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DNA Vaccination Breaks Tolerance for a Neo-Self Antigen in Liver: A Transgenic Murine Model of Autoimmune Hepatitis

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Understanding the pathogenesis of autoimmune hepatitis requires an animal model in which chronic progressive immune injury develops spontaneously or with minimal manipulations. The new transgenic mouse model proposed in this study is based on the hypothesis that infectious agents have the potential to initiate autoreactivity through molecular mimicry. A transgenic mouse expressing lymphocytic choriomeningitis virus nucleoprotein (NP) in a H-2b background developed liver injury when vaccinated with plasmids expressing NP as an intracellular or a secretory protein. Coinjection of plasmids coding for NP and IL-12 facilitated the induction of a TH1 phenotype as detected by a specific B lymphocyte response characterized by a predominance of IgG2 subclass anti-NP Abs. CTLs activated in peripheral lymphoid organs by DNA vaccination migrated to the periportal and lobular areas of the liver. Their presence was associated with a significant degree of cytolysis, as evidenced by elevated transaminases several weeks after immunization. As activated specific T lymphocytes proliferated in the periphery and caused cytolysis of target cells, this study suggests that autoimmune hepatitis can be triggered by molecular mimicry, and that local injury may not be essential to initiate autoreactivity in the liver. The Journal of Immunology, 2002, 169: 4889–4896.

Knowledge of the homeostatic mechanisms responsible for equilibrium between immune tolerance and autoimmunity is essential for the comprehension of autoimmune disease and for the development of specific therapies. B and T lymphocyte immune responses can be affected by clonal deletion, anergy, and ignorance taking place in central immune organs or in the periphery. The trafficking, fate, and consequences of lymphocyte accumulation in different organs are not well understood. The liver has a particular role in regulation of the immune response. Naive and memory T cells migrate physiologically through the liver and localize preferentially in the perportal areas (1, 2). Similarly, activated T lymphocytes proliferate and colonize the periportal areas (2). In the liver, viral, tumor, and normal self Ags are readily accessible to class I-restricted T lymphocytes (3). Different concentrations of neo-self Ag in the liver lead to tolerance or ignorance, when these Ags are expressed at high or low levels, respectively (4, 5).

Several transgenic murine models of immune related liver diseases have been designed to understand the mechanisms involved in the development of the tissue injury and of the breakdown of tolerance, but these models have many drawbacks. A case in point is the transgenic mouse expressing a short peptide (aa 1–60) of the lymphocytic choriomeningitis virus (LCMV) glycoprotein under the control of the albumin promoter (5). These animals need to be bred with mice that exhibit increased susceptibility to liver disease and require injection of specific T cells before developing a transient elevation of serum transaminase levels (5). An important determinant of the organ-specific immune response is the promoter use for neo-self Ag expression. The albumin promoter is responsible for high levels of neo-self Ag expression in the liver, but also for low levels of expression in the thymus (5). The expression of a neo-self Ag in this central immune organ led to negative selection of most high affinity/avidity self-reactive T cells. The fact that hepatitis can be induced by neonatal thymectomy in mice confirms the importance of T cell selection in this organ (6). Mouse models expressing IFN-γ in the liver or deficient in TGF-β1 have been reported to develop liver tissue injury, thereby showing the significance of regulatory cytokines in pathogenesis of cytolysis (7, 8). TGF-β1-deficient mice developed multiorgan inflammation, including the liver, but only presented necrosis of hepatocytes in a BALB/c genetic background, establishing the importance of genetic susceptibility (8).

Autoimmune diseases have been generated after infection by LCMV in transgenic mice expressing LCMV-nucleoprotein (NP) or glycoprotein in β cells of the islets of Langerhans or their oligodendrocytes (9–12). These transgenic mice did not develop any immunopathology in the absence of the LCMV challenge (9–12). Such experiments showed that molecular mimicry between self peptides and viral proteins can be responsible for initiating and maintaining the autoimmune process. Diabetes and CNS autoimmune disease in transgenic mice were mediated by CD8+ cells, and a critical number of activated CTLs were necessary to induce the disease (9–12).

In view of the limitations of the models studied until now, a new transgenic murine model is presented in this study. A B and T lymphocyte-mediated immune response against a liver neo-self Ag is generated by DNA vaccination. To avoid local modifications produced in the liver by LCMV, a plasmid expressing LCMV-NP was delivered by i.m. injection to transgenic mice. Taking previous experience into consideration, it was elected to produce transgenic mice expressing LCMV-NP in their liver under the control of a liver-specific promoter. In this situation, the transthyretin (TTR) liver-specific minimal enhancer promoter sequence appeared to be...
the appropriate choice (13, 14). LCMV infection produces CD8+ mediated acute hepatitis, showing that it is a hepatotropic virus (15). DNA vaccination protects against LCMV infection, and even more interesting, generates a B and T lymphocyte response similar to that induced by live virus infection. The CTLA-4 extramembrane region was fused to NP in the expression vector, to improve the delivery of the neo-self Ag to APCs and to enhance the immune response. Several months after the vaccination, liver inflammation causing an increase of alanine aminotransferase (ALT) levels was recorded. In the conception of the LCMV transgenic mouse model of autoimmune hepatitis (AIH), at least two factors relevant to the pathogenesis of the human disease were considered: 1) the importance of a particular genetic background (it is known that mice with different background respond to distinct epitopes of LCMV proteins), and 2) the presence of an environmental trigger factor (such as immunization against LCMV proteins or the LCMV infection). The DNA transgene fragment originated from C3H (H-2k) eggs from C3H (H-2k) mice. Genomic DNA was prepared from founder mice and their offspring tail, and screened for the presence of NP DNA by PCR amplification, using specific primers. The 5' primer sequence was 5'-GGT GTA GTA AGG GTT TGG G-3' and the 3' primer sequence was 5'-CCT GCC CCC AAG GAC TGC-3'. Founder mice showing integration of the transgene by PCR amplification and confirmed by Southern blotting were bred with C57BL/6 mice to establish transgenic lines with a H-2b background. Tissue-specific NP expression was proved by RT-PCR amplification of total RNA and indirect immunofluorescence of cryostat sections (see below). β-Actin mRNA was amplified with specific primers and used as control for RT-PCR. Two lines, designated LCMV-NP-S1 and LCMV-NP-S2, were kept for use in future experiments.

DNA vaccination

Plasmids for vaccination were constructed in pRc/CMV (Invitrogen, Carlsbad, CA), with the human CMV promoter for expression in eukaryotic cells. Plasmid pCMV-NP was obtained by introducing NP DNA in the NotI-Apal cloning site of pRc/CMV. The capability of this construct to express NP was tested by transient transfection of HepG2 cells.

cDNA coding for the mouse CTLA-4 extracellular region was obtained by PCR amplification of a fragment of full-length cDNA in pUC9 American Type Culture Collection (ATCC, Manassas, VA). The following primers were used: 5'-GCT CTA CAA CTA GTG AGG CCC TCC CGG CGG-3' and 5'-CCA AGA ATG CAC AGT AGC GCC ATG 3' of the chicken β-globin domain (HS4; kindly offered by G. Felsenfeld, Bethesda, MD) to generate the construct pHS4-TRL-NP-SV40-HS4. The capability of this construct to generate the Apal-NotI site of pCMV. The insertion of CTLA-4 cDNA created a single open reading frame encoding the fusion protein CTLA-4-NP.

Plasmids were propagated in Escherichia coli by standard techniques and purified with a Qiagen Endoffree Plasmid Maxi kit (Qiagen), according to the manufacturer’s instructions. Plasmid preparation purity was confirmed by spectrophotometric analysis and electrophoresis. Six- to 8-wk-old transgenic mice (TTR-NP-S1) were injected under general anesthesia in the internal tibial muscle with 100 μg (50 μl) of plasmid DNA dissolved in saline buffer. The plasmids pRc/CMV, pCMV-NP, or pVR-IL-12 (kindly provided by G. Prad’homme, Montreal, Quebec, Canada) were injected alone, respectively. In addition, two groups each containing six mice were vaccinated with pCMV-NP and pVR-IL-12 or pCMV-CTL-4-NP and pVR-IL-12.

Transgenic mouse model of autoimmune hepatitis (AIH)

Materials and Methods

Transgene construction

Full-length LCMV-NP cDNA (a gift from R. Zinkernagel, Zurich, Switzerland) was subcloned in the BamHI cloning site of pHuescript-KS II (Stratagene, La Jolla, CA), designated as pBS-NP. SV40 t intron and poly(A) sequences were isolated from the pPAP-GLVP (obtained from B. O’Malley, Houston, TX), and subcloned as a 850-bp BamHI-Spl fragment into pBS-NP. The TTR minimal enhancer/promoter region was isolated from pTTR-CAT (kindly offered by R. Costa, Chicago, IL); this 330-bp HindIII fragment was subcloned in the pBS-NP to create pBS-TTR-NP-SV40. Subsequently, the fragment containing TTR-NP-SV40 was inserted between two copies of the 5' element of the chicken β-globin domain (HS4; kindly offered by G. Felsenfeld, Bethesda, MD) to generate the construct pHS4-TTR-NP-SV40-HS4. The capability of this construct to allow NP expression in hepatocytes was tested by transient transfection of HepG2 cells (see below).

Transgenic mice generation

The DNA transgene fragment originated from NosI endonuclease restriction of pBS-HS4-TTR-NP-SV40-HS4. The 7.8-kb DNA obtained was isolated by gel electrophoresis and purified with a QiAgel quick gel electrophoresis kit (Qiagen, Santa Clarita, CA). The transgene was injected in fertilized eggs from C3H (H-2b) × C57BL/6 (H-2b) mice. Genomic DNA was prepared from founder mice and their offspring tail, and screened for the presence of NP DNA by PCR amplification, using specific primers. The 5' primer sequence was 5'-GGT GTA GTA AGG GTT TGG G-3' and the 3' primer sequence was 5'-CCT GCC CCC AAG GAC TGC-3'. Founder mice showing integration of the transgene by PCR amplification and confirmed by Southern blotting were bred with C57/BL/6 mice to establish transgenic lines with a H-2b background. Tissue-specific NP expression was proved by RT-PCR amplification of total RNA and indirect immunofluorescence of cryostat sections (see below). β-Actin mRNA was amplified with specific primers and used as control for RT-PCR. Two lines, designated LCMV-NP-S1 and LCMV-NP-S2, were kept for use in future experiments.

ELISA

NP cDNA was inserted in the pGEX-4T3 (Pharmacia Biotech, Montreal, Quebec, Canada) cloning site to prepare GST-NP fusion protein in isopropyl β-D-thiogalactoside-induced bacterial cultures. The fusion protein was purified using a glutathione Sepharose column (Pharmacia Biotech). The purity of the preparation was assessed by SDS-PAGE, and the protein was quantified by UV spectroscopy with BSA as standard. Microwell plates were coated with 0.2 μg/well of GST-NP fusion protein in 0.1 M NaHCO3, pH 8.6, overnight at 4°C. After blocking with 2% BSA in PBS for 1 h at 37°C, 200 μl of 1/100 dilution of mice sera or standard dilution of mice anti-NP serum with highest titer was added. Mouse sera were obtained before and 1, 2, and 3 mo after vaccination. The presence of anti-NP Ab was revealed by incubation with anti-mouse IgG alkaline phosphatase-conjugated Ab at a dilution of 1/2000 (Bioresearch). Alkaline phosphatase was developed by incubation with p-nitrophenyl phosphate, and the result read at 405 nm. ODs were compared for sera from different groups of vaccinated TTR-NP-S1 mice. An antiserum was considered positive if its specific OD was at least 2.5 times higher than the mean OD of the premunition sera from transgenic and nontransgenic mice. The same technique was applied to establish the IgG subclass. Briefly, mice sera were diluted to 1/50, and second Abs, conjugated anti-IgG1, anti-IgG2a, anti-IgG2b, and IgM alkaline phosphatase (Santa Cruz Biotechnology), were used at a dilution of 1/1000.

CTLs against NP-dominant epitope

EL4 cells, an H-2b lymphoma T cell line (ATCC), served as targets. Briefly, 1 × 104 target cells coated with NP6-myc peptide (FQFQNGQFI) were incubated with serial dilutions of between 1 × 104 and 5 × 108 splenocytes (effector cells) in a final volume of 200 μl. After 5 h of incubation at 37°C, the release of lactate dehydrogenase was measured at 490 nm using the CytoTox 96 assay kit (Promega), according to the manufacturer’s instructions. Percent lysis was calculated by the formula: 100 × (A – B – C)/(D – C), in which A is experimental value (test release), B

In vitro transcription-translation and immunoprecipitation

In vitro transcription-translation of pCMV-CTL-4-NP was undertaken using the RiboMax large scale RNA production system and the rabbit reticulocyte lysate system (Promega, Madison, WI), according to the manufacturer’s instructions. In vitro synthesized and [35S]methionine-labeled CTLA-4-NP fusion protein was immunoprecipitated with rabbit anti-LCMV serum or anti-CTL-4-NP polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA), according to a previously published protocol (16). The immunoprecipitate was analyzed by 10% SDS-PAGE, followed by autoradiography.

Immunohistochemistry and histopathology

Liver samples from transgenic mice were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) and frozen in liquid nitrogen. Cryostat sections of these livers were fixed in acetone at 4°C for 10 min, and then treated by indirect immunofluorescence, as described above.

Transgenic LCMV-NP-S1-vaccinated mice were sacrificed at the fifth month after the last plasmid injection, and their livers were dehydrated, embedded in paraffin, sectioned, and stained with H&E. Semiquantitative grading of liver biopsies was assessed according to previously described numerical score (17, 18).
is spontaneous background signal value from effector cells, C is spontaneous background signal value from target cells, and D is the target maximum signal value. Maximum release and spontaneous release were determined by incubating the cells with lysis solution and culture medium, respectively.

Serum ALT activity
Serum ALT levels were measured in a Beckman-Synchron CX9 apparatus, from blood taken each month after the last plasmid injection.

Statistical analysis
Statistical significance was evaluated by the Student’s t test, and p < 0.05 was considered significant.

Results
Generation of TTR-NP transgenic mice
A murine-specific liver promoter (liver-specific TTR promoter) was fused to cDNA coding for LCMV-NP (Fig. 1A) and injected into fertilized mouse embryos. Before injection, the construct was transiently transfected into HepG2 cells, and NP expression was demonstrated by indirect immunofluorescence (Fig. 1B). In 4 of 21 mice tested, the presence of the transgene was detected by Southern blot analysis (data not shown) and PCR amplification (Fig. 1C). All such mice passed the TTR-NP transgene to their offspring. Specific expression of LCMV-NP in the liver was evaluated by RT-PCR amplification of liver-extracted total RNA and by indirect immunofluorescence of liver cryostat sections (Fig. 2). Transcription of the NP transgene was not detectable by RT-PCR in spleen, thymus, muscle, or kidney (Fig. 2A). Two founder lines, designated TTR-NP-S1 and TTR-NP-S2, showed NP expression in their livers. TTR-NP-S1 was used for further studies described in this work. The NP-dominant epitope differs according to the mouse H-2 haplotype. In the H-2b background, the epitope was located in the C-terminal region of the protein and was constituted of 9 aa. To work on a homozygous H-2b background, TTR-NP-S1 were bred with C57BL/6 to the B-haplotype. In the H-2b background, the epitope was located in the C-terminal region of the protein and was constituted of 9 aa. To work on a homozygous H-2b background, TTR-NP-S1 were bred with C57BL/6 to the fifth generation. The transgenic offspring were genotyped for H-2b by PCR amplification, followed by dot blot hybridization using sequence-specific oligonucleotide probes for H-2b and H-2k. Observation of TTR-NP-S1 for >1 year showed that none of these mice had any sign of spontaneous liver or extrahepatic disease.

Construction of expression vectors and DNA vaccination
Similar immune responses to Ags produced by the host were induced by viral infection and DNA vaccines. For DNA vaccination, two vectors were constructed, using the human CMV promoter and a SV40 poly(A) sequence. One vector coded for NP, and the other for CTLA-4-NP (Fig. 3A), pCMV-NP coded for an intracellular, cytoplasmic form of the LCMV-NP (Fig. 3B). TTR-NP-S1 mice were immunized between the ages of 6 and 8 wk. Vaccination took place every 15 days, for a total of three i.m. injections. The animals were sacrificed 5 mo after the last plasmid injection, at the age of 8 mo, for analysis of serum ALT levels, histology, and cytotoxicity assay.

FIGURE 1. A, Transgene construct. The LCMV-NP transgene was produced by ligating the TTR minimal liver-enhancer/promoter sequence to NP cDNA and SV40 t intron poly(A). The TTR-NP-SV40 construct was flanked by a chromosomal insulator (HS4) for integration site-independent expression. B, Transgene transient expression. The transgene construct was transfected into HepG2 cells, and transient expression was detected by indirect immunofluorescence, as described in Materials and Methods. C, PCR amplification analysis of mouse DNA. The transgene was detected in transgenic mice (Tg S1 and Tg S2) tail DNA by PCR amplification. Negative control (−) and nontransgenic mice (N-Tg) did not show any LCMV-NP-specific products. The pCMV-NP plasmid served as positive control (+). TTR-NP-S1 transgenic mice (Tg S1) were used for additional experiments.

FIGURE 2. Detection of transgene expression in the liver. In A, LCMV-NP expression in the various tissues was assessed by RT-PCR amplification of total RNA extracted from transgenic mice. Two bands were observed, corresponding to the spliced and nonspliced mRNA of NP in the liver from TTR-NP-S1 mice. β-actin mRNA was expressed in all tissues examined. In B, indirect immunofluorescence on liver cryostat sections, using nonimmune serum (a) or anti-NP serum (b), showed the expression of NP in TTR-NP-S1 mice at relatively low levels throughout the liver lobule. L (liver); S (spleen); T (thymus); M (muscle); K (kidney).

FIGURE 3. A, Plasmids used for vaccination. A cytoplasmic and a secreted form of the neo-self Ag LCMV-NP were used to vaccinate TTR-NP-S1 mice. The latter form contains CTLA-4 coding for the extracellular region of this protein. The expression of both genes was under control of the human CMV promoter. B, Transient expression of pCMV-NP. HepG2 cells were transfected with pCMV-NP, and expression of the protein was detected by indirect immunofluorescence. C, Immunization protocol. TTR-NP-S1 mice were immunized between the ages of 6 and 8 wk. Vaccination took place every 15 days, for a total of three i.m. injections. The animals were sacrificed 5 mo after the last plasmid injection, at the age of 8 mo, for analysis of serum ALT levels, histology, and cytotoxicity assay.
mainly cytosolic, protein, and was tested by transient transfection of HepG2 cells, followed by indirect immunofluorescence (Fig. 3B). pCMV-CTLA-4-NP coded for a secretory protein and was tested by in vitro transcription and translation, followed by immunoprecipitation, using anti-NP and anti-CTLA-4 Abs as well as gel electrophoresis (data not shown). TTR-NP-S1 mice were injected between the ages of 6 and 8 wk to obtain an optimal response (see protocol in Fig. 3C). Intracellular and secretory forms of NP encoded by pCMV-NP or pCMV-CTLA-4-NP, respectively, were expressed after i.m. injections, as shown by the production of anti-NP-specific Abs (see below).

Characterization of the B lymphocyte-mediated immune response

Every month after the last injection, serum samples were obtained for quantification of anti-NP Abs against purified NP Ag and compared with preimmune sera. Three groups of mice sera injected with different DNA preparations were compared; two of them were coinjected with a plasmid encoding for IL-12 to enhance IgG production. Sera drawn at different postinjection times were matched. Ab response was significantly higher in mice injected with pCMV-NP compared with pCMV-CTLA-4-NP (mean ± SD; 650 ± 150 vs 300 ± 200, p = 0.0002). In mice immunized with pCMV-CTLA-4-NP, anti-NP Abs were detected at similar levels at 1 and 3 mo after the last injection. In contrast, a maximum level was found at the second month, but did not reach statistical significance. The levels of these Abs were similar during the 3 mo following vaccination with pCMV-NP (Fig. 4A). Immunization did not induce Abs against NP in the sera of transgenic mice vaccinated with pCMV (blank vector) alone or IL-12-expressing plasmid alone (data not shown).

Predominance of the IgG1 subclass is an indication of a Th2-prevailing immune response, whereas predominant IgG2 subclass production reflects a Th1 immune response. The presence of the

FIGURE 4. A, ELISA for anti-NP autoantibodies. IgG anti-NP Abs were detected 1 mo after the last injection, and similar levels were found throughout the study period. Values are expressed as mean ± SD for six mice for each group (n = 6). Mice vaccinated with plasmid expressing the intracellular form of the neo-self Ag LCMV-NP (n = 6) displayed a significantly higher level of anti-NP Abs at 1 and 3 mo after vaccination when compared with mice injected with pCMV-CTLA-4-NP (n = 6). No anti-NP Ab production was detected (data not shown) in both groups of mice receiving pCMV alone (n = 6) or pVR-IL-12 alone (n = 6), *p < 0.001; **p < 0.003. B, IgG subclass of anti-NP Abs. TTR-NP-S1 mice coinjected with pCMV-CTLA-4-NP + pVR-IL-12 did not show any anti-NP Ab of the IgG1 subclass. Statistically significant differences were observed in IgG2a anti-NP production when coinjection of IL-12 with pCMV-NP (n = 6, p = 0.01) or pCMV-CTLA-4-NP (n = 6, p = 0.03) was compared with pCMV-NP alone (n = 4). IgG1 or IgG2b levels were not significantly different between mice immunized with pCMV-NP alone and those that received pCMV-NP plus pVR-IL-12.
local IL-12 production at the site of the immune response led to a
dominant Th1 phenotype. Analysis of the IgG isotype in mice
immunized with different protocols showed that in pCMV-NP-in-
jected animals, coinjection of a plasmid encoding for IL-12 in-
creased the production of IgG2a (Fig. 4B). It is noteworthy that in
sera from pCMV-CTLA-4-NP-injected mice, IgG1 was absent,
leading to the conclusion that in these mice the immune response
was of the Th1 type. In addition, the absence of IgG1 can explain
the lower level of IgG observed with sera from mice injected with
pCMV-CTLA-4-NP, when compared with those injected with
pCMV-NP (Fig. 4A).

Detection of the CTL response against the NP-dominant epitope
CTLs play an important role in immune responses against virally
infected or tumoral cell as well as in tissue attack in autoimmune
diseases. CTL activation is mediated by specific cytokines, and in
our protocol is influenced by coinjection of DNA encoding for
IL-12. Specific cell lysis in the presence of the dominant epitope
was provoked by T cells isolated from the spleen of immunized
mice (Fig. 5). Splenocytes from vaccinated TTR-NP-S1 mice were
harvested at the second or fifth month after the last plasmid injec-
tion and cultured in the presence of NP aa 396–404 synthetic
peptide for 5 days. Clearly, a linear rise in target cell lyses was
observed after incubation with an increasing number of effector
splenocytes, keeping stable the concentration of target cells coated
with the peptide representing the NP-dominant epitope (Fig. 5). T
lymphocytes analyzed in this cytotoxicity assay were from mice
coinjected with pVR-IL-12 and either pCMV-CTLA-4-NP or
pCMV-NP and showing the stronger Th1 immune response.
pCMV-CTLA-4-NP and pCMV-NP induced comparable levels of
CTL response at the second or fifth month after the last plasmid
injection. In contrast, no CTL activity was detected in splenocytes
from naive TTR-NP-S1 mice or immunized with pVR-IL-12 alone
(Fig. 5).

Autoimmune liver disease
The presence of immune-mediated liver disease in the mouse
model was detected by repeated measurements of ALT levels and
microscopic assessment of liver injury.

In TTR-NP-S1 mice immunized with pCMV-NP and IL-12, an
increase of serum ALT was observed at the fifth month after the
last injection. However, in the pCMV-CTLA-4-NP- and the IL-
12-injected mice, ALT elevation was noted at the fourth month
after the third DNA injection (Fig. 6). It is therefore clear that
cytolysis appeared to be an earlier event in mice producing a se-
cretery protein targeted to APCs. In contrast, ALT levels were not
increased in TTR-NP-S1 mice injected with pCMV alone or pVR-IL-12
alone plasmids (Fig. 6).

Histopathology examination of livers at the fifth month after
DNA vaccination showed inflammatory infiltrates in the portal
tracts and within lobules in mice with abnormal levels of serum
ALT. Interface hepatitis was present in the portal tracts (Fig. 7).
Semiquantitative grading score of liver biopsies from pCMV-
CTLA-4-NP- and the IL-12-injected mice was between 9 and 12,
according to Knodell et al. (17). Scarce images of bridging necro-
sis were observed, but no fibrosis was found. Therefore, biopsies
of these mice were considered as showing moderate chronic hepatitis (18). Histology of the liver from all control groups did show neither inflammatory infiltrate nor fibrosis.

Discussion

The development of an autoimmune hepatitis model is an essential step in understanding its pathogenesis. Knowledge of AIH is far behind advances made in the comprehension of other autoimmune diseases such as diabetes, multiple sclerosis, and lupus (9–12, 19), mainly because of the lack of appropriate animal models developing chronic progressive immune injury. The complexity of the regulation of the immune response in the liver and the multiplicity of possible triggers of autoimmunity contribute to the difficulties found in understanding the pathogenesis of AIH. To date, reported transgenic and nontransgenic mice models show some shortcomings, such as the need for repeated Ag injections to induce and sustain liver inflammation (20), the transfer of specific T cells and their activation (5), or the production of toxic products in the hepatocytes (7, 21). The development of a simple new model that will help elude these problems is necessary to identify specific therapeutic strategies.

A transgenic mouse expressing a neo-self Ag in the liver is an attractive model that has already been applied successfully to other autoimmune diseases (9–12). The first problem to solve is the level of expression of any Ag in the liver, and to do so specifically in this organ, by avoiding the simultaneous expression of Ag in immune system central organs. To solve part of the problem, cDNA encoding for the neo-self Ag LCMV-NP was inserted between insulators (HS4). Frequently in the liver, transgenes are located in heterochromatin regions or at sites influenced by neighboring negative regulatory elements, and fail to be expressed. Insulator sequences have been shown to block the suppressive effects of the surrounding chromosome and direct integration site-independent expression of the transgene in mice (22, 23). To target transgene expression in mice specifically to the liver, a 300-bp enhancer/promoter region of TTR was a preferential choice for the expression of a neo-self Ag (13, 14). The TTR enhancer and promoter proximal DNA sequence direct transcription of minigenes in the liver, even in low copy number (13). Taking all these facts into consideration, it is possible that the success of the transgenic mouse model presented in this study is at least partially due to transgene construction.

DNA vaccination induces specific humoral and cellular mediated immune responses in animal models and humans (24, 25). The type of immune response obtained by DNA vaccination is qualitatively the same as that observed after a viral infection (24, 25). This immune response is protective against a persistent or acute, even otherwise lethal infection (26, 27). The strong B and T cell response can be optimized by i.m. injection. In muscle, proteins are rapidly released in the circulation, and their expression is longer than in other tissues. At least three injections of plasmids are necessary to generate CTL responses (28). This methodology allowed a vigorous and durable level of anti-NP Abs and CTL activity to be induced against the NP main epitope in TTR-NP-S1 mice. In TTR-NP-S1 mice injected with DNA coding for CTLA-4-NP fusion protein, liver inflammation was detected earlier than in mice injected with cDNA coding only for NP. When plasmid-coded proteins were secreted and especially when they were targeted to APCs, an increase in the Ab and cellular response was observed (29, 30). This was the case of the fusion protein in which the extracellular part of the CTLA-4 sequence was included, because CTLA-4 is a ligand of B7-1 and B7-2 receptors expressed on the surface of APCs.

Immune responses are influenced quantitatively and qualitatively by cytokines. IL-12 is a 70-kDa protein produced by APCs that boost the B and T cell-mediated response, even in neonates, driving differentiation of T cells toward a Th1 phenotype (28, 31–33). IL-12 acted locally and not systemically through an adjuvant effect to induce Th1 activity (28). Plasmid-producing IL-12 injected with DNAs modified the immune response to a Th1 phenotype and enhanced CTL activity (28). This known property of IL-12 led to the coinjection of a plasmid producing this cytokine with NP or CTLA-4-NP DNAs. The high levels of anti-NP Abs of the IgG2 subclass observed after vaccination with both plasmids in this model were interpreted as proof of the induction of Th1 cells. This particular phenotype was predominant in TTR-NP-S1 mice injected with CTLA-4-NP, showing that targeting of the neo-self Ag to IL-12-activated APCs was the best combination to ensure activation of the Th1 response. Protective immune responses during viral infections as well as pathological responses against self Ags in several autoimmune diseases are of the Th1 type (34–38). Therefore, the activity of this CD4+ pathway could be essential to induce AIH in the TTR-NP-S1 mouse model.

DNA vaccines are relatively easy to prepare, heat stable, and inexpensive to produce. In addition, one vaccine can be produced against multiple pathogens. However, occasionally DNA vaccination in some animal models has produced unexpected effects (39, 40). Clearly, an autoimmune process is triggered by molecular mimicry between vaccine DNA code Ags and mammalian proteins, as was shown in the case of TTR-NP-S1 mice. Moreover, because cross-reactivity between Ags does not always imply sequence similarity, there remains a potential for the development of unexpected immune responses directed toward endogenous protein (41, 42).

Previously described models of autoimmunity have shown similar delays between the induction of a specific immune response and consistent necrosis of the targeted cells (9–12). Following serum ALT levels as a marker of hepatocyte lysis, it was apparent that this process started several weeks after the third plasmid injection, that it was only observed in mice vaccinated with plasmids containing NP cDNA, and that it was found earlier in mice injected with the CTLA-4-NP construct than in mice expressing an intracellular NP form. Altogether these results showed that hepatitis in TTR-NP-S1 vaccinated only with plasmids expressing NP was a

![FIGURE 7. Histological examination of the liver from transgenic mice.](http://www.jimmunol.org/)

a. Liver tissue section from TTR-NP-S1 transgenic mice vaccinated with pCMV alone did not show any inflammatory cells in the portal tract. b. In livers of transgenic mice vaccinated with pCMV-CTLA-4-NP and pVR-IL-12, portal tract, interface, and intralobular lymphocytic infiltration were observed. Enlarged and inflamed portal tract with spikes of inflammation extending into the parenchyma (arrows) is shown. Tissue sections were stained by H&E, and examined by standard optical microscopy at low magnification (×20).
specific event. The delay between the last injection and the increase in serum transaminases was probably due to the time necessary for activated T cells to migrate, divide, and follow avidity maturation in the liver to reach a critical number, as was described in the progression of autoimmune diabetes (43). Moreover, it is not known how many hepatocytes should be lysed to elevate serum ALT above normal limits.

The liver plays a particular role in T cell differentiation in humans and mice. This pathway is activated during infections, malignancies, and autoimmune diseases, acquiring a predominant place in athymic mice (44, 45). Naive and memory T lymphocytes migrate through the liver, showing a preference for the periporal field (2). Activated T lymphocytes proliferate in the periporal tract (2, 46), in which predominance of inflammation is found in chronic hepatitis. In addition, hepatocyte Ags in a HLA class I context are readily accessible to CTLs, mainly because of the lack of basement membranes in hepatic sinusoids and the discontinuity of the endothelial layer (2, 3). Altogether, these previously published results explained why in our model, CTLs specific for NP, activated in the peripheral lymphatic organs by DNA vaccination, could be responsible for tissue injury. Inflammation and cell necrosis were observed in the portal tracts and in the periporal areas, as well as intralobularly, findings consistent with those observed in patients with AIH. It will be particularly interesting in any future work to follow, for a longer time, the evolution of this chronic hepatitis in a TTR-NP-S1 mouse model, and to document the chronicity of the disease and the progressive development of liver fibrosis.

Break of tolerance and ignorance to a self liver Ag are absolutely necessary to induce AIH. The fate of specific T cells in the liver depends on the level of Ag expression (4) and on the simultaneous presence of inflammatory signals (47). High levels of Ag expression would lead to tolerance, and low levels would result in ignorance (4, 5). In the TTR-NP-S1 mouse model, the level of liver NP expression, even if not quantified, was not very high, as shown by a mediocore signal in immunofluorescence screening, and by its light signal detection in liver homogenates tested by Western blotting (data not reported). This level of NP expression probably prevented peripheral anergy or tolerance from developing in TTR-NP-S1 mice. Recently, it was shown that break of tolerance in the liver needs the transfer and strong activation of specific T cells to mice expressing a neo-self Ag under the control of the albumin promoter (5). However, in this model, high affinity T cells were mostly deleted in the thymus, in which low expression of the neo-self Ag was detected (5). In the transgenic mice described in this work, NP expression in the thymus from TTR-NP-S1 mice was not detected. In addition, taking into consideration the specificity of the TTR enhancer/promoter sequence used (48, 49), it could be expected that no major central deletion of specific T cells occurred in these animals. CTL sp. act. against cells presenting the NP main epitope in an H-2b background was found in vaccinated TTR-NP-S1 mice, concurrently with the development of hepatitis. These specific T cells were probably activated in the peripheral lymph nodes by Th1-induced lymphocytes, and migrated through the liver, in which the neo-self Ag peptide H-2Db on the surface of the hepatocytes became the target. Recently, it was shown that T lymphocytes, stimulated in distant lymphoid sites, migrated to the liver, in which they provoke hepatocyte injury (50).

In conclusion, in TTR-NP-S1 mice expressing LCMV-NP in their liver, tolerance to the neo-self Ag was broken by DNA vaccination. The immune response appears to be Th1 mediated, and resulted in high levels of IgG autoantibodies and activated CTLs specific for the neo-self Ag-dominant epitope. This model showed several of the characteristics of AIH, namely elevation of serum ALT, hepatitis characterized by lymphocytic infiltration, and a predominance of Th1 phenotype leading to the presence of autoantibodies and the activation of specific CTLs.

Analysis of the breakdown of tolerance to a liver self Ag and specifically the role of CD4+ (Th1/Th2) and CD8+ lymphocytes into the liver should help to elucidate pathogenic mechanisms leading to tissue destruction. Furthermore, study of different triggering mechanisms (LCMV infection) and the role of cytokines on the chronic tissue injury will orientate the exploration of factors involved in human AIH.

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References


