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Monoclonal Antibodies to a 55-Kilodalton Protein Present in Duck Liver Inhibit Infection of Primary Duck Hepatocytes with Duck Hepatitis B Virus

JU-TAO GUO AND JOHN C. PUGH*
Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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As an approach to identifying hepatocyte receptors for the avian hepadnavirus duck hepatitis B virus (DHBV), hybridomas were prepared from mice immunized with permissive duck hepatocytes. Monoclonal antibodies (MAbs) were screened for the ability to inhibit binding of DHBV particles to primary duck hepatocytes and to block infection. We identified two MAbs which partially blocked binding and caused marked inhibition of infection of primary duck hepatocytes with DHBV. Lack of cross-reactivity with DHBV envelope proteins suggested that inhibition of infection was due to specific interaction between the antibodies and a host cell surface molecule. Both MAbs immunoprecipitated a 55-kDa protein (p55) expressed in duck liver and several other duck tissues. p55 homologs were also identified in other birds and mammals. We predict from our data that only a small proportion of total cellular p55 molecules are expressed at the surfaces of hepatocytes and that p55 is involved in some early step in the infectious pathway.

Several candidate hepatocyte receptors for hepatitis B virus (HBV) have been reported (1, 5, 11–13). All of these were able to bind virus envelope protein, though none have been proven to actually have a role in initiating HBV infection of hepatocytes. Indeed, there are no reports of genetic transfer of susceptibility for any hepadnavirus or of acquisition of virus binding ability by resistant cells after introduction of DNA from permissive hepatocytes. In light of these findings and of the recent discovery of virus receptor cofactors (2, 3), it is possible that expression of more than one hepatocyte protein will be needed to confer susceptibility upon a resistant cell type. Elucidation of the hepadnavirus infectious pathway will most probably require identification of the hepatocyte molecules that are responsible for adsorption, uptake, and uncoating of the virus, using assays specific to each of these steps.

Duck HBV (DHBV) is an attractive model with which to pursue the identification of hepadnavirus receptor molecules, because the avian virus offers several unique advantages for such studies. For example, there exists a convenient experimental infection system which uses cultures of primary duck hepatocytes (PDHs) (18). By using this system, the roles of different regions of the virus envelope proteins upon virus infectivity can be readily tested (10, 16, 17). We and others have shown that Pekin duck PDHs have high-affinity binding sites for both DHBV and DHBV surface antigen (DHBsAg) particles (7, 14). Furthermore, the virus binds specifically only to susceptible hepatocytes and not to resistant chicken hepatocytes or duck embryo fibroblasts (14). Hence, the presence of specific hepatocyte binding sites for DHBV is associated with susceptibility to virus infection. The N-terminal pre-S region of the DHBV large (L) envelope protein appears to perform an important role in specific interaction with the hepatocyte cell surface (6, 7, 10, 15). gp180, a carboxypeptidase expressed in duck liver and several other tissues, has been shown to bind the pre-S region of the DHBV L protein with high affinity (8, 9). A role for gp180 in the DHBV infectious pathway has been proposed; however, gp180 by itself is unable to confer susceptibility or virus binding capacity to resistant cells, and anti-gp180 antibodies have not been reported to block infection.

As an approach to identifying other hepatocyte molecules that bind DHBV envelope proteins and which may be components of a functional receptor complex, we have used a method which selects for antibodies that interfere with DHBV infection of PDHs. We immunized BALB/c mice with PDHs and Pekin duck liver plasma membrane preparations in the absence of adjuvant. After five to six monthly intraperitoneal injections with material from about 5×10^6 hepatocytes, mice were sacrificed and splenocytes were used for preparation of hybridomas. Our primary screen was designed to identify monoclonal antibodies (MAbs) that inhibit adsorption of DHBV particles to intact PDHs by binding to virus receptor sites on the cell surface. We applied a radioimmunoassay that uses radiolabeled immunoglobulin G (IgG) specific for DHBV small (S) envelope protein to determine the amount of virus particles bound to the surfaces of viable hepatocytes (14). Because noninfectious DHBsAg particles are secreted into the blood in vast excess compared with DHBV, the assay primarily detects binding of DHBsAg (14). Using this approach we screened almost 2,000 individual supernatants and isolated two MAbs, 3H6 and 3H9, which inhibited binding of DHBsAg by more than 50% compared with a control MAb (Fig. 1). Both 3H6 and 3H9 were of the IgM subclass. An obvious concern was that the inhibitory effect we observed might be due to a virus-neutralizing antibody. To address this concern, we showed by immunoblotting that neither 3H6 nor 3H9 recognized DHBV L or S envelope protein, nor could the antibodies immunoprecipitate native virus particles (Fig. 2). We concluded, therefore, that virus binding inhibition was not due to the direct interaction of the MAbs with the DHBV envelope proteins. We then asked if these antibodies would also inhibit infection of PDHs with DHBV. Southern analysis of intracellular DHBV DNA harvested 6 days after infection showed that preincubations of PDHs with MAbs 3H6 (Fig. 3) and 3H9 (data not shown) each resulted in an approximately fourfold reduction in the level of intracellular replicative DHBV DNA. Immunofluorescence staining for DHBV core protein at 6 days

* Corresponding author. Mailing address: Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111. Phone: (215) 728-4780. Fax: (215) 728-3574. E-mail: johnpugh@sprintmail.com.

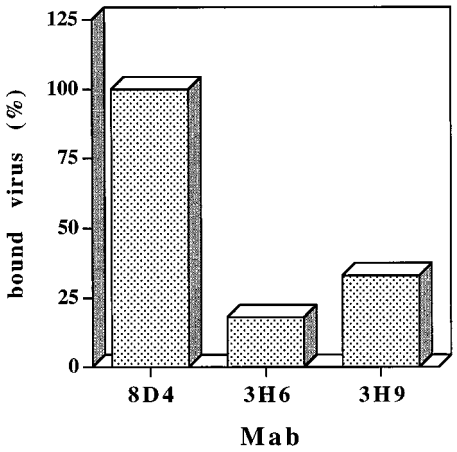

FIG. 1. MAbs 3H6 and 3H9 inhibit binding of DHBV to PDHs. PDH monolayers were incubated with hybridoma supernatant at room temperature for 1 h, followed by a further 1-h incubation with DHBV-containing duck serum diluted 1:100 in Optimem (Gibco-BRL), pH 7. Hepatocytes were incubated with ^{125}I-labeled anti-DHBV S IgG (MAb 7C.12) for 30 min, and after the hepatocytes were washed, the relative amount of bound virus was determined by gamma counting of cell lysates prepared in 1% (wt/vol) SDS. Results are expressed as the percentages of counts obtained with a control MAb, 8D4.

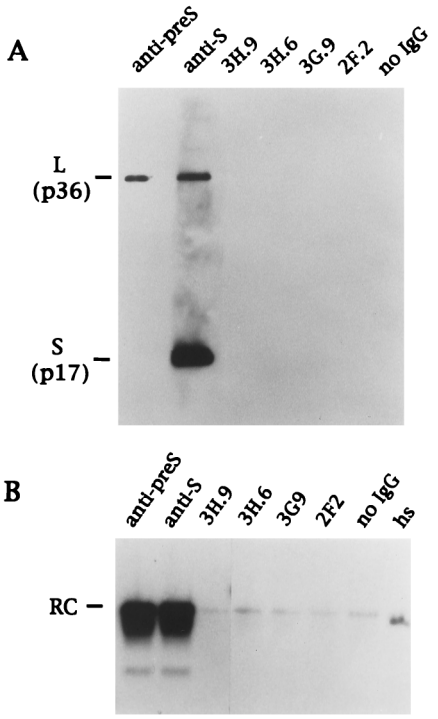

FIG. 2. (A) MAbs 3H6 and 3H9 do not recognize DHBV L and S envelope proteins, as shown by immunoblot analysis. DHBV particles partially purified from duck serum were separated by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell), and strips of the membrane were incubated with MAbs as indicated. Bound antibody was detected by using HRP-conjugated goat anti-mouse IgG (Cappel) and chemiluminescence (Pierce). MAbs specific for DHBV envelope proteins and MAbs 3H6 and 3H9 are described in the text. 3G9 and 2F2 are unrelated MAbs used as controls. (B) MAbs 3H6 and 3H9 do not immunoprecipitate DHBV particles. Five microliters of DHBV-positive duck serum was diluted to 100 μl with phosphate-buffered saline, and after incubation with MAbs as indicated, DHBV particles were immunoprecipitated with Pansorbin (Calbiochem) preincubated with rabbit anti-mouse IgG. The DHBV DNA was extracted by SDS-pronase digestion, resolved on a 1.5% (wt/vol) agarose gel, and detected by Southern hybridization with a radiolabeled RNA complementary to DHBV minus-strand DNA (18). hs, hybridization standard of 10 pg of linearized, full-length, cloned DHBV DNA; RC, relaxed circular DHBV DNA. MAbs are the same as for panel A.

postinfection confirmed that this result was due to approximately fourfold-fewer hepatocytes being infected with the original inoculum (data not shown).

We predicted from the above-described data that MAbs 3H6 and 3H9 inhibited DHBV infection by binding to molecules on the surfaces of PDHs. However, attempts to demonstrate specific cell surface binding of 3H6 and 3H9, either by immunofluorescence staining or by radioimmunoassay with radiolabeled anti-mouse IgG (NEN), were not successful (data not shown). Since 3H6 and 3H9 apparently confer the phenotype we observed by binding to cell surface molecules, we predict that 3H6 and 3H9 recognize molecules that are expressed on the surfaces of hepatocytes at such low levels that they were undetectable by our assays. Interestingly, Kuroki and coworkers were unable to demonstrate the presence of gp180 on the surfaces of PDHs, though the protein was readily detected in cell lysates (8). MAbs 3H6 and 3H9 were used to screen polypeptides present in duck liver plasma membrane preparations and total duck liver lysates by immunoblot analysis, but this failed to identify a specific protein. We therefore used immunoprecipitation of biotinylated liver lysates, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer, and detected proteins with horseradish peroxidase (HRP)-conjugated avidin (Neutravidin; Molecular Probes) and by chemiluminescence (Supersignal; Pierce) (8) (details available upon request). Lysates were prepared from 200 mg of tissue, and a volume equivalent to 10 mg was used for immunoprecipitation. This method identified a 55-kDa protein that was specifically precipitated from duck liver lysates by both 3H6 and 3H9 (Fig. 4). It appears, therefore, that 3H6 and 3H9 have the same specificity, though we have not determined if the MAbs compete for the same epitope on p55. Immunoprecipitation of biotinylated duck liver lysates with an anti-DHBV S IgG in the presence of DHBV-positive duck serum did not coprecipitate p55, though gp180 was readily detected by this method (data not shown). This result indicates that p55 does not bind to detergent-solubilized DHBV envelope proteins, but the finding does not discount binding of p55 to native virus particles.

We used the assay described above to determine the pattern of distribution of p55 in various duck tissues (Fig. 5). p55 was detected at approximately equivalent levels in liver, kidney, pancreas, heart, and brain and at lower levels in skeletal mus-

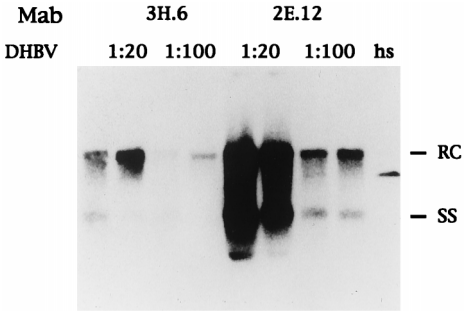

FIG. 3. MAb 3H6 inhibits DHBV infection of PDHs. PDHs were incubated with 3H6 or 2E12 (control) hybridoma supernatants at 37°C for 30 min. The antibody was then removed, and the cells were incubated with DHBV-positive duck serum, diluted as shown, for a further 30 min at 37°C. Cells were maintained in culture for 6 days, and then total intracellular DNA was prepared and DHBV DNA was analyzed by Southern blot hybridization (Fig. 2B). hs and RC are the same as for Fig. 2B. SS, single-stranded DHBV DNA.

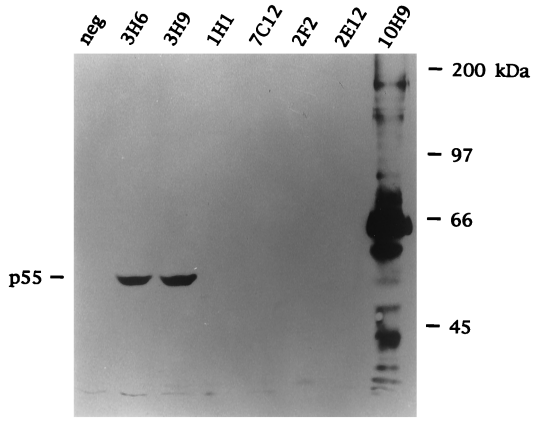

FIG. 4. MAbs 3H6 and 3H9 immunoprecipitate a 55-kDa cellular protein from biotinylated duck liver lysate. See the text for details of preparation of biotinylated duck liver lysates. An immunoprecipitation assay was performed by using the indicated MAbs and Pansorbin (Calbiochem) preincubated with rabbit anti-mouse IgG. Precipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell), and detected by using HRP-Neutravidin (Molecular Probes) and chemiluminescence (Supersignal; Pierce). MAb 10H9 has no effect upon DHBV infection but recognizes an abundant 65-kDa duck hepatocyte membrane protein and was included here as a positive control; 2F2 and 2E.12 are unrelated MAbs used as negative controls. neg, no first antibody.

cle, lung, intestine, and spleen. Hence, p55, like gp180, is widely distributed in duck tissues and expressed at high levels in liver, pancreas, and kidney, which are all established sites of DHBV replication (4). Interestingly, MAbs 3H6 and 3H9 recognized p55 homologs in liver lysates prepared from Muscovy ducks, chickens, and woodchucks (data not shown), suggesting that the 3H6/3H9 epitope is preserved on p55 from each of these animals. This result also suggests that 3H6 and 3H9 may exert an inhibitory effect upon woodchuck hepatitis virus infection of primary woodchuck hepatocytes, and this possibility is currently being tested.

In summary, we have isolated two MAbs which partially inhibit binding of DHBsAg to PDHs and infection of PDHs with DHBV. Both MAbs specifically recognize a 55-kDa protein expressed in liver and several other duck tissues. We have not been able to demonstrate cell surface expression of p55, and we predict that, as for gp180, only a small proportion of the total number of p55 molecules present in hepatocytes are expressed at the surface. Our data are consistent with a role for p55 in the pathway of adsorption and uptake of DHBV into hepatocytes. The possibility that p55 represents one component of a receptor complex appears to be unlikely, because we detected only p55 in immunoprecipitates prepared from duck liver lysates with MAbs 3H6 and 3H9 (Fig. 4). However, the small fraction of p55 molecules which we predict are present at the cell surface may be complexed with other proteins. Identification of p55 by amino acid analysis of immunoaffinity-purified p55 peptides or by cDNA cloning may help to clarify the role of p55 during infection of hepatocytes with DHBV.

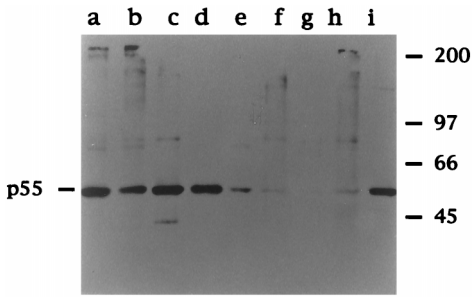

FIG. 5. p55 is widely expressed in most duck tissues. See the text and Fig. 4 for details of the immunoprecipitation assay with MAb 3H6. Approximately 10 mg of tissue was used to prepare each of the lysates used for immunoprecipitation. Lanes: a, liver; b, kidney; c, pancreas; d, heart; e, skeletal muscle; f, lung; g, intestine; h, spleen; i, brain. Molecular masses (in kilodaltons) are indicated on the right.

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