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Fibronectin of Human Liver Sinusoids Binds Hepatitis B Virus: Identification by an Anti-Idiotypic Antibody Bearing the Internal Image of the Pre-S2 Domain

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Anti-idiotypic antibodies (anti-Ids) have been successfully used to characterize and isolate receptors of several cell ligands. To prepare an immunological probe for identification of cellular components interacting with the hepatitis B virus (HBV), polyclonal antisera against a panel of five HBV-specific monoclonal antibodies (MAbs) were produced in syngeneic BALB/c mice. MAbs to HBV used for immunization (Ab1) recognized biologically important and potentially neutralizing epitopes, located in the pre-S1, pre-S2, or S region-encoded domains of HBV proteins. All the anti-Ids (Ab2) were specific to idiotopes of the homologous Ab1 and inhibited their interaction with the corresponding viral epitopes, suggesting that they recognized unique determinants on the paratope of each immunizing Ab1. Therefore, all five generated polyclonal anti-Ids were of the Ab2 β type and could represent internal images of viral epitopes. Ab2 raised against the pre-S2 region-specific MAb F124 bound to the extracellular matrix fibronectin of human liver sinusoids. Immunohistochemical studies demonstrated the attachment of viral and recombinant (S, M) hepatitis B surface antigen particles with the pre-S2 region-encoded epitopes to the fibronectin of human liver sinusoids. In contrast, recombinant (S, L*) hepatitis B surface antigen particles, in which the epitope recognized by F124 MAb was not expressed, did not show any binding capacity. These findings suggest that human liver fibronectin may bind HBV in vivo by the pre-S2 region-encoded epitopes in a species-restricted manner. Furthermore, binding of the circulating virus to liver sinusoids could facilitate its subsequent uptake by hepatocytes.

Specific antibodies (Ab1) produced during immune responses to an antigen possess two functionally distinct areas: an antigen-binding site (paratope) and a set of immunogenic determinants (idiotopes) capable of induce anti-idiotypic antibodies (anti-Ids) (Ab2). Anti-Ids of the A2β type, also referred as internal image antibodies, reactive with a paratope of an immunizing antibody, have the capacity to mimic the original antigen used to generate Ab1.

It has been postulated that antibodies can mimic the binding of a receptor to its ligand and that the anti-Id raised against such antibodies might be used to identify the receptor (40, 42). Several investigators have recently used the vast molecular repertoire of antibody molecules as mimics of ligands for a variety of hormonal and neurotransmitter receptors (12, 23, 41, 42). Induction of anti-Ids has also been considered as an alternative approach for identification of viral receptors (5, 12, 19, 23, 24, 41, 42, 45). However, this question remains open, since in other studies, anti-Ids failed to identify viral receptors (1), and in the reciprocal system, anti-Ids produced against anti-receptor antibodies did not recognize viral attachment proteins (6).

Hepatitis B virus (HBV) shares structural, molecular, and biological properties with other members of the hepadnavirus family, which are mainly hepatotropic and may lead to persistent infection. As well as the prominent disease manifestations in the liver, several other tissues, such as the pancreas, kidneys, bile duct, spleen, and peripheral blood mononuclear cells, can apparently support HBV replication, since viral antigens and replicative intermediates have been localized in most of these tissues (25). Functional studies of the infection of susceptible cells by HBV have been hampered by the restricted host range of HBV and by the failure to establish an in vitro tissue culture system in which the mechanism of infection could be investigated under controlled conditions.

Several candidates for HBV receptors have recently been proposed (2, 11, 15, 26, 31, 33, 36). Nevertheless the exact nature of cellular components interacting with HBV and the mechanism of viral attachment and of subsequent uptake by hepatocytes remain unknown.

The envelope of HBV contains three distinct coterminal proteins, known as large (L), middle (M), and major (S), which are the translation products of the same open reading frame with different initiation sites (13). The domains of these proteins encoded by the pre-S region of the viral genome represent putative HBV attachment sites identified by studies of the binding of HBV to cells or to isolated plasma membranes (29, 35). Synthetic peptide vaccines, encompassing fragments of the pre-S2 (16) or pre-S1 (30) domains, have been shown to be protective in chimpanzees. The S protein is the major component of all current HBV vaccines and elicits a protective antibody response. These data are consistent with an essential role of the three protein domains of the HBV envelope in virus neutralization in vivo. Antibodies directed to these domains may prevent different sequential steps of viral attachment, entry, or uncoating or may act by different neutralization mechanisms as aggregation, complement activation, or steric hindrance.

In this study we raised polyclonal anti-Ids against a panel of monoclonal antibodies (MAbs) directed to five nonoverlap-

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TABLE 1. Specificity of Ab1 MAbs used for preparation of anti-Ids

MAb	Immunizing antigen	Specificity	Epitope recognized	Subtype specificity	Epitope location
5a19	HBV	Anti-pre-S1	Pre-S1(36–43)	*ay*	HBV cell attachment site (29)
F124	HBV	Anti-pre-S2	Pre-S2(120–126)	*ay* > *ad*	Pre-S2 glycosylation site
C-BE	HBV	Anti-pre-S2	Pre-S2(132–145)	*ay*, *ad*	Pre-S2 protective peptide (17)
F376	(S, M) HBsAg	Anti-pre-S2	Pre-S2(132–145)	*ay* > *ad*	
39-10	HBV	Anti-S	*a* determinant	*ad*; *ay*	Conformational *a* determinant

ping epitopes of the putative HBV-neutralizing domains with the purpose of identifying the cellular components that interact with HBV. We demonstrate that one of these anti-Ids identifies the extracellular matrix fibronectin of human liver sinusoids as a potential HBV-binding protein via the pre-S2 domain. The in vivo interaction of HBV with liver fibronectin may play a role in the initial steps of virus entry into its target cell, subsequently mediated by specific hepatocyte receptors.

MATERIALS AND METHODS

Reagents. Reference preparations of human fibronectin, rabbit polyclonal anti-fibronectin antibodies, mouse anti-fibronectin MAbs, and mouse anti-laminin MAbs were from Sigma Chemical Co., St. Louis, Mo. Mouse fibronectin was from United States Biochemical Corp., Cleveland, Ohio. Rabbit polyclonal anti-collagen IV was from Monosan, Uden, The Netherlands. *N*-Glycanase (endoglycosidase F) was from Genzyme, Boston, Mass.

Preparation of anti-Ids. Mouse MAbs to five nonoverlapping epitopes, located in the pre-S1, pre-S2, or S domain of the HBV envelope, were used for preparation of anti-Ids (Table 1). All MAbs except for MAb CB-E were prepared in our laboratory. MAb C-BE was kindly provided by B. Porstmann, Humboldt University, Berlin, Germany (37).

Mouse immunoglobulins (Igs) were purified by protein A chromatography (8) and were coupled to keyhole limpet hemocyanin (KLH; Sigma) as the carrier protein by chemical cross-linking in the presence of glutaraldehyde (42). Five groups of three BALB/c mice were immunized subcutaneously with 0.4 mg of each MAb linked to keyhole limpet hemocyanin emulsified with complete Freund's adjuvant. Further booster injections of the same keyhole limpet hemocyanin-MAb were given on days 21, 35, and 47. At the end of the immunization procedure, ascitic fluids were prepared by injecting 1.5×10^6 Ehrlich's cells intraperitoneally.

Assays for detection of anti-Ids. Two types of enzyme-linked immunosorbent assays (ELISA) were used to study the anti-Id activity: a direct ELISA to detect binding of Ab2 to Ab1 and an inhibition ELISA to determine the ability of each anti-Id to inhibit the binding of Ab1 to the corresponding viral epitope.

For direct ELISA, microtiter plates (Nunk, Roskilde, Denmark) were coated with each Ab1 in a concentration of 1 μg/ml (purified by protein A chromatography), and the plates were overcoated with 1% bovine serum albumin (BSA)–0.05% Tween 20 in phosphate-buffered saline (PBS). The Ab1-coated plates were incubated with Ab2 diluted in PBS, and anti-Id bound to Ab1 was detected by addition of the homologous peroxidase-conjugated Ab1 and subsequently revealed with *o*-phenylenediamine as a substrate.

For the inhibition ELISA, the wells were coated overnight with 1 μg of viral antigens per ml at 4°C. Recombinant pre-S2–hepatitis B surface antigen (HB sAg) particles were used for analysis of anti-Ids to pre-S2 and S-specific MAbs. A synthetic peptide analog of the pre-S1 sequence (amino acids 12 to 49) of the *ay* antigenic subtype (kindly provided by P. Coursaget) was used for characterization of anti-Id directed to anti-pre-S1 MAb. Nonspecific protein binding was blocked with 1% BSA–0.05% Tween 20 in PBS for 2 h at 37°C. A constant amount of each peroxidase-labeled Ab1 was preincubated for 1 h at 37°C with appropriate dilutions of either anti-Ids or preimmune sera or with a diluent (1% BSA–0.05% Tween 20 in PBS) followed by incubation with the antigen-coated plates for 1 h at 37°C. After washing and reaction with *o*-phenylenediamine, the peroxidase-labeled Ab1 bound to the plate was measured by its A_{492}. Results were expressed as percent inhibition of the binding of labeled MAb to the corresponding antigen by the anti-Id, compared with inhibition by the preimmune mouse serum as a negative control.

HBV and recombinant HBsAg particles. HBV particles were purified from the plasma of a virus carrier by a sequence of ultracentrifugations as previously described (2). Recombinant (S, M) HBsAg–pre-S2 particles containing the middle and the major HBV proteins (27) were kindly provided by R. Vinas, Pasteur Mérieux. Recombinant (S, L*) particles composed of the major S protein and of a truncated large protein (L*) containing the S domain, pre-S1(12–52), and pre-S2(133–145) amino acid sequences (34) were kindly provided by P. Hauser, SmithKline Beecham Pharmaceuticals. Both types of recombinant particles were purified by the manufacturers.

Cell cultures. All adherent cell lines were grown as monolayer cultures at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) inactivated fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Nonadherent cell lines were grown in RPMI 1640 medium supplemented with fetal calf serum, L-glutamine, and antibiotics as above.

Immunofluorescence. An indirect immunofluorescence assay was used to investigate anti-Id reactivity with different continuous cell lines. The adherent cells were grown to 50 to 75% confluency on glass coverslips. The cell monolayers were washed three times with cold PBS and fixed with 3% (vol/vol) paraformaldehyde for 20 min at room temperature. After washing, the cells were reacted for 10 min with 50 mM NH_4Cl. After subsequent washings, the monolayers were incubated for 1 h at room temperature with anti-Ids (ascitic fluids) diluted 1:200 in 0.5% (wt/vol) BSA in PBS. The cell-bound mouse immunoglobulins were revealed with rhodamine-labeled anti-mouse Ig (Dako) diluted in 0.5% (wt/vol) BSA in PBS. Fluorescence was observed with a confocal laser scanning microscope (Leïca).

Immunofluorescence analysis of nonadherent cell lines was carried out with a fluorescence-activated cell analyzer (FACsCan; Becton-Dickinson, Grenoble, France). Fluorescence intensity was distributed on logarithmic scales, and 10,000 events were recorded in list mode on a FACsCan program. The percentage of positive cells was determined by setting the lower limit above that of the negative controls (no serum or preimmune sera).

Immunoblot analysis. Human and mouse liver extracts were prepared by homogenization of the tissue (0.6 g) in cold PBS containing 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at $10,000 \times g$, and the liver pellets as well as the pellet of PCL/PRF/5 cells were solubilized for 2 min at 100°C in Tris-HCl buffer containing 2% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) 2-mercaptoethanol, 0.01% (wt/vol) bromophenol blue, and 20% (vol/vol) glycerol. The proteins were separated on 5 to 15% polyacrylamide gels and electroblotted to nitrocellulose. The membrane strips were postcoated with 5% skim milk powder in PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween), washed, and probed with anti-Ids diluted in PBS-Tween. Peroxidase labeled anti-mouse Ig (heavy and light chains; Dako) served as a second antibody. After final rinses, blots were visualized with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, United Kingdom).

Dot blot assay. Anti-Ids were tested by dot blot for their reactivity with denatured homologous Ab1. Aliquots of an untreated sample (10 μl, at a concentration of 10 μg/ml) or of a sample treated for 2 min at 100°C with 2% SDS and 5% 2-mercaptoethanol were deposited on a nitrocellulose membrane and air dried. The membranes were postcoated overnight with 5% skim milk, washed, and incubated with the corresponding anti-Id diluted in PBS-Tween. The antibodies bound to the strips were detected with horseradish peroxidase-labeled anti-mouse Ig and revealed by the enhanced chemiluminescence detection system.

Absorption and enzymatic digestion procedures. Ab2 to F124 (diluted 1:10 in PBS) was absorbed for 1 h at 37°C either with a pellet prepared from the PCL/PRF/5 cell line or with protein A-Sepharose 6B (Pharmacia) saturated with MAb F124. Before and after this procedure, the samples (diluted 1:50 in PBS) were tested for anti-Id activity and for their reactivity against fibronectin by a solid-phase ELISA.

Fibronectin and (S, M) HBsAg particles were digested with *N*-glycanase for 21 h at 37°C in 0.5 M acetate buffer. The effect of digestion of these antigens on the reactivity of Ab2 to F124 and MAb F124 compared with the samples incubated in parallel without the enzyme was determined by solid-phase ELISA.

Immunohistochemistry. The reactivity of Ab2 with normal human liver components was investigated with frozen liver sections. Samples of normal human liver were obtained from patients who underwent surgical resection for a benign tumor. Liver tissue was embedded in OCT Compound (Miles Diagnostic Division, Elkhart, Ind.), frozen in liquid nitrogen, and stored at −80°C. Serial 5-μm-thick sections were cut, fixed for 5 min with cold acetone, and air dried. Immunohistochemical staining was carried out by the avidin-biotin peroxidase complex method with the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, Calif.) as specified by the manufacturer. The tissue sections were preincubated with 3% normal sheep serum diluted in PBS to block nonspecific binding.

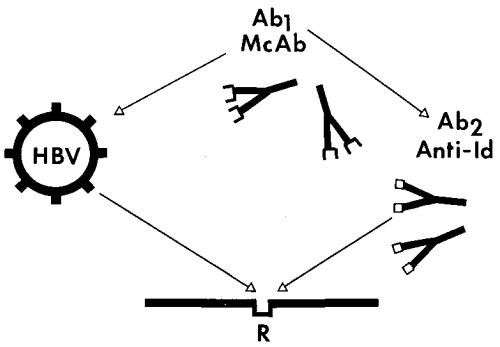

FIG. 1. Rationale of the anti-Id approach for identification of the HBV receptor(s). McAb, monoclonal antibody.

Sections were then incubated with the anti-Id (serum or ascitic fluid) diluted 1:50 in PBS for 30 min at room temperature. Biotinylated anti-mouse IgG (Vector) was used as a second antibody. Detection was carried out with 3-amino-9-ethylcarbazole as the chromogenic substrate. Controls included sections on which the primary antibody was omitted or replaced by preimmune sera or irrelevant ascitic fluids. The reactivity of Ab2 with mouse or rabbit livers was tested by the same technique on frozen sections of normal mouse or rabbit liver tissue.

For binding studies, either HBV purified from serum or recombinant (S, M) HBsAg or (S, L*) HBsAg particles were incubated for 1 h at room temperature on human or mouse liver sections prefixed with cold acetone. Virions or HBsAg particles bound to liver tissue were revealed with anti-HBs MAb and biotinylated anti-mouse IgG as described above. For inhibition experiments, the sections were preincubated with rabbit antibody to fibronectin or collagen IV or normal rabbit globulins as control, before incubation either with HBV or with recombinant HBsAg particles.

RESULTS

The rationale behind the experiments reported here is schematically presented in Fig. 1. Cellular receptors can display structural features of paratopes of antibodies directed to cell attachment proteins of the virus. Consequently, some of the anti-idiotypes raised against virus-neutralizing antibodies may be similar enough in their combining sites to the viral attachment proteins to recognize virus-binding components on the cells.

Ab2 recognize idiotypic determinants related to the antigen-binding sites of Ab1 MAbs. Anti-Ids were screened by direct ELISA with homologous and heterologous Ab1 MAb on the solid phase. Each Ab2 was reactive with its corresponding immunizing Ab1 and did not cross-react with heterologous Ab1, suggesting that all five of the generated anti-Ids recognized unique idiotopes on each immunizing Ab1 (Fig. 2).

A series of inhibition ELISAs was carried out to investigate if Ab2 were reactive with the paratopes of their homologous Ab1. In these assays, recombinant particles expressing pre-S2 and S epitopes or synthetic pre-S1 peptide were used as viral antigens and the labeled Ab1 MAbs were used as tracers. Each Ab2 inhibited the binding of labeled homologous Ab1 to the corresponding viral antigen in a dose-dependent manner (Fig. 3), while the preimmune sera had no effect. Moreover, when used in constant dilution, each Ab2 inhibited 70 to 90% of the binding of its homologous Ab1 to the corresponding viral epitope whereas it did not interfere with the binding of any heterologous Ab1 to viral antigens (Fig. 4).

The idiotypes on Ab1 recognized by Ab2 were apparently conformational, since no Ab2 reacted with its homologous Ab1 by dot blot under denaturing conditions (data not shown).

Reactivity of Ab2 to F124 with the PCL/PRF/5 cell line. Using anti-Id reagents, we searched for cellular structures that

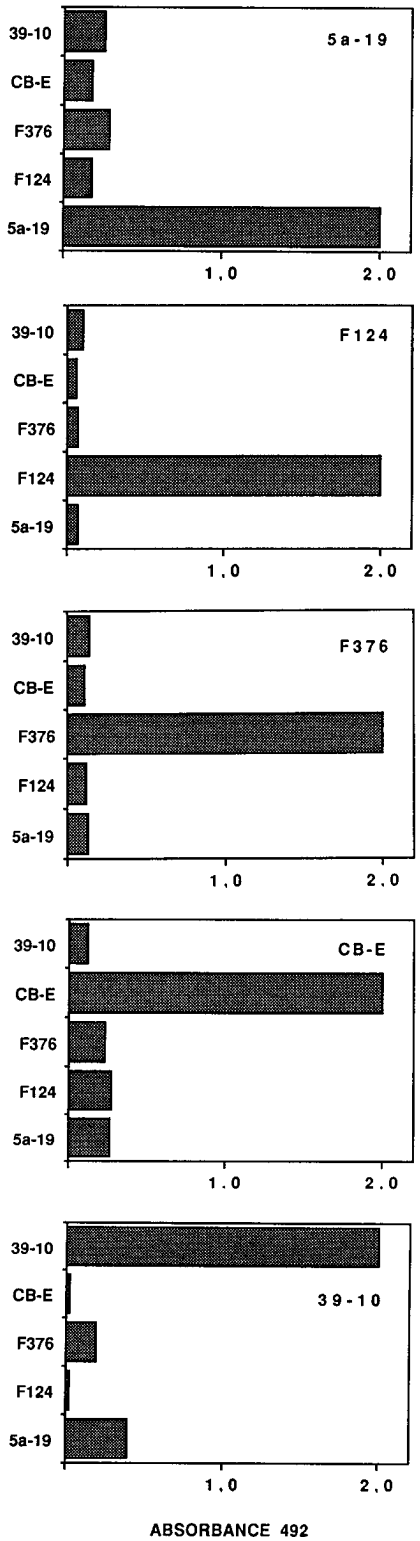

FIG. 2. Direct ELISA for determination of the specificity of Ab2. The plates were coated with homologous and heterologous Ab1 (1 μg/ml) and reacted with each Ab2 diluted 1:50. The anti-Ids bound to the plate were revealed with peroxidase-labeled Ab1 as indicated in each panel.

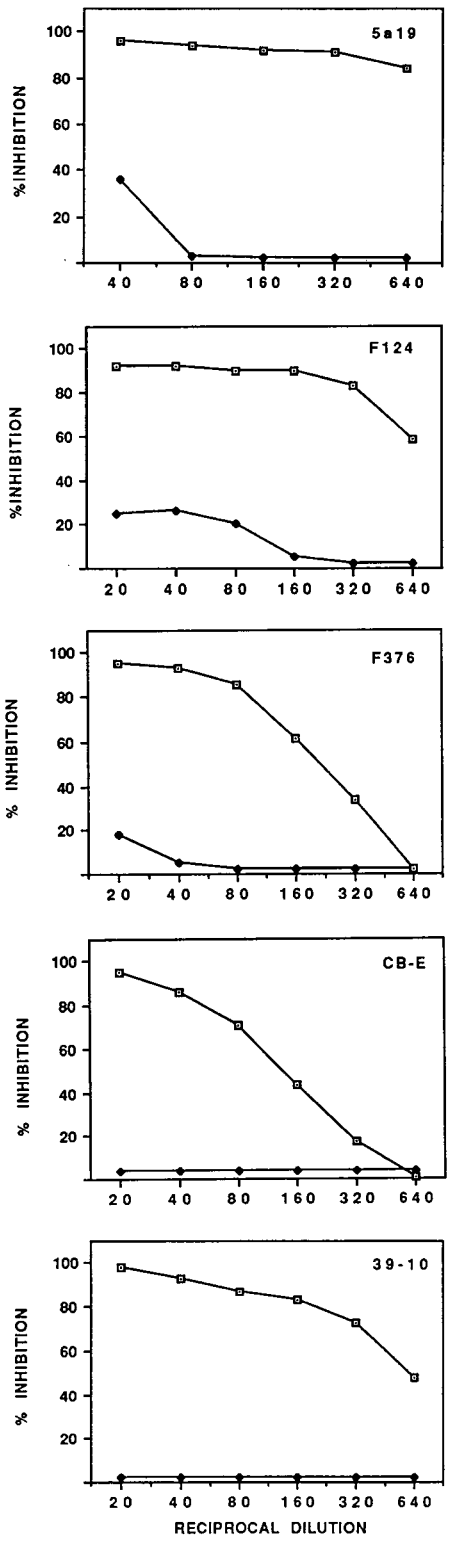

FIG. 3. Inhibition of the reactivity of labeled Ab1 with the corresponding viral antigen by homologous Ab2. The reaction was carried out on ELISA plates coated with recombinant (S, M) particles serving as the pre-S2 and S antigens (for Ab2 to F124, CB-E, F376, and 39-10) or with pre-S1(12–49) synthetic peptide as the pre-S2 antigen (for Ab2 to 5a19). Labeled Ab1 (indicated in each panel) were preincubated with serial dilutions of homologous Ab2 (□) or with the corresponding preimmune mouse sera (◆). Results were expressed as percent inhibition compared with the sample incubated with PBS.

potentially interacted with HBV. For this purpose, all anti-Ids were reacted in the indirect fluorescence test with monolayers of different adherent cell lines: HepG2 (hepatoma), PCL/PRF/5 (hepatoma), WRL-68 (fetal hepatoma), HeLa (cervical adenocarcinoma), HEp-2 (laryngeal carcinoma), and ARL-6 (rat hepatoma). The anti-Ids were also analyzed by FACsCan for their reactivity with nonadherent cell lines: U 937 (promonocytic cell line), CEM (T-cell leukemia) J-JHAN (T-cell lymphoblastoma), and THP1 (monocytic cell line). Of the different human cell lines tested, only the PCL/PRF/5 human hepatoma cell line was found to be reactive with anti-Id raised with pre-S2-specific MAb F124, whereas no reactivity of other anti-Ids could be found on this and other tested cell lines. Ab2 to F124 strongly stained pericellular structures, delineating individual cells and filaments sparsely located between the cells (Fig. 5A). The distribution pattern of immunofluorescence suggested that the component stained by this anti-Id can be localized in the extracellular matrix produced by the PCL/PRF/5 cell line (Fig. 5C). The fluorescence pattern of Ab2 to F124 corresponded to that obtained after staining of this cell line with MAb to human fibronectin (Fig. 5B), whereas no reactivity of reference anti-laminin antibody could be evidenced on the PCL/PRF/5 hepatoma cell line.

Immunohistochemical staining of human liver. Immunohistochemical studies were performed to examine whether the anti-idiotypic antisera contained antibodies reactive with the normal human liver tissue. No cytoplasmic or membranous staining could be seen in or on hepatocytes with all anti-Ids tested. However, with ascitic fluids of all three mice immunized with pre-S2 region-specific MAb F124, a continuous linear staining was observed along the liver sinusoids (Fig. 6A). The reactivity of Ab2 to F124 was apparently restricted to human tissues, since no staining of sinusoids was observed when mouse or rabbit livers were used as controls (data not shown).

A similar staining pattern was obtained with MAb to fibronectin (Fig. 6B), suggesting that Ab2 to F124 was reactive with a component located in the extracellular matrix. Indeed, the reactivity of this Ab2 with liver perisinusoidal regions was abolished by the preincubation of the liver tissue with rabbit antibody to fibronectin but not influenced by preincubation either with antibody to collagen IV or with normal rabbit globulins.

Identification of the component recognized by Ab2 to F124 as human fibronectin. Ab2 to F124 identified a 220-kDa protein band corresponding to fibronectin when analyzed by immunoblotting with the extract from human liver, extract from the PCL/PRF/5 cell line, and a reference preparation of fibronectin (Fig. 7). No protein bands were detected when other anti-Ids or normal mouse serum were used as controls.

The lack of reactivity of Ab2 to F124 with either mouse or rabbit liver or with a rat hepatoma cell line (ARL-6) known to produce an extracellular matrix (10) suggested that the component reactive with Ab2 to F124 was specific to human tissue. Indeed, results of both ELISA and immunoblotting confirmed the specificity of this antibody to fibronectin of human origin (Fig. 7).

Analysis of anti-fibronectin activity of Ab2 to F124. Immunochemical staining and ELISA showed the reactivity of Ab2 to F124 produced in all immunized mice with human fibronectin. However, neither ELISA nor Western immunoblot revealed the presence of fibronectin in the immunizing preparation of MAb F124, and mouse preimmune sera were negative for anti-fibronectin antibody.

To further analyze the specificity of Ab2 to F124, we attempted to absorb the anti-fibronectin activity of this anti-Id with protein A-Sepharose saturated with MAb F124. This

treatment completely abolished the reactivity of Ab2 to F124 with MAb F124 and induced a fourfold reduction of its reactivity with fibronectin (Fig. 8).

In another series of experiments, we attempted to absorb the anti-fibronectin activity with a pellet of PCL/PRF/5 cell line, rich in the extracellular matrix. This treatment completely abolished anti-fibronectin activity of Ab2 to F124 and diminished by 30% its reactivity with F124 MAb in inhibition ELISA (data not shown), indicating that about one-third of Ab2β to F124 was reactive with fibronectin. These results suggested that immunization with MAb F124 could induce an anti-fibronectin response.

To investigate the role of the sugar moieties in the binding of Ab2 to F124 to fibronectin, human serum fibronectin was digested with *N*-glycanase. In parallel, the reactivity of McAb F124 with the pre-S(120–126) epitope was determined following removal of sugar moieties. The effect of this treatment was tested by solid-phase ELISA with corresponding native and treated antigens. These experiments revealed that the removal of the sugar component of fibronectin did not influence its recognition by Ab2 to F124. In contrast, the deglycosylation of (S, M) HBsAg considerably decreased the reactivity of MAb F124 with its corresponding pre-S2 epitope (data not shown).

Fibronectin of liver sinusoids binds HBV and recombinant (S, M) HBsAg particles. From the reasoning in Fig. 1, it seemed probable that human liver fibronectin could represent a component that interacted with the N-terminal pre-S2 epitopes of HBV envelope, recognized by this immunizing MAb. To investigate this possibility, tissue slides of normal human liver were fixed and incubated with the HBV concentrate or with two types of recombinant HBsAg particles: (S, M) HBsAg, expressing the complete pre-S2 domain, and (S, L*) HBsAg particles, in which the pre-S2 epitope reactive with MAb F124 was deleted.

The attachment of both HBV and (S, M) HBsAg was observed on human liver sinusoids (Fig. 9A and B). The staining pattern was similar to that obtained with Ab2 to F124 and with the reference antibody to fibronectin but of lower intensity. The binding was apparently restricted to human tissue, since no attachment of the viral and recombinant HBsAg particles could be shown on mouse liver. The interaction of both HBV and (S, M) HBsAg particles with the liver perisinusoidal area was completely abolished by preincubation of the liver tissue with rabbit antibodies to human fibronectin (Fig. 9D). Recombinant (S, L*) particles, lacking the domain reactive with F124 MAb, did not show any binding to liver sinusoids (Fig. 9C).

No binding of viral or recombinant (S, M) HBsAg particles could be evidenced in vitro either to soluble human and mouse fibronectins on an ELISA plate or to liver proteins separated in gels and blotted onto the nitrocellulose (data not shown).

DISCUSSION

This report describes the characterization of anti-Ids produced against a panel of potentially neutralizing MAbs to the HBV surface proteins. The anti-Ids were elicited in a syngeneic system by immunization of inbred BALB/c mice with MAbs produced in the same strain to generate antibodies to unique idiotopes of immunizing Ab1. This approach was developed to produce internal-image antibodies that could mimic antigenic determinants of the viral envelope and thus identify cellular components capable of interacting with the HBV. Internal-image anti-Ids have been considered as an alternative vaccine against HBV infection (4, 20) and have been previously used in a study on HBV receptor in a xenogeneic system (immunization of rabbits with mouse Igs [33]).

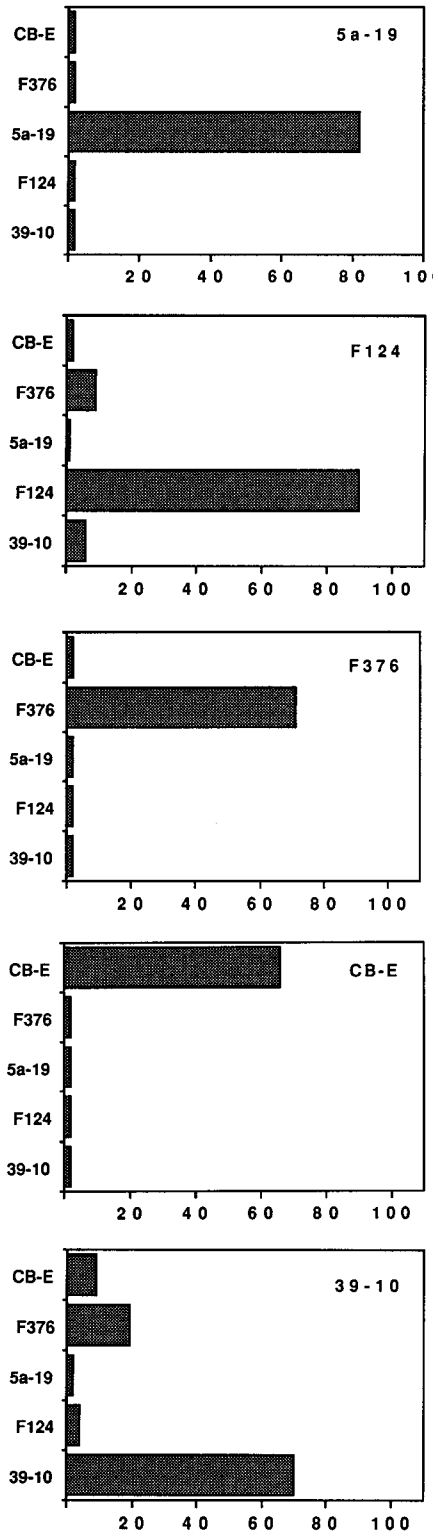

FIG. 4. Cross-inhibition ELISA to determine the reactivity of Ab2 with the antigen-binding site of Ab1. Labeled Ab1 (indicated in each panel) were preincubated with homologous or heterologous Ab2 (at a constant dilution of 1:40) before the reaction with the corresponding viral antigen on the plate. Results are expressed as percent inhibition calculated as for Fig. 3.

FIG. 5. Reactivity of Ab2 to F124 (A and C) and MAb to fibronectin (B) with the PCL/PRF/5 hepatoma cell line.

Epitope mapping of Ab1 MAb demonstrated that each of the five MAbs selected to produce anti-Ids reacted with a distinct, biologically important, potentially neutralizing epitope of the viral envelope. MAb 5a19 recognized the pre-S1(36–43) amino acid sequence (results obtained in collaboration with R. Neurath and C. Howard) located in the main HBV receptor-binding site (29). The pre-S2 region-specific MAbs were reactive with the pre-S2(120–145) amino acid sequence encompassing the second putative HBV receptor-binding site (29): MAb F376 (28) and CB-E were specific for apparently nonoverlapping epitopes in the pre-S2(133–153) amino acid sequence, which has been shown to elicit protective antibodies in chimpanzees (16). F124 anti-pre-S2 MAb (3) recognized the pre-S2(120–126) epitope of the middle HBV protein and was preferentially reactive with the pre-S2 domain glycosylated at Asn-123 (13a; also see above). This MAb is unreactive with the corresponding sequence in the large HBV protein because of the difference either in conformation or in glycosylation of these proteins. MAb F124 inhibited the binding of recombinant and HBV particles to isolated human hepatocyte mem-

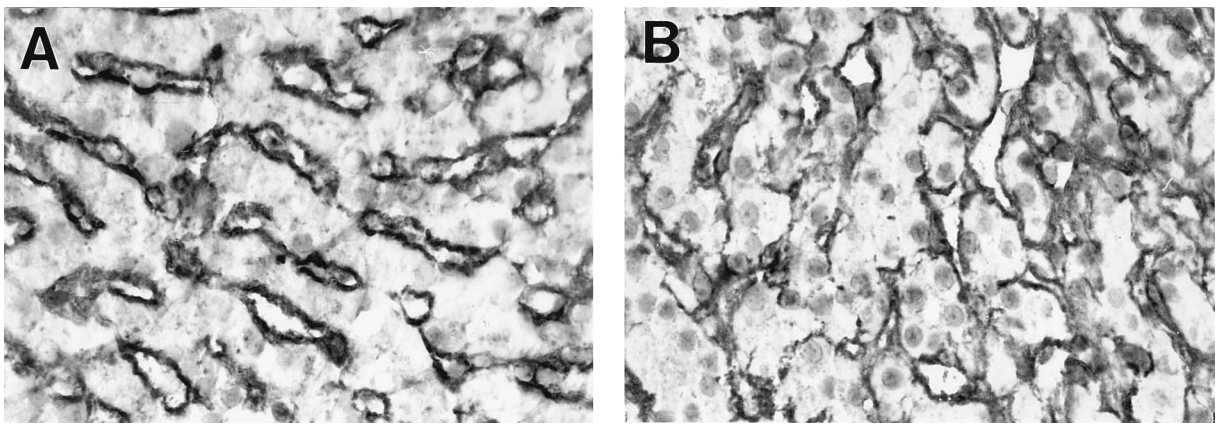

FIG. 6. Immunohistochemical staining of human liver sinusoids with Ab2 to F124 (A) and with the reference MAb to fibronectin (B). Magnification, ×400.

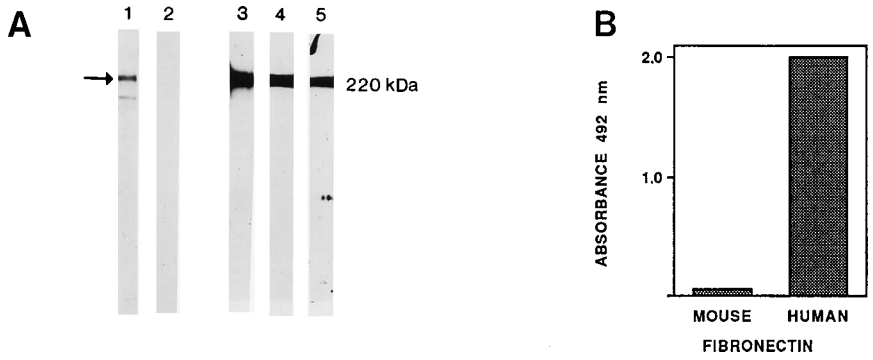

FIG. 7. Reactivity of Ab2 to F124 with human fibronectin. (A) Western blot analysis. Lanes: 1, extract from human liver; 2, extract from mouse liver; 3, extract from PCL/PRF/5; 4 and 5, human fibronectin. Lanes 1 to 4 were revealed with Ab2 to F124, and lane 5 was revealed with MAb to fibronectin followed by peroxidase-labeled anti-mouse Ig. The immunoreactive bands were visualized by enhanced chemoluminescence. (B) Reactivity of Ab2 to F124 with fibronectin of human origin as shown by ELISA.

branes (35). Finally, MAb 39-10 was directed to the conformational epitope located in the group *a* specific determinant of the major S protein, an antigenic domain known to elicit a protective antibody response and to interact with human hepatocyte membranes (15). All these Ab1 MAbs were inhibited by the antibodies of humans convalescent from acute infection (unpublished observations).

All five generated Ab2 were highly specific for their respective immunizing MAb in accordance with the different epitope specificity of each Ab1. All the Ab2 were reactive with the conformational idiotypes on Ab1 MAbs, since they did not recognize their respective immunizing Ab1 under denaturing conditions. Moreover, each anti-Id inhibited the binding of its homologous Ab1 with the corresponding viral epitopes but did not interfere with the reactivity of heterologous Ab1 MAbs. Therefore, these criteria appear to show that all polyclonal Ab2 produced in syngeneic, inbred BALB/c mice were real anti-Ids that were reactive only with the idiotopes of the corresponding immunizing Ab1 but not with other parts of the mouse antibody molecule. Moreover, the subpopulation of these anti-Ids was reactive with the idiotopes located inside or near the antigen-binding site of the respective Ab1. This type of anti-Ids, classified as anti-Ids Ab2β (7), could represent the internal image of viral antigens. Since these anti-Ids were generated by immunization with Ab1, some of which are potentially neutralizing, these anti-Ids might recognize structures capable of interacting with HBV.

Anti-Id produced by immunization with MAb F124, directed against an epitope located in the N-terminal part of the pre-S2 region, recognized the human fibronectin of the extracellular matrix. This anti-Id, raised against mouse MAb, was apparently reactive with a human-specific part of the protein, which was never used in the immunization procedure. It is reasonable to assume that F124 recognizes the pre-S2 epitope of the HBV in the same way in which fibronectin recognizes HBV. The interaction of HBV with fibronectin should be confined to the part of the viral envelope which binds the F124 MAb. Indeed, HBV and recombinant (S, M) HBsAg particles with corresponding pre-S2 epitopes attached to human liver sinusoids, whereas recombinant (S, L*) HBsAg particles lacking this region failed to interact. Fibronectin was apparently involved in virus and HBsAg–pre-S2 binding, since it was completely abolished by preincubation of liver tissue with anti-fibronectin antibodies. Moreover, the affinity of the extracellular matrix for HBV seems to be species restricted, since no binding of viral and recombinant particles to sinusoids was observed with mouse liver.

Besides viral attachment to the perisinusoidal area, the binding of viral particles to hepatocyte membranes could be evidenced by the immunochemical staining on some human liver tissue slides. This finding is in accordance with the presence of putative HBV receptors on plasma membranes of human liver cells (15, 26, 29, 31, 33, 35). A candidate for a receptor protein, located on the hepatocyte membrane, that could be implicated in the entry of HBV into hepatocytes has recently been described by our group (2). This protein and its potential role for HBV infection are under investigation in our laboratory.

A number of pathogenic microrganisms have been shown to bind fibronectin with a high degree of specificity and with high affinity (44). For some microorganisms, the interaction with fibronectin may represent a crucial step in the colonization of host tissue and development of infection. A fibronectin-binding protein has been isolated from *Staphylococcus aureus* (9). Other pathogens like *Pneumocystis carinii* (38), *Trypanosoma cruzi* (32), and *Candida albicans* (22) have been shown to bind to fibronectin. Some of them utilize the RGD amino acid sequence for association with host cells (32, 38). In addition, fibronectin is capable of interacting directly with various viruses such as HIV (43), Rous sarcoma virus (41), myxoviruses and paramyxoviruses (17, 18, 21), and HAV (39). The affinity of viral envelope glycoproteins of influenza A virus, parainfluenza virus type 1, and mumps virus for fibronectin are impor-

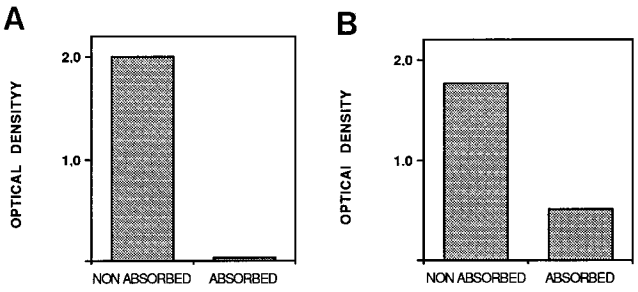

FIG. 8. Analysis of the anti-fibronectin activity of Ab2 to F124. Reactivity of Ab2 to F124 with MAb F124 (A) and with human fibronectin (B) was determined by ELISA before and after absorbtion of Ab2 to F124 with insoluble MAb F124.

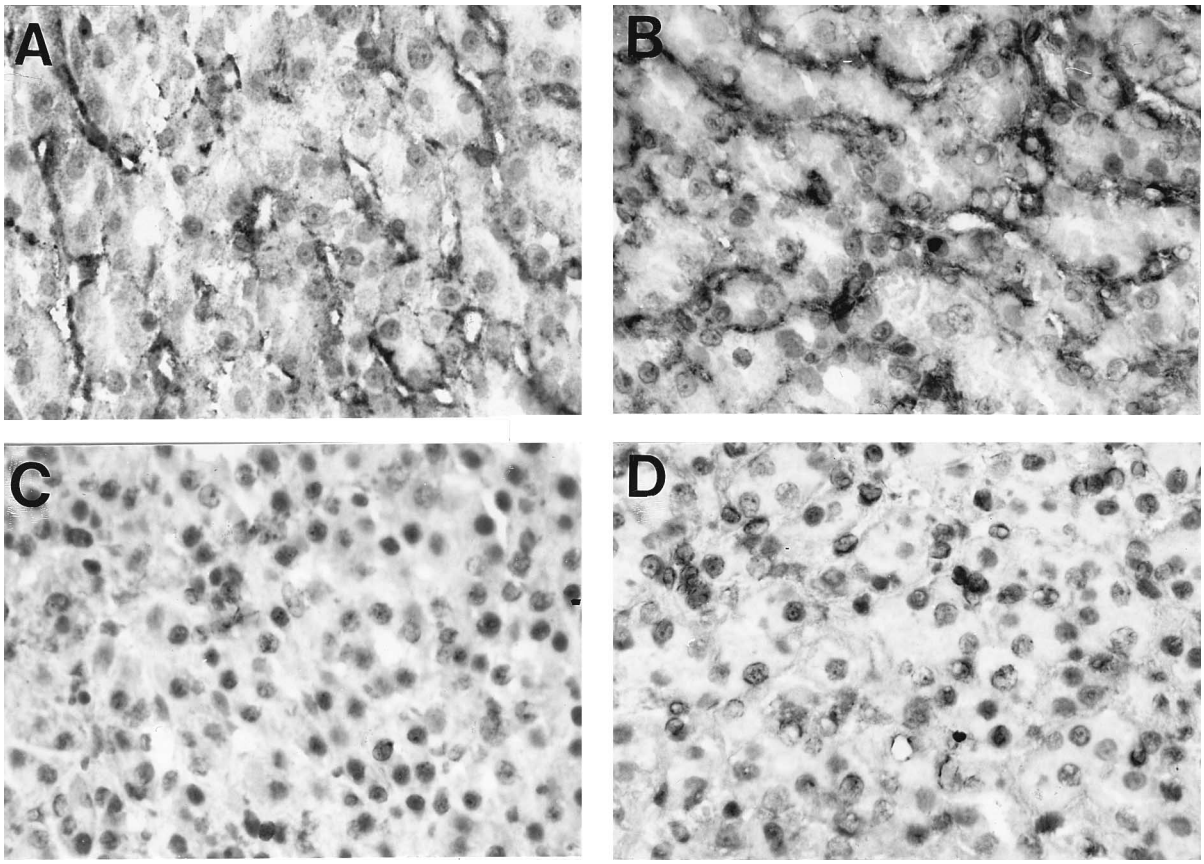

FIG. 9. (A and B) Attachment of serum HBV (A) and of recombinant (S, M) HBsAg particles bearing the complete pre-S2 domain (B) to human liver sinusoids as evidenced by peroxidase staining. (C) Absence of binding of recombinant (S, L*) particles lacking N-terminal pre-S2 epitopes. (D) Inhibition of HBV attachment by rabbit antibodies to human fibronectin.

tant for pathogenesis of viral infection and have been suggested to play a role in virus-cell interaction (17, 21).

There is increasing evidence that many viruses may use multiple receptors for cell entry. Multiple receptors could be coreceptors and act together, or, alternatively, the initial binding step could be followed by a secondary binding and subsequent viral penetration by fusion or by endocytosis (14). For viruses circulating in body fluids such as blood or respiratory secretions, the initial binding must be able to effect rapid docking of the virus to its host cell. In such situations, the binding rate can be more important than affinity, and consequently a virus might bind weakly to an abundant receptor until it finds a second receptor (14). The sinusoidal region of the hepatocyte plasma membrane has a direct contact with the bloodstream, and many receptors for growth factors and hormones are located in this area. It is tempting to suggest that the fibronectin of human liver sinusoids might contribute to the initial binding of the circulating virus, with its subsequent uptake into hepatocytes mediated by a specific receptor molecule located on the cell membrane.

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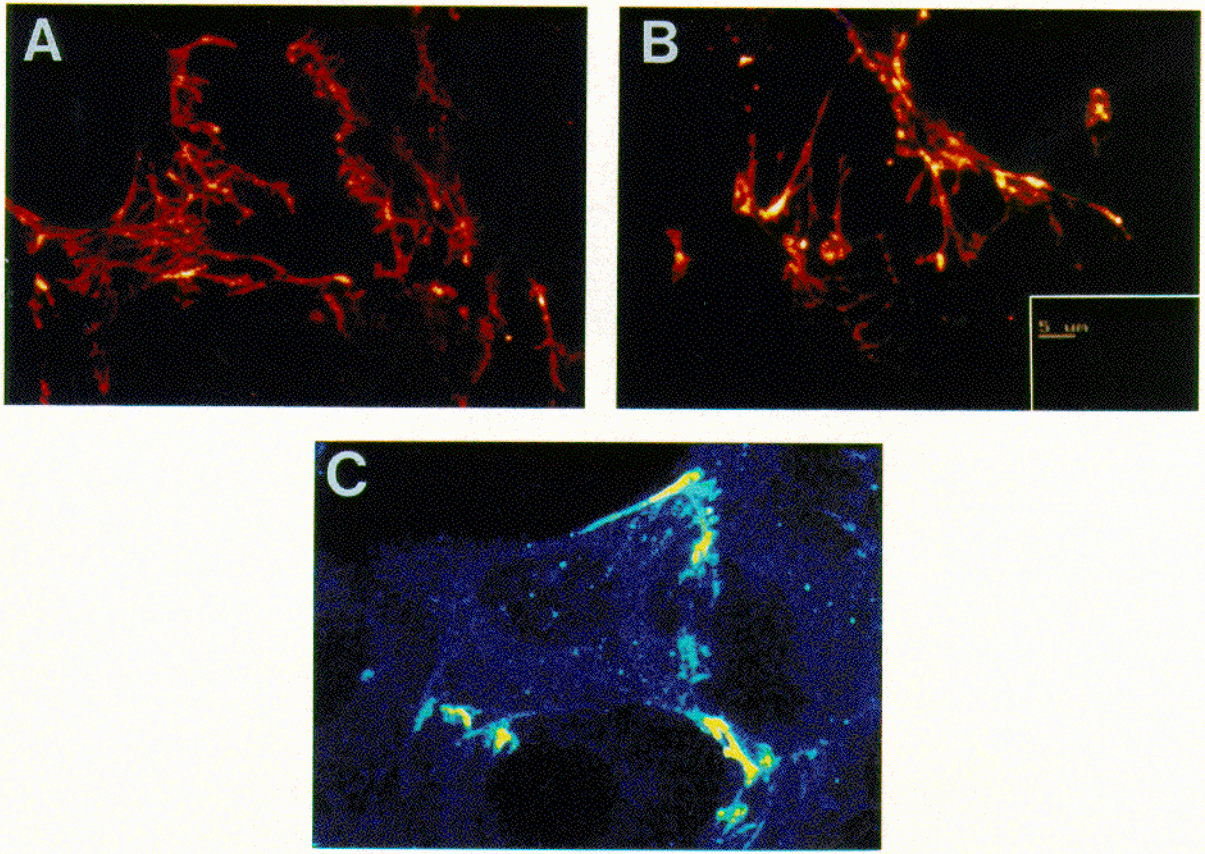
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