# Compartmentalization of T Lymphocytes to the Site of Disease: Intrahepatic CD4+ T Cells Specific for the Protein NS4 of Hepatitis C Virus in Patients with Chronic Hepatitis C

By Maria A. Minutello,* Piero Pileri,* Derya Unutmaz,* Stefano Censini,* George Kuo,‡ Michael Houghton,‡ Maurizia R. Brunetto,§ Ferruccio Bonino,§ and Sergio Abrignani*

*From the *Immunobiology Research Institute Siena, Siena 53100, Italy; the ‡Chiron Corporation, Emeryville, California 94608; and the §Division of Gastroenterology, Ospedale Le Molinette, Torino 10126, Italy*

## Summary

The adult liver is an organ without constitutive lymphoid components. Therefore, any intrahepatic T cell found in chronic hepatitis should have migrated to the liver after infection and inflammation. Because of the little information available on the differences between intrahepatic and peripheral T cells, we used recombinant proteins of the hepatitis C virus (HCV) to establish specific T cell lines and clones from liver biopsies of patients with chronic hepatitis C and compared them with those present in peripheral blood mononuclear cells (PBMC). We found that the protein nonstructural 4 (NS4) was able to stimulate CD4+ T cells isolated from liver biopsies, whereas with all the other HCV proteins we consistently failed to establish liver-derived T cell lines from 16 biopsies. We then compared NS4-specific T cell clones obtained on the same day from PBMC and liver of the same patient. We found that the 22 PBMC-derived T cell clones represent, at least, six distinct clonal populations that differ in major histocompatibility complex restriction and response to superantigens, whereas the 27 liver-derived T cell clones appear all identical, as further confirmed by cloning and sequencing of the T cell receptor (TCR) variable and hypervariable regions. Remarkably, none of the PBMC-derived clones has a TCR identical to the liver-derived clone, and even with polymerase chain reaction oligotyping we did not find the liver-derived clonotypic TCR transcript in the PBMC, indicating a preferential intrahepatic localization of these T cells. Functionally, the liver-derived T cells provided help for polyclonal immunoglobulin (Ig)A production by B cells in vitro that is 10-fold more effective than that provided by the PBMC-derived clones, whereas there is no difference in the help provided for IgM and IgG production. Altogether these results demonstrate that the protein NS4 is highly immunogenic for intrahepatic CD4+ T cells primed by HCV in vivo, and that there can be compartmentalization of some NS4-specific CD4+ T cells to the liver of patients with chronic hepatitis C.

Hepatitis C virus (HCV)[1] is an RNA virus responsible for the majority of blood-borne non-A, non-B hepatitis (1), which induces chronic hepatitis in at least half the infected patients (2). At present, it is not known what role the immune response plays in the course of HCV infection. We have previously reported that PBMC from HCV-infected individuals proliferate in response to HCV recombinant proteins and that peripheral responses to the Core protein correlate with a benign course of infection (3). However, the study of T cell responses in the peripheral blood, although critical to the understanding of both the immunogenicity of viral proteins and the immune status of the individual, does not necessarily reflect the pattern of T cell responses present in the liver. Information on phenotype and function of intrahepatic T cells may be relevant to the understanding of the preferential presence, i.e., compartmentalization, of some T cells into the liver and can therefore help in elucidating the immunopathogenesis of chronic hepatitis C. Since the adult liver is an organ without constitutive lymphoid components (4), any intrahepatic T cell found in chronic hepatitis should have migrated to the liver after infection and inflammation. Once in the tissue, T cells can be restimulated by Ag to un-

[1] *Abbreviations used in this paper:* E, envelope; HCV, hepatitis C virus; HS, human serum; NS, nonstructural; SE, staphylococcal enterotoxin; SOD, superoxide dismutase.


J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/07/0017/09 $2.00
Volume 178 July 1993 17–25

dergo further clonal expansion and can exert their effector functions, whether they are cytotoxic or delayed-type hypersensitivity (DTH) responses. Generally, $CD4^+$ T cells are critical to the accomplishment of any effector function of the immune system, since they can act both directly as DTH inducers (5) and may be killer cells (6), and indirectly helping both B lymphocytes to produce neutralizing Ab (7) and $CD8^+$ T cell precursors to mature to effector killer cells (8).

We have studied the specificity and function of $CD4^+$ T lymphocytes infiltrating the liver of patients with chronic hepatitis C. Using recombinant proteins of HCV, we established T cell lines and clones from both liver biopsies and, when possible, the corresponding PBMC. To study T cell compartmentalization, $CD4^+$ T cell clones obtained from PBMC and liver of the same individual and specific for the same HCV protein were compared for MHC restriction, TCR reactivity to bacterial superantigens, lymphokine production, killing ability, and helper activity for Ig production by B cells in vitro. Here we show evidence that some nonstructural 4 (NS4)-specific $CD4^+$ T cells, with a peculiar ability to help IgA production, can compartmentalize to the liver of patients with chronic hepatitis C.

## Materials and Methods

*Patients with Chronic Hepatitis C.* The three patients studied had HCV-specific serum antibodies, detected by two second-generation ELISAs (Abbott Laboratories, North Chicago; and Ortho Diagnostic Systems, Raritan, NJ), specific for the Core, NS3, and NS4 proteins. Two patients (nos. 13 and 14) had ongoing chronic hepatitis demonstrated by elevated serum levels (>40 U/liter) of alanine aminotransferase (ALT) for >1 yr. No. 13 is a 61-yr-old female with a histologic diagnosis of chronic active hepatitis, and no. 14 is a 53-yr-old male with a histologic diagnosis of nonspecific reactive hepatitis. The third study subject is a patient (no. 71) whose chronic hepatitis C went into clinical remission upon IFN-$\alpha$ treatment, as demonstrated by the range values of serum ALT (i.e., 8–14 U/liter) in 12 monthly tests and by the absence of symptoms of liver disease for 12 mo since IFN-$\alpha$ treatment. No. 71 is a 36-yr-old female with a histologic diagnosis of fibrosis.

*HCV Recombinant Proteins.* The HCV proteins Core, envelope 1 (E1), envelope 2 (E2), NS3–4, NS4, and NS5 were expressed as COOH-terminal fusions with human superoxide dismutase (SOD) in yeast ( *Saccharomyces cerevisiae*) using the method described earlier for the expression of the C100–3 HCV-Ag (1) and purified as previously reported (3).

*Cell Cultures.* Culture medium was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 5% human serum (RPMI-HS). For the growth of T cell lines and clones, RPMI-HS was supplemented with 50 U/ml rIL-2 (Cetus Corp., Emeryville, CA).

*Establishment of Liver-derived T Cell Lines.* Liver biopsies (~2-cm length and 1.5-mm diameter), taken from patients who gave informed consent, were put in petri dishes with RPMI-HS and washed vigorously with a Pasteur pipette, to eliminate contaminating blood lymphocytes. They were manually homogenized in glass mortars (Bellco International, Feltham, UK) in the presence of 5 ml RPMI-HS, and mononuclear cells were separated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density-gradient centrifugation, washed twice in RPMI-HS, and counted. Generally, we recovered 3–8 $\times$ $10^4$ mononuclear cells/biopsy. Approximately 6 $\times$ $10^3$ liver-derived mononuclear cells were cultured with $10^5$ irradiated (3,000 rad) autologous PBMC in 0.2 ml of RPMI-HS in 96-well flat-bottomed microplates in the presence of 9 $\mu$g/ml of recombinant HCV proteins. After 7 d, the growing cultures were supplemented with rIL-2 (50 U/ml) and, after an additional 15 d, they were tested in Ag-specific proliferation assays.

*PBMC Proliferation Assay.* Heparinized venous blood drawn from patients who gave informed consent was diluted (vol/vol) in PBS. PBMC were separated by Ficoll-Hypaque density-gradient centrifugation. For the proliferation assays, PBMC (2 $\times$ $10^5$ cells) in 0.2 ml of RPMI-HS were cultured in 96-well flat-bottomed microplates in the presence or absence of HCV recombinant proteins at 1, 3, and 9 $\mu$g/ml final concentrations in triplicate wells. After 6 d 1 $\mu$Ci of [$^3$H]thymidine (sp act, 5 Ci/mmol; Amersham Corp., Amersham, UK) was added in each well and DNA-incorporated radioactivity was measured after an additional 16 h by liquid scintillation counting. The SD was <40% in all cases. Proliferation was considered positive when stimulation index (cpm incorporated in response to antigen/cpm incorporated in the absence of antigen) was $\geq$4.

*Establishment of PBMC-derived T Cell Lines.* PBMC ($10^5$) were cultured in 0.2 ml RPMI-HS in 96-well flat-bottomed microplates in the presence of 9 $\mu$g/ml of recombinant HCV proteins. After 7 d, rIL-2 (30 U/ml) was added and after an additional 15 d cultures were tested in Ag-specific proliferation assays.

*T Cell Line Proliferation Assay.* T cell lines (4 $\times$ $10^4$ cells) were cultured with $10^5$ irradiated (3,000 rad) autologous PBMC in 0.2 ml of RPMI-HS in 96-well flat-bottomed microplates in triplicate wells in the presence or absence of HCV recombinant proteins at 1, 3, and 9 $\mu$g/ml. After 2 d, 1 $\mu$Ci of [$^3$H]thymidine/well was added and DNA-incorporated radioactivity was measured after an additional 16 h by liquid scintillation counting. The data are expressed as mean cpm $\pm$ SD of triplicate wells.

*Establishment of T Cell Clones.* T cell clones were established as previously described (9). Briefly, NS4-specific T cell lines, both from PBMC and liver, were cloned by limiting dilution (0.3 cell/well) in the presence of irradiated (3,000 rad) allogeneic PBMC (5 $\times$ $10^5$/ml), PHA (1 $\mu$g/ml; Wellcome, Dartford, UK), and rIL-2 (100 U/ml) in 20-$\mu$l cultures in Terasaki trays. The T cell clones obtained were screened for their capacity to proliferate in response to the HCV protein used as stimulator. EBV-transformed B (EBV-B) cell lines were obtained as described (9). HLA-DR homozygous EBV-B cell lines were obtained from the European Collection of Animal Cell Cultures (ECACC; Salisbury, UK).

*Proliferation Assay of T Cell Clones.* T cells (2 $\times$ $10^4$) were cultured with irradiated (6,000 rad) EBV-B cells (1.5 $\times$ $10^4$) in 0.2 ml RPMI-HS in 96-well flat-bottomed microplates in triplicate wells in the presence or absence of 1, 3, and 9 $\mu$g/ml of the Ag. After 2 d, 1 $\mu$Ci of [$^3$H]thymidine was added and the radioactivity incorporated was measured after an additional 16 h by liquid scintillation counting. For the proliferative responses to superantigens, experiments were performed as above but antigens were substituted with 1 ng/ml of staphylococcal enterotoxin (SE) A, SEB, SEC1, SEC2, SEC3, SED, SEE, toxic shock syndrome toxin (TSST), and exfoliative toxin (ExT) (Toxin Technology Inc., Sarasota, FL).

*Flow Cytometric Analysis.* The following FITC- or PE-conjugated mAbs, all purchased from Becton Dickinson & Co. (Mountain View, CA), were used in various combinations for double staining: Leu-5b (CD2), Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), Leu-M3 (CD14), Leu-16 (CD20), WT31 (anti-TCR $\alpha/\beta$). Cells (2 $\times$ $10^4$) from liver T cell lines or PBMC cultured for 7 d with HCV proteins were incubated for 30 min at 4°C with

mAbs at 2 μg/ml. Cells were then washed twice with cold PBS and analyzed on a FACStar® flow cytometer (Becton Dickinson & Co.). Blast cells were gated according to forward and side scatter parameters. For the analyses of fresh liver-derived mononuclear cells, ~6 × $10^3$ cells were stained with the same procedure described above.

*TCR Cloning and Sequencing.* Total RNA was isolated from T cell clones according to Chomczynski and Sacchi (10). cDNA synthesis and PCR were carried out as described by Uematsu (11). Briefly, oligo(dT)-primed double-stranded cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus (M-MLV)-derived reverse transcriptase, RNase H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase, followed by incubation with T4 DNA polymerase for blunt-end formation. The blunt-ended cDNA was circularized with T4 DNA ligase in a volume of 10 μl. The ligated material was used as template for the PCR. Amplifications of α and β chains were performed separately in 50-μl reaction mixtures containing primers either for Cα or for Cβ regions (each at 300 nM), 67 mM Tris HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, BSA (100 ng/μl), 2 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, Taq polymerase (0.05 U/μl). The primers used are as follows: Cα forward primer (5'-GGGTCGACGACCTCATGTCTAGCACAGT), Cα inverse primer (5'-GCATGCGGCCGCCCTGCTATGCTGTGTGTCT), Cβ forward primer (5'-GGGTCGACCTGTGCACCTCCTTCCCATT), and Cβ inverse primer (5'-CATGCGGCCGCATGGCCATGGTCAAGAGA). Each forward and inverse primer contains artificial SalI and NotI sites, respectively. After 35 cycles of PCR (denaturation at 94°C for 40 s, annealing at 65°C for 40 s, and extension at 72°C for 60 s), the Klenow fragment of *E. coli* DNA polymerase I was added to ensure full-length DNA synthesis. PCR products were purified by phenol-chloroform extraction, precipitated with ethanol, and digested with restriction endonuclease in excess amounts of SalI and NotI. Fragments of the expected sizes for the cDNAs were separated by preparative agarose gel electrophoresis and purified by adsorption on glass beads. The material was ligated into pBluescript previously digested with SalI and NotI, and the obtained recombinants were used to transform XL1 blue *E. coli* cells. The colonies obtained were screened using a colony hybridization technique and an internal Cα (TCTCTCAGCTGGTACACGGCAGGGTCAGGG) or Cβ (GGACCTGAACAAGGTGTTCCCACCCGAGGT) $^{32}P$-labeled oligonucleotide as probe. Single plaques were picked and grown up, and recombinant phage DNA was purified for DNA sequence determination. Sequencing reactions were done using the Sequenase sequencing system (United States Biochem. Corp., Cleveland, OH).

*TCR Transcript Identification by PCR Oligotyping.* PBMC (3 × $10^6$) were stimulated with NS4 (5 μg/ml) in 15 replicate microcultures (2 × $10^5$ cells/0.2 ml per well) in 96-well flat-bottomed microplates, grown to ~4 × $10^6$ cells, and five pools were made, each of cells from three wells. Each pool was processed as follows: total RNA was extracted and 0.5 μg of it was reverse transcribed. 5% of the cDNA was amplified in 50 μl, using 30 pmol of each primer. For the TCR β chains amplification, the following primers were used: Cβ 5'-GGTGTGGGAGAATTCTGCTTCTGA-3' and Vβ3 5'-CGCTTCTCCCGGATTCTGGAGTCC-3'. The PCR reactions were heated once for 4 min at 94°C, followed by 20 s at 62°C, 2 min at 72°C, and 1 min at 94°C for 35 cycles, followed by a 10-min extension at 72°C. 10 μl of each PCR reaction was fractionated on a 2% agarose gel, alkaly blotted onto N-Hybond nylon membrane, and hybridized overnight at 45°C with $^{32}P$-labeled N region–specific oligonucleotide (5'-GCAGTCCCGGGGGGCCCTATTCAC) in 6× SSC, 6× Denhardt's, and 0.2% SDS. The filters were washed twice in 6× SSC, 0.2% SDS at 45°C for 10 min, and twice at 65°C 2× SSC and 0.2% SDS. Optimal hybridization and washing temperatures were determined in cell mixing experiments in which the PCR oligotyping was performed on scaled numbers of the relevant clone mixed with a constant number of PBMC from unrelated donors.

*PCR-assisted Amplification of Lymphokine mRNA.* Lymphokine-specific primer pairs for IL-2, -3, -4, -5, TNF-α, -β, GM-CSF, TGF-β, IFN-γ, and β-actin mRNA were purchased by Clontech (Palo Alto, CA). T cell clones were cultured with EBV B cells that have been preincubated in the presence or absence of NS4. After 12 h, total RNA was isolated by the RNAzol™ B method (Biotecx Laboratories, Houston, TX). First-strand cDNA copies were synthesized in a 20-μl volume containing 1 μg of total RNA, 200 ng oligo(dT) 12–18, and 200 U M-MLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD), and 25 U RNAsin (Promega Biotech, Madison, WI) in 50 mM Tris-Cl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.5 mM each dGTP, dATP, dCTP, dTTP for 1 h at 37°C. The cDNA preparation was then diluted to 100 μl. Subsequently, 5 μl of cDNA was amplified in 50 μl in the presence of 200 nM 5' and 3' primers, 200 μM dNTPs, 1 U Taq polymerase (Perkin-Elmer Cetus, Emeryville, CA), 0.1 μg BSA, and PCR buffer containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$. For IL-4 and IFN-γ the buffer was 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$. PCR was performed in a DNA thermal cycler (Perkin-Elmer Cetus) for 30 cycles: 45-s denaturation at 94°C, 45-s annealing at 60°C, and 90-s extention at 72°C. The reaction product was visualized by electrophoresis in 2% agarose gel in 0.5× TBE buffer containing 0.5 μg/ml ethidium bromide. 1 μg of HaeIII-digested øx174 cDNA (New England Biolabs Inc., Beverley, MA) was run in parallel as molecular weight markers.

*Helper Assay.* Noncognate T cell helper assays were performed with modifications of the method described by Lanzavecchia et al. (12). Activated T cell clones specific for NS4 were used as helper T cells for Ig production by allogeneic B cells purified from PBMC of healthy donors. For T cell activation, 5 × $10^5$ T cells/ml were cultured in anti-CD3-coated 24-well plates (Costar, Cambridge, MA) at 37°C in 5% $CO_2$ for 8 h. T cells were collected, washed three times, and irradiated (1,200 rad). To purify B cells, PBMC were incubated with FITC-labeled mAbs anti-CD2, anti-CD16, anti-CD56, anti-CD57, and anti-CD14 (all mAbs from Becton Dickinson & Co.) at 4°C for 30 min, washed, and negatively sorted at the FACS®. The sorted population was >96% $CD20^+$ and $CD2^-$. T-B cell cocultures were set up in 96-well flat-bottomed microplates in 200 μl total volume per well. Various numbers of irradiated T cells (0.3, 1, and 3 × $10^4$) were cultured with 3 × $10^3$ purified B cells in RPMI-FCS at 37°C in 5% $CO_2$. After 10 d IgM, IgG, and IgA were measured in the culture supernatants.

*Assays for Ig.* IgM, IgG, and IgA in the culture supernatants were measured with ELISA. Briefly, polystyrene microplates were coated with goat anti-human μ or γ, or α antiserum (10 μg/ml) in coating buffer ($Na_2CO_3$ 0.18 g/liter, $NaHCO_3$ 2.1 g/liter, NaCl 9 g/liter, $NaN_3$ 0.2 g/liter), pH 8.6, at 4°C overnight. The plates were washed and appropriate dilutions of the culture supernatants in PBS-FCS were added for 2 h at room temperature. After washing, a goat anti-human μ or γ or α antiserum, coupled to alkaline phosphatase, was added for a further 2 h. After a final wash, the enzyme bound to the wells was determined using 1 mg/ml *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, MO) in 1 M ethanol amine buffer, pH 9.6. The absorbance at 405 nm was read with an automated photometer. The amount of Ig was determined by comparison with a standard curve constructed using known

amounts of purified IgM, IgG, and IgA. Our ELISAs have the following sensitivity: IgM, 10–3,000 ng/ml; IgG, 1–500 ng/ml; IgA, 30–2,000 ng/ml.

## Results

*Liver-derived T Cell Lines Specific for HCV Proteins.* To assess the specificity of intrahepatic T cells primed by HCV infection in vivo, we studied, in patients with chronic hepatitis C, the response of liver-derived T cells to six HCV recombinant proteins corresponding to the putative Core, E1, E2, and the nonstructural proteins NS3, NS4, and NS5 (13).

The low number of T cells recovered from liver biopsies (3–8 × $10^4$/specimen) did not allow direct proliferation experiments. Therefore, we tried to isolate liver-infiltrating T cells specific for HCV proteins by culturing low numbers (~5 × $10^3$) of liver-derived mononuclear cells with recombinant HCV proteins in the presence of irradiated autologous PBMC. The growing cultures were then tested in Ag-specific proliferation assays using autologous irradiated PBMC as APC.

We obtained, from three patients, specific T cell lines from liver biopsies cultured with the protein NS4. Fig. 1 shows dose-response curves to NS4 of T cell lines from the patients nos. 13, 14, and 71. None of the lines responded to recombinant SOD expressed in yeast or to a crude yeast extract (data not shown), indicating that these T cell lines were truly specific for NS4.

To determine the phenotype of T cells proliferating in response to NS4 in vitro, we determined by immunofluorescence the phenotype of blast cells present in the cell lines and compared it with the phenotype analyses performed on the fresh biotic material from the same patients. The mononuclear cells isolated from the three biopsies were 50–75% TCR $\alpha/\beta^+$, 15–35% $CD4^+$, and 20–50% $CD8^+$, whereas the blast cells present in the NS4-specific lines were >97% TCR $\alpha/\beta^+$, 80–90% $CD4^+$, and only 10–15% $CD8^+$ (data not shown), indicating that only $CD4^+$ T cells were stimulated by NS4.

With the other HCV proteins (Core, E1, E2, and NS5) we failed to obtain Ag-specific T cell lines from any of the three liver samples. Furthermore, we consistently failed to obtain T cell lines specific for these antigens also with liver biopsies from 16 other patients with chronic hepatitis C. This is in contrast with what is found in PBMC, since we obtained T cell lines and clones specific for either Core, E1, E2, or NS5 from PBMC of these patients (S. Abrignani, manuscript in preparation). We therefore conclude that NS4 is the only HCV Ag that allows growth in vitro of HCV-specific $CD4^+$ T cells from the liver.

*Comparison of the NS4-specific $CD4^+$ T Cells from Liver and PBMC.* To investigate functional and structural differences between local and peripheral T cell response to NS4, we studied the PBMC of the three patients from whom we isolated liver-derived T cell lines specific for NS4. We first measured the proliferative response of their PBMC to the HCV proteins, and Fig. 2 shows that PBMC from patients nos. 13 and 14 did not respond to any HCV protein, while PBMC from no. 71 proliferated in response to E2, NS3, NS4, and NS5. We therefore established NS4-specific T cell lines from the PBMC of patient no. 71, which were collected on the same day when liver biopsy was taken. To have the widest spectrum of specific clones, the PBMC-derived lines were established starting from $10^6$ mononuclear cells. We then cloned by limiting dilution the NS4-specific T cell lines iso-

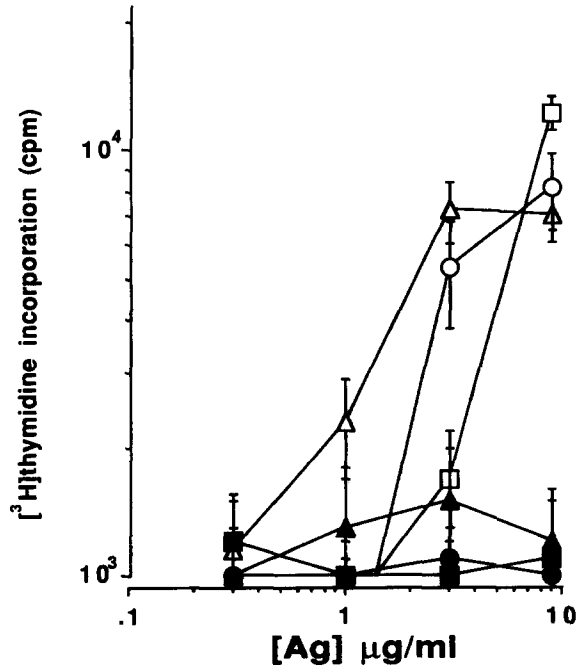


**Figure 1.** Liver-derived T cells recognize NS4. Proliferation assay of T cell lines derived from liver of patient nos. 13 (*triangles*), 14 (*circles*), and 71 (*squares*) in the presence of different concentrations of the HCV protein NS4 (*open symbols*) or the control protein SOD (*closed symbols*).

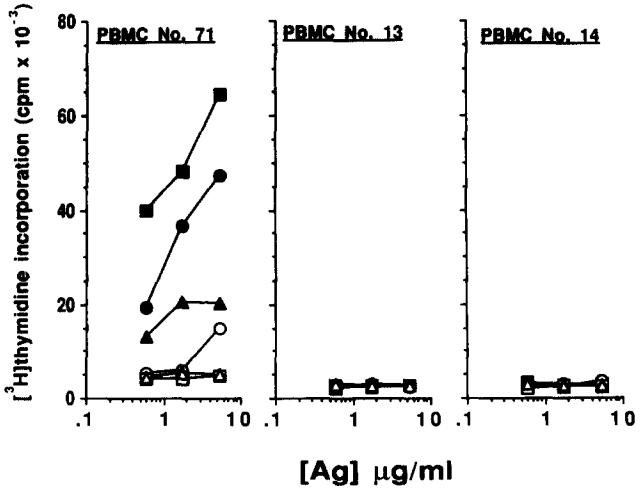


**Figure 2.** Peripheral responses to HCV proteins. Proliferation of PBMC from patient nos. 13, 14, and 71 in response to various concentrations of Core (*open triangles*), E1 (*open squares*), E2 (*open circles*), NS3–4 (*closed squares*), NS4 (*closed circles*), and NS5 (*closed triangles*) recombinant antigens.

**Table 1.** *Ag Specificity, MHC Restriction, and TCR Reactivity to SEs of PBMC- and Liver-derived T Cell Clones*

| Clone* | NS4‡ | DR§ | SEA‖ | SEB | SEC1 | SEC2 | SEC3 | SED | SEE | TSST | EXT | Medium |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *cpm* × $10^{-3}$ | | | | | | *cpm* × $10^{-3}$ | | | | | |
| PBMC-derived T cell clones ($n$ = 22) | | | | | | | | | | | | |
| B1 (9) | 107 | 2w15, 4w4 | **73** | 1 | 1 | 1 | 1 | 1 | **87** | 1 | 1 | 1 |
| B38 (1) | 52 | 2w15, 4w4 | 3 | 1 | 1 | 1 | **32** | 1 | **66** | 1 | 1 | 1 |
| B14 (1) | 78 | 2w15, 4w4 | **42** | 1 | 1 | 3 | 1 | 1 | 1 | 1 | 4 | 1 |
| B2 (9) | 96 | 2w15 | **155** | 1 | 1 | 1 | 1 | 1 | **149** | 1 | 1 | 1 |
| B91 (1) | 138 | 2w15 | **192** | **55** | 4 | 7 | 2 | 3 | 8 | 8 | 2 | 1 |
| B10 (1) | 12 | ND | 3 | 3 | 4 | **63** | **15** | 2 | 2 | 1 | 1 | 1 |
| Liver-derived T cell clones ($n$ = 27) | | | | | | | | | | | | |
| L4 (27) | 68 | 2w15, 4w4 | 2 | **24** | 1 | 1 | 1 | **21** | 1 | 1 | 1 | 2 |

* Numbers in parentheses indicate the number of individual T cell clones isolated for each group.
‡ T cell proliferation in response to NS4 presented by autologous EBV B cells.
§ HLA-DR present on the DR-homozygous EBV-B cell lines that are able to present NS4 to the T cell clones. The indication of two DR alleles means that the T cell clone recognized NS4 presented both by a DR2w15 and by a DR4w4 homozygous EBV B cell line.
‖ T cell proliferation in response to the indicated SEs in the presence of autologous EBV B cells.

lated from both liver and PBMC of patient no. 71, and obtained 22 specific clones from the PBMC and 27 from the liver that proliferate in response to NS4 in the presence of irradiated autologous PBMC or EBV-B cells as APC. These clones do not recognize either recombinant SOD or a control yeast extract, and all are $CD3^+$, TCR $\alpha/\beta^+$, $CD4^+$, and $CD8^-$ (data not shown).

To investigate whether NS4-specific T cells present in the liver differed from those present in PBMC, the T cell clones were compared for MHC restriction and response to superantigens, which can indicate the type of V$\beta$ genes used by T cells (14). Table 1 shows, for all the clones, the proliferative response to NS4 presented by autologous EBV-B cells, the MHC restriction assessed with EBV-B cell lines homozygous for DR, and the proliferative response to various SEs. On the basis of the DR restriction and the response to toxins, we could identify six different groups of T cell clones specific for NS4 from the PBMC of patient no. 71. In contrast, all the liver-derived T cell clones appeared identical in DR restriction and superantigen responses.

To investigate further the monoclonality of liver T cells, we took randomly 4 of the 27 liver-derived T cell clones and sequenced their TCR $\alpha$ and $\beta$ genes. Fig. 3 shows that they all use V$\alpha$8.1 (15), J$\alpha$25 (16), V$\beta$3.1 (17), J$\beta$1.6 (18), and D$\beta$ 1.1 (18) genes, and that the sequences of the N regions are identical in all four clones. This demonstrates that the four clones are identical and suggests that all the 27 liver clones derive from a single precursor.

Given the low number (1–2 × $10^3$) of $CD4^+$ T cells we used to establish liver cell lines, it is not very surprising that all the 27 liver-derived T cell clones are identical. However, it is remarkable that the pattern of toxin response (SEB and SED) found with the liver clones was not present in any blood-derived NS4-specific T cell clones. To investigate further the compartmentalization to the liver of this clone, and having the complete TCR $\alpha$ and $\beta$ sequence of the liver T cell clone, we attempted to detect the presence in the PBMC of the clonotypic TCR transcripts using a PCR oligotyping technique that is very sensitive, allowing the detection of ~10 cells among $10^6$ irrelevant lymphocytes, and is highly specific. Therefore, 2 × $10^6$ PBMC were stimulated with NS4 and expanded up to 4 × $10^6$ cells. RNA was extracted, reverse transcribed, and amplified using V$\beta$ + C$\beta$ oligonucleotides; the amplified products were fractionated on a gel, blotted, and hybridized with N region–specific oligonucleotides. Fig. 4 shows that liver clonotypic oligonucleotides hybridize to

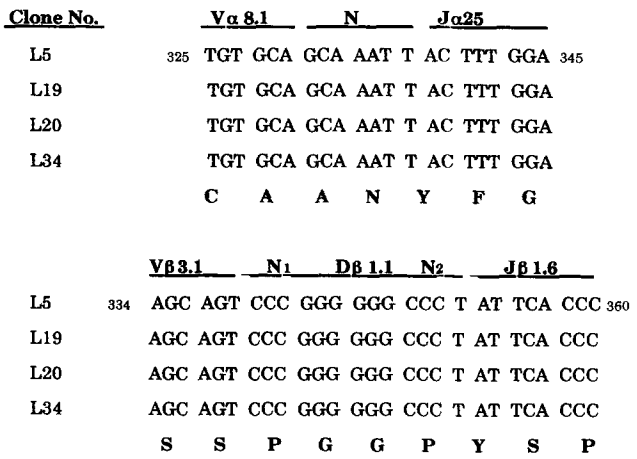


**Figure 3.** Comparison of nucleotide and deduced amino acid sequences of variable and hypervariable regions of TCR from four liver-derived T cell clones specific for NS4.

amplified products of a control cell mixture containing 10 or 100 liver T cell clones and $10^6$ irrelevant PBMC, whereas they do not hybridize to the amplified products from different pools of the PBMC no. 71.

From all the above results we conclude that there is compartmentalization to the liver of a T cell clone that is either absent or present at a very low frequency in the PBMC.

*Functional Differences between Liver- and Blood-derived T Cell Clones.* We then asked whether there were functional differences between liver- and blood-derived T cells. For this, we studied the killing ability, the helper activity, and the lymphokine produced by the two sets of clones.

The killing ability of the clones was tested against autologous EBV-B cells incubated overnight with NS4 and then labeled with $^{51}Cr$. We found that all the clones from both blood and liver were able to induce specific killing of 30–60% of the Ag-pulsed EBV-B cells (data not shown). We therefore conclude that the killing assay is not discriminating between liver- and PBMC-derived T cells.

The ability of T cells to help Ig production by B cells was assessed by coculturing activated and irradiated T cell clones with purified peripheral B cells for 10 d and then measuring IgM, IgG, and IgA in the culture supernatant. Table 2 shows that six PBMC-derived clones (each representing a distinct population) and two liver-derived T cell clones (which are identical) induce comparable amounts of IgM and IgG, but they differ in their ability to induce IgA production. Indeed, the liver-derived T cell clone induces levels of IgA produc-

**Table 2.** *Ig Production by Purified PBMC-derived B Cells Cocultured for 10 d with Different Numbers of Activated and Irradiated T Cell Clones*

| Clone | T cells | IgM | IgG | IgA |
|---|---|---|---|---|
| | × $10^3$ | | *ng/ml* | |
| None | 0 | 63 | 20 | 47 |
| PBMC-derived clones | | | | |
| B13 | 3 | 640 | 1,080 | 697 |
| | 10 | 5,512 | 9,965 | 10,401 |
| | 30 | >44,464 | 81,115 | 73,468 |
| B38 | 3 | 214 | 220 | 121 |
| | 10 | 1,230 | 2,109 | 3,849 |
| | 30 | 8,973 | 9,127 | 26,516 |
| B14 | 3 | 524 | 1,664 | 421 |
| | 10 | 5,529 | 6,768 | 6,599 |
| | 30 | 19,883 | 16,359 | 57,710 |
| B11 | 3 | 115 | 464 | 177 |
| | 10 | 3,984 | 4,933 | 4,970 |
| | 30 | >44,464 | 12,202 | 51,103 |
| B91 | 3 | 692 | 2,999 | 3,667 |
| | 10 | 1,490 | 5,272 | 13,167 |
| | 30 | 3,588 | 28,269 | 43,418 |
| B10 | 3 | 121 | 61 | 33 |
| | 10 | 2,234 | 7,760 | 4,300 |
| | 30 | 10,487 | 35,136 | 44,488 |
| Liver-derived clones | | | | |
| L29 | 3 | 1,946 | 4,246 | 18,721 |
| | 10 | 6,051 | 18,168 | 177,657 |
| | 30 | 22,611 | >55,711 | 1,704,715 |
| L36 | 3 | 1,336 | 4,067 | 30,621 |
| | 10 | 7,212 | 23,015 | 428,256 |
| | 30 | 24,046 | >55,711 | 813,225 |

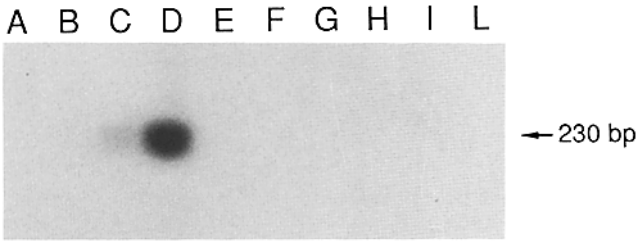


**Figure 4.** TCR clonotype of liver T cells is not detected in PBMC. Oligotyping for the liver clonotype performed on PCR-amplified $\beta$ gene products of 10 (lane *C*) or 100 (lane *D*) cells from the liver clone no. 19 mixed with $10^6$ unrelated PBMC. The size of the amplified product is shown on the right. (Lane *A*) Nothing; (lane *B*) unrelated PBMC alone; (lane *E*) mock RNA extract from culture supernatant; (lanes *F–L*) the five pools from PBMC of patient no. 71 cultured for ~10 d with NS4.

tion that are ~10-fold higher than those induced by PBMC-derived T cell clones.

We then asked whether this different help to B cells was due to differences in the type of lymphokines produced. We had quantitative assays available only for IL-2 (bio-assay on CTLL) and IFN-$\gamma$ (ELISA), and for both lymphokines we did not find significant differences among blood and liver clones (data not shown). For other lymphokines, IL-2, -3, -4, -5, TNF-$\alpha$, -$\beta$, GM-CSF, TGF-$\beta$, and IFN-$\gamma$, we measured the mRNA with PCR. T cells were activated with antigen-pulsed B cells, and after 12 h RNA was extracted and the presence of lymphokine mRNA assessed. A representative experiment in Fig. 5 shows that mRNA for all the lymphokines is present both in three PBMC- and in two liver-derived T cell clones. As a control, Fig. 5 shows that one representative blood T cell clone cultured with B cells without Ag has mRNA only for IL-3, GM-CSF, TGF-$\beta$, and TNF-$\beta$. We therefore conclude that there are no qualitative differences in the type of lymphokines produced by blood- and liver-derived T cell clones specific for NS4, and that all the T cell clones analyzed are classifiable as Th0, the most common type of human T helper cells (19).

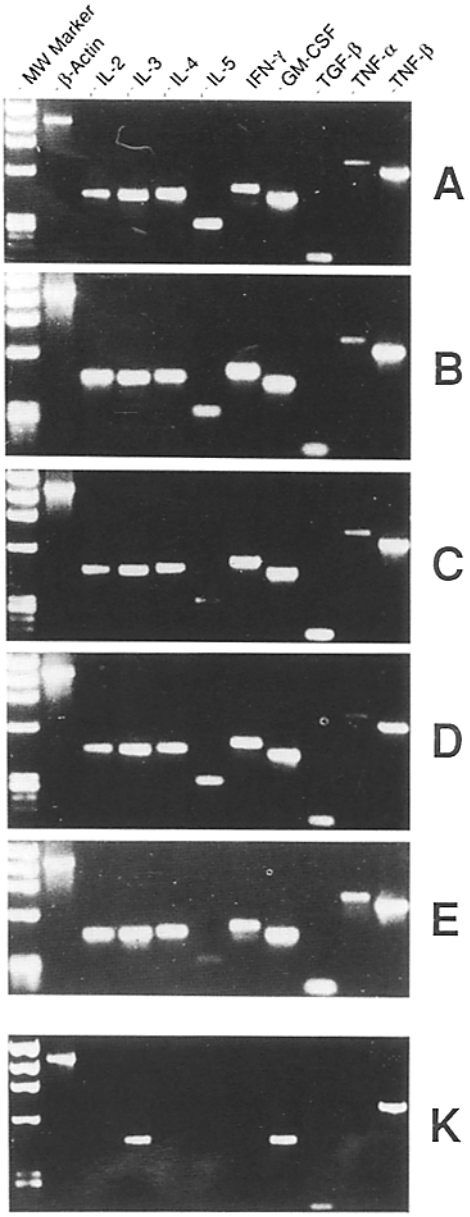


**Figure 5.** Peripheral and intrahepatic T cells are both of the Th0 type. PCR-assisted mRNA for lymphokine in three PBMC- and two liver-derived T cell clones 12 h after activation with Ag. PBMC-derived clones are: (*A*) B13; (*B*) 38; (*C*) B91. Liver-derived clones are: (*D*) L29; (*E*) L36. (*K*) Control showing that most lymphokine mRNA are not present in T cells from clone B13 cultured for 12 h with B cells in the absence of Ag.

## Discussion

In the present study we addressed the issue of the local T cell responses into the liver of patients with chronic hepatitis C. We have found that the only HCV-specific T cell lines we could establish from liver biopsies were specific for NS4. This result strengthens our previous finding that NS4 is the most immunogenic HCV protein for peripheral $CD4^+$ T cells (3). Indeed, NS4 can be considered as an immunodominant region of HCV for liver-derived $CD4^+$ T cells primed in vivo.

Although HCV particles have not yet been isolated, NS4 is considered a nonstructural protein of the virus (13) that should be in the infected cells but not in the virion particles. The high frequency of intrahepatic $CD4^+$ T cells specific for NS4 could imply either a very effective charging of MHC class II molecules by peptides derived from endogenously synthesized NS4 (20) in infected hepatocytes that express MHC class II molecules during chronic hepatitis (21), or that NS4 is a membrane protein, as suggested by its high hydrophobicity (13), which recycles in the MHC class II pathway. Alternatively, it is possible that this protein is shed by infected cells, internalized by hepatocytes, macrophages, or specific B cells, and presented to $CD4^+$ T cells. This last possibility would be consistent with the serology data demonstrating that NS4 (i.e., the C100–3 Ag) is a highly immunogenic protein also for antibody responses (1).

We failed to isolate, in 19 liver biopsies, T cells specific for HCV proteins other than NS4. This failure was not related to a defect of antigenicity of the proteins, since we obtained T cell lines and clones specific for either Core, E1, E2, NS3, or NS5 from PBMC of these patients (S. Abrignani, manuscript in preparation). One obvious possibility is that specific T cells were not detected because in most livers they are present at a frequency <1:1,000–2,000 $CD4^+$ T cells, i.e., the number of liver $CD4^+$ cells used for culturing with each single antigen. Alternatively, since proteins, such as E1 and E2, show considerable sequence heterogeneity among HCV isolates (22), one could speculate that $CD4^+$ T cells are present but recognize variable regions of HCV proteins. Such T cells would not be stimulated by recombinant proteins from HCV isolates different from the patients' isolates. Finally, we cannot rule out the possibility that these $CD4^+$ T cells are present in the liver biopsies but are anergic or unresponsive to antigenic stimuli in vitro.

When we compared the NS4-specific responses in the liver and in the corresponding PBMC, we found a compartmentalization to the liver of patient no. 71. In this case, we obtained on the same day NS4-specific T cell lines both from PBMC and liver. These lines were cloned and PBMC- and liver-derived T cell clones specific for NS4 were compared for MHC restriction and response to superantigens. Furthermore, TCR from four liver T cell clones were sequenced. Given the low number ($\sim 10^3$) of $CD4^+$ T cells we used to establish liver cell lines, it is not surprising that all the 27 liver-derived T cell clones are identical, whereas the 22 PBMC-derived T cell clones, originated from $3 \times 10^5$ $CD4^+$ T cells, are heterogeneous and represent at least six distinct clonal populations that differ in the MHC restriction and/or superantigen responses. However, it is remarkable that none of the PBMC clones is identical to the liver-derived clone, and even using a PCR oligotyping technique we did not detect the liver clonotypic TCR transcripts in the PBMC, demonstrating that this clone is in fact compartmentalized to the liver. Interestingly, the liver clone provides help for polyclonal IgA production by B cells that is far more effective than that provided by the PBMC-derived clones, whereas there is no difference in the help provided for IgM and IgG production. We did not find qualitative differences in the mRNA of T cell lymphokines that could account for the difference in the help for IgA production between PBMC- and liver-derived T cells. Further studies are required both to elucidate the mechanisms responsible for this preferential help for IgA production and to investigate whether this is a general feature of intrahepatic T cells.

In the case of chronic hepatitis B, isolation of intrahepatic $CD4^+$ T cell clones specific for either Core (23) or envelope (24) of hepatitis B virus has been reported. $CD8^+$ T cell clones specific for the envelope of HCV have been isolated from the liver of patients with chronic hepatitis C (25). Our study shows that it is possible to establish liver-derived $CD4^+$ T cell clones specific for the protein NS4 of HCV from patients with chronic hepatitis C. Furthermore, we have shown data very suggestive that there can be preferential sequestration into the liver of some T cell clonotypes that are not detectable in the PBMC. We cannot differentiate whether intrahepatic T cells are the cause (26–28) or the result of the liver damage, but their presence at the site where chronic infection and inflammation occur suggests they are effector cells of the immune response to HCV. Further long-term studies on biopsies and PBMC from the same patients will investigate the dynamics of intrahepatic effector T cells in the course of disease and their eventual appearance as memory cells in PBMC.

We thank G. Casorati e P. Dellabona for teaching us the PCR oligotyping technique, R. Rappuoli and T. De Magistris for critical reading of the manuscript, and P. Botarelli for contributions at earlier stages of this project.

Address correspondence to Sergio Abrignani, Immunobiology Research Institute Siena, IRIS, Via Fiorentina 1, 53100 Siena, Italy.

*Received for publication 21 December 1992 and in revised form 16 March 1993.*

## References

1. Kuo, G., Q.-L. Choo, H.J. Alter, G.L. Gitnick, A.G. Redeker, R.H. Purcell, T. Miyamura, J.L. Dienstag, M.J. Alter, C.E. Stevens, G.E. Tegtmeier, et al. 1989. An assay for circulating Ab to a major etiologic virus of human non-A, non-B hepatitis. *Science (Wash. DC).* 244:362.
2. Dienstag, J.L. 1990. Hepatitis non-A, non-B: C at last. *Gastroenterology.* 99:1177.
3. Botarelli, P., M.R. Brunetto, M. Minutello, P. Calvo, D. Unutmaz, A.J. Weiner, Q.-L. Choo, J.R. Shuster, G. Kuo, F. Bonino, M. Houghton, and S. Abrignani. 1993. T lymphocyte response to Hepatitis C virus in different clinical courses of infection. *Gastroenterology.* 104:580.
4. Bianchi, L. 1983. Liver biopsy interpretation in hepatitis. *Pathol. Res. Pract.* 178:180.
5. Bianchi, A.T.J., H. Hooijkaas, R. Benner, R. Tees, A.A. Nordin, and M.H. Schreier. 1981. Clones of helper T cells mediate antigen-specific, H-2 restricted DTH. *Nature (Lond.).* 290:62.
6. Muller, D., B.H. Koller, J.L. Whitton, K.E. LaPan, K.K. Brigman, and J.A. Frelinger. 1992. LCMV-specific, class II-

restricted cytotoxic T cells in $\beta_2$-microglobulin deficient mice. *Science (Wash. DC).* 255:1576.
7. Sprent, J., and S.R. Webb. 1987. Function and specificity of T-cell subsets in the mouse. *Adv. Immunol.* 41:39.
8. Guerder, S., and P. Matzinger. 1992. A fail-safe mechanism for maintaining self-tolerance. *J. Exp. Med.* 176:553.
9. Abrignani, S., D. Montagna, M. Jeannet, J. Wintsch, N.L. Haigwood, J.R. Shuster, K.L. Steimer, A. Cruchaud, and T. Staehelin. 1990. Priming of $CD4^+$ T-cells specific for conserved regions of HIV-gp120 in humans immunized with a recombinant envelope protein. *Proc. Natl. Acad. Sci. USA.* 87:6136.
10. Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
11. Uematsu, Y. 1991. A novel and rapid cloning method for the T-cell receptor variable region sequences. *Immunogenetics.* 34:174.
12. Lanzavecchia, A., B. Parodi, and F. Celada. 1983. Activation of human B lymphocytes: frequency of antigen-specific B cells triggered by alloreactive or by antigen-specific T cell clones. *Eur. J. Immunol.* 13:733.
13. Choo, Q.-L., K.H. Richman, J.H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, A. Medina-Selby, P.J. Barr, et al. 1991. Genetic organization and diversity of the hepatitis C virus. *Proc. Natl. Acad. Sci. USA.* 88:2451.
14. Marrack, P., and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. *Science (Wash. DC).* 248:705.
15. Yoshikai, Y., N. Kimura, B. Toyonaga, and T.W. Mak. 1986. Sequences and repertoire of human T cell receptor $\alpha$ chain variable region genes in mature T lymphocytes. *J. Exp. Med.* 164:90.
16. Uematsu, Y., H. Wege, A. Straus, M. Ott, W. Bannwarth, J. Lanchbury, G. Panayi, and M. Steimetz. 1991. The T-cell-receptor repertoire in the synovial fluid of a patient with rheumatoid arthritis is polyclonal. *Proc. Natl. Acad. Sci. USA.* 88:8534.
17. Plaza, A., D.H. Kono, and A.N. Theofilopoulos. 1991. New human V$\beta$ genes and polymorphic variants. *J. Immunol.* 147:4360.
18. Toyonaga, B., Y. Yoshikai, V. Vadasz, B. Chin, and T.W. Mak. 1985. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor $\beta$ chain. *Proc. Natl. Acad. Sci. USA.* 82:8624.
19. De Vries, J.E., R. de Waal Malefyt, H. Yssel, M.-G. Roncarolo, and H. Spits. 1991. Do human TH1 and TH2 $CD4^+$ clones exist? *Res. Immunol.* 142:59.
20. Van den Oord, J., R. de Vos, and V.J. Desmet. 1986. In situ distribution of MHC products and viral antigen in chronic HBV infection: evidence that HBc-containing hepatocytes may express HLA-DR antigens. *Hepatology.* 6:981.
21. Jin, Y., J.W.K. Shih, and I. Berkower. 1988. Human T-cell response to the surface Ag of hepatitis B virus (HBsAg). Endosomal and nonendosomal pathways are accessible to both endogenous and exogenous Ag. *J. Exp. Med.* 168:293.
22. Weiner, A.J., M.J. Brauer, J. Rosenblatt, K.H. Richman, J. Tung, K. Crawford, F. Bonino, G. Saracco, Q.-L. Choo, M. Houghton, and J.H. Han. 1991. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology.* 180:842.
23. Ferrari, C., M.U. Mondelli, A. Penna, F. Fiaccadori, and F.V. Chisari. 1987. Functional characterization of cloned intrahepatic, hepatitis B virus nucleoprotein-specific helper T cell lines. *J. Immunol.* 139:539.
24. Barnaba, V., A. Franco, A. Alberti, C. Balsano, R. Benvenuto, and F. Balsano. 1989. Recognition of hepatitis B virus envelope proteins by liver-infiltrating T lymphocytes in chronic HBV infection. *J. Immunol.* 143:2650.
25. Koziel, M.J., D. Dudley, J.T. Wong, J. Dienstag, M. Houghton, R. Ralston, and B.D. Walker. 1992. Intrahepatic cytotoxic T lymphocytes specific for Hepatitis C virus in persons with chronic hepatitis. *J. Immunol.* 149:3339.
26. Zinkernagel, R.M., E. Haenseler, T.P. Leist, A. Cerny, H. Hengartner, and A. Althage. 1986. T cell mediated hepatitis in mice infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* 164:1075.
27. Barnaba, V., A. Franco, A. Alberti, R. Benvenuto, and F. Balsano. 1990. Selective killing of hepatitis B envelope Ag-specific B cells by class I-restricted, exogenous Ag-specific T lymphocytes. *Nature (Lond.).* 345:258.
28. Moriyama, T., S. Guilhot, K. Klopchin, B. Moss, C.A. Pinkert, R.D. Palmiter, R.L. Brinster, O. Kanagawa, and F.V. Chisari. 1990. Immunobiology and pathogenesis of hepatocellular injury in hepatitis B virus transgenic mice. *Science (Wash. DC).* 248:361.