Patr-A and B, the Orthologues of HLA-A and B, Present Hepatitis C Virus Epitopes to CD8+ Cytotoxic T Cells From Two Chronically Infected Chimpanzees

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Summary

Common chimpanzees (Pan troglodytes) infected with hepatitis C virus (HCV) show a disease progression similar to that observed for human patients. Although most infected animals develop a chronic hepatitis, virus persistence is associated with an ongoing immune response, for which the beneficial or detrimental effects are uncertain. Lines of virus-specific cytotoxic CD8+ T lymphocytes (CTL) have been previously established from liver biopsies of two common chimpanzees chronically infected with HCV-1. The viral epitopes recognized by six lines of CTL have been defined using synthetic peptides and shown to consist of 8 to 9–residue peptides derived from various viral proteins. Five of the epitopes derive from sequences that vary among strains of HCV. The majority of the corresponding variant epitopes from different HCV strains were either recognized less efficiently or not at all by the CTL, suggesting their response may have limited potential for controlling replication of HCV variants. Complementary DNAs encoding the class I alleles of the two common chimpanzees, Patr-A, -B, and -C were cloned, sequenced, and transfected individually into a class I–deficient human cell line. Analysis of peptide presentation by the class I transfectants to CTL identified the Patr class I allotypes that present the six epitopes defined here and an additional epitope defined previously. The assignment of epitopes to class I allotypes based upon analysis of the transfected cells correlates precisely with the segregation of antigen-presenting function within a panel of common chimpanzee cell lines and the expression of class I heavy chains as defined by isoelectric focusing. Five of the HCV-1 epitopes are presented by Patr-B allotypes, two epitopes are presented by a Patr-A allotypes, and none is presented by Patr-C allotypes.

Hepatitis C virus (HCV)[1] is the principal cause of community-acquired and posttransfusion non-A, non-B hepatitis worldwide (1, 2). HCV is a small, enveloped, positive-strand RNA virus assigned to the family of Flaviviridae (3). HCV causes acute hepatitis and >50% of infected individuals subsequently develop chronic hepatitis with 10–20% eventually becoming cirrhotic (4). The pathogenic mechanisms that lead to the high rate of chronic liver disease are poorly understood. CTL provide a beneficial immune response against many viral infections (5), but their role in HCV infection remains unknown. CD8+ and CD4+ CTL with specificity for HCV have been isolated from the livers of persistently infected individuals (6–8).

Uncertain is whether these CTL act beneficially to limit viral replication or exacerbate tissue damage as has been shown for another hepatotropic virus, the hepatitis B virus (HBV) (9).

Chimpanzees are the species most closely related to humans and potentially the best animal model for the study of many diseases. HCV infection of chimpanzees leads to a course of disease similar to that seen for human patients. Most animals (50–70%) fail to resolve the acute infection and develop persistent or intermittent viremia after challenge (10, 11). Chronicity results in necroinflammatory and ultrastructural changes in the liver that are like those typically observed for humans chronically infected with HCV (12, 13).

Recently, we initiated a prospective study of the CTL response in common chimpanzees (Pan troglodytes) infected with the HCV-1 strain of virus. Several CD8+ CTL lines have been generated from liver-infiltrating lymphocytes obtained from two chronically infected chimpanzees and

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[1]Abbreviations used in this paper: B-LCL, B-lymphoblastoid cell lines; β₂m, β₂-microglobulin; HBV, hepatitis B virus; HCV, hepatitis C virus; Patr-A, -B, -C, common chimpanzee (Pan troglodytes) A, B, and C locus class I MHC antigens; VV, vaccinia virus.

their antigen specificity was mapped, using recombinant vaccinia viruses, to different structural and nonstructural proteins of HCV (14). Despite the presence of HCV-specific CD8$^+$ CTL, infection became persistent in both chimpanzees. Subsequent study showed that a CTL epitope "fine mapped" to amino acids 1446–1454 of the NS3 protein of HCV-1 was mutated with 100% penetration in all viral isolates derived from the liver and serum of one infected animal (15). This observation implies viral escape from CTL-mediated immunity might contribute to disease progression.

CTL of the CD8$^+$ phenotype typically recognize viral peptides of 8–10 amino acids in length that are presented by MHC class I molecules (16). Analysis of CTL specificity, primarily in humans and mice, has shown that the nature of viral epitopes presented is determined by substitutions within the peptide binding site that distinguish class I heavy chain allotypes (17). This property serves to diversify the CTL response within individuals of a population or species.

The multigene family encoding class I heavy chains is in evolutionary terms, highly dynamic (18, 19). Unlike most other mammalian species, chimpanzees have class I genes that closely resemble the HLA class I genes of humans. In particular the highly polymorphic HLA-A, -B, and -C genes which are most strongly implicated in the immune response have their orthologues Patr-A, -B, and -C in the common chimpanzee (Pan troglodytes) (20–24). Indeed, there are no species-specific differences that distinguish alleles of orthologous human and chimpanzee class I loci. Despite the similarities, no individual class I allele is shared by the two species, although certain pairs of alleles, particularly A locus alleles, are very similar (25).

As yet there is little knowledge of the functional properties of chimpanzee class I MHC molecules with which to complement the structural comparisons. The two chimpanzees chronically infected with HCV, and from which various CD8$^+$ CTL lines have been derived, provided an opportunity to investigate antigen presentation by chimpanzee class I molecules during an immune response to a natural pathogen of humans. Furthermore, such analysis is likely to provide insight as to the role of class I allotypes and CD8$^+$ CTL in disease progression and the desirability of influencing such responses through vaccination. To these ends we have defined the peptide epitopes recognized by six HCV-specific CTL lines derived from the two chronically infected chimpanzees and determined the identities and primary structures of the autologous Patr class I allotypes that present them.

Materials and Methods

Cell Lines. Immortalized B-lymphoblastoid cell lines (B-LCL) were generated from Ficoll-Hypaque–separated chimpanzee PBMC by transformation with supernatant from the EBV-secreting marmoset cell line B95-8 (American Type Culture Collection, Rockville, MD) in RPMI 1640 medium containing 20% FCS and 1 μg/ml cyclosporin A (Sandoz Research Institute, East Hanover, NJ) as described (14). B cell lines were grown in RPMI

1640 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO), 2 mM L-glutamine, 1.4 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (Irvine Scientific, Santa Ana, CA).

Virus-specific CTL Clones. HCV-specific CD8$^+$ CTL lines were generated as described previously (14). In summary, liver biopsies obtained using the modified Klatskin technique were homogenized in PBS supplemented with 1% FCS. CD8$^+$ cells were isolated using anti-CD8 antibodies coupled to magnetic beads (Dynabeads; Dynal Inc., Great Neck, NY) and cloned by limiting dilution in 96-well round bottom plates containing irradiated human PBMC as feeder cells and in the presence of medium containing RPMI 1640, 10% FCS heat-inactivated FCS (Hyclone Laboratories, Inc., Logan, UT), 50 U/ml recombinant human IL-2, 10% (vol/vol) IL-2 (Pharmacia, Silver Springs, MD), antibiotics, and 0.1 mg/ml anti-CD3 mAb (AMAC, Westbrook, ME). All CTL lines consisted of >98% CD8$^+$ cells as assessed by flow cytometry. HCV-specific CD8$^+$ lines were maintained by periodic restimulation using anti-CD3 antibodies in medium containing feeder cells.

Cytotoxicity Assay. CTL lines were tested for cytotoxic activity against LCL targets and transfectants using a standard 4-h Na$_2$^{51}CrO$_4$ (^{51}Cr) release assay (26). Target cells were prepared by incubating LCL with synthetic peptides (10 μg/ml unless indicated otherwise) and ^{51}Cr (50 μCi) for 1 h and then washing them three times. The radioactivity released during the 4-h incubation of CTL and target cells was measured from 100-μl samples of culture supernatant and the percent specific lysis calculated using the formula: 100 × [(experimental cpm − spontaneous cpm)/ (total cpm − spontaneous cpm)] = % specific lysis. Spontaneous release was determined from targets incubated in medium alone and maximal release from targets incubated with 5% Triton X-100. CTL assays were replicated with similar results.

Peptides. Peptides were synthesized by Chiron mimotopes (Clayton, Australia) or Research Genetics (Huntsville, AL) using Fmoc solid phase methods. All peptides had free NH$_2$ and COOH termini.

Cloning, Sequencing, and Analysis of Patr Class I cDNA. Total RNA was prepared from 3 × 10^7 LCL using RNAzol (Tel-Test, Inc., Friendswood, TX) and purified as described (27). First-strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) according to the vendor's instructions. Chimpanzee Patr class I cDNA was amplified using the PCR and oligonucleotide primers (5' UT and either 3' UTA, UTB, or UTC depending upon the class I locus targeted) that we have used routinely for the amplification of human HLA-A, -B, and -C alleles (28). The conditions were as described (27) except the number of PCR cycles was increased to 30. The products were purified using the Wizard kit (Promega Corp., Madison, WI) and subcloned into the replicative form of the phage vectors M13mp18 and M13mp19. Partial sequencing was performed manually using the Sequenase kit (Sequenase v2; United States Biochemical Co., Cleveland, OH) and sequencing primers S3/N3 and/or S4/N4 (28) in order to identify and distinguish alleles. The sequence of selected clones was completed using a (model 373; Applied Biosystems, Foster City, CA) DNA automated sequencer as described (27). A consensus sequence was derived from the sequence of at least three clones. Analysis of the sequences was performed using the GCG software package (Genetics Computer Group, Madison, WI). Newly identified Patr-A, -B, and -C alleles were named in accordance with the published guidelines (29).

Preparation of Class I Constructs for Transfection. Class I cDNA

clones identical to the consensus sequence or differing only by synonymous substitutions were identified for certain Patr-A, -B, and -C alleles. These inserts were removed from the replicative form of the M13 phage vector by digestion with SalI and HindIII and subcloned into the dephosphorylated expression vector pBJ1-neo (30) cut with XhoI and HindIII.

For those alleles for which all clones characterized had nonsynonymous substitutions, a strategy of splicing together the error-free segments from two imperfect clones was adopted. One of the cDNA was cut out of the phage vector and ligated into dephosphorylated pBluescript·KS(+) (Stratagene, La Jolla, CA) cut with SalI and HindIII. The region containing PCR-induced errors (one or a localized cluster of nucleotide substitutions) was then removed by digestion with either SalI or HindIII (depending upon whether the 5' or 3' part of the insert was to be kept in the vector) and a restriction enzyme selected to cut once in the insert sequence and place all the errors in one of the two resulting fragments. The vector containing the error-free segment was isolated by gel purification using the BIO-101 kit (BIO 101 Inc., La Jolla, CA) and dephosphorylated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) according to Sambrook et al. (31). An error-free fragment corresponding to the region removed from the first cDNA clone was isolated from the replicative form of a second cDNA clone using the same restriction enzymes used to divide the first insert. The second fragment was gel purified and ligated to the vector containing the first fragment using T4-DNA-Ligase (New England Biolabs, Inc., Beverly, MA) as described (31). The sequences of the recombinants thus produced were determined for the previously mutated regions. Clones having the correct sequence were subcloned into the expression vector pBJ1-neo as described above.

Preparation and Characterization of Class I Transfectants. The HLA class I-deficient human B cell line 721.221 (32) was transfected with individual Patr-A, -B, or -C alleles cloned into the pBJ1-neo vector using the BTX electroporation system (BTX Inc., San Diego, CA). 3 × 10^6 cells were resuspended in 200 μl of 1× HeBS (20 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, and 6 mM dextrose) mixed with 20 μg DNA and electroporated in a 2-mm gap cuvette with a 350-V pulse at 360 mΩ and 50 μF followed by a second pulse of the same duration after a 30-s pause. After resuspension in culture medium containing 30% FCS, 10^6 aliquots of cells were transferred to the wells of a 24-well plate. 3 d later, medium containing G418 was added (1 mg/ml active drug, GIBCO BRL).

Cultures of G418 resistant cells were sorted on the basis of class I expression. Five million cells were incubated for 60 min at 4°C with fluorescein-conjugated W6/32 antibody, which binds all human and chimpanzee class I allotypes (33). The cells were washed with sterile ice-cold PBS supplemented with 1 μg/ml propidium iodide (PI; Sigma Chemical Co.) and sorted using a FACStar$^{PLUS®}$ flow cytometer (Becton Dickinson & Co., San Jose, CA). Cultures developed from the sorted cells were used for analysis.

Flow Cytometric Analysis and Antibodies. Surface expression of MHC class I molecules was determined by indirect immunofluorescence. Approximately 10^6 cells were incubated with W6/32 antibody at 4°C for 30 min, washed with PBS, and then similarly incubated with fluoresceinated goat anti–mouse antibody (Biosource International, Camarillo, CA). Cells were washed with PBS containing PI at a final concentration of 1 μg/ml to facilitate the exclusion of dead cells. Analysis was performed using a FACScan® flow cytometer (Becton Dickinson & Co.). Background fluorescence was evaluated using an irrelevant isotype-matched first antibody (CVC.7) (34). Transfectants expressing human class I

molecules were used as positive controls (35); for the analysis of transfectants expressing chimpanzee class I molecules, untransfected 721.221 cells were used as the negative control.

Immunoprecipitation and IEF. Immunoprecipitation of class I molecules and their analysis by one-dimensional IEF gels were performed as described (36). Briefly, LCL or transfected cells were biosynthetically labeled with [^{35}S]Cys and [^{35}S]Met (Amersham Corp., Arlington Heights, IL). The cells were lysed with detergent and the lysates were precleared. Chimpanzee class I molecules were immunoprecipitated using W6/32, treated with Neuraminidase type VI (Sigma Chemical Co.), and analyzed on one-dimensional IEF gels under denaturing conditions. After electrophoresis, the gel was fixed in 15% acetic acid, treated with Amplify (Amersham Corp.), dried and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

EMBL/GenBank/DDBJ Accession Numbers. The accession numbers for the Patr sequences cited in this paper are X13114 (Patr-A04), U10540 (Patr-A11), U10544 (Patr-A14), X13115 (Patr-B01), U05586 (Patr-B13); the nucleotide sequences described in this report for Patr-A11, Patr-A14, Patr-B01, and Patr-B13 cover the complete coding region of these class I molecules and extend the previous entries. These extended sequences and the newly identified Patr sequences (Patr-B16, Patr-B17, Patr-C03, Patr-C04, Patr-C05, and Patr-C06) have been submitted to the Genome Sequence Data Base (National Center for Genome Resources, Santa Fe, NM) and will become available under the following accession numbers: L47291 (Patr-A11), L47292 (Patr-A14), L47293 (Patr-B01), L47297 (Patr-B13), L47296 (Patr-B16), L47348 (Patr-B17), L47294 (Patr-C03), L47347 (Patr-C04), L47298 (Patr-C05), and L47299 (Patr-C06).

Results

CD8$^+$ Cytotoxic T Cells from the Livers of Chronically Infected Chimpanzees Recognize HCV Peptides of Eight to Nine Amino Acid Residues in Length. The goal of these first experiments was to characterize the HCV-1 epitopes recognized by six HCV-1–specific cytotoxic CD8$^+$ T cell lines established from intrahepatic lymphocytes of two chronically infected chimpanzees. Using recombinant vaccinia viruses (VV) as a source of antigen, previous analysis had localized the epitopes to subsets of viral proteins (14). We now describe the use of synthetic peptides to define short peptide epitopes that serve as potent antigens in the sensitization of autologous B cell lines for CTL-mediated lysis.

Two CTL lines from chimpanzee Ch-458 (458/14B, 458/5F) and one from chimpanzee Ch-503 (503/11.3) were known to be specific for epitopes of either the E2 or NS2 proteins that localize to residues 364–906 of the HCV-1 polyprotein (14). The specificity of CTL line 458/14B was further analyzed by examining its capacity to lyse autologous B cell targets sensitized with synthetic peptides representing a series of overlapping 20-residue sequences within residues 364–906 of HCV-1. As shown in Fig. 1 *A*, CTL line 458/14B recognized peptides p54 and p55 derived from residues 531–550 and 541–560, respectively. Lysis of target cells sensitized with peptides corresponding to the flanking sequences was less efficient and peptides derived from other regions were inactive. These results indicate the target epitope for 458/14B is contained in the se-
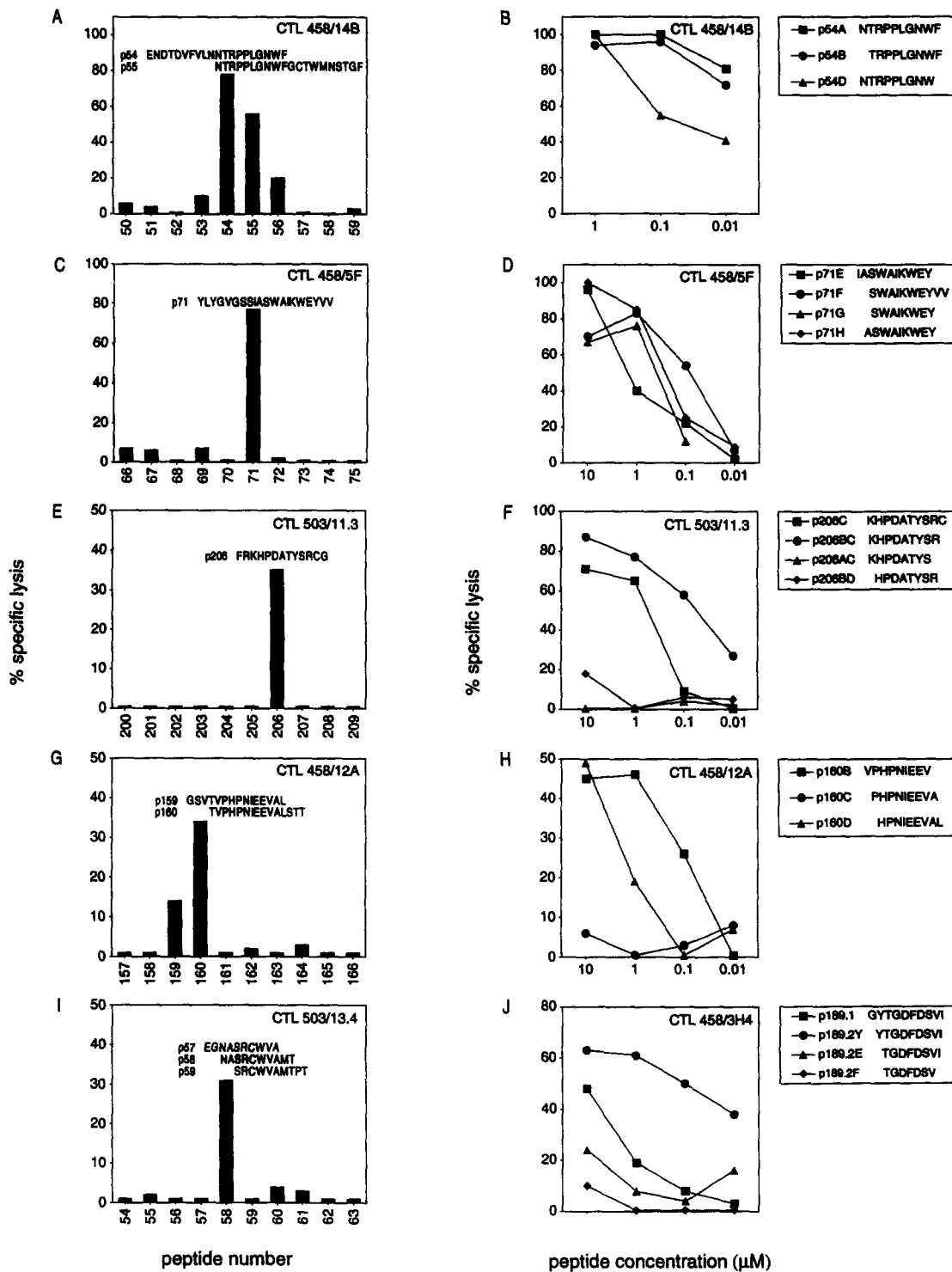
Figure 1. Fine mapping of CTL epitopes recognized by HCV-specific, chimpanzee class I–restricted CTL lines reveals epitopes contained in peptides of eight to nine amino acid residues length. The CTL lines 458/14B, 458/5F, 503/11.3, 458/12A, 503/13.4, and 458/3H4 were previously broadly mapped with recombinant VV expressing core E1, E2/NS1, NS2, and NS3 proteins (14). Fine mapping was initially performed by testing each line's ability to lyse autologous B-LCL targets individually incubated with overlapping synthetic peptides spanning the HCV antigens as identified with recombinant VV-infected targets (*A, C, E, G,* and *I*). The epitope contained in the peptides (*p54, p71, p206,* and *p160*) recognized by the individual clones (*14B, 5F, 11.3,* and *12A*) was further mapped with a series of smaller, overlapping peptides (not shown). Each peptide in a series was tested at a final concentration of 10 µg/ml. Those peptides giving the highest specific lysis were further truncated and tested over a range of concentrations as shown on each x-axis (*B, D, F, H,* and *J*). The E/T ratio shown is 20:1.

quence NTRPPLGNWF, common to peptides p54 and p55. A peptide corresponding to this sequence (p54A) was synthesized and shown to sensitize target cells for CTL recognition over a range of concentrations (Fig. 1 B). A peptide lacking the NH$_2$-terminal asparagine residue (p54B) was equally potent, whereas removal of the COOH-terminal phenylalanine residue resulted in a peptide (p54D) that was somewhat less effective (Fig. 1 B). These data suggest that the nonameric peptide p54B most likely represents the epitope recognized by CTL line 458/14B, although further truncations of NH$_2$- and COOH-terminal residues will be required to establish this point.

The set of 20-residue peptides covering amino acids 364-906 of HCV-1 was similarly used to characterize the epitope recognized by CTL line 458/5F. Only the peptide corresponding to residues 701-720 (p71) stimulated lysis by this CTL line (Fig. 1 C). The epitope recognized by 458/5F is therefore a product of the COOH-terminal region of the E2 protein. Further definition of the epitope was achieved by testing a series of overlapping 10-residue peptides spanning the sequence of p71. Peptides p71E (IAS-WAIKWEY) and p71F (SWAIKWEYVV) sensitized target cells for lysis, showing the epitope lies within their shared sequence (data not shown). Indeed, p71G a synthetic peptide corresponding to this octamer sequence (SWAIK-WEY) was equally efficient for sensitization of target cells, indicating that it contained all critical residues for class I MHC binding and T cell recognition (Fig. 1 D).

The epitope recognized by CTL line 503/11.3 was analyzed using synthetic 13-amino acid peptides encompassing residues 364-906 of HCV-1. The only active peptide was p206 (Fig. 1 E), which corresponds to residues 586-598, a part of the E2 protein close to, but distinct from, the epitope recognized by CTL line 458/14B. Further definition of the epitope was obtained using synthetic peptides corresponding to overlapping 10-residue sequences spanning the p206 sequence. That peptides p206B (RKHP-DATYSR) and p206C (KHPDATYSRC) sensitized target cells for lysis by CTL line 503/11.3 (data not shown) pointed to the epitope being their shared nonamer sequence. This thesis was supported by analysis of synthetic peptides corresponding to the nonamer sequence or to octamer sequences in which either the NH$_2$- or COOH-terminal residues were missing: the nonamer peptide (p206BC) was clearly most effective for target cell sensitization (Fig. 1 F).

Previous characterization of CTL line 458/12A showed that it recognizes an epitope within residues 907-1618 of the HCV-1 NS3 protein (14). Individual 15-residue synthetic peptides derived from the sequence of NS3 were used to further define the epitope. Two peptides (p159 and p160), which share the sequence of residues 1356-1367 of NS3, sensitized autologous targets for lysis by 458/12A (Fig. 1 G). When a series of nonameric peptides offset by one residue that represented the overlap between peptides p159 and p160 were tested, peptides p160B (VPHP-NIEEV) and p160D (HPNIEEVAL) had activity whereas p160C (PHPNIEEVA) and other peptides representing the flanking sequences did not (data not shown). On titration

of the p160B, C, and D peptides, the best sensitization of targets for killing was obtained with p160B (Fig. 1 H). Further truncation of COOH- or NH$_2$-terminal residues of p160B abrogated target cell sensitization (data not shown), and thus the minimum, optimal epitope probably includes amino acids 1357-1365 of the NS3 protein. That p160D also has activity might be due to cross-reaction with this peptide, which also contains a residue identical to that observed in p160B, a proline, as putative position 2 anchor.

The epitope recognized by CTL line 503/13.4 was previously mapped to either the core or E1 proteins of HCV (14). Analysis of synthetic peptides representing overlapping decamer sequences within the target region (residues 1-340 of the polyprotein) revealed a single antigenically active peptide (p58, residues 234-243) (Fig. 1 I). Comparison of shorter synthetic peptides derived from the sequence of p58 identified the nonamer GNASRCWVA (p58D) as the minimal epitope for 503/13.4 (data not shown). Of note, this epitope from the E1 protein overlaps with an octamer (ASRCWVAM) presented by HLA-B35 to CTL from a patient with chronic HCV (6).

We find that CTL line 458/3H4, as also reported for CTL line 458/13F, is specific for an epitope of the NS3 protein contained within the 15-residue peptide p189 (residues 1443-1457 of the HCV polyprotein) (14). Analysis of 10-residue peptides derived from within the p189 sequence revealed a single peptide (p189.1: GYTGDFDSVI) with antigenic activity (data not shown). A related nonamer peptide, which lacked the NH$_2$-terminal glycine residue of p189.1, was found to be more potent than p189.1 (Fig. 1 J), whereas peptides lacking the COOH-terminal isoleucine or additional residues at the NH$_2$ terminus were inactive. Thus the minimum, optimal epitope for CTL line 458/3H4 appears to be the nonamer peptide YTGDFDSVI.

This analysis of chimpanzee CD8$^+$ CTL lines with specificity for the HCV-1 strain of human HCV has identified six epitopes derived from three viral proteins. A seventh epitope has already been described (14, 15). Six of the epitopes correspond to nonamer peptides and one to an octamer (summarized in Table 1). The length of peptides recognized by chimpanzee CTL are thus characteristic of those presented by class I MHC molecules as defined from the study of humans and mice (37, 38).

Correlation of Presentation of HCV-1 Epitopes with the Segregation of Class I Heavy Chain Allotypes in a Population of Common Chimpanzees. Identification of synthetic peptides corresponding to the HCV epitopes recognized by the CTL from chimpanzees Ch-458 and Ch-503, enabled us to correlate presentation of individual target epitopes with particular MHC class I allotypes. The six CTL lines were tested for their capacity to lyse 22 chimpanzee EBV-transformed B-LCL, including 458LCL and 503LCL, in the presence of a mixture of peptides representing the six epitopes (Fig. 2 A). Lytic reactions were then correlated with the presence of MHC class I heavy chains as revealed by isoelectric focusing gels of class I immunoprecipitates obtained from each cell line with the monomorphic antibody W6/32 (33) (Fig. 2 B).

The pool of peptide epitopes sensitized cells of chimpan-

Table 1. *Antigen Specificity and Restriction Elements of Intrahepatic CD8⁺ CTL Lines from Ch-458 and Ch-503*

		Peptide epitope			
CTL line	Antigenic protein	Name	Residues	Sequence	Restriction element
458/5F*	E2/NS1‡	p71G§	711–718‖	SWAIKWEY¶	Patr-A11?**
458/14B	E2/NS1	p54B	542–550	TRPPLGNWF	Patr-B13
458/12A	NS3	p160B	1357–1365	VPHPNIEEV	Patr-B13
458/3H4‡‡	NS3	p189.2Y	1444–1452	YTGDFDSVI	Patr-B01
503/10D§§	NS3	p189.2A	1446–1454	GDFDSVIDC	Patr-B16
503/13.4	E1	p58D	233–241	GNASRCWVA	Patr-B16
503/11.3	E2/NS1	p206BC	588–596	KHPDATYSR	Patr-A04

*Intrahepatic CD8⁺ CTL lines from chimpanzees Ch-458 and Ch-503.
‡HCV-1 target protein.
§Designation of the synthetic HCV peptide epitope.
‖NH₂- and COOH-terminal amino acids (aa) of the HCV-1 peptide epitopes.
¶Complete amino acid sequence of the HCV-1 peptide epitopes.
**The presentation of p71H by Patr-A11 is based on exclusion as described in the text.
‡‡Clone 458/3H4 has the same specificity as clone 458/13F published previously (14) and was used throughout this study.
§§The mapping of the epitope recognized by line 503/10D has been described in Erickson et al. (14) and Weiner et al. (15).

A

CTL	Peptide		Cytotoxic Activity
503/10D	p189.2	○	— — — — — — — — — — — — + — — — + — — — — —
503/11.3	p206C	□	— — — — — — — — + — — + — — + — + + — — — +
458/5F	p71F	●	— — — — — — — — — — — — — — — + — — — — — —
458/12A	p160D	■	— — — — — — — + —· — — — — — — + + — — — — —
458/14B	p54	◆	— — — — — — — + — — — — — — — + + — — — — —
458/3H4	p189.2E	△	NT NT NT NT NT NT NT NT + NT NT + — + + — — — + + — +

B
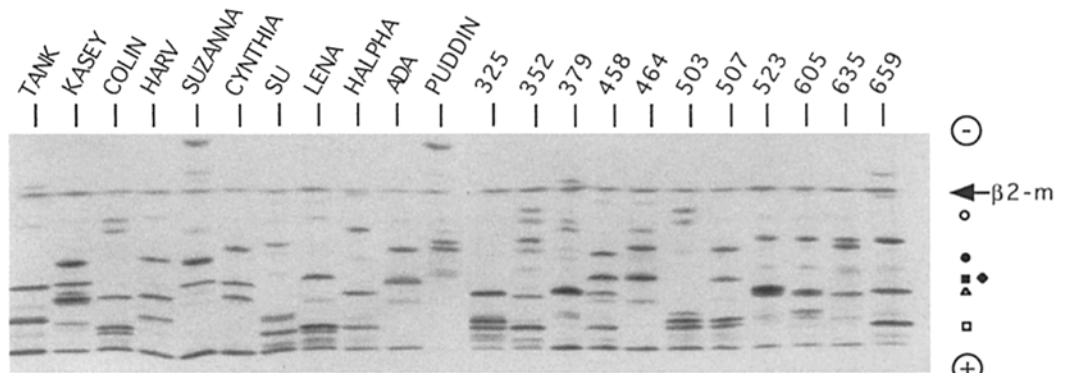
Figure 2. Presentation of HCV-1 peptide epitopes correlates with segregation of class I heavy chain allotypes in a cohort of common chimpanzees. (*A*) Target cells consisting of 20 allogeneic and 2 autologous EBV-transformed chimpanzee B-LCL were pulsed with a pool of synthetic HCV peptide epitopes (*p189.2, p206C, p71F, p160D, p54*, and *p189.2E*) for 1 h at the time of ⁵¹Cr labeling and tested against the CD8⁺ CTL lines 503/10D, 11.3; 458/5F, 12A, 14B, and 3H4. The results are shown at an E/T ratio of 20:1 and were regarded positive for a given target and CTL line if the specific lysis was ≥40% (indicated by + in the matrix topping the gel, at the intersection of a CTL row with the column of a target cell). (*B*) One-dimensional IEF pattern of chimpanzee MHC class I molecules (Patr-A, -B, and -C) immunoprecipitated with mAb W6/32 from [³⁵S]methionine-labeled cellular lysates of the same 22 chimpanzee B-LCL tested in the cytotoxicity assay. Significant lysis by a particular HCV-specific CD8⁺ CTL line was observed only for those cells that shared a band(s) and therefore allele(s) in the IEF as indicated by the symbols (○, ●, ■, ◆, △, and □). Band (●) is only present in B-LCL from chimpanzee Ch-458, but is shared with B-LCL from chimpanzee Ch-335 not run on this gel, and 458LCL and 335LCL are both lysed by CD8⁺ CTL line 458/5F in the presence of p71F as explained in the main text. (*Arrow*) Invariant β₂m. NT, not tested.

zees Ch-458 and Ch-503 from which the CTL were derived in the expected manner. Thus 458LCL were lysed by CTL lines 458/5F, 458/12A, 458/14B, and 458/3H4, and 503LCL were lysed by CTL lines 503/10D and 503/11.3. In addition, CTL line 503/11.3 lysed cells from Ch-458, suggesting chimpanzees Ch-458 and Ch-503 share a MHC class I allotype. Supporting this hypothesis was the finding that CTL line 503/11.3 lysed 6 of the 22 chimpanzee LCL in a pattern that correlated precisely with the presence of one of the class I heavy chain bands seen in the IEF gels. The exception was LCL from chimpanzee Ch-352. This cell line has a class I heavy chain band with the same isoelectric point as the band shared between 458LCL and 503LCL, but was not lysed by CTL line 503/11.3. This may be due to the expression by chimpanzee Ch-352 of a distinct allele, which fortuitously has the same electrophoretic properties as the allele common to Ch-458 and Ch-503 (Fig. 2).

In a similar manner, class I heavy chain bands correlating with the presentation of all six of the HCV epitopes, were identified (Fig. 2). CTL lines 458/12A and 458/14B correlated with the same class I heavy chain band, suggesting their target epitopes are presented by the same MHC class I allotype. CTL line 458/5F lysed the autologous cell line (458LCL) but no other one of the 22 chimpanzee LCL. Analysis of EBV-transformed B cell lines from additional chimpanzees revealed a single allogeneic cell line (from chimpanzee Ch-335) that is lysed by CTL line 458/5F in the presence of the peptide pool. Correlating with the pattern of lysis, cell lines 458LCL and 335LCL share a class I heavy chain band not seen in the other cell lines (Fig. 2 and data not shown). These results are consistent with the six HCV epitopes being presented to CTL by MHC class I molecules and identify candidate class I allotypes for each epitope.

Definition of the MHC Class I Alleles (Patr-A, -B, and -C) Expressed by the Two Chimpanzees from which the HCV-specific Cytotoxic T Cell Lines Were Derived. To define the chimpanzee class I allotypes that present the six HCV epitopes, we cloned and sequenced cDNA encoding the classical MHC class I alleles from the two chimpanzees (Ch-458 and Ch-503) from which the CTL were derived. For the common chimpanzee the classical class I loci comprise Patr-A, Patr-B, and Patr-C which are orthologous to the human HLA-A, -B, and -C loci, respectively. Both chimpanzees are heterozygous for each locus, and cDNA corresponding to two Patr-A, -B, and -C alleles were obtained from each animal (Fig. 3). These cDNA gave 11 different sequences, one Patr-A allele being common to Ch-458 and Ch-503.

The three Patr-A alleles and two of the four Patr-B alleles correspond to alleles defined previously (21, 23, 24). The four Patr-C sequences and two of the Patr-B sequences correspond to previously undiscovered alleles. One of the "new" Patr-B alleles (Patr-B17) is distinctive, having various amino acid substitutions not found in the known chimpanzee and human MHC class I molecules. On the basis of a series of linked substitutions, human HLA-C alleles can be divided into two groups (39). Three of the Patr-C alleles (Patr-C03, Patr-C05, and Patr-C06) are similar to HLA-C alleles of one group, whereas the

fourth allele (Patr-C04) is similar to HLA-C alleles of the second group. Thus, the division of C alleles into these two groups antedates separation of the human and chimpanzee lineages some 5 million years ago (40).

Identification of the Chimpanzee Patr-A and -B Allotypes that Present Individual HCV Peptide Epitopes to CTL. Complementary DNA clones corresponding to the Patr-A, -B, and -C alleles of chimpanzees Ch-458 and Ch-503 were subcloned into the eukaryotic expression vector pBJ1-neo and transfected into the class I–deficient human B cell line 721.221 (32). In the transfected cells, chimpanzee class I heavy chains associate with endogenous human β_2-microglobulin (β_2m), which is identical to chimpanzee β_2m in the amino acid sequence (25). Flow cytometric analysis of the transfectants using the W6/32 mAb showed that 9 of the 11 transfected alleles had levels of class I expression comparable to those obtained with HLA class I cDNA clones (Fig. 4 A). No expression was obtained for cells transfected with two Patr-A alleles (Patr-A11 and Patr-A14 from chimpanzees Ch-458 and Ch-503, respectively) which may be due to artefactual mutations in the cDNA clones (data not shown).

The class I molecules expressed by the transfectants were immunoprecipitated and analyzed on IEF gels. Each transfectant expressed a single band not seen in untransfected 721.221 cells, and their isoelectric points each corresponded to one of the class I heavy chain bands seen in either the "parental" EBV-transformed B cell line from Ch-458 or Ch-503 (Fig. 4 B). This analysis, therefore, permitted assignment of alleles and sequences to the bands seen on IEF gels.

The panel of nine transfected cell lines expressing single Patr class I alleles was tested for its capacity to present HCV peptide epitopes to CTL. CTL specific for the seven epitopes summarized in Table 1 were examined and for six of them, a single transfectant proved capable of presenting the target epitope (Fig. 5). Moreover, the transfectants were as active in presentation as the autologous B cell lines. One epitope was presented by a Patr-A allotype, the other five by Patr-B allotypes. No epitope was presented by Patr-C.

CTL line 503/11.3 lyses both 458LCL and 503LCL cells for which the only shared class I allotype is Patr-A04. Consistent with these observations, the transfectant expressing Patr-A04 presented peptide p206BC to CTL line 503/11.3. That the same subset of autologous EBV-transformed chimpanzee B cell lines presented peptides p54 to CTL 458/14B and peptides p160D to 458/12A suggested these epitopes were presented by the same class I allotype. This was confirmed by demonstrating that the transfectant expressing Patr-B13 presented both epitopes to their respective CTL. Similarly, CTL lines 503/13.4 and 503/10D were both demonstrated to recognize peptides presented by Patr-B16. The Patr-B01 allotype presented the relevant epitopic peptide to CTL line 458/3H4. The only Patr-B allotype that presented none of the epitopes studied here is the unusual Patr-B allele (Patr-B17) of chimpanzee Ch-503 (Table 1). For all CTL, the patterns of antigen presentation by the transfectants confirmed the provisional assignments of antigen-presenting allotypes based on the panel analysis shown in Fig. 2.

leader

	-24	-10	-1

Consensus MRVMAPRTLLLLLSGALALTETWA
Patr-A04   - a - - - - - - - v - - - - - - - - - - q - - -
Patr-A11   - a - - p - - - - - - - - - - - - - - - q - - -
Patr-A14   - a - - - - - - - v - - - - - - - - - - q - - -
Patr-B01   - q - t - - - - v - - - - - a - - - - - - - - -
Patr-B13   - q - t - - - - v - - - - - a - - - - - - - - -
Patr-B16   - q - t - - - - v - - - - - a - - - - - - - - -
Patr-B17   - m - i - - - - v - - - - - a - - - - - - - - -
Patr-C03   - - - - - - - - - - i - - - - - - - - - - - - -
Patr-C04   -
Patr-C05   - - - - - - - - - - i - - - - - - - - - - - - -
Patr-C06   - - - - - - - - - - l - - - - - - - - - - - - -

Ch-458	
Patr-A04	Mayer et al. 1988
Patr-A11	McAdam et al. 1995
Patr-B01	Mayer et al. 1988
Patr-B13	McAdam et al. 1994
Patr-C03	new
Patr-C04	new

Ch-503	
Patr-A04	Mayer et al. 1988
Patr-A14	McAdam et al. 1995
Patr-B16	new
Patr-B17	new
Patr-C05	new
Patr-C06	new

α 1 domain

Consensus GSHSMRYFYTAVSRPGRGEPRFIAVGYVDDTQFVRFDSDAASPRMEPRAPWIEQEGPEYWDRETQIVKANTQTDRESLRNLRGYYNQSEA
Patr-A04   - - - - - - - - s - s - q - e - - r s - - - s a - - - - v d - g t - - - - - - - - - d
Patr-A11   - - - - - - - - s - q - q - - r n m - - s a - - - - a n - g t - - - - - - - - - d
Patr-A14   - - - - - - - - s - s - q - e - - r s - - - f a - - - - a n - g t - - - - - - - - - d
Patr-B01   - - - - - - - - s - - - - - - - - - - - t - r n m - - s a - - - - - - n - - i a l r - - - - - - -
Patr-B13   - - - - - - - - s - - - - - - - - - - - s - - - - - - - - - - - - - - - - - - e - - - - - m - - - - - - - - - - - n - - - y - - q a - - - - v - - g - - - - - - - - -
Patr-B16   - - - - - k - - - - - - - - - - - - - - t - - - - - - - - - - - w - - - - - - - - k - - t - - - - - - - - - - - - - - - - s - t - a - - - v - - - - - l r - - - - - - -
Patr-B17   - - - - - k - - - - s - - - - - - - - - s - - - - - - - - - - - - - - - - - - v - - - - - m - - - - - - - - c - - - - - - f - t - - - - - v - g - - g - - - - - - - -
Patr-C03   - - - - - - - - - - h - g - - - - - v - - - - - - - - - - - - k y - r q a - a - - v n - - k - - - - - - - - - d
Patr-C04   - g - - - - - v - - - - - - - - - - - - k y - r q a - a - - v n - - k - - - - - - - - - d
Patr-C05   - - - - - - - - - - h - g - - - - - v - - - - - - - - h - - - - k y - r q a - a - - v - - - - - - - - - - - - d
Patr-C06   - - - - - - - - - - h - g - - - - - v - - - - - - - - h - - - - k y - r q a - a - - v - - - - - - - - - - - - d

α 2 domain

Consensus GSHTLQRMYGCDVGPDGRLLRGYDQYAYDGKDYIALNEDLRSWTAADTAAQITQRKWEAARVAEQLRAYLEGTCVEWLRRYLENGKETLQRA
Patr-A04   - - - - i - l - f - - - - - - s - - - f - - - - r - d - m - - - - - - - h a - t
Patr-A11   - - - - i - i - - - - - - - - s - - - f - - - - r - d - m - - - - - k - - - - - h a - - - r - - - - - - - - - - d g - - - - - - - - - - t
Patr-A14   - - - - i - l - f - - - - - - s - - - f - - - - e - h - m - - - - - - - c - - - h a - t
Patr-B01   - - - - w - t - - - - m - - - - - - - - g - s - - - - - - - - - - - - - - e - - - r -
Patr-B13   - - - - - s - - - - - - - - - - - - - e - - - - - - - - - - - - - q - - s - r -
Patr-B16   - - - - w - t - f - - - - - - - - - - r - - - - - - - - - - - - q - - s - r -
Patr-B17   - - - - - - t - - - - m - - - - - - - y - - - - - - - - - l - - - - - - - - p - - - s - - - f - - - g a - m - - - -
Patr-C03   - - - - - s - - - - l - - - - - - - g - e - - - r - - - - - l - - - - - - - - - - - - - - - r - - - - -
Patr-C04   - - - - - k - - - - l - - - - - - - e - f - a - - - - - - - - - l -
Patr-C05   - - - - - w - - - - l - l - - - - a -
Patr-C06   - - - - - s - - - - l - - - - - - - - - - - - - - - - l - - - - - - - - - - - - - - l - - - a - - - - - - - - - - l - - - - - - - - - - r - - - - -

α 3 domain

Consensus DPPKTHVTHHPISDHEATLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSGEEQRYTCHVQHEGLPKPLTLRW
Patr-A04   - - - - - - m - - - - - - - r - g -
Patr-A11   - - - - - - m - g -
Patr-A14   - - - - - - m - - - - - - - r - g -
Patr-B01   - e -
Patr-B13   -
Patr-B16   -
Patr-B17   - - - - - - - - - - - - - - - v -
Patr-C03   e h - - - - - - - - - v - g - e - - - - - -
Patr-C04   e - - - - - - - - - l - g - - - - - - - - - - q - - - - - - - m - - - - - e - - - - - -
Patr-C05   e h - - - - - - - - - v - g - e - - - - - -
Patr-C06   e h - - - - - - - - - v - g - e - - - - - -

transmembrane domain

Consensus EPSSQSTIPIVGIVAGLAVLAVLVVIGAVVAAVMCRRKSS
Patr-A04   - - - - - p - - - - - - - - t - - - - - l g a - i t - - - - - - - - w - - - - -
Patr-A11   - - - - - p - - - - - - - - t - - - - - l g a - i t - - - - - - - - w - - - - -
Patr-A14   - - - - - p - - - - - - - - t - - - - - l g a - i t - - - - - - - - w - - - - -
Patr-B01   - v - t - a v v - - - - - - - - - - - - -
Patr-B13   -
Patr-B16   -
Patr-B17   - - - - - - v - - - - - - - - - - - - - , - - - - - - - - - - - - - - - - - - -
Patr-C03   g - - - - p - - - - - - - a - - - - - - - - a - l - - - - - v - - - - - - -
Patr-C04   - - - - - p - - - - m - - - - - - - v - a - l - - - - t - m - - - - -
Patr-C05   g - - - - p - - - - - - - a - - - - - - - a - l - - - - - v - i - - - -
Patr-C06   g - - - - p - - - - - - - a - - - - - - - - a - l - - - - - v - - - - - - -

cytoplasmic domain

Consensus GGKGGSYSQAASSDSAQGSDVSLTACKV
Patr-A04   d r -
Patr-A11   d r - - - - - t -
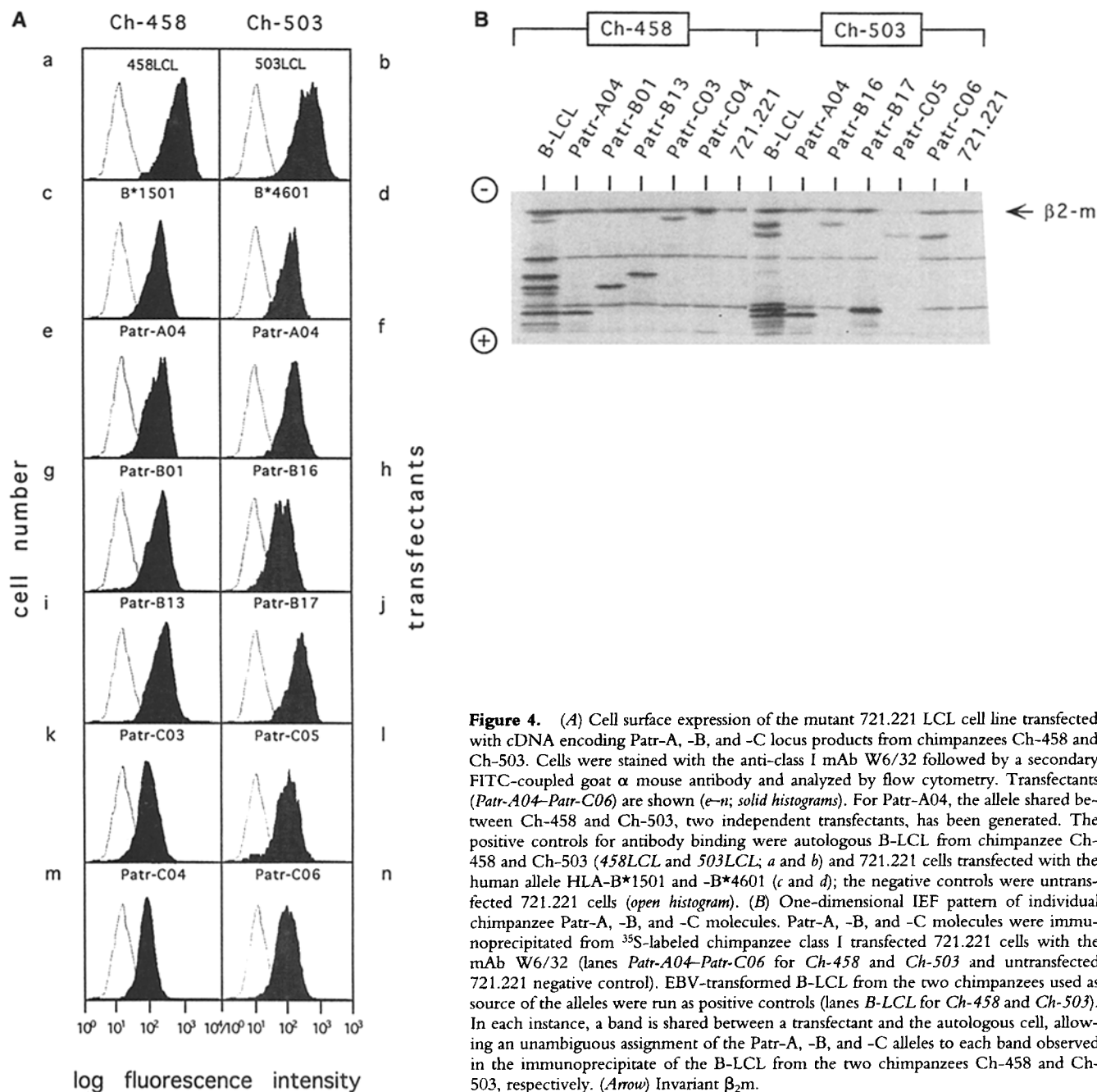Patr-A14   d r -
Patr-B01   -
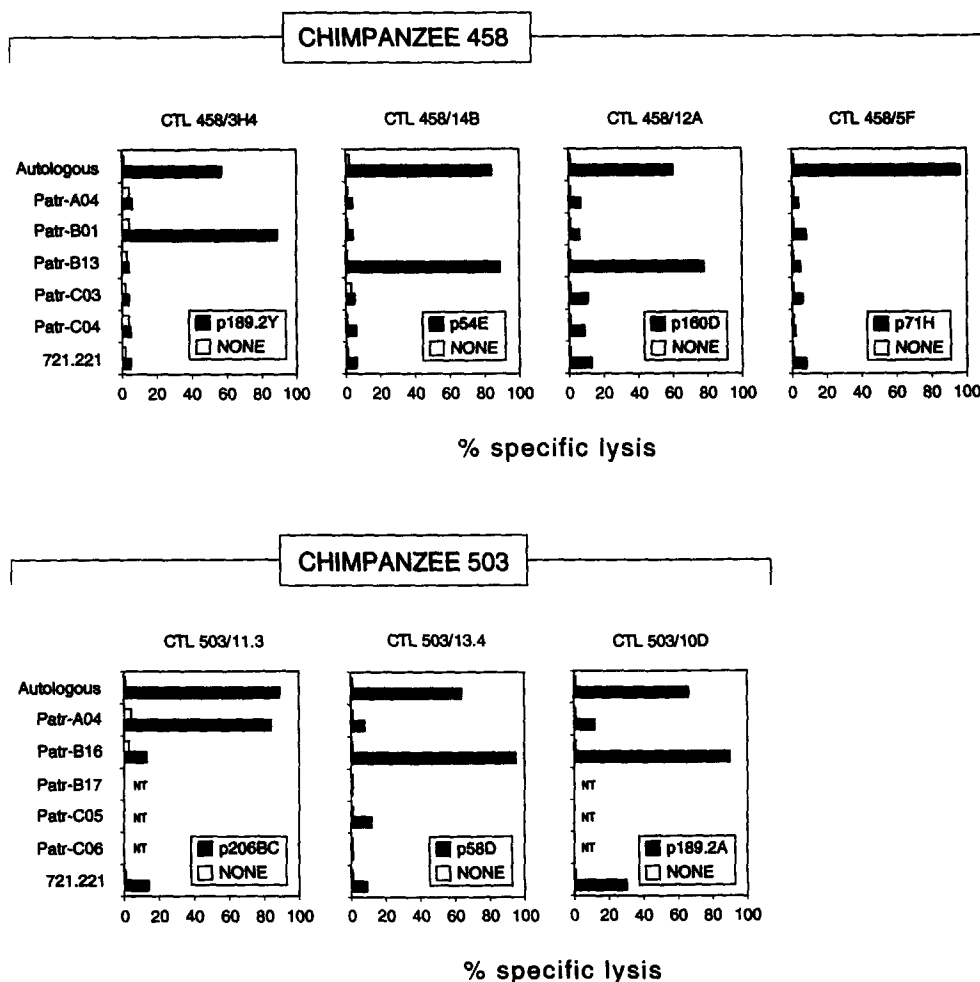Patr-B13   -
Patr-B16   -
Patr-B17   -
Patr-C03   - - - - - - c - - - - - - n - - - - - - e - - i - - - a
Patr-C04   - - - - - - c - - - - c - n - - - - - - e - - i t - - a
Patr-C05   - - - - - - c - - - - - - n - - - - - - e - - i - - - a
Patr-C06   - - - - - - c - - - - - - n - - - - - - e - - i - - - a

Figure 4. (*A*) Cell surface expression of the mutant 721.221 LCL cell line transfected with cDNA encoding Patr-A, -B, and -C locus products from chimpanzees Ch-458 and Ch-503. Cells were stained with the anti-class I mAb W6/32 followed by a secondary FITC-coupled goat α mouse antibody and analyzed by flow cytometry. Transfectants (*Patr-A04–Patr-C06*) are shown (*e–n; solid histograms*). For Patr-A04, the allele shared between Ch-458 and Ch-503, two independent transfectants, has been generated. The positive controls for antibody binding were autologous B-LCL from chimpanzee Ch-458 and Ch-503 (*458LCL* and *503LCL; a* and *b*) and 721.221 cells transfected with the human allele HLA-B*1501 and -B*4601 (*c* and *d*); the negative controls were untransfected 721.221 cells (*open histogram*). (*B*) One-dimensional IEF pattern of individual chimpanzee Patr-A, -B, and -C molecules. Patr-A, -B, and -C molecules were immunoprecipitated from ^{35}S-labeled chimpanzee class I transfected 721.221 cells with the mAb W6/32 (lanes *Patr-A04–Patr-C06* for *Ch-458* and *Ch-503* and untransfected 721.221 negative control). EBV-transformed B-LCL from the two chimpanzees used as source of the alleles were run as positive controls (lanes *B-LCL* for *Ch-458* and *Ch-503*). In each instance, a band is shared between a transfectant and the autologous cell, allowing an unambiguous assignment of the Patr-A, -B, and -C alleles to each band observed in the immunoprecipitate of the B-LCL from the two chimpanzees Ch-458 and Ch-503, respectively. (*Arrow*) Invariant β$_2$m.

None of the nine transfectants presented peptide p71H to the 458/5F CTL line. By a process of elimination, this result suggested the Patr-A11 allotype, for which a transfectant was not available, is the antigen-presenting mole-cule. Supporting this assignment is correlation of the iso-electric point of Patr-A11, calculated on the basis of the deduced amino acid sequence, with that of the observed heavy chain band shared by the cell lines (458LCL and

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Figure 3. Amino acid sequence predicted from the complete nucleotide sequence of the Patr-A, -B, and -C alleles from chimpanzees Ch-458 and Ch-503 compared to the consensus sequence of all currently identified HLA-A, -B, and -C alleles. Amino acids are indicated by single letter code. Differences with the consensus are given by lowercase letter substitutions, whereas identity is indicated by a dash. Codon numbers are given above the consensus. The alleles are also tabulated in the box and have been denoted as new or with their recommended name (29), followed by the reference if already described in the literature. These sequence data are available from EMBL/GenBank/DDBJ under the accession numbers as given in Materials and Methods.

1769   Kowalski et al.

CHIMPANZEE 458

CTL 458/3H4   CTL 458/14B   CTL 458/12A   CTL 458/5F

Autologous
Patr-A04
Patr-B01
Patr-B13
Patr-C03
Patr-C04
721.221

■ p189.2Y ☐ NONE   ■ p54E ☐ NONE   ■ p160D ☐ NONE   ■ p71H ☐ NONE

0 20 40 60 80 100   0 20 40 60 80 100   0 20 40 60 80 100   0 20 40 60 80 100

% specific lysis

CHIMPANZEE 503

CTL 503/11.3   CTL 503/13.4   CTL 503/10D

Autologous
Patr-A04
Patr-B16
Patr-B17   NT
Patr-C05   NT
Patr-C06   NT
721.221

■ p206BC ☐ NONE   ■ p58D ☐ NONE   ■ p189.2A ☐ NONE

0 20 40 60 80 100   0 20 40 60 80 100   0 20 40 60 80 100

% specific lysis

Figure 5. Lysis of chimpanzee MHC class I transfected 721.221 by HCV-specific CD8+ CTL lines is peptide and allele specific. 721.221 cells expressing single MHC class I alleles from chimpanzees Ch-458 (*Patr-A04, -B01, -B13, -C03,* and *-C04*) and Ch-503 (*Patr-A04, -B16, -B17, -C05,* and *-C06*) were tested for lysis by the CD8+ CTL lines 458/3H4, 14B, 12A, 5F, and 503/11.3, 13.4, 10D in the presence (*solid bars*) or absence (*open bars*) of cognate HCV-1 peptide epitope (*p189.2Y, p54E, p160D, p71H, p206BC, p58D,* and *p189.2A*) in a 4-h ^{51}Cr-release assay at an E/T ratio of 20:1. The concentration of the HCV-1 peptides was 10 μg/ml except for lines 503/13.4 and 458/3H4, where a concentration of 100 μg/ml and 0.01 μg/ml HCV-1 peptide, respectively, was used. Each assay included autologous targets from chimpanzees Ch-458 and Ch-503 as positive controls and untransfected 721.221 cells as negative controls. Note that Ch-458 and Ch-503 share the A-allele Patr-A04. *NT*, not tested.

335LCL) that present antigen to CTL line 458/5F.

Natural Variation in HCV-1 Epitopes often Causes Loss of Recognition by CD8+ CTL. The majority of epitopes defined in this study involve sequences that vary between strains of HCV. We therefore examined how natural variation within these epitopes affected CTL recognition. Peptides having variant sequences were synthesized and compared to "wild-type" HCV-1 peptide epitopes in their capacity to sensitize targets for lysis by the cognate CD8+ CTL line.

Only two epitopes, both located in NS3, were conserved in all HCV genotypes. These include the 189.2A epitope recognized by CTL line 503/10D, which was completely conserved in sequence (15), and the 189.2Y epitope recognized by CTL line 458/3H4, which shows variable usage of a tyrosine or phenylalanine residue at amino acid position 1. This variability in p189.2Y did not affect recognition by CTL line 458/3H4 (Fig. 6 E). CTL 503/11.3 recognized only one of four natural variants of the E2 protein sequence. Unknown is whether the failure to recognize variants is due to poor binding of the variant peptides to class I, or poor recognition of the peptide–class I complex by CTL. The single amino acid substitution that distinguishes the inactive variants is lysine for arginine at

position 9 in the peptide (Table 2). However, when this substitution alone was introduced into the HCV-1 epitope, it caused no reduction in activity (Fig. 6 A). Thus, this single substitution cannot alone account for the lack of recognition of the three variant epitopes. Analogous results were obtained with natural variants of the epitopes recognized by three other CTL. 2 of the 11 variants examined showed reduced antigenic activity and 8 gave no activity (Fig. 6, B–D). These results indicate naturally occurring variation in most epitopes described here limits cross-reactivity of class I MHC–restricted CTL for other HCV subtypes.

Discussion

Investigation of the specificity of the CD8+ CTL response in two chimpanzees chronically infected with HCV-1 provides further evidence suggesting that the host response of *Pan troglodytes* and *Homo sapiens* to HCV is similar. The seven CTL lines we studied were all specific for 8 to 9–residue viral peptides presented by chimpanzee class I MHC molecules. With few exceptions (41–43), human CD8+ CTL are directed towards peptide epitopes presented by allotypes of the HLA-A and B loci. For example,
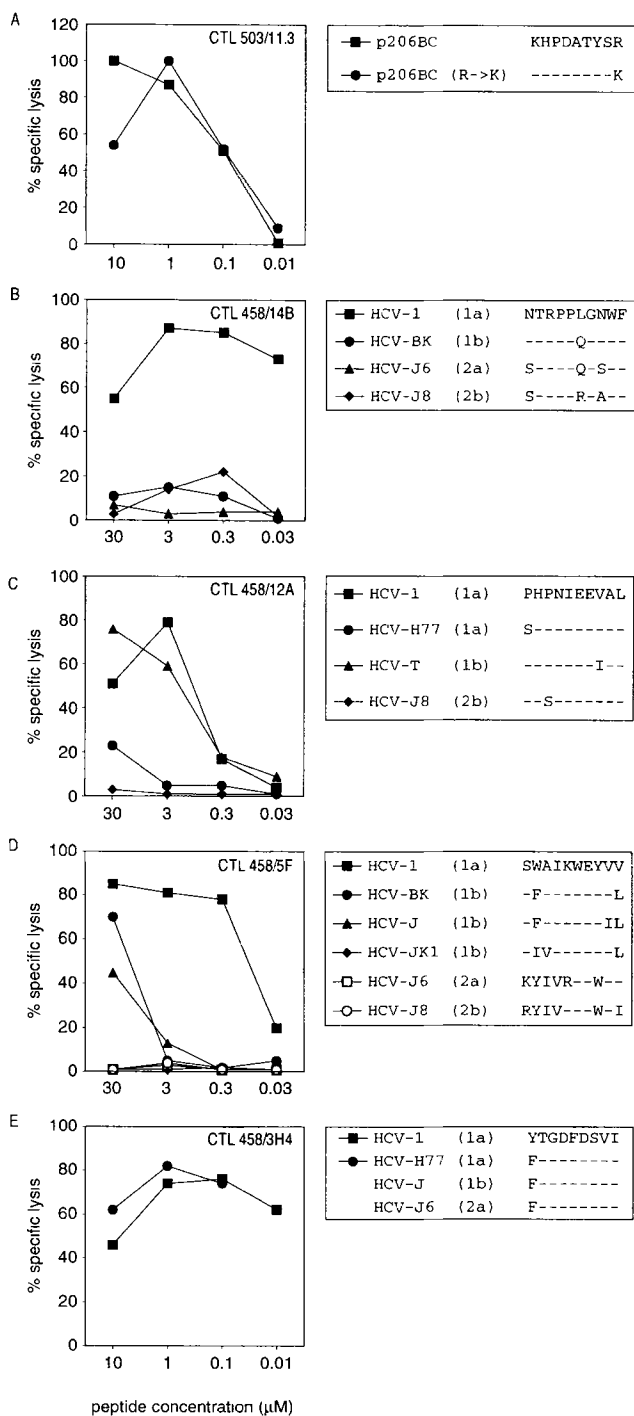
Figure 6. Effect of natural sequence variation on recognition of HCV-specific CD8+ CTL lines. Autologous B-LCL were sensitized with the indicated concentration of the HCV-1 or variant epitopic peptide and tested against the cognate CD8+ CTL line (A) 503/11.3; (B) 458/14B; (C) 458/12A; (D) 458/5F; and (E) 458/3H4 at an E/T ratio of 10:1. The HCV peptides were either minimum epitopes or contained the minimum epitope in their core sequence except for line 458/12A, where these residues represented the suboptimal epitope p160D. The epitope represented by p206BC (R→K) is a synthetic variant with a substitution of a lysine for an arginine at the COOH terminus of the HCV-1 epitope p206BC. Lysis was measured in a standard 4-h ^{51}Cr-release assay and was always <5% for B-LCL treated with medium only. The sequence of the epitopes for the HCV genotypes (subtype is indicated in parentheses) obtained from

Table 2. *HCV Subtype Specificity of CD8+ CTL Line 503/11.3*

Peptide	HCV-strain (subtype)	Sequence	Percent specific lysis of targets at E/T ratio of		
			40:1	10:1	2.5:1
			%		
None*	—‡	—§	<1‖	<1	<1
p206BC	HCV-1 (1a)	KHPDATYSR	98	94	77
p206BC1	HCV-H77 (1a)	K y P e A T Y S R	100	100	76
p206BC2	HC-J6 (2a)	KHPD t TY i k	4	7	4
p206BC3	HCV-JK1 (1b)	KHP e ATYt k	4	4	2
p206BC4	HC-J8 (2b)	KHPDATY l k	2	3	2

*Autologous EBV-transformed B-LCL from chimpanzee Ch-503 were untreated or pulsed with 10 μg/ml of synthetic peptide epitopes from wild-type or variant viruses for 1 h and tested against the p206BC-specific CTL line 503/11.3.

‡HCV genotypes and subtypes are according to the nomenclature of Simmonds et al. (63, 64).

§The sequence of HCV genotypes between aa 588–596 obtained from GenBank/EMBL/DDBJ database is shown in single letter code. p206 is the parental peptide. Lower case letter indicates substitution.

‖Values represent percent specific 51Cr-release in a 4-h assay.

antigen presentation by various HLA-A (A2, A3, A11, A23, and A29) and HLA-B (B7, B8, B35, B44, B50, B51, and B53) allotypes has stimulated a CTL response in patients infected persistently with HCV (6, 7, 44–48). Chimpanzees Ch-458 and Ch-503 appear to have similarly focused their CD8+ CTL response upon HCV-1 peptides presented by allotypes of Patr-A and -B loci, the orthologues of HLA-A and HLA-B. In neither species do allotypes of the orthologous HLA-C and Patr-C loci seem prominent in stimulating T cells. HLA-C is effective in regulating the activities of human natural killer cells (49, 50) and similarities with HLA-C raise the possibility that Patr-C also performs this function.

Five of the seven HCV-1 epitopes we analyzed are presented by Patr-B allotypes. This bias may be the result of sampling CTL from only two chimpanzees, and analysis of further animals is essential to see if dominance of HCV antigen presentation by Patr-B is a more general phenomenon. Moreover, the potential for HCV to mutate and diversify within infected animals could result in CTL responses to variant epitopes presented by Patr-A or C allotypes that were invisible to screening assays using constructs and peptides derived from the wild-type HCV-1 sequence.

EMBL/Genbank/DDBJ databases is shown in single letter code. Dashes represent identity with the published HCV-1 sequence (1) and amino acid substitutions are indicated (63, 64).

Whereas we acknowledge the limitations of our data, it is worth considering the possibility that Patr-B allotypes may be better suited for HCV antigen presentation, as has been recently proposed for the allo- and virus-specific CTL response in humans (51, 52). Indeed, some of the strongest associations of class I HLA with disease susceptibility are with alleles of HLA-B (53, 54). The functional propensities of the HLA-A, -B, and -C loci can be correlated with differences in their polymorphism. By comparison to HLA-A or -C. the HLA-B locus exhibits greater functional variation between individual alleles, a larger number of alleles, and a faster rate of generating new alleles (55–57). Although information on Patr-A, -B, and -C polymorphism is less comprehensive than for HLA-A, -B, and -C, similar patterns are emerging for these chimpanzee class I loci (23, 25).

Localization of epitopes within the envelope and NS3 proteins of HCV-1 suggests that most viral proteins are processed for class I MHC presentation. Indeed, the CTL lines described here were identified using only recombinant VV expressing the COOH-terminal half of the HCV polyprotein (14), but more recent data indicate that other nonstructural proteins such as NS4 are also recognized by intrahepatic CTL from Ch-458 and Ch-503 (our unpublished observations). Thus, the epitopes defined here probably represent a subset of those recognized by HCV-specific CTL from these animals. Nevertheless, it is noteworthy that with the exception of two NS3 epitopes, naturally occurring variation among HCV subtypes limited cross-reactivity of HCV-1 (subtype 1a)–specific CTL lines. This is likely to pose a challenge for control of multiple HCV types by vaccine-induced CTL responses. Moreover, the limited ability of the CTL lines to recognize the variant epitopes could explain in part the reoccurrence of viremia and hepatitis in humans (58) and chimpanzees (59–61) after reexposure to other strains or subtypes of HCV.

Another consequence of virus variation may be escape from CTL surveillance. Previous studies (15) demonstrated that the dominant HCV quasi-species detected in chimpanzee Ch-503 contained a single amino acid substitution in the p189.2A epitope in NS3 that prevented recognition by CTL against the index peptide. The finding that naturally occurring variation in other epitopes reduces efficiency of CTL recognition suggests the potential for mutation in multi-

ple epitopes that would facilitate persistence of the virus.

Although chimpanzees Ch-458 and Ch-503 share the Patr-A04 allele, we have only been able to detect a response to the E2 peptide p206BC presented by this allotype in animal Ch-503. There are various possible explanations for this result, of which the most trivial is the failure to culture such cells from the tissue of chimpanzee Ch-458. More interesting is the possibility that Ch-458 failed to make a response, or that the tempo of the response was different in the two animals. Future analysis of the kinetics of the CD8+ CTL response during the course of an infection should address this issue and examine whether the apparent dominance of Patr-B presentation is a general feature of the chimpanzee response to HCV or a particular feature of chronic infection.

Orthologous chimpanzee and human MHC class I loci are so alike that there are no shared "species-specific" features within the primary structures that permit the alleles of one species to be distinguished from those of the other species (25, 62). Neither have any alleles shown to be identical in the two species, a generalization that remains unaltered by the characterization in this study of six previously undiscovered Patr class I alleles. Although lineage relationships can be discerned between Patr-A and HLA-A alleles, and Patr-B and HLA-B alleles share many of the same motifs, pairwise comparison of chimpanzee and human allotypes always reveals differences within the peptide binding site. It is therefore likely that in the details of their peptide binding specificity and of the precise HCV peptides presented, individual chimpanzee allotypes will in general not show a one-to-one correspondence with their most closely related human allotypes. Evidence consistent with this view are the differences in the HCV peptides presented by HLA-A*0301 and A*1101 and the related chimpanzee alleles, Patr-A04 and Patr-A11. The distinctions, however, are similar to those encountered when comparing HLA class I antigen presentation in different human populations or different individuals. Arguably, both the similarities and differences between class I HLA and Patr molecules contribute to the value of chimpanzees for the study of HCV infection and of vaccination strategies aimed at diverse human populations.

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Note added in proof: Since acceptance of this manuscript for publication, a transfected cell expressing the Patr-All allotype has been made. Incubation of this transfectant with peptide p71H renders it susceptible to lysis by CTL 458/5F. This positive data confirms the conclusion, previously based on negative evidence (see Table I, Fig. 5, and associated text), that Patr-All presents peptides p71E, F, G, and H (Fig. 1 D).

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