Ae2$_{a,b}$-Deficient Mice Develop Antimitochondrial Antibodies and Other Features Resembling Primary Biliary Cirrhosis

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Background & Aims: **Cl^-/HCO_3^- anion exchanger 2 (AE2) is involved in intracellular pH (pH_i) regulation and transepithelial acid-base transport, including secretin-stimulated biliary bicarbonate excretion. *AE2* gene expression was found to be reduced in liver biopsy specimens and blood mononuclear cells from patients with primary biliary cirrhosis (PBC), a disease characterized by chronic nonsuppurative cholangitis associated with antimitochondrial antibodies (AMA) and other autoimmune phenomena. In mice with widespread *Ae2* gene disruption, we previously reported altered spermiogenesis and reduced gastric acid secretion. We now describe the hepatobiliary and immunologic changes observed in these *Ae2*$_{a.b}$-deficient mice.** ***Methods:*** **In this murine model, splenocyte pH_i and T-cell populations were studied by flow cytometry. CD3-stimulated cytokine secretion was estimated using cytokine arrays. AMA were evaluated by immunoblotting and proteomics. Hepatobiliary changes were assessed by immunohistopathology, flow cytometry, and serum biochemistry. Cholangiocyte gene expression was analyzed by real-time polymerase chain reaction.** ***Results:*** ***Ae2*$_{a,b}^{-/-}$ mice exhibit splenomegaly, elevated pH_i in splenocytes, increased production of interleukin-12p70 and interferon gamma, expanded $CD8^+$ T-cell population, and under represented $CD4^+FoxP3^+$/regulatory T cells. Most *Ae2*$_{a,b}^{-/-}$ mice tested positively for AMA, showing increased serum levels of immunoglobulin M and G, and liver-specific alkaline phosphatase. About one third of *Ae2*$_{a,b}^{-/-}$ mice had extensive portal inflammation with $CD8^+$ and $CD4^+$ T lymphocytes surrounding damaged bile ducts. Cholangiocytes isolated from *Ae2*$_{a,b}^{-/-}$ mice showed gene expression changes compatible with oxidative stress and increased antigen presentation.** ***Conclusions:*** **Ae2 deficiency alters pH_i homeostasis in immunocytes and gene expression profile in cholangiocytes, leading to immunologic and hepatobiliary changes that resemble PBC.**

Cl^-/HCO_3^- anion exchanger 2 (AE2; also known as SLC4A2) mediates electroneutral Na^+-independent Cl^-/HCO_3^- exchange across the plasma membrane.[1,2] This widely expressed exchanger is a relevant acid loader involved in the regulation of intracellular pH (pH_i).[1,2] Moreover, AE2 is involved in transepithelial acid-base transport, including proton gastric secretion[3,4] and secretin-stimulated biliary HCO_3^- excretion.[5]

Alkalinization of pH_i in lymphocytes has been reported to affect their proliferation, differentiation, and activation status.[6] Because AE2 activity might influence acid-base equilibrium and cell function, not only in lymphocytes but also in biliary epithelial cells, alterations of this transporter were postulated to play an etiopathogenic role in primary biliary cirrhosis (PBC).[7–9] PBC is an autoimmune disease of unknown pathogenesis that results in destruction of small- and medium-sized bile ducts.[10,11] The disease occurs mainly in middle-aged women and is characterized by portal inflammation surrounding interlobular bile ducts and the presence of antimitochondrial antibodies (AMA). Indeed, up to 90%–95% of patients develop AMA against some components of the 2-oxo dehydrogenase complexes, mainly the inner lipoyl domain in the E2 component of the pyruvate dehydrogenase complex (PDC-E2).[12] Although AMA might not be involved in the pathogenesis of the disease, T-cell immune responses against PDC-E2 determinants seem to be implicated in organ damage.[11,13]

Despite the strong association with autoimmune phenomena, PBC responds poorly to classic immunosuppressants. The fact that ursodeoxycholic acid, a bile acid that induces bicarbonate-rich choleresis, may improve the clinical course of PBC[14] is compatible with the concept that defective Cl^-/HCO_3^- exchange may play a role in the pathogenesis of the disease.[9,15] Consistent with this view, *AE2* gene expression has been reported to be

Abbreviations used in this paper: AE2, Cl^-/HCO_3^- anion exchanger 2; AMA, antimitochondrial antibodies; FACS, fluorescence-activated cell sorter; hALP, hepatic alkaline phosphatase; IL, interleukin; PBC, primary biliary cirrhosis; PCR, polymerase chain reaction; PDC-E2, E2 component of the pyruvate dehydrogenase complex; pH_i, intracellular pH; Q-TOF/MS, quadrupole/time-of-flight mass spectrometry; Treg, regulatory T cells.

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reduced in liver biopsy specimens and blood mononuclear cells from patients with PBC.[7,8]

Although regulation of pH_i appears to be important to modulate lymphocyte function, there is no information on the consequences of altered Cl^-/HCO_3^- exchange activity on the homeostasis of the immune system. Similarly, it is not known whether defective AE2 function may be damaging for bile duct cells. Previously we had generated $Ae2_{a,b}^{-/-}$ mice with a widespread disrupted expression of the 3 major Ae2 variant isoforms Ae2a, Ae2b1, and Ae2b2.[16] Early in life, these mice show increased perinatal mortality and male sterility due to azoospermia,[16] as well as impaired stimulated gastric acid secretion,[4] growth retardation, bone abnormalities, and deafness (see Recalde et al[4]). We now describe that mature/elderly $Ae2_{a,b}^{-/-}$ mice show immunologic and hepatobiliary features similar to those found in PBC. Our present data therefore indicate that the universal deficiency of Ae2 function in $Ae2_{a,b}^{-/-}$ mice may eventually disturb the immune system and the biliary epithelium besides other tissues, predisposing the animals to develop a PBC-like syndrome in addition to the previously documented PBC-unrelated phenotypic alterations.

Materials and Methods

Mice

$Ae2_{a,b}$-targeted mice were generated as described.[16] Most experimental animals were bred in heterozygous couples against an FVB/N background. Confirmatory experiments were also performed in mice bred in heterozygous couples against different backgrounds (129/Sv, Balb/c, and SJL). Breeding was performed at the CIMA animal facility (University of Navarra). Each $Ae2_{a,b}^{-/-}$ mouse was housed together with respective control littermates of the same sex in a separated wire mesh cage. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Biochemical Monitoring of Mouse Serum

Blood was extracted from anesthetized mice at 6, 12, and 15 months of age, and serum aliquots were stored at −20°C for simultaneous analysis. Serum levels of alanine aminotransferase and total alkaline phosphatase were determined in a Hitachi 911 analyzer (Roche/Hitachi, Indianapolis, IN), including a normalizing external control. Total alkaline phosphatase values were corrected for the hepatic fraction of alkaline phosphatase (hALP) by determining in each mouse the relative levels of hALP after its separation from the bone-derived alkaline phosphatase by automated gel electrophoresis in Hydragel ISO-PAL agarose gel in the Hydrasys system (Sebia, Norcross, GA). For visualization and densitometric analysis of separated bands, we used a Hyrys-2 densitometer (Sebia). Total immunoglobulin (Ig) M and IgG levels were measured with mouse enzyme-linked immunosorbent assay kits (Assay-Designs, Ann Arbor, MI, and Bethyl Laboratories, Montgomery, TX, respectively).

Detection of AMA Against Mouse PDC-E2

After preparation of PDC extracts from mouse hearts as described,[17] extract proteins were electrophoresed (10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis; 15 μg/lane) and electrotransferred to nitrocellulose membranes. Blocked membrane strips were incubated with 1:3000 diluted mouse sera (room temperature, 1 hour) and washed and incubated with 1:10,000 diluted peroxidase-labeled goat anti-mouse IgG plus IgM antibodies (Pierce, Rockford, IL). Once Western Lightning Chemiluminescence Reagent (Perkin Elmer, Boston, MA) was added, chemiluminescent bands were detected with an ImageQuant ECL system (GE Healthcare, Buckinghamshire, England). For quadrupole/time-of-flight mass spectrometry (Q-TOF/MS) of the immunoreactive PDC band, extract aliquots were loaded in parallel gels (10% sodium dodecyl sulfate/polyacrylamide gel electrophoresis): one for band visualization with SimplyBlue Safestain (Invitrogen, Carlsbad, CA) and the other for excision of the 65-kilodalton band. After proteolytic digestion and reverse-phase separation of tryptic digests, peptides were analyzed by mass spectrometry followed by data processing with MassLynx 4.0 and ProteinLynx Global Server 2.0 (Waters, Manchester, UK) connected to the Swiss-Prot database.[18]

Recombinant Mouse Peptide With the PBC-Specific PDC-E2 Epitope

We expressed in *Escherichia coli* the mouse PDC-E2 inner lipoyl domain peptide 170–313 (National Center for Biotechnology Information protein database NP_663589), fused to an N-terminal His-Patch thioredoxin sequence and a C-terminal 6xHis tag (total mol wt, 31,340). It contains the 24-aa sequence GDLLAEIETDKATIGFEVQEEGYL, which encompasses the major PBC-specific autoantigens (previously referred to as peptides 167–186, 163–176, and 165–174).[11,13] PDC-E2 complementary DNA fragment (432 base pairs long) produced by polymerase chain reaction (PCR) on mouse liver complementary DNA (see Table 1 for primers used) was subcloned into pBAD/Thio TOPO vector (ThioFusion Expression Kit; Invitrogen) for L-arabinose–induced expression in *E coli*. After fast protein liquid chromatography purification, recombinant peptide was electrophoresed (1 μg/lane) and electrotransferred. Immunoblots with 1:3000 diluted mouse sera and chemiluminescence detection were performed as described previously with mouse PDC extract (see detailed supplementary methodology; supplementary material available online at www.gastrojournal.org). Type determination of AMA was performed by using peroxidase-labeled secondary antibodies against either mouse IgM or IgG (Pierce).