α-Enolase Autoantibodies Cross-Reactive to Viral Proteins in a Mouse Model of Biliary Atresia

BRANDY R. LU,* STEPHEN M. BRINDLEY,‡ REBECCA M. TUCKER,‡ CHERIE L. LAMBERT,‡ and CARA L. MACK*,‡

*Section of Pediatric Gastroenterology, Hepatology, and Nutrition, The Children's Hospital, University of Colorado Denver School of Medicine, Aurora; and ‡Section of Allergy and Clinical Immunology, University of Colorado Denver School of Medicine, Aurora, Colorado

See editorial on page 1461.

BACKGROUND & AIMS: Biliary atresia (BA) is a neonatal cholangiopathy of unknown etiology. The bile duct injury that occurs in patients with BA might result from a hepatobiliary viral infection followed by an autoimmune response against the bile duct epithelia. We aimed to identify autoantigens recognized by serum antibodies in the Rhesus rotavirus (RRV)-induced mouse model of BA; findings were correlated with BA in humans. **METHODS:** Bile duct epithelial proteins were screened for their reactivity with serum antibodies from the mouse model of BA using immunoblot assays. Unique proteins that reacted with sera antibodies were identified by mass spectrometry and verified using enzyme-linked immunosorbent assay (ELISA) and immunoblot analyses. Candidate autoantibodies in BA patient sera were analyzed by ELISA. **RESULTS:** A bile duct epithelial antigen that reacted strongly with serum immunoglobulin (Ig) G from the mouse model of BA was identified as α-enolase. α-Enolase autoantibody specificity was confirmed by ELISA and immunoblot analyses. Anti-RRV and anti-enolase antibodies cross-reacted with enolase and RRV proteins; we identified regions of sequence homology between RRV and enolase. Serum samples from patients with BA had increased levels of anti-enolase IgM and IgG. **CONCLUSIONS: We have identified autoantibodies against α-enolase in a mouse model of BA (infected with RRV) and in serum samples from patients, indicating a role of humoral autoimmunity in disease pathogenesis. The cross-reactivity between an anti-enolase antibody and RRV proteins indicates that molecular mimicry might activate humoral autoimmunity in BA patients; further studies are required.**

Keywords: Neonatal Immunity; Neonatal Cholestasis; Biliary Obstruction.

Biliary atresia (BA) is a progressive, inflammatory cholangiopathy characterized by periductal inflammation with fibrosis and bile duct obstruction culminating in cirrhosis and the need for pediatric liver transplantation in the majority of patients.[1–4] The proposed pathogenesis of BA involves an initial perinatal hepatobiliary viral infection followed by a cellular and humoral autoimmune-mediated response targeting bile duct epithelia.[5] To test this hypothesis, work has focused on the Rhesus group A rotavirus (RRV)-induced murine model of BA. Newborn BALB/c mice infected with RRV develop hyperbilirubinemia, acholic stools, and progressive inflammation and obstruction of the extrahepatic bile duct by 2 weeks of age.[6] Despite documented clearance of the virus from the liver, the inflammatory injury of the bile ducts continues until death from malnutrition and liver failure by 3 to 4 weeks of life.[6–8]

Support for an autoimmune-mediated etiology derives from 2 recent studies in this murine model that identified autoreactive T cells specific to bile duct epithelia.[9,10] In these studies, adoptive transfer of hepatic T cells from BA mice into naïve immunodeficient recipients produced bile duct specific inflammation and injury. In humans, oligoclonal expansion of $CD4^+$ and $CD8^+$ T cells was identified in the livers of BA patients, suggesting an antigen-driven cellular immune response.[11] However, little is known about the potential role of humoral autoimmunity in BA. Previous studies of murine and human BA have detected immunoglobulin deposits colocalizing and surrounding bile duct epithelia.[9,12] The aim of this study was to investigate serum autoantibodies in BA to identify potential bile duct epithelial protein targets of the antibodies in murine BA and to correlate these findings with human BA.

Materials and Methods

RRV-Induced Murine Model of BA

All animals received humane care in accordance with criteria from the National Institutes of Health

Abbreviations used in this paper: ALT, alanine aminotransferase; BA, biliary atresia; BLASTp, basic local alignment search tool for proteins; BSS, Hank's balanced salt solution; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; Ig, immunoglobulin; MOWSE, molecular weight search; NMC, normal mouse cholangiocyte; RRV, Rhesus rotavirus; VP, viral protein.

0016-5085/$36.00
doi:10.1053/j.gastro.2010.07.042

"Guide for Care and Use of Laboratory Animals" (publication No. 86-25 1985). Timed-pregnant female BALB/c mice were purchased from rotavirus-free colonies of Jackson Laboratory (Bar Harbor, ME). Newborn pups were injected with either Hank's balanced salt solution (BSS controls) or RRV as previously described.[7] Animals were sacrificed on day 14 of life, and livers and blood were collected at the time of death for analysis. Because of the small quantity of sera from each pup, sera were pooled from an average of 5–10 pups.

Immunofluorescence

Chamber slides (Nalge Nunc Int, Naperville, IL) were plated to confluency with either the BALB/c normal mouse cholangiocyte (NMC) cell line (provided courtesy of Yoshiyuki Ueno, MD, Tohoku University School of Medicine, Sendai, Japan) or the human cholangiocyte cell line (provided courtesy of Gregory Gores, Mayo Clinic, Rochester, MN). The cells were fixed with 2% paraformaldehyde, bathed in 6% hydrogen peroxide (to remove endogenous peroxidases), and blocked with Fc gamma receptor antibody (BD Biosciences, Rockville, MD). Confirmation of bile duct epithelial nature was determined with anti-cytokeratin 19-AlexaFlor555[13] for murine studies, and anti-cytokeratin 7 antibody (Dako Cytomation, Glostrup, Denmark) for human studies. For serum immunoglobulin (Ig) G studies, pooled murine BA or BSS sera (1:100 dilution) or individual human BA or control sera (n = 5 each) was added to appropriate cholangiocyte slides followed by goat anti-mouse IgG-fluorescein isothiocyanate (FITC) or mouse anti-human IgG-FITC, respectively (Sigma-Aldrich, St. Louis, MO) and the FITC signal enhanced with the Tyramide Signal Amplification fluorescein system (PerkinElmer, Boston, MA). Cells were counterstained with Hoechst dye and visualized using the Olympus 1X81 inverted motorized microscope (Center Valley, PA). Digital photographs were obtained with the Hamamatsu ORCA IIER monochromatic CCD camera (Bridgewater, NJ) with identical exposure times for all samples.

Western Immunoblot Analysis

The cultured NMC cell line was separated into the cytosolic and membrane components using the Ready-Prep Protein Extraction Kit (Bio-Rad, Hercules, CA), and protein concentrations were determined with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Cytosolic NMC proteins, cesium gradient-purified RRV (courtesy of Harry Greenberg, MD, Stanford University, CA), purified rabbit muscle enolase (Sigma-Aldrich; high isoform homology with α-enolase[14]), or control ovalbumin (Sigma-Aldrich) was separated onto a NuPAGE Bis-Tris acrylamide gel (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with powdered milk, and diluted sera (1:100) from BA or BSS mice was incubated with the membrane, followed by goat-anti-mouse IgG-peroxidase (KPL, Gaithersburg, MD). The membranes were developed with the Western Lightning Reagent (Millipore, Billerica, MA) and exposed to autoradiographic film. Western blots were repeated 5 times with different pools of sera. For anti-RRV reactivity studies, commercially prepared sheep antiserum to RRV (AbD Serotec, Oxford, UK) was affinity purified in the following manner. Cesium gradient purified RRV was coupled to agarose per manufacturer's protocol (MicroLink Protein Coupling Kit; Pierce Biotechnology), RRV antiserum was bound to the column, and purified anti-RRV antibody was eluted. For some studies, affinity-purified polyclonal anti-enolase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was utilized. Other controls included mouse anti-vimentin antibody (NeoMarkers, Fremont, CA) and goat anti-actin antibody (Santa Cruz Biotechnology).

Mass Spectrometry Analysis

Candidate protein bands identified by Western blot were stained with Bio-safe Coomassie (Bio-Rad), excised, trypsin digested, and prepared for mass spectrometry analysis using standard techniques.[15] Samples were loaded into a Finnigan Deca LCQ liquid chromatography-tandem mass spectrometry, and results were analyzed with the Mascot search engine. Mascot is a proprietary identification program available from Matrix Science (Boston, MA). It performs mass spectrometry data analysis through a statistical evaluation of matches between observed and projected peptide fragments. Probability-based molecular weight search (MOWSE) scores >33 and peptide coverage $>20\%$ were considered significant.[16] Amino acid homology between RRV and murine α-enolase was assessed by basic local alignment search tool for proteins (BLASTp) search. Protein crystal structures were obtained from the Research Collaboratory for Structural Bioinformatics protein data bank.

Enzyme-Linked Immunosorbent Assay

Proteins were plated onto a Immulon 2B flat bottom microtiter plate (Thermo, Milford, MA), blocked with enzyme-linked immunosorbent assay (ELISA) diluent solution (eBioscience, San Diego, CA), incubated with goat anti-enolase antibody (Santa Cruz Biotechnology) at increasing dilutions, followed by donkey anti-goat IgG peroxidase to generate a standard curve (each dilution was assigned an arbitrary number in units/milliliters). For serum studies, total IgG levels were measured, and equal amounts of total IgG were compared between groups. Serum control groups included BSS; noncholestatic, RRV infected siblings of BA mice; mothers of BA mice; and cholestatic adult mice that had undergone bile duct ligation (courtesy of R. Sokol, University of Colorado Denver, Aurora, CO). A secondary anti-mouse IgG peroxidase was added followed by tetramethylbenzidine