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Development and characterization of a rodent model of immune-mediated cholangitis

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ABSTRACT **The cholangiopathies are a group of hepatobiliary diseases in which intrahepatic bile duct epithelial cells, or cholangiocytes, are the target for a variety of destructive processes, including immune-mediated damage. We tested the hypothesis that cholangitis could be induced in rodents by immunization with highly purified cholangiocytes. Inbred Wistar rats were immunized with purified hyperplastic cholangiocytes isolated after bile duct ligation from either syngeneic Wistar or allogeneic Fischer 344 rats; control rats were immunized with bovine serum albumin (BSA) or hepatocytes. After immunization with cholangiocytes, recipient animals developed histologic evidence of nonsuppurative cholangitis without inflammation in other organs; groups immunized with BSA or hepatocytes showed no cholangitis. Immunohistochemical studies revealed that portal tract infiltrates around bile ducts consisted of CD3-positive lymphocytes, some of which expressed major histocompatibility complex class II antigen; B cells and exogenous monocytes/macrophages were essentially absent. Transfer of unfractionated Con-A-stimulated spleen cells from cholangiocyte-immunized (but not BSA-immunized) rats into recipients also caused nonsuppurative cholangitis. Moreover, these splenocytes from cholangiocyte-immunized (but not BSA-immunized) rats were cytotoxic *in vitro* for cultured rodent cholangiocytes; no cytotoxicity was observed against a rat hepatocyte cell line. Also, a specific antibody response in sera of cholangiocyte-immunized rats was demonstrated by immunoblots against cholangiocyte proteins. Finally, cholangiograms in cholangiocyte-immunized rats showed distortion and tortuosity of the entire intrahepatic biliary ductal system. This unique rodent model of experimental cholangitis demonstrates the importance of immune mechanisms in the pathogenesis of cholangitis and will prove useful in exploring the mechanisms by which the immune system targets and damages cholangiocytes.**

Human cholangiopathies represent a diverse group of liver diseases in which the intrahepatic bile duct epithelial cell, or cholangiocyte, is thought to be the target of destruction (1). These diseases can be grouped into two general categories: non-immune-mediated and immune-mediated. The former result from a variety of causes, including genetic predisposition (e.g., cystic fibrosis), infections (e.g., AIDS cholangiopathy), or ischemia; the latter include graft-versus-host disease (GVHD), liver allograft rejection, primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). Immune-mediated cholangiopathies are progressive diseases which eventually lead to death from liver failure unless liver transplantation is performed (2, 3). While the role of the immune system in GVHD and allograft rejection is clear, the immune mechanisms involved in the pathogenesis of PBC and PSC are obscure, in part because of lack of suitable animal models (2, 3).

Animal models that mimic human immune diseases have contributed to a better understanding of the pathogenesis of these diseases. For example, experimental allergic encephalomyelitis (4, 5), type II collagen-induced arthritis (6), and experimental thyroiditis (5) have all been induced by immunization with exogenous immunogen.

Using highly purified rat cholangiocytes (7) as a source of antigen, we now describe an experimental approach which leads to the successful induction of rodent immune cholangitis.

MATERIALS AND METHODS

Rats and Reagents. Twelve-week-old male inbred Wistar/NHd (*RT1^u*) and Fischer (*RT1^{v1}*) rats were purchased from Harlan–Sprague–Dawley. Unless otherwise stated, reagents were purchased from Sigma.

Cell Isolation. Three weeks after bile duct ligation, hyperplastic cholangiocytes were isolated from Wistar or Fischer rats and purified as previously described (7). Final cholangiocyte purity was 75.6% ± 4.2% [n = 8; assessed by staining for γ-glutamyl transpeptidase (γGT), an enzyme which in the liver is specific for rat cholangiocytes (8)]; contaminating cells were almost entirely endothelial cells and essentially no hepatocytes were present. Hepatocytes were isolated from Wistar rats as described and were ≥99% pure (9).

Immunization Scheme. Ten million purified hyperplastic cholangiocytes from Wistar (syngeneic group, n = 12) or Fischer (allogeneic group, n = 12) rats were emulsified in Freund's complete adjuvant and injected three times subcutaneously into Wistar rats at 14-day intervals. Control Wistar rats were immunized with either syngeneic hepatocytes (10 million cells per rat; n = 9) or bovine serum albumin (BSA, 100 μg per rat; n = 9). Five rats in each group received an additional immunization 28 days after the third immunization.

Histological and Immunohistochemical Evaluation. Animals from each group were sacrificed 1 week after each immunization. For histological studies, liver, kidney, colon, pancreas, and salivary gland were fixed with 10% buffered Formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin, and samples were evaluated under code. The severity of the portal inflammation and cholangitis was graded from 0 to +++ (1).

To identify the cells infiltrating the portal tracts, fresh frozen liver sections were stained by the indirect immunoperoxidase technique. Mouse anti-CD3 monoclonal antibody (IF4, Serotec) was used as a primary antibody (1:200) to detect T cells. To detect macrophages, B lymphocytes, major histocompatibility complex (MHC) class II antigen, or cytokeratin 19 (CK19), mouse monoclonal antibodies MRC OX-41, MRC OX-33, MRC OX-6 (Serotec), and BA17 (Dako), respectively, were used as primary antibodies, over a range of dilutions (1:20–1:200). Horseradish peroxidase (HRPO)-conjugated goat antibodies to mouse IgM (Tago) for CD3 or HRPO-

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Abbreviations: GVHD, graft-versus-host disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; γGT, γ-glutamyl transpeptidase; BSA, bovine serum albumin; MHC, major histocompatibility complex; CK19, cytokeratin 19; CA II, carbonic anhydrase II.

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conjugated goat antibodies to mouse IgG (Tago) were used as second antibodies and developed with aminoethylcarbazole substrate. In all experiments, negative controls using normal goat serum instead of primary antibody were employed.

Adoptive Transfer Studies. Spleen cells from either cholangiocyte-immunized or BSA-immunized rats were harvested 1 week after the third immunization. Following red blood cell lysis by hypertonic shock, splenocytes were incubated for 72 hr in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and Con A at 5 mg/ml. Ten million Con-A-stimulated splenocytes were subsequently injected i.p. into naive (i.e., unimmunized) syngeneic recipients rats; tissue was harvested 1 week later and processed as described above.

***In Vitro* Cytotoxicity Assay.** Primary cultures of normal rat cholangiocytes (NRC-1) served as target cells in the *in vitro* cytotoxicity assay and were prepared by a modification of the method of Yang *et al.* (10). A rat hepatocyte cell line (BRH) (ATCC no. CRL1422) was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 media containing 10% fetal bovine serum. Their phenotype was confirmed as being hepatocytic by demonstrating both the presence of glucose-6-phosphatase, albumin, and aminotransferase (hepatocyte-specific markers) and the absence of CK19 (cholangiocyte-specific marker). These cells were cultured ($\approx 5 \times 10^5$ cells per well) and served as a control (i.e., noncholangiocyte) target cell.

Con A-Activated Splenocytes. These cells, which serve as effector cells in the *in vitro* cytotoxicity assay, were prepared from cholangiocyte- or BSA-immunized rats as described (11). Two hours before mixing, target cells (NRC-1 or BRH) were labeled with 10 μCi (1 μCi = 37 kBq) of ^{51}Cr (sodium chromate; Amersham) per 10^6 cells. After several washings with cold Hepes-buffered saline, splenocytes (effector cells) were added to radiolabeled target cells over a range of effector-to-target ratios (0.1:1 to 100:1) and incubated for 8 hr at 37°C. The supernatants were collected and detergent (1% Nonidet P-40) was added to lyse the cells (11, 12); the supernatant and cell-associated radioactivities were determined with a γ counter, and the cytotoxicity was calculated as percent release of ^{51}Cr.

Cholangiograms. Cholangiograms were prepared as described (12) with livers from rats 1 week after final immunization with purified cholangiocytes (n = 4) or BSA (n = 3).

Immunoblotting. Immunoblots were performed by the method of Towbin *et al.* (13). Briefly, 100 μg of protein from homogenized hyperplastic cholangiocytes or 5 μg of human carbonic anhydrase II (CA-II) was electrophoresed in an SDS/10% polyacrylamide gel according to Laemmli (14). Proteins were then electrophoretically transferred to Nitro Bind nitrocellulose filters (Micron Separations, Westboro, MA), and diluted (1:100) preimmune or immune sera from cholangiocyte-immunized rats was incubated with filters for 1 hr at 37°C. After several washings with 0.3% Tween 20 in phosphate-buffered saline (PBS), filters were incubated with alkaline phosphatase-labeled goat anti-rat immunoglobulin (Cappel) for 1 hr at 37°C and developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

Serum Liver Enzymes. Serum from each group of rats was harvested prior to immunization and then weekly for 4 weeks after initial immunization and stored at −20°C until assayed. Serum alkaline phosphatase, alanine aminotransferase, γGT, and direct bilirubin were measured with a clinical autoanalyzer (Becton Dickinson) set for human parameters, an approach whose validity we had confirmed with sera from rats made cholestatic by bile duct ligation.

Statistics. All data were expressed as the mean ± SD. Statistical analysis of data was performed using the Student t test, and differences were considered significant at $P < 0.05$.

RESULTS

Histological Changes Following Immunization with Cholangiocytes. *Liver.* One week after the third immunization with purified hyperplastic cholangiocytes, virtually all portal tracts in rat liver showed diffuse inflammation consisting predominantly of mononuclear cells (Fig. 1*A*), and the infiltrating mononuclear cells surrounded intrahepatic bile ducts (Fig. 1*B*), findings consistent with nonsuppurative cholangitis. As early as 7 days after initial immunization, cholangiocyte-immunized animals showed histological evidence of cholangitis which persisted over the first three immunizations (Table 1). However, after a fourth booster immunization, liver specimens showed increased inflammation with destructive cholangitis (Table 1). Surprisingly, the degree of cholangitis did not differ between the allogeneic and syngeneic immunization groups. No inflammation was observed in the parenchymal areas of cholangiocyte-immunized rats. Liver specimens from BSA- or hepatocyte-immunized rats showed no or minimal histological abnormalities (Table 1; Fig. 1*C*).

Nonliver. Tissue specimens from kidney and colon showed no inflammation after immunization with purified cholangiocytes, hepatocytes, or BSA (results not shown). Pancreas, salivary glands, and extrahepatic bile ducts also showed no histological abnormalities in any immunization groups (results not shown).

Immunohistochemistry. Stains for the cholangiocyte-specific protein CK19 demonstrated unequivocally that after cholangiocyte immunization, the structures surrounded by infiltrating mononuclear cells were bile ducts (Fig. 2*A*); this was also observed in biliary epithelia after the booster immunization. Moreover, immunohistochemistry with specific monoclonal antibodies revealed that the majority of the portal tract infiltrates were positive for CD3 and MHC class II antigen (Fig. 2 *B* and *C*) but negative for B-cell and macrophage epitopes (results not shown).

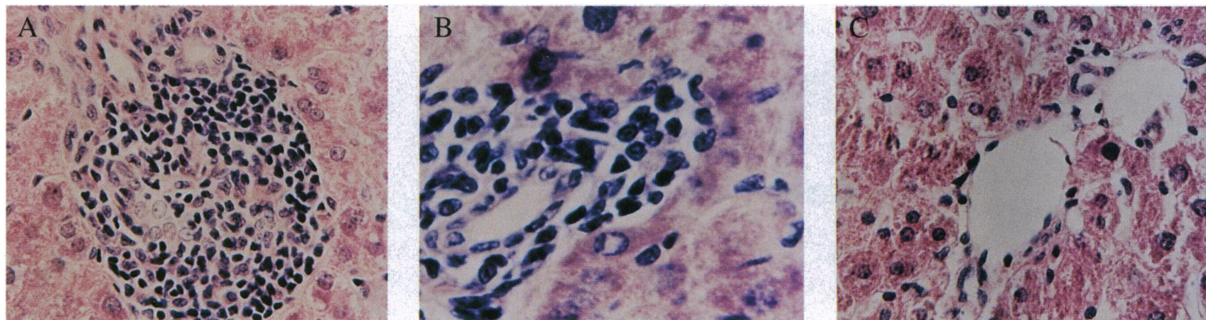

FIG. 1. Rat liver. (Hematoxylin and eosin.) (*A*) Liver obtained after immunization with cholangiocytes. (×120.) (*B*) Same as *A* with higher magnification of portal tract. (×190.) Note mononuclear cell in bile duct. (*C*) Liver after immunization with BSA; no inflammation is observed. (×120.)

Table 1. Histological grading of cholangitis

Immunogen	Grade after initial immunization				
	Day 7	Day 21	Day 35	Day 70	Day 70 (booster)
Syngeneic cholangiocytes	++	+	++	0	+++
Allogeneic cholangiocytes	++	++	++	0	+++
Hepatocytes	±	0	0	0	±
BSA	0	0	0	0	0

0, No change; ±, minimal change; +, mild cholangitis; ++, moderate cholangitis; and +++, severe cholangitis. Each time point included three animals from each group with the exception of the hepatocyte and BSA group at day 70 (booster), in which there were two animals.

Adoptive Transfer Experiments. One week after the i.p. injection of Con-A-activated splenocytes from cholangiocyte-immunized rats into naive animals, recipients developed nonsuppurative cholangitis (Fig. 3*A*). The inflammation was liver specific; kidney and colon did not show any histological changes (results not shown). Immunohistochemistry of liver infiltrates indicated that these mononuclear infiltrates were predominantly composed of T lymphocytes (Fig. 3*B*) surrounding CK19-positive bile ducts (Fig. 3*C*). The adoptive transfer of Con-A-activated splenocytes from BSA-immunized rats showed no infiltrate in liver, kidney, or colon (results not shown).

***In Vitro* Cytotoxicity Assay.** ^{51}Cr-labeled NRC-1 or BRH cells were incubated with splenocytes from cholangiocyte- or BSA-immunized rats to test whether the splenocytes were directly cytotoxic for the radiolabeled target cells. Splenocytes from cholangiocyte-immunized rats caused lysis of ^{51}Cr-labeled NRC-1 cells in a dose-dependent fashion (Fig. 4*A*). This effect was cell specific—that is, splenocytes from cholangiocyte-immunized rats did not cause lysis of ^{51}Cr-labeled BRH cells (Fig. 4*B*). Splenocytes from BSA-immunized rats caused no increased lysis of ^{51}Cr-labeled NRC-1 or BRH cells over the same range of effector-to-target ratios (Fig. 4).

Cholangiograms. No abnormalities were observed on cholangiograms of rats immunized with BSA (Fig. 5*A*). In contrast, after immunization with cholangiocytes, cholangiograms showed tortuous intrahepatic ducts and localized areas of duct ectasia (Fig. 5*B*). No abnormalities were observed in the extrahepatic ducts of either group.

Immunoblotting. As shown in Fig. 6*A*, sera from cholangiocyte-immunized rats detected several protein bands on immunoblots of cholangiocyte proteins which were not detected by preimmune sera. Additional experiments indicated that the humoral response included antibodies against CA-II, an antigen found in liver uniquely on cholangiocytes (Fig. 6*B*). These antibodies were absent from preimmune sera. While no distinct band was observed at 32.5 kDa in lane 2 of Fig. 6*A* where CA-II might be expected, this may reflect either insufficient material for detection or differential glycosylation of native and purified CA-II.

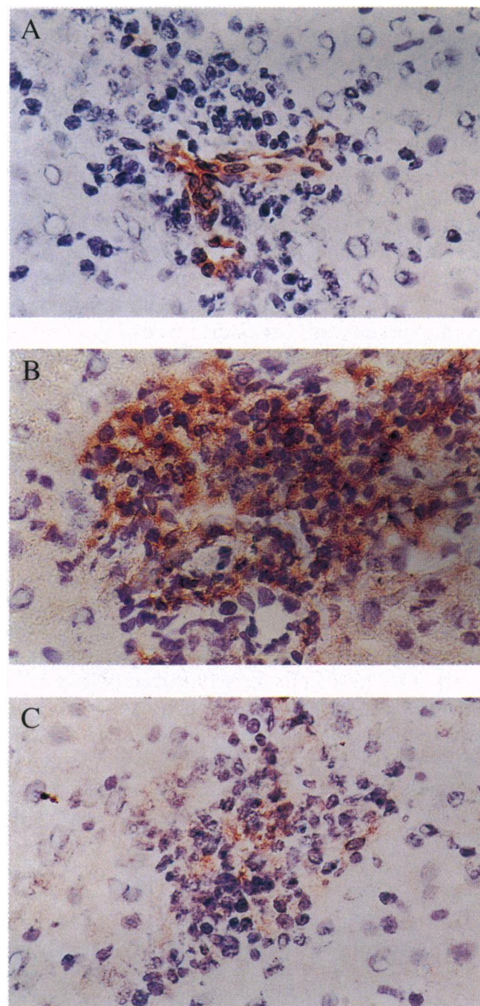

FIG. 2. Immunohistochemistry from liver specimen obtained from a cholangiocyte-immunized rat. (×120.) (*A*) Anti-CK19 antibody (BA17) staining shows that the interlobular bile duct is surrounded by inflammatory infiltrates. (*B*) Anti-CD3 (IF4) staining shows infiltrates consist primarily of T cells. (*C*) Some of these infiltrates are positive for MHC class II protein, suggesting the presence of activated T cells.

Serum Enzyme Assays. Serial levels of serum liver enzymes, including alkaline phosphatase, alanine aminotransferase, and γGT, and total bilirubin did not show any significant change throughout the experimental period in any immunization group (data not shown).

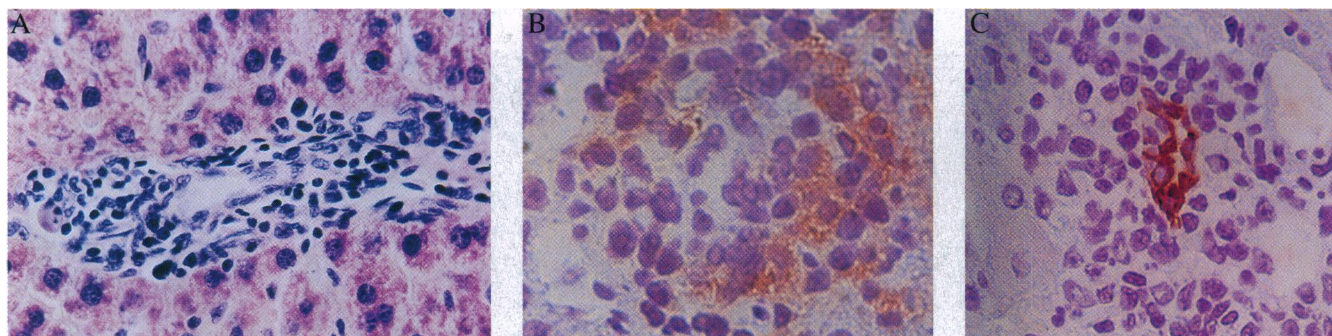

FIG. 3. Histology and immunohistochemistry from adoptive transfer experiments. (*A*) Liver from rat transferred with cholangiocyte-immunized splenic lymphocytes. Note mononuclear cells are causing cholangitis around interlobular bile duct. (Hematoxylin and eosin; ×120.) (*B* and *C*) Anti-CD3 (IF4) (*B*) and anti-CK19 (BA17) (*C*) staining from same animal. (×120.) These serial sections show that the bile duct is surrounded by CD3-positive T cells.

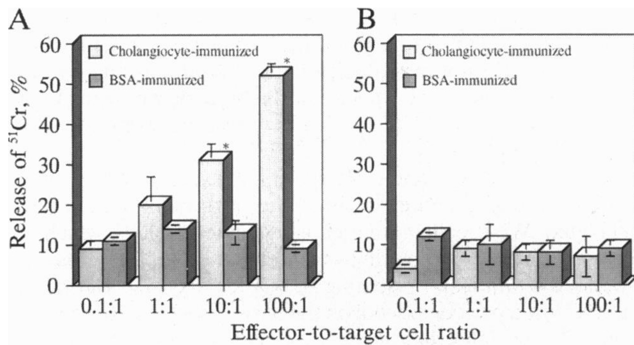

FIG. 4. *In vitro* cytotoxicity assays. Results are mean ± SD; $n = 3$. (*A*) Syngeneic ^{51}Cr-labeled cultured cholangiocytes were used as target cells, and splenic lymphocytes from cholangiocyte-immunized rats or BSA-immunized rats were mixed in various effector-to-target cell ratios. *, Splenic lymphocytes from cholangiocyte-immunized rats show significantly higher cytotoxicity compared with that of BSA-immunized rats ($P < 0.05$). (*B*) ^{51}Cr-labeled hepatocyte cell line as target cells. Splenic lymphocytes from neither cholangiocyte-immunized nor BSA-immunized animals show cytotoxicity.

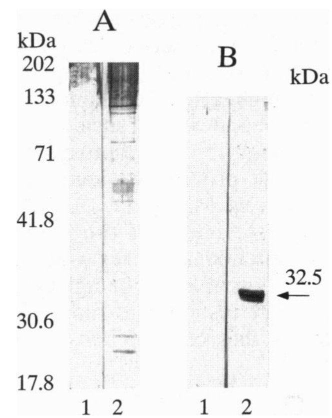

FIG. 6. (*A*) Immunoblots for detecting crude cholangiocyte protein by using pooled sera from preimmune animals (lane 1) and those from cholangiocyte-immunized animals (lane 2). While equal amounts (20 μg) of protein extracted from hyperplastic cholangiocytes were loaded and identical dilutions of sera (1:100) were used, only immune sera show several reactive proteins, ranging from 19 to 120 kDa. (*B*) Immunoblots for detecting CA-II. Human CA-II (5 μg) was added prior to electrophoresis and immunoblotting. Immune sera (lane 2), but not preimmune sera (lane 1), showed reaction product of the expected molecular size (arrow).

DISCUSSION

We describe and characterize an animal model of immune cholangitis with similarities to several immune-mediated cholangiopathies. Following immunization with rat cholangiocytes, inflammation developed in the liver in an organ-specific fashion and consisted of mononuclear cells, composed predominantly of T lymphocytes, infiltrating portal tracts and surrounding bile ducts. Furthermore, adoptive transfer of unfractionated Con-A-stimulated spleen cells from cholangiocyte-immunized rats into naive recipients produced a cholangitis similar to that observed after primary immunization. Splenocytes from rats immunized with cholangiocytes showed selective and dose-dependent *in vitro* cytotoxicity against cholangiocytes. Immunization with rat cholangiocytes also led to the development of cholangiocyte-specific antibodies in the serum of immunized animals. Finally, immunization with cholangiocytes caused cholangiographic evidence of distortion and tortuosity of the intrahepatic biliary ductal system. These results establish and provide initial characterization of a rodent model of immune-mediated cholangitis.

Immunization with cholangiocytes leads to portal tract inflammation predominantly composed of T lymphocytes; B cells, neutrophils, and macrophages appear to be absent. These results suggest that the T-cell infiltrates are reacting to cholangiocyte antigens. Two sets of experiments support this idea. First, mitogen-activated splenocytes from cholangiocyte-immunized animals, but not from control-immunized animals, caused specific, dose-dependent cytolysis of primary cultures of normal rat cholangiocytes, but not rat hepatocytes. Of relevance, others have demonstrated that splenocytes from patients with PBC but not from healthy controls have cytotoxic activity against cholangiocytes (15). Second, the ability to demonstrate portal tract inflammation following the adoptive transfer of mitogen-stimulated splenocytes from cholangiocyte-immunized animals into naive recipients supports the cellular immune reactivity against cholangiocytes. Aberrant MHC class II expression by bile duct epithelial cells is well described in human cholangiopathies (3). While MHC class II expression by biliary epithelia was not observed in the model, the expression of MHC class II antigen by cells infiltrating the portal tracts is consistent with the possibility that they are activated T cells. The presence of T-cell-predominant infiltrates in the portal tracts of patients with PBC is well recognized (16).

Not surprisingly, immunization with cholangiocytes also led to the development of serum antibodies which react against several cholangiocyte proteins. The identities of these antigens are unknown; however, one of these antibodies appears to be against CA-II, which has been reported to be present in the sera of patients with autoimmune diseases (17). More recently, it has been shown that, in patients with immune-mediated liver diseases (18), CA-II is expressed only by cholangiocytes in liver (19). However, the significance of as well as the mechanisms involved in the production of these antibodies following cholangiocyte immunization is unclear.

Several features observed in our model of rodent immune cholangitis contrast with abnormalities seen in the human immune cholangiopathies. First, the inflammation observed in our model is self-limited. In addition, the portal tract inflam-

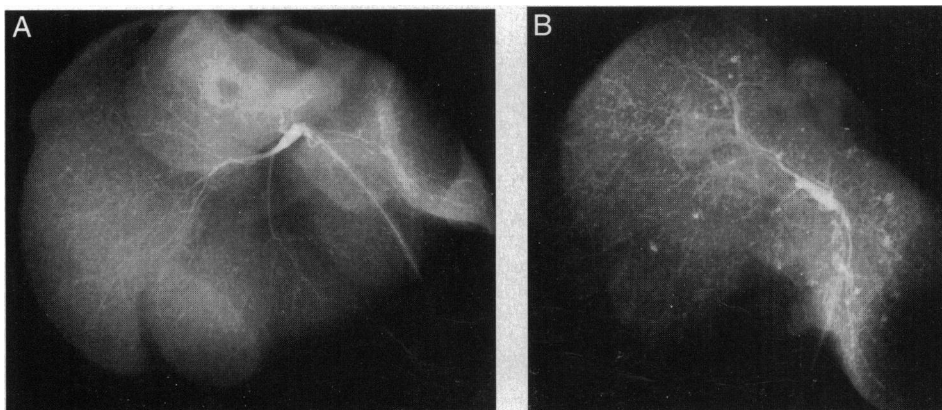

FIG. 5. Representative cholangiograms of BSA-immunized (*A*) and cholangiocyte-immunized (*B*) rats. The BSA-immunized rat shows a normal-appearing intrahepatic biliary tree. The cholangiocyte-immunized rat shows tortuous intrahepatic ducts and localized areas of ectasia.

mation is mild to moderate, and no enzyme abnormalities were observed. These results likely reflect several variables, including the immunization scheme. The limited nature of the immune cholangitis might also be explained by the presence of a negative-feedback phenomenon, such as clonal elimination of autoreactive lymphocytes or apoptosis, the lack of which might account for the progressive immune cholangitis observed in human diseases. Manipulation of the immunization regimen—including assessment of other sources of T cells (e.g., mesenteric lymph nodes or liver)—and further purification and characterization of key cholangiocyte antigens might alter the inflammatory response to more closely mimic the destructive processes that characterize the human immune cholangiopathies.

Other animal models of human autoimmune disease, such as experimental allergic encephalomyelitis (EAE) and arthritis induced by type-II collagen, have similarities with our immune cholangitis model. Experimental animals develop organ-specific inflammation consisting predominantly of T lymphocytes following active immunization with exogenous proteins. In both models, antigen-specific T-cell clones have been established and T cells have been proven to play a central role in the development of disease-specific inflammation. Also, adoptive transfer of the inflammation and direct cytotoxicity toward the target cells has been reported in EAE (20).

Initially, animals were immunized with allogeneic cholangiocytes to maximize the host immunological response. Since normal cholangiocytes express MHC class I, but not MHC class II, molecules, we were surprised that a greater degree of portal tract inflammation was not observed with allogeneic than with syngeneic immunization. This observation suggests that our immunization protocol breaks tolerance to self-cholangiocytes.

Several other animal models of cholangitis have been reported. For example, cholangitis was induced after surgical construction of self-filling blind loops of intestine, which leads to bacterial overgrowth (12). The findings support an association between intestinal inflammation and hepatobiliary diseases, and they may help to explain why the vast majority of patients with PSC have associated inflammatory bowel disease (3). However, an immunologically mediated mechanism seems unlikely because immunosuppressive agents (e.g., cyclosporin A, methotrexate, and prednisone) fail to prevent hepatobiliary injury in this model (21). Moreover, the portal tract infiltrate in this model includes neutrophils, and it has not been characterized further. Another model of cholangitis is the murine model of GVHD (22). Although this rodent model clearly mimics human GVHD and leads to T-cell-predominant portal tract infiltrates, the relationship between this inflammation and the immune cholangitis observed in other cholangiopathies, such as PBC, is still speculative. Moreover, the inflammatory infiltrate which characterizes the model of GVHD is the product, not of active immunization with cholangiocytes, but of seeding irradiated mice with allogeneic lymphocytes. Nevertheless, this model supports the concept that an alteration in the immune system of rodents can result in cholangitis. Two reports have previously described the induction of cholangitis in animals after injection of potential biliary antigens derived from crude rat liver (23) or rabbit gallbladder (24) homogenates. These models have not been studied further, possibly because they were poorly reproducible, and/or the putative antigens were not derived from purified cholangiocytes; moreover, the nature of the cellular infiltrates was not fully defined. More recently, a model of autoimmune cholangitis in neonatally thymectomized mice stimulated with biliary antigens derived from partially purified porcine intrahepatic bile duct cells was reported (25). In these last three reports (23–25), investigators did not perform adoptive transfer experiments and did not characterize the specificity and direct cytotoxicity of the lymphocytes purported to be involved in the inflammatory liver infiltrate, since techniques for primary culture of rodent cholangiocytes as target cells were not available to them. By establishing primary cultures of rat cholangiocytes in our laboratory, we were able to utilize a unique tool to directly demonstrate cytotoxicity by sensitized lymphocytes.

The specific details and molecular mechanisms involved in the development of immune cholangitis in our model remain to be elucidated. We speculate that cholangiocyte antigens, which are normally sequestered within intracellular compartments, are processed by antigen-presenting cells after exposure during the immunization period; indeed, on the basis of recent data from our group (26), we infer that the cholangiocyte itself has the capacity to present exogenous antigens. Presentation of such antigens in conjunction with MHC class II proteins to CD4-positive T lymphocytes could initiate release of cytokines, leading to antibody production and the recruitment of cytotoxic T lymphocytes to biliary epithelia. While this proposed sequence of events is currently hypothetical, the availability of a reproducible model of immune cholangitis such as described by us will allow the experimental dissection of this paradigm.

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