SDS)–0.2 mg of proteinase K (Sigma) per ml, and high-molecular-weight DNA was sheared by repeated passage of the lysate through an 18-gauge needle. After incubation at 42°C for 1 h, the NaCl concentration of the lysate was adjusted to 0.5 M, oligo(dT)-cellulose (Stratagene) was added (approximately 5 mg/60-mm-diameter dish of hepatocytes), and the mixture was incubated for a further hour at room temperature with agitation. The oligo(dT) was washed three times with 5 ml of 10 mM Tris-HCl (pH 7.5)–0.5 M NaCl–1 mM EDTA and then transferred to a spin column (Millipore Ultrafree-MC). mRNA was eluted by washing the oligo(dT)-cellulose twice with 0.2 ml of 10 mM Tris-HCl (pH 7.5)–1 mM EDTA at 45°C. mRNA was recovered by ethanol precipitation, resolved on a 1.5% (wt/vol) agarose formaldehyde gel, and transferred to nylon membrane (Amersham Hybond-N). DHBV mRNA was detected with a full-length, cloned DHBV DNA radiolabeled with ^{32}P by nick translation.

Immunofluorescence to detect DHBcAg in infected hepatocytes. Hepatocytes were fixed with methanol-glacial acetic acid (95:5) at −20°C and stained with a rabbit antiserum to DHBV core particle proteins. Core protein was detected with a goat anti-rabbit–fluorescein isothiocyanate conjugate (Cappel) (18). Cells were photographed with a Nikon Diaphot fluorescence microscope.

Virus for infections and binding assays. The source of DHBV for infection and binding studies was serum from Pekin ducklings from a flock congenitally infected with DHBV maintained by the Fox Chase Cancer Center laboratory animal facility. DHBV-containing serum pools were aliquoted and stored at −70°C.

Assay to detect DHBV bound to hepatocytes. Radioimmunoassay and autoradiography techniques were combined to provide both quantitative and qualitative information on binding of virus to different cell types.

The assay used a microscopic autoradiography technique to detect cells that bound virus and was an adaptation of a method used to detect cells which express growth factor receptors (2). Hepatocytes were cultured for 1 to 4 days after plating, and then the medium was replaced with DHBV-containing duck serum diluted with Optimem medium (Gibco-BRL), pH 7 (0.8 to 1.0 ml/60-mm-diameter dish). Cells were incubated for 1 h with gentle rocking. All incubations and washes were at room temperature. The monolayer was washed twice with Dulbecco's PBS for 5 min each time, incubated with radiolabeled anti-DHBV IgG (see below) diluted in blocker (5% [vol/vol] normal Pekin duck serum in Dulbecco's PBS) for 1 h with gentle rocking, and then washed as described above. Alternatively, infected cells were incubated with unlabeled anti-DHBV IgG followed by ^{125}I goat anti-mouse IgG (NEN) diluted in blocker. Cells were fixed with freshly prepared glutaraldehyde (2.5% [vol/vol] in PBS) for 30 min at room temperature, then washed with water, and air dried. To determine relative amounts of bound radioactivity, the sides were removed from the plastic dishes and the monolayers were exposed to a Fuji phosphoimaging screen for approximately 30 min. The screen was then read in a Fujix model BAS1000 bioimager. For autoradiography, monolayers were coated with Kodak NTB2 nuclear emulsion, air dried, and incubated in a lightproof box containing Drierite for 2 to 8 days at 4°C. The emulsion was developed with Kodak D19 developer (40 g/500 ml) at 20°C for 8 min, and dishes were then immersed in Kodak fixer for approximately 2 min and washed in water for at least 5 min. Cells were photographed with a Nikon Optiphot microscope under dark-field illumination.

RESULTS

Derivation of MAbs for the detection of DHBV particles bound to intact hepatocytes. DHBV expresses two envelope proteins from a single open reading frame (ORF); the larger, termed L, spans the entire ORF, and the smaller, termed S, initiates at an internal methionine and extends to the end of the ORF. Thus, the amino-terminal region of L is not found in S. This amino-terminal region is usually referred to as pre-S.

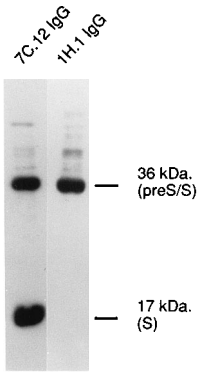


FIG. 1. Characterization of anti-DHBV MAbs. DHBV was partially purified from duck serum as described in Materials and Methods. DHBV proteins were resolved on an SDS–12.5% polyacrylamide gel and transferred to nitrocellulose (13). After fixation, filters containing identical DHBV samples were incubated with 7C.12 and 1.H1 anti-DHBV monoclonal IgGs, and bound IgG was detected by autoradiography after incubation with ^{125}I-labeled goat anti-mouse IgG (NEN).

Pre-S is believed to contain those determinants which specify virus host range, and pre-S determinants may react with the cell surface during initiation of viral infection (5). Both S and pre-S are probably exposed on the surface of virions, as some MAbs reactive to pre-S or S domains have been found to neutralize infectivity (reference 1; see below). For the purposes of this study, we sought MAbs that could react with virus particles bound to the surface of hepatocytes without causing the particles to dissociate. As described below, two such antibodies, reactive to S and pre-S, respectively, were derived and used for this purpose.

A screen of MAbs derived from splenocytes isolated from a mouse immunized with a mixture of DHBV and DHBsAg particles yielded approximately 100 stable hybridomas secreting anti-DHBV IgG. Characterization of MAbs by Western blotting revealed that one of these antibodies was specific for the 17-kDa S surface polypeptide of DHBV, whereas all of the remainder were directed against the pre-S region of the 36-kDa L envelope polypeptide (Fig. 1). The high yield of MAbs directed against the pre-S domain is most likely a consequence of the immunodominance of the hydrophilic pre-S region of the larger of the two DHBV envelope polypeptides (1).

The studies described below used the anti-S IgG (7C.12) and one of the anti-pre-S IgGs (1H.1), which are of the IgG2b and IgG2a subclasses, respectively. Both MAbs inactivated DHBV infectivity when incubated with virus prior to infection of primary duck hepatocytes (data not shown). Interestingly, the anti-pre-S IgG (1H.1 IgG) exhibited only a moderate neutralizing effect compared with the anti-S IgG (7C.12) when DHBV was adsorbed onto hepatocytes before incubation with antibody (Fig. 2). One interpretation of this result is that the pre-S region of the large DHBV envelope protein is involved in the initial binding event during DHBV infection (5) and might therefore be inaccessible to the 1H.1 IgG.

Detection of DHBsAg associated with a subset of primary duck hepatocytes by using radiolabeled anti-DHBV immunoglobulin. As a first approach to detecting DHBV particles bound to duck hepatocytes, we tried immunofluorescence staining with fluorescein isothiocyanate-labeled anti-DHBV IgG. This method was associated with high levels of background signal and was not used further. Instead, we examined the use of radiolabeled anti-DHBV IgG and autoradiography to detect virus binding. This method was associated with very

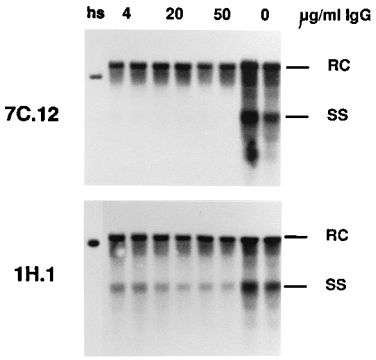


FIG. 2. Neutralizing activities of anti-DHBV MAbs 7C.12 and 1H.1 on virus preadsorbed to primary Pekin duck hepatocytes. Equivalent amounts of DHBV in L15 medium were adsorbed to duck hepatocytes on duplicate 60-mm-diameter dishes at 4°C for 1 h. Monolayers were washed twice with PBS and then incubated for a further hour with 7C.12 or 1H.1 IgG at the concentrations shown. Cells were maintained in L15 medium for 6 days, after which total nucleic acids were prepared and DHBV was DNA analyzed by Southern blot hybridization (see Materials and Methods). RC and SS represent the positions of relaxed circular and single-stranded DHBV DNA, respectively; hs is a hybridization standard of 10 pg of linearized full-length, cloned DHBV DNA.

low background signal without loss of antibody specificity and was used for all subsequent studies. Figure 3 shows an application of the method to detect DHBsAg in infected cells. Frozen sections prepared from the pancreas of a Pekin duckling congenitally infected with DHBV were incubated with ^{125}I-labeled anti-S (7C.12) IgG, washed, and processed for autoradiography (Fig. 3). The method detected envelope protein in a colony of endocrine cells and in a discrete subset of exocrine cells, in agreement with previous reports (3, 4).

To ascertain if binding sites for DHBV were present on cells that are highly susceptible to infection with DHBV but not on cells that cannot be infected with DHBV, we initially compared levels of binding of virus particles to primary Pekin duck hepatocytes and primary Pekin duck embryo fibroblasts. The resistance of duck embryo fibroblasts to infection with DHBV was demonstrated by the absence of detectable CCC DHBV DNA in the nuclei of these cells 24 h after infection with a high-titer DHBV inoculum and by the absence of DHBV DNA replicative intermediates in infected cultures at later time points (data not shown). To assay for the binding of DHBV particles (virions and DHBsAg), cell cultures were incubated with various amounts of duck serum containing these virus particles, and the relative amount of bound viral particles was determined

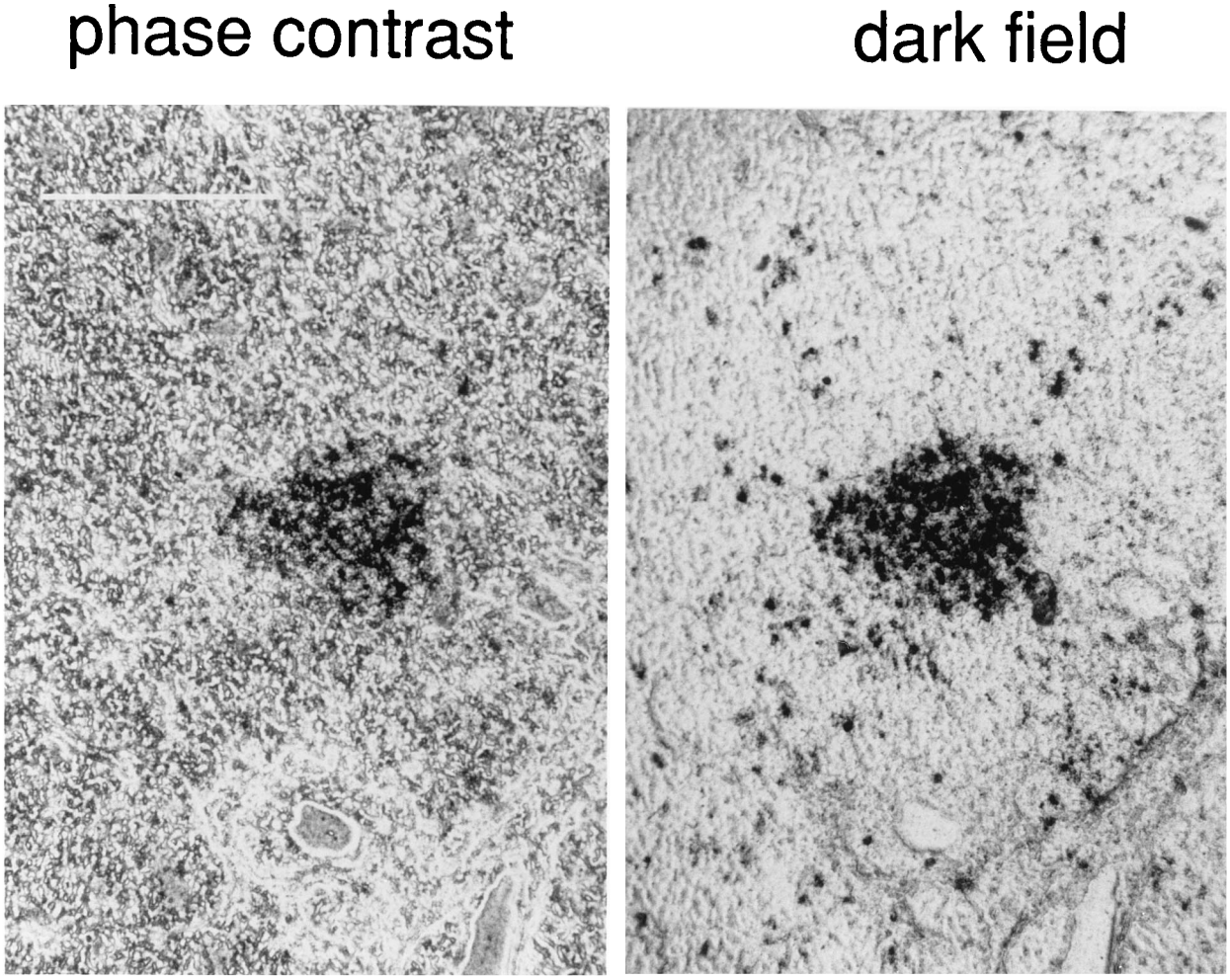


FIG. 3. Detection of DHBV envelope proteins in DHBV-infected pancreas, using radiolabeled anti-S IgG. Frozen sections of pancreas from a Pekin duckling congenitally infected with DHBV were incubated with ^{125}I-labeled 7C.12 IgG, then fixed with glutaraldehyde, and processed for autoradiography (see Materials and Methods). The same field was photographed under both phase-contrast and dark-field illumination. The bar represents approximately 1 mm.

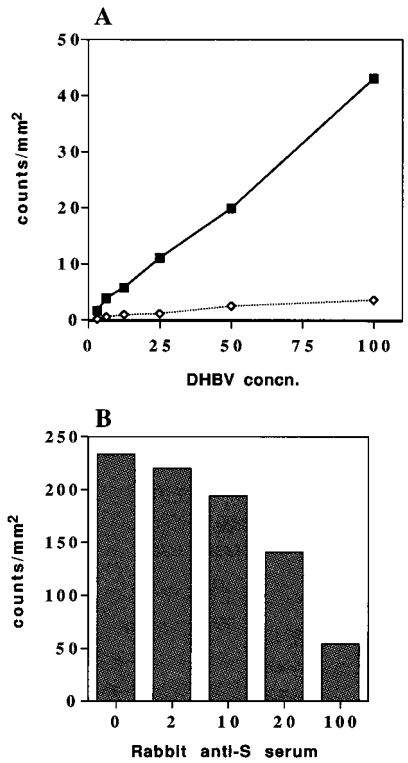


FIG. 4. DHBV binds to permissive Pekin duck hepatocytes but not to duck embryo fibroblasts. (A) Sixty-millimeter-diameter dishes of Pekin duck hepatocytes (squares) or Pekin duck embryo fibroblasts (diamonds) were incubated with serial dilutions of DHBV-positive duck serum (1:20 to 1:640) in Optimem (pH 7), and radiolabeled 7C.12 IgG was used to detect bound virus. Counts were determined with a Fuji Bioimager and corrected for the background observed when cells were incubated with 1:20 normal duck serum under identical conditions. A 1:20 dilution of DHBV stock is expressed as 100, a 1:40 dilution is expressed as 50, etc. (B) Pekin duck hepatocytes were incubated with equivalent amounts of DHBV-positive duck serum. Bound virus was detected with ^{125}I-labeled 7C.12 IgG in the presence of increasing amounts (microliters) of rabbit antiserum generated to purified DHBV S envelope protein. Relative counts were determined with a Fuji Bioimager.

after incubating cells with radiolabeled anti-DHBV IgG and counting as described in Materials and Methods. The amount of virus particles bound to the Pekin duck hepatocytes was directly proportional to the amount of DHBV-positive duck serum applied to the cells (Fig. 4A). The receptor sites did not appear to be saturated even after incubation with the highest-titer virus inoculum, containing ca. 10^9 virions and 10^{11} or more DHBsAg particles per ml (i.e., a few thousand particles per cell). When cells were incubated with a DHBV inoculum of 100-fold-lower titer, a significant level of specific binding could still be detected. Moreover, no significant binding of label was detected when hepatocytes were incubated with equivalent amounts of serum from an uninfected duck (data not shown). The specificity of anti-S MAb 7C.12 for bound DHBV was further demonstrated by competition with a rabbit antiserum raised to the purified S envelope protein of the virus (13). Addition of increasing amounts of rabbit anti-S serum resulted in a marked reduction in binding of radiolabeled 7C.12 IgG to DHBV-infected primary duck hepatocytes (Fig. 4B). This result showed that the radiolabeled 7C.12 IgG was highly specific for binding to cell-associated DHBV. In contrast to the results obtained for duck hepatocyte cultures, only very low amounts of radiolabel were found associated with duck embryo fibroblast cultures incubated with equivalent amounts of virus under the same conditions. These results were consistent, therefore, with the hypothesis that the ability of DHBV to infect a cell, as assessed by appearance of viral CCC DNA in the nucleus, was dependent upon the presence of a specific cell surface receptor.

We next examined whether the overall binding to a culture reflected the binding to individual cells in the culture or whether most of the label was bound by only a small fraction of the cells. To determine the fraction of primary Pekin duck hepatocytes that bound virus and to confirm that radiolabeled antibody was in fact associated with viable cells, dishes were processed for autoradiography after counting. Autoradiography of infected monolayers revealed grains specifically associated with a subset of hepatocytes, indicating the presence of DHBV particles bound to these cells. Approximately half of the hepatocytes bound relatively large amounts of virus, while the remainder exhibited signals comparable with those observed on cells exposed to normal duck serum alone (Fig. 5). Hepatocytes exposed to normal duck serum had a uniformly low number of associated grains, indicating that the signal was dependent on addition of serum containing viral particles (Fig. 5). Hepatocytes that bound large amounts of virus sometimes appeared in clusters, but these cells were not morphologically distinct from those that appeared negative for binding. Thus, the degree of binding was consistent with the high degree of susceptibility of Pekin duck hepatocytes to infection by DHBV.

Muscovy duck hepatocytes do not express abundant binding sites for DHBV. Though DHBV-resistant duck embryo fibroblasts did not bind DHBV, it was still possible that DHBV was bound by a conserved hepatocyte membrane protein that was present on the surfaces of both virus-susceptible and virus-resistant hepatocytes. To examine this possibility, we first examined whether virus binding receptors could be detected on hepatocytes of Muscovy ducks, which are closely related to Pekin ducks, as shown by their ability to interbreed to produce infertile progeny. Muscovy ducks are resistant to infection by DHBV, as assessed by their failure to develop a viremia after virus inoculation. However, we had previously reported that ca. 1% of the cells in hepatocyte cultures prepared from Muscovy ducklings can be infected with DHBV (12). This low-level susceptibility to infection was again consistent with the close relationship of these two duck species. However, our assay did not detect binding of DHBV particles to primary Muscovy duck hepatocytes (Fig. 5). Unfortunately, there is a background grain count in this assay which precludes distinguishing whether the ability of DHBV to infect a small fraction of Muscovy duck hepatocytes might be due to efficient binding of virus particles to rare cells (<1%) or to low-affinity binding events that can occasionally facilitate the initiation of infection but which are not detected by our assay. Nonetheless, the results were again consistent with the hypothesis that susceptibility to DHBV correlates with the presence of a receptor for virus particles at the cell surface and that this receptor is species specific. In support of this view, we also failed to detect significant binding to chicken hepatocytes, which are completely resistant to DHBV infection (data not shown).

Progressive loss of virus binding capacity is coincident with increased resistance to infection in Pekin duck hepatocyte cultures. We next wished to determine whether virus binding correlated with susceptibility to infection within Pekin duck hepatocyte cultures. As a first step, we took advantage of the fact that primary Pekin duck hepatocytes maintained in medium supplemented with fetal calf serum rapidly lose susceptibility to infection by DHBV (18). Hepatocytes isolated from ducks that are congenitally infected with DHBV continue to

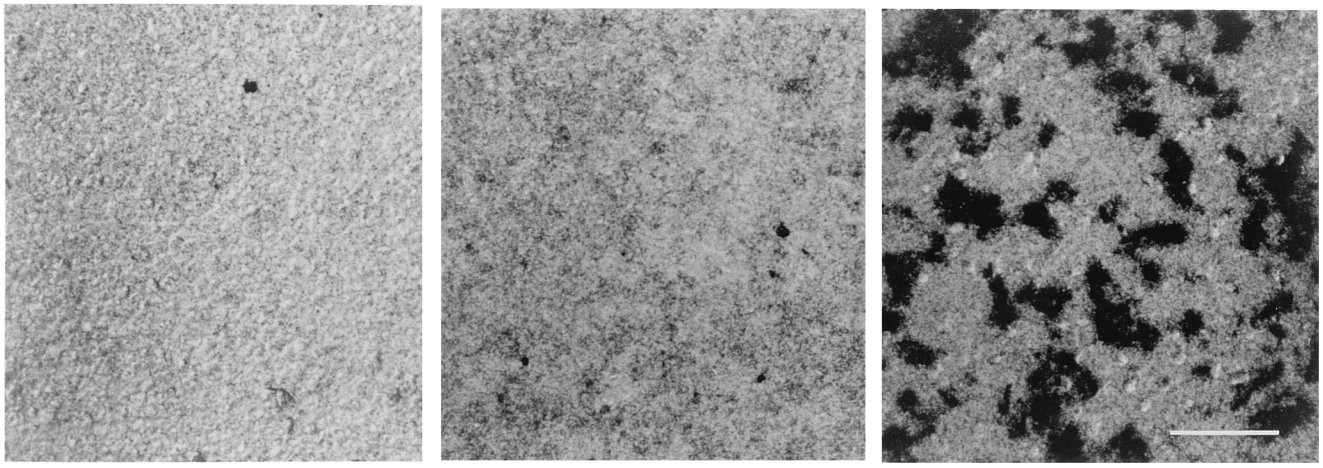


FIG. 5. DHBV can readily be detected bound to permissive Pekin hepatocytes but not to partially resistant Muscovy duck hepatocytes. One milliliter of a 1:20 dilution of DHBV or normal duck serum (NDS) was incubated with Pekin or Muscovy duck hepatocytes for 1 h, washed, incubated with radiolabeled 7C.12 IgG, and processed to detect binding of DHBV by autoradiography (see Materials and Methods). The Muscovy and Pekin duck hepatocytes were exposed to photographic emulsion for equivalent periods and developed under the same conditions. The bar represents approximately 200 μm.

support virus DNA replication and produce virus long after cells become refractory to infection in vitro (18). It appears, therefore, that some function required for initiation of DHBV infection, such as expression of a suitable cell surface receptor for virus, is lost during the first few days of culture in serum-supplemented medium. We exploited this fact in the present study to determine whether the appearance of resistance in duck hepatocyte cultures is associated with loss of virus binding capacity.

Primary Pekin hepatocytes were compared at 2 and 10 days after plating both for ability to bind DHBV and for susceptibility to infection with DHBV (Fig. 6). Virus infection was monitored by assaying DHBV CCC DNA and DHBV mRNAs in cells 24 h after infection. Almost all of the CCC DNA present in cells at this early time point must have arisen by conversion of virion DNA, and consequently the level of CCC DNA directly reflects the amount of DHBV that was internalized. Both CCC DNA and mRNA levels were approximately 10-fold lower when cells were infected at 10 days after plating than when they were infected on day 2, indicating that DHBV was not taken up into cells efficiently at the later time (Fig. 6A). We also showed that these cells were highly susceptible to DHBV infection 2 days after plating by using the more standard infectivity assay for DHBV, which measures the relative amount of replicative DHBV DNA intermediates in cultures 6 days after infection. Here again, the amount of replication was approximately 10-fold lower in cells infected at day 10 than in cells infected at day 2 after plating, allowing for loss of cells in cultures between 8 and 16 days after plating. This reduction therefore cannot be attributed to a decline in the capacity of duck hepatocytes to support DHBV DNA replication after 10 days in culture but rather is a consequence of infection of a reduced number of cells. We then examined how the capacity of hepatocytes to bind viral particles changed between 2 and 10 days postplating. Counting of bound radiolabeled IgG (Fig. 6B) and autoradiography (Fig. 6C) showed that viral particles bound efficiently to approximately half of the hepatocytes at 2 days after plating, as previously described, but that after 10 days, essentially all of the virus binding capacity of the culture had disappeared, with only rare cells binding detectable amounts of virus. Therefore, as in the experiments described above, the capacity of primary duck hepatocytes to bind DHBV particles appears to be closely associated with susceptibility of cells to infection with DHBV.

DHBsAg may compete with DHBV for binding sites on primary Pekin duck hepatocytes. DHBV surface antigen particles and virions both contain S and L envelope proteins on their surfaces (5, 13). Antibodies to S or pre-S will therefore detect both types of virus particles equally well. The source of DHBV for experimental infections in this study was serum isolated from congenitally infected Pekin ducklings, which contains at least a 100-fold excess of noninfectious DHBsAg over DHBV. Consequently, if both virus particles bind primary Pekin duck hepatocytes with similar efficiencies, the DHBV binding assay may principally detect DHBsAg. To test this hypothesis, we combined an infectivity assay with the virus binding assay described above. Primary duck hepatocyte cultures were incubated with equivalent amounts of serum-derived DHBV and then assayed directly for the presence of bound virus particles or incubated for a further 6 days and stained for DHBV core protein to determine what proportion of cells in the culture had become infected (see Materials and Methods). Approximately 10-fold more cells scored positive for binding than were infected by the inoculum (Fig. 7, 1:20 DHBV). A similar proportion of hepatocytes scored positive for virus binding after incubation of cultures with a low-titer inoculum, although the signal associated with these cells was reduced, indicating that fewer virus particles were bound (Fig. 7, 1:100 DHBV). This result is consistent with our hypothesis that the binding assay principally detects DHBsAg. However, our data do not rule out the possibility that the assay exclusively detects binding of mature virions, since only a small proportion of DNA-containing particles may be infectious (14).

An obvious question is whether virus and DHBsAg particles

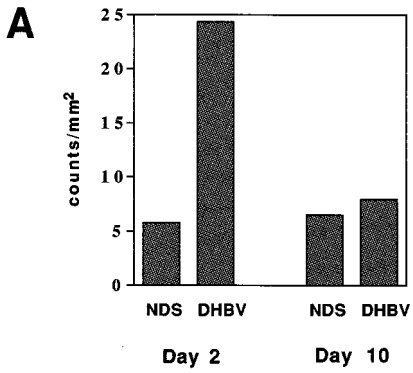
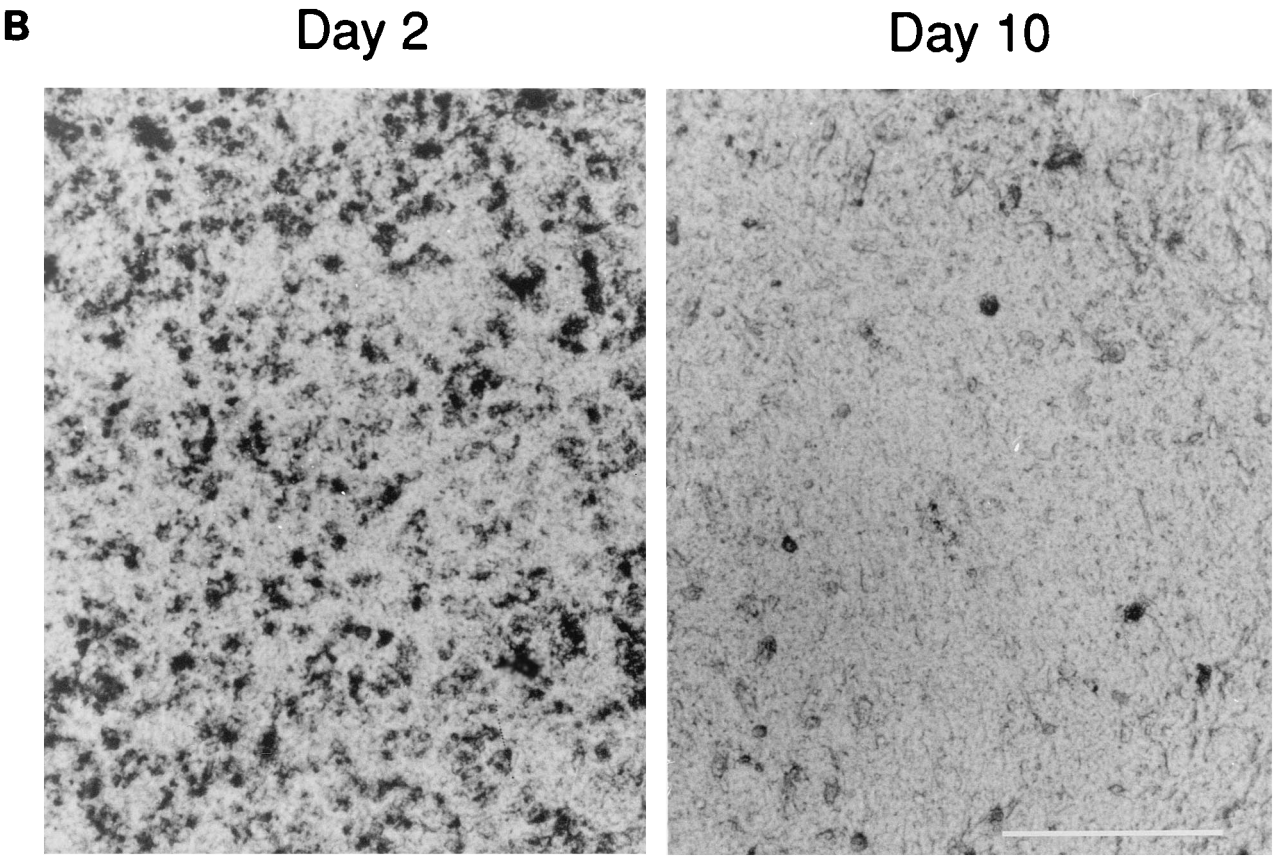
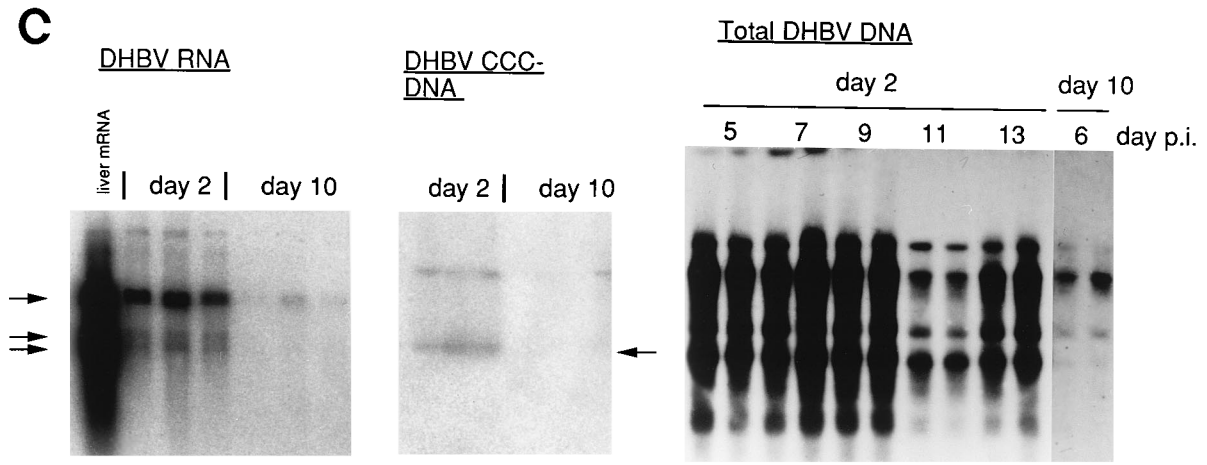


FIG. 6. Loss of virus susceptibility during prolonged culture of Pekin duck hepatocytes is associated with a decline in virus binding activity. (A) Hepatocytes maintained in L15 supplemented with 5% fetal bovine serum were incubated with equivalent amounts of the same virus inoculum, or with normal duck serum (NDS), at 2 or at 10 days after plating. Binding of virus was detected by using ^{125}I-labeled 7C.12 IgG, and counts were determined with a Fuji Bioimager. (B) Autoradiography was performed on the same dishes to determine the relative proportion of cells which bind virus at day 2 versus day 10. The bar represents approximately 500 μm. (C) Dishes infected at 2 or 10 days after plating were harvested 24 h after infection for isolation of DHBV CCC DNA or DHBV mRNA and at various times postinfection (p.i.) for total DHBV DNA. DHBV nucleic acids were detected by filter hybridization as described in Materials and Methods. The relative amount of each species at day 10 versus day 2 was determined by counting filters in a Fuji Bioimager.





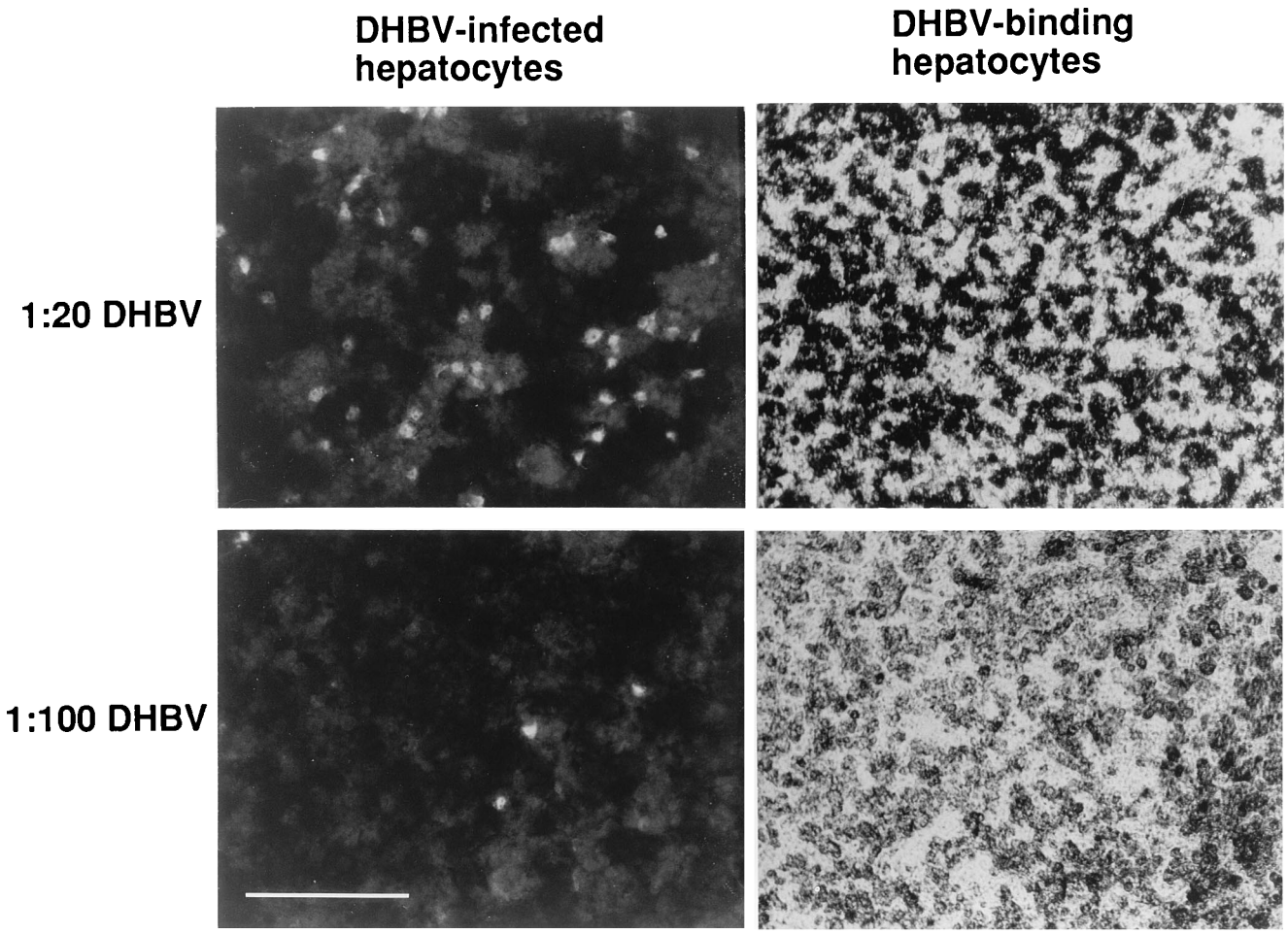


FIG. 7. Comparison of infection and binding efficiency of DHBV. Dishes of primary Pekin duck hepatocytes were incubated with equivalent amounts of DHBV-positive duck serum (1:20 or 1:100 dilution of virus) and then either processed to detect bound virus particles as described in Materials and Methods (right panels) or incubated for a further 6 days, after which the number of productively infected cells was determined by immunofluorescence staining for DHBV core protein (left panels). The bar represents approximately 1 mm.

bind to the same receptor. The correlation between infectivity and particle binding suggests that they do, an interpretation that is supported by several additional findings. First, we previously reported that maximal infection of hepatocytes requires prolonged incubation with virus, and second, we found that only a very small proportion of susceptible hepatocytes are infected when exposure to virus is carried out solely at 4°C, a condition under which recycling of cell surface receptors would presumably be inhibited (14). Third, in a recent study using recombinant DHBsAg particles from yeast cells, it was proven that DHBsAg particles can block infections (5). Taken together, these data indicate that surface antigen particles compete with infectious virions for available receptor sites on the surface of susceptible hepatocytes. In summary, all of the results described above support the conclusion that the assay described here detects binding of viral particles to cell surface receptors that are needed for a successful initiation of infection.

DHBV binding sites are retained on the surfaces of infected hepatocytes. DHBV-infected hepatocyte cultures are highly resistant to superinfection by a second, genetically marked variant of DHBV (15). If the resistance to superinfection is due to the fact that all available receptor sites are already occupied by endogenously produced viral particles, we might expect to see a high level of bound particles with the radiolabeled antibodies to S or pre-S. We therefore carried out the assay with primary hepatocytes isolated from Pekin ducks that were congenitally infected with DHBV. Bound virus was detected by using either anti-S or anti-pre-S IgG followed by radiolabeled anti-mouse IgG. The effect of adding additional virus to the DHBV-infected cells was also investigated. Approximately 60% of the DHBV-infected cells had large numbers of associated silver grains (Fig. 8), similar to the pattern that we observed when uninfected cells were incubated with virus. No difference was observed in either the number of virus-binding cells or the amount of radiolabel associated with these cells (data not shown) if cultures were preincubated with exogenous virus. This result suggests that all available virus binding sites on the surfaces of DHBV-infected hepatocytes were occupied. Thus, at least one mechanism for superinfection resistance appears to occur, as in many other viral systems, by blocking of cell surface receptors for the superinfecting virus.

DISCUSSION

Identification of the hepatocyte receptor(s) for HBV may facilitate the development of improved in vivo models for study of HBV pathogenesis as well as novel antiviral strategies to

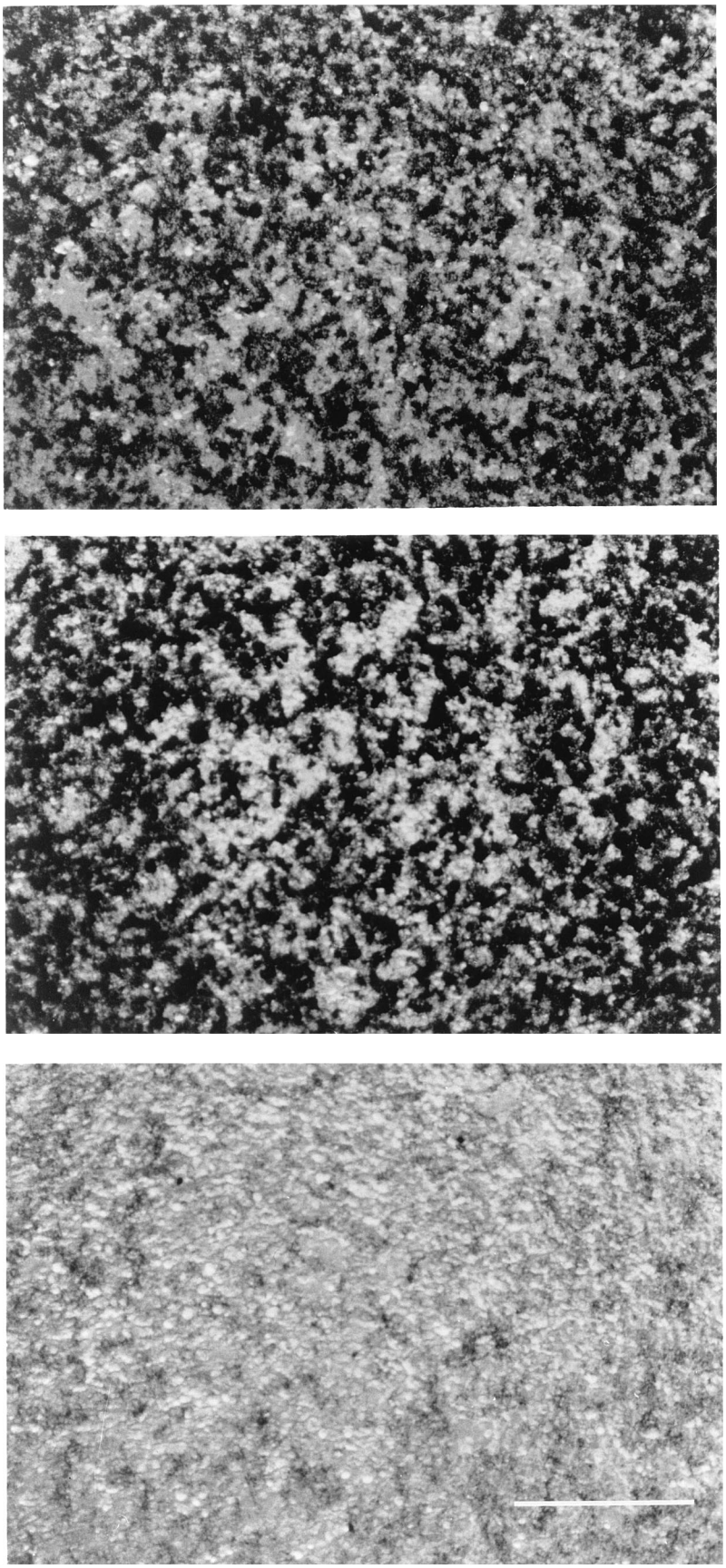


FIG. 8. Binding sites on DHBV-infected hepatocytes are occupied. Identification of DHBV-binding hepatocytes by autoradiography. Hepatocytes isolated from DHBV-infected ducks were incubated with approximately 5 μg of anti-DHBV S IgG (7C.12), anti-DHBV pre-S IgG (1.H1), or an unrelated IgG of the same isotype (anti-Rous sarcoma virus integrase; gift of Ann Skalka) per ml. Bound antibody was detected by using ^{125}I-labeled goat anti-mouse IgG (NEN) and autoradiography. The bar represents approximately 1 mm.

limit spread of HBV infection in chronic carriers. DHBV infection of primary Pekin duck hepatocytes provides a convenient model system for the study of hepadnavirus infections. The similarity in tissue tropism exhibited by DHBV and HBV indicates that they may bind to related cell surface molecules in order to initiate infection. A corollary of this premise is that identification of the DHBV receptor may reveal the nature of the receptor for HBV. While attempting to identify these molecules, we have begun to investigate the distribution of receptors on susceptible and resistant cell types by using an assay that relies on their ability to bind virus particles. The goal of the current study was to demonstrate the existence of specific binding sites for DHBV on the surfaces of Pekin duck hepatocytes and to examine whether binding was restricted to susceptible cells. To approach this problem, we developed a sensitive binding assay that uses radiolabeled MAbs specific for the DHBV envelope proteins.

The virus binding assay revealed that approximately 50% of primary Pekin duck hepatocytes express abundant receptors for DHBV. We did not detect binding to Muscovy duck hepatocytes, which, as we recently reported, are relatively resistant to DHBV infection (12). We were also unable to detect binding to Pekin duck embryo fibroblasts or to chicken hepatocytes, both of which are resistant to DHBV infection at the level of sensitivity of our assays (<0.01% cells infected). These results suggest that the cell surface binding which we detected is a property of cells that are highly susceptible to DHBV infection. Further evidence for this conclusion was obtained by taking advantage of the observation that primary hepatocytes lose susceptibility to DHBV infection after about 6 days when maintained in serum-supplemented medium (14, 18). We found that this resistance was associated with the loss of virus binding capacity on all but a small fraction (<1%) of hepatocytes and, as predicted from this finding, occurs at a step before CCC DNA formation.

The number of cells that scored positive for virus binding was approximately 10-fold greater than the number of cells which became infected with DHBV in the same experiment. While one explanation for this result is that the assay exclusively detects binding of DHBV but 90% of DNA containing particles are uninfectious, a more plausible interpretation is that the virus binding assay principally detects binding of DH BsAg particles which display the same envelope proteins as DHBV but which are present in vast excess in infectious duck serum. However, our previous data strongly suggest that DH BsAg inhibits infection by DHBV, a conclusion supported by results of a recent study that used recombinant DHBsAg to compete for binding of DHBV to primary duck hepatocytes (5, 14). While we have not yet demonstrated saturation of virus binding sites by addition of viral particles to uninfected hepatocytes, all binding sites on hepatocytes isolated from DHBV-infected ducklings appeared to be occupied. Taken together, these data imply that DHBV and DHBsAg bind to the same receptor and that our assay therefore detects the presence of a receptor for DHBV. This assay should facilitate development of screening procedures to isolate MAbs (blocking antibodies) that react with the cell surface receptor for DHBV and, ultimately, the identification of cells expressing the cloned receptor, irrespective of their ability to support subsequent steps in hepadnavirus replication.

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