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Molecular mimicry rather than identity breaks T-cell tolerance in the CYP2D6 mouse model for human autoimmune hepatitis

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ABSTRACT

In our novel mouse model for autoimmune hepatitis (AIH), wildtype FVB mice infected with an Adenovirus (Ad) expressing the major AIH autoantigen human cytochrome P450 2D6 (hCYP2D6) show persistent histological and immunological features associated with AIH, including the generation of anti-hCYP2D6 antibodies with an epitope specificity identical to LKM-1 autoantibodies in AIH-patients. Since FVB mice do not express hCYP2D6, the immune response was directed against mouse CYP (mCYP) homologues. Additional expression of hCYP2D6 in transgenic mice resulted in amelioration of the liver disease. In the present study we used the CYP2D6 model to assess why tolerance breakdown and induction of autoimmune liver disease is more efficient if the triggering antigen is similar but not identical to the target autoantigen. We found that in contrast to the specificity and magnitude of anti-hCYP2D6 antibody responses, T-cell responses differ profoundly between wildtype and transgenic mice. Detailed T-cell epitope mapping studies show a robust, antigen-specific T-cell reactivity in FVB mice largely directed against one CD4 and three CD8 epitopes, activating a total of approximately 1% CD4 and 10% CD8 T-cells, respectively, while infected hCYP2D6 mice generated almost no hCYP2D6-specific T-cells. The frequency of hCYP2D6-specific T-cells was approximately 3-fold higher in the liver compared with the spleen. Amino acid sequence comparison revealed that the immunodominant epitopes were located in hCYP2D6-segments of intermediate homology between hCYP2D6 and its mCYP homologues. Our data indicate that self/non-self molecular mimicry, rather than molecular identity, is a prerequisite for breaking T-cell tolerance in the liver.

1. Introduction

Molecular mimicry of host structures by invading pathogens has been suggested as a plausible mechanism for the induction of autoimmune disease. Many associations between pathogen infections and autoimmune diseases have been found and structural similarities between antigenic components of invading pathogens and host structures have been demonstrated [1,2]. Definite proof for molecular mimicry as the causative mechanism for the development of autoimmune disease is difficult to obtain but strong evidence in support of its decisive role has been demonstrated for some autoimmune-mediated diseases such as Guillain-Barré Syndrome [3], primary biliary cirrhosis (PBC) [4] and acute rheumatic fever [5]. Several animal models have been developed based on the concept of molecular mimicry but often use identical rather than similar antigens as triggers and targets. Such an approach was successful in generating animal models for type 1 diabetes (T1D) or rheumatoid arthritis (RA). An example is the rat insulin promoter (RIP)-lymphocytic choriomeningitis virus (LCMV) mouse model for T1D, which uses transgenic mice that express the glycoprotein (GP) or nucleoprotein (NP) of LCMV specifically in the islets of Langerhans. Infection of RIP-LCMV mice with a virus (LCMV) expressing an identical antigen as trigger results in the development of T1D [6].

In contrast, the generation of an animal model for autoimmune liver diseases, such as AIH, is hampered by the immunotolerant state of the liver [7–10]. Attempts to target liver antigens in a similar manner as pancreatic islet antigens usually required additional transfer of antigen-specific T-cells or additional

Abbreviations: Ad, adenovirus; AIH, autoimmune hepatitis; CYP, cytochrome P450; hCYP2D6, human cytochrome P450 2D6; mCYP, mouse Cyp homologues of hCYP2D6 (Cyp2D8, Cyp2D11, Cyp2D22, and Cyp2D26); LKM-1, liver kidney microsomal-1; T1D, type 1 diabetes.

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activation by pro-inflammatory cytokines, indicating that infection with a pathogen carrying a trigger identical with the target autoantigen in the liver *per se* is not sufficient to overcome the unresponsiveness of the immune system [11–14]. Thus, we wanted to investigate whether infection of mice with a trigger that is similar but not identical with the target autoantigen might be more effective.

We have previously demonstrated that infection of wildtype FVB mice with an Adenovirus (Ad) expressing human cytochrome P450 2D6 (hCYP2D6), the major autoantigen in human AIH type 2 (AIH-2) results in an autoimmune-mediated liver damage resembling the human disease [15,16]. Briefly, Ad-hCYP2D6-infected FVB mice developed persistent features characteristic for liver damage associated with AIH, including massive hepatic fibrosis, cellular infiltrations, and focal to confluent necrosis. Importantly, Ad-hCYP2D6-infected FVB mice generated anti-hCYP2D6 antibodies specific for an epitope that is identical to the immunodominant hCYP2D6 epitope recognized by anti-LKM-1 antibodies from AIH-2-patients [15,16]. Interestingly, when transgenic hCYP2D6 mice were infected with Ad-hCYP2D6 the liver damage was reduced and its onset delayed compared to wildtype FVB mice, indicating a certain unresponsiveness to the challenging Ad-hCYP2D6-infection in mice expressing the identical autoantigen. However, only a minor reduction of the anti-hCYP2D6 antibody titre has been detected in hCYP2D6 mice and the fine specificity of antibodies generated in either FVB or hCYP2D6 mice was identical focussing on the same immunodominant epitopes recognized by sera of AIH-patients [15].

Here we performed a detailed T-cell epitope mapping in Ad-hCYP2D6-infected FVB and hCYP2D6 mice. We observed that in contrast to a robust T-cell reactivity directed against several epitopes found in FVB mice, the hCYP2D6-specific T-cell response was almost absent in hCYP2D6 mice. Further, we found that in FVB mice the T-cell reactivity was highest to epitopes with intermediate homology between hCYP2D6 and its mCYP homologues. Our data indicate that pathogen-infection is more likely to induce autoimmunity in a host carrying a similar rather than an identical target antigen.

2. Material & methods

2.1. Mice and virus

All animal experiments have been approved by the local Ethics Animal Review Board, Darmstadt, Germany. hCYP2D6 mice, generated as described elsewhere [17], were kindly provided by Frank Gonzalez (National Institutes of Health, Bethesda, USA). FVB/NHsd mice were from Harlan (Indianapolis, USA). Adenovirus-vector Ad5-hCYP2D6 (Ad-hCYP2D6) was created as described [15]. Adenovirus-vector Ad5-GFP (Ad-GFP) was from Vector BioLabs. Unless described otherwise, mice were injected with 2×10^8 pfu of either Ad-hCYP2D6 or Ad-GFP (i.p. and i.v.). Virus titres were determined by a cytopathic effect assay using serial dilutions of the adenovirus to infect HEK-293 target cells [18].

2.2. Peptides

Sixty-one staggered 20mer peptides overlapping by 12 amino acids (aa) and spanning the full-length hCYP2D6 molecule have been described elsewhere [19]. Mouse peptides 6 (aa44-63), 21 (aa163-183) and 25 (aa196-215) corresponding to the mouse *Cyp* (mCYP) aa sequences obtained from the databank of the National Center for Biotechnology Information (NCBI) (mCYP2D9: NP_034136.2; mCYP2D11: NP_001098001; mCYP2D22: AAH16256; mCYP2D26: AAH23241) were synthesized by GenScript Corporation.

2.3. Isolation of liver lymphocytes

Livers were perfused *in situ* with PBS to remove contaminating blood and then processed as described [16].

2.4. Flow cytometry

For intracellular cytokine stains, splenocytes or liver lymphocytes were stimulated overnight with 2 μg/ml hCYP2D6 peptides in presence of 1 μg/ml Brefeldin A. Cells were stained for surface expression of CD4 and CD8, fixed, permeabilized, and stained for intracellular IFNγ (CD4-FITC, CD8-PerCp/Cy5.5 and IFNγ-PE antibodies were used, BD Biosciences) and acquired using a FACS Canto II (BD Biosciences) as described [16].

2.5. In vivo cytotoxicity assay

Splenocytes of FVB mice were resuspended at 5×10^6 cells/ml and pulsed overnight with 5 μg/ml of the indicated peptides. Cells were then washed two times with RPMI1640 and labelled with CFSE (Sigma) for 10 min at room temperature. For differential CFSE fluorescence intensities, peptide-pulsed cells were labelled at a final concentration of 0.2 μM ($CFSE^{low}$) and un-pulsed cells at 2 μM ($CFSE^{high}$). Cells were washed with RPMI1640 supplemented with 10% FCS two times and resuspended (1.5×10^8 cells/ml). Equal amounts of $CFSE^{high}$ and $CFSE^{low}$ cells were combined and i.v. injected into sex-matched recipients. Specific *in vivo* cytotoxicity was determined by analysis of the differentially labelled target cell populations in the blood by flow cytometry (FACSCanto II, BD Biosciences). The ratio between $CFSE^{low}$ and $CFSE^{high}$ cells 10 min after transfer were used as 100% and all consecutive time points were calculated as percentages of that ratio.

2.6. Histology

Liver tissue was quick-frozen in Tissue-Tek O.C.T. (Sakura Finetek) on dry ice. Seven μm sections were cut, fixed in EtOH at −20 °C and washed in PBS. An avidine-biotin blocking step (Vector laboratories) was included and rat anti-mouse CD3 antibody (BD Biosciences) and biotinylated secondary antibodies (Vector laboratories) were incubated with the sections for 60 min each and colour reaction was obtained by sequential incubation with avidine-peroxidase conjugate (Vector laboratories) and diaminobenzidine–hydrogen peroxide. Sirius Red staining was done by incubating EtOH-fixed cryosections for 1 h in Sirius Red solution containing 0.1% saturated picric acid (Electron Microscopy Sciences). Sections were washed in 2 changes of 0.01 N HCl for 2 min, rinsed in water, dehydrated in 3 changes of absolute EtOH for 1 min each, incubated in 2 changes of xylol and mounted in Roti-Histokit (Roth).

2.7. ELISA

96-well microtiter plates were coated overnight at 4 °C with 0.25 μg/ml recombinant hCYP2D6 (Invitrogen), in 100 mM carbonate-buffer (pH 9.6) and plates were blocked with 2% FCS in PBS for 90 min at room temperature. Sera were added in PBS containing 2% FCS and were incubated for 90 min at 37 °C. Dilution series for each serum started at 1:50 followed by 1:2 dilution. Alkaline-phosphatase-labelled goat anti-mouse IgG antibody (1:2000, Southern Biotech, Birmingham, USA) was added for 90 min and the reaction was developed by addition of ECF substrate (GE Healthcare Bio-Sciences). Fluorescence intensity was determined using a Pharos FX molecular imager (Bio-Rad).