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Expression of Hepatitis C Virus Proteins Does Not Interfere With Major Histocompatibility Complex Class I Processing and Presentation *In Vitro*

DARIUS MORADPOUR,[1] BENNO GRABSCHEID,[2] ANDREAS R. KAMMER,[2] GUNTER SCHMIDTKE,[3] MARCUS GROETTRUP,[3] HUBERT E. BLUM,[1] AND ANDREAS CERNY[2]

Hepatitis C virus (HCV) infection takes a chronic course in the majority of patients. The mechanisms underlying the evasion of the host immune response and viral persistence are poorly understood. In this context, we investigated interactions of HCV proteins with major histocompatibility complex (MHC) class I processing and presentation pathways using cell lines that allow the tetracycline-regulated expression of viral structural and nonstructural proteins. These well-characterized inducible cell lines were found to efficiently process and present endogenously synthesized HCV proteins via MHC class I. Functional MHC class I cell-surface expression and intracellular proteasome activity were not affected by the expression of HCV proteins. These results suggest that viral evasion of the host immune response does not involve interactions of HCV with MHC class I processing and presentation. Other mechanisms, such as interference with the interferon system, may be operative in HCV infection, leading to viral persistence. (HEPATOLOGY 2001;33:1282-1287.)

In the majority of individuals, hepatitis C virus (HCV) infection takes a chronic course that may progress to cirrhosis and eventually hepatocellular carcinoma.[1,2] The pathogenesis of HCV-induced liver disease and the mechanisms underlying viral persistence are poorly understood.[3,4] Experimental and clinical evidence suggests that CD4- and CD8-positive T lymphocytes are involved in viral clearance and pathogenesis of liver disease in hepatitis C.[5-11] However, the lack of an efficient cell-culture system or a suitable small-animal model permissive for HCV infection and replication has thus far limited the systematic investigation of the mechanisms of viral persistence and pathogenesis.[12]

The aim of this study was to evaluate cell lines inducibly expressing HCV proteins as target cells for human HCV-specific cytotoxic T lymphocytes (CTL), and to examine whether the expression of HCV proteins interferes with major histocompatibility complex (MHC) class I processing and presentation as a potential mechanism of immune evasion. A number of viruses, most notably members of the herpesvirus family, use this strategy to establish persistent infection.[13,14] Using a tetracycline-regulated gene-expression system,[15] we have previously generated a comprehensive panel of continuous human cell lines that allow the tightly regulated expression of HCV structural and nonstructural proteins[16-19] (Moradpour D, et al., unpublished data, March 2001). The cellular and viral proteolytic machineries and posttranslational modification pathways were found to be fully functional in these cell lines. This well-characterized and highly reproducible model system, therefore, provides the unique opportunity to investigate structural and functional properties of viral proteins and their interactions with cellular proteins and pathways in the biological context of the entire HCV polyprotein.[19-22] Here, we demonstrate that these cell lines can efficiently process and present endogenously synthesized HCV proteins via MHC class I, and that expression of HCV proteins does not interfere with this important antigen processing and presentation pathway.

Abbreviations: HCV, hepatitis C virus; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; aa, amino acid; PBMC, peripheral blood mononuclear cell; rIL-2, recombinant interleukin 2; EBV, Epstein-Barr virus; PBS, phosphate-buffered saline; MeOSuc-GLF-AMC, methoxysuccinyl-glycine-leucine-phenylalanine-amido-4-methylcoumarin.

From the [1]Department of Medicine II, University of Freiburg, Freiburg, Germany; [2]Department of Internal Medicine, University Hospital, Berne, Switzerland; and [3]Research Department, Cantonal Hospital, St. Gallen, Switzerland.

MATERIALS AND METHODS

Tetracycline-Regulated Cell Lines. Tetracycline-regulated cell lines were established by stable transfection of U-2 OS human osteosarcoma cells (American Type Culture Collection, Rockville, MD), first with the tetracycline-controlled transactivator,[23] and second with different HCV cDNA constructs under the control of a tetracycline-controlled transactivator–dependent promotor.[15] UHCV-11 and -32 cells[18] allow the inducible expression of the entire open reading frame, UNS3-4A-24 cells[19] of the NS3-4A complex derived from a prototype HCV H strain cDNA.[24] Western blot analyses of cell lysates were performed in parallel to the immunologic assays to confirm the tightly regulated expression of HCV proteins.

CTL Lines. CTL lines specific for HCV amino acid (aa) residues 1073-1081 (CINGVCWTV), an immunodominant HLA-A2–restricted epitope in the NS3 serine protease domain,[25] were isolated

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from 3 HLA-A2–positive patients (P04, P52, P54) with chronic hepatitis C. Briefly, peripheral blood mononuclear cells (PBMC) were isolated on Ficoll-Paque density gradients and cultured in the presence of peptide-pulsed autologous PBMCs and 20 U per milliliter of recombinant interleukin 2 (rIL-2) (EuroCetus, Amsterdam, the Netherlands). Bulk cultures were restimulated on a weekly basis with autologous peptide-pulsed PBMCs and rIL-2. To generate CTL lines, peptide-specific cultures were identified by ^{51}Cr-release assay, depleted of CD4^{+} cells using immunomagnetic negative selection, and seeded at 100 cells per well in 96-well plates in the presence of 1 μg per milliliter of phytohemagglutinin and 60 U per milliliter of rIL-2. ^{51}Cr-release assays were performed as previously described.[25,26] Briefly, target cells were incubated overnight with synthetic peptides at 10 μg/mL, labeled with 100 μCi of ^{51}Cr (Amersham, Arlington Heights, IL) for 1 hour, and washed 3 times with HEPES-buffered saline. Cytolytic activity was determined in a 4-hour ^{51}Cr-release assay using U-bottom 96-well plates containing 5,000 target cells per well. All assays were performed in duplicate. Percent cytotoxicity was determined by the formula: 100 × ([experimental release − spontaneous release]/[maximal release − spontaneous release]). Maximal release was determined by lysis of target cells with 1% Triton X-100. HCV-specific CTL lines were expanded by restimulation with allogeneic feeder cells and peptide-pulsed EBV-JY cells, an HLA-A2–positive Epstein-Barr virus (EBV)-immortalized B lymphoblastoid cell line (American Society for Histocompatibility and Immunogenetics, Boston, MA).

HLA Typing. Cell lines were typed by polymerase chain reaction sequence-specific primer typing using the Cyclerplate Protrans System (Quest Biomedical, West Midlands, UK) following the manufacturer's instructions.

Synthetic Peptides. Synthetic peptides were obtained from Chiron Mimotopes (Clayton, Australia).

Vaccinia Viruses. Recombinant vaccinia viruses were generated as described.[27] The vaccinia virus SC59 NNRd, which allows expression of aa 364-1619 (E2 through most of NS3) derived from the HCV-1 strain,[28] and wild-type WR vaccinia virus were kindly provided by Dr. Michael Houghton, Chiron Corp., Emeryville, CA. Vaccinia virus infection of target cells was performed as previously described.[25,26]

Flow Cytometry. Cells were detached from culture dishes using calcium- and magnesium-free medium, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS supplemented with 2% fetal calf serum at a concentration of 10^7 per milliliter. The monoclonal antibodies, 0791HA (anti–HLA-A2), 0735AHA (anti–HLA-B12, 76+), and 0792AHA (anti–HLA-B27, 44, 47+) (all from One Lambda, Canoga Park, CA), were used for MHC class I cell-surface staining. A fluorescein isothiocyanate–conjugated anti-mouse F(ab′)2 fragment (Morwell Diagnostics, Egg, Switzerland) was used as secondary antibody. Incubations with primary and secondary antibodies were performed for 20 minutes at 4°C, followed by 3 washing steps with PBS supplemented with 2% fetal calf serum after each incubation. Flow cytometry was performed with a Becton Dickinson FACS-SCAN flow cytometer and Cellquest software.

Proteasome Activity Assay. The method described by Harding et al.[29] was used with minor modifications to measure intracellular proteasome activity. Cells were harvested, washed twice with PBS, and resuspended in HEPES-buffered saline at a concentration of 10^6 per milliliter. Triplicates of 100 μL were distributed into the wells of a 96-well plate. One hundred microliters of methoxysuccinyl-glycine-leucine-phenylalanine-amido-4-methylcoumarin (MeO-Suc-GLF-AMC) solution, a membrane-permeant fluorogenic substrate of the chymotrypsin-like proteasome activity, was added to each well at the indicated concentrations, and the plate was incubated at 37°C for the indicated times. Fluorescence was measured with a SPECTRFluorPLUS Fluorimeter (Tecan, Gröding, Austria) using 360 nm as excitation and 465 nm as emission wavelengths.

RESULTS

HLA Typing. Polymerase chain reaction sequence-specific primer typing of the U-2 OS–derived cell line UHCV-32, revealed the following HLA class I and II alleles: HLA-A2, A32 (19), B12, B44, DRB1*0901, and DRB1*1401.

U-2 OS–Derived Inducible Cell Lines Are Targets for HLA-A2–Restricted Human CTL. To examine whether U-2 OS–derived inducible cell lines could serve as CTL targets, cells were exogenously loaded with synthetic peptides corresponding to previously identified CTL epitopes and analyzed by ^{51}Cr-release assays with epitope-specific HLA-A2–restricted human CTL lines as effector cells. In the experiment shown in Fig. 1, UHCV-32 cells cultured in the presence of tetracycline were pulsed with 10 μg per milliliter of HCV aa 1073-1081 peptide and analyzed by a 4-hour ^{51}Cr-release assay with the P04 and P52 CTL lines as effector cells. The HLA-A2–positive EBV-immortalized B lymphoblastoid cell line EBV-JY was identically loaded with synthetic peptides and used as a reference in these experiments. U-2 OS–derived inducible cell lines grow as adherent monolayer cultures. For use as CTL targets, cells were detached by a brief incubation with 0.25% trypsin, 1 mmol/L ethylenediaminetetraacetic acid and transferred to non–tissue culture–treated petri dishes, where they remained non- or only slightly adherent. Under these experimental conditions, UHCV-32 and other U-2 OS–derived inducible cell lines were specifically and efficiently lysed by peptide-specific human CTL lines.

U-2 OS–Derived Inducible Cell Lines Process and Present Endogenously Synthesized HCV Proteins Via MHC Class I. We examined whether U-2 OS–derived inducible cell lines could process and present endogenously synthesized HCV proteins via MHC class I. In these cells, steady-state HCV protein expression is reached 24 to 48 hours after tetracycline withdrawal, while no expression of HCV proteins is observed in the pres-

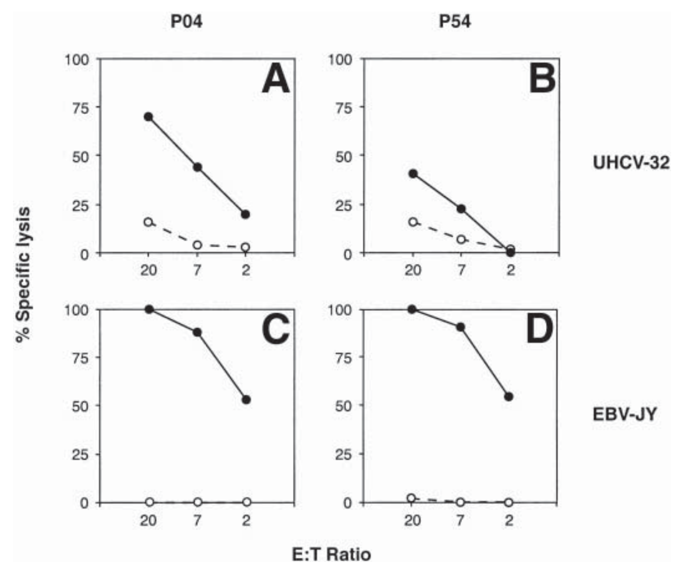

FIG. 1. U-2 OS–derived inducible cell lines are targets for HLA-A2–restricted human CTL. (A) and (B) UHCV-32 cells cultured in the presence of tetracycline or (C) and (D) EBV-JY cells were exogenously loaded with a synthetic peptide corresponding to HCV aa 1073-1081 (CINGVCWTV) and analyzed by a 4-hour ^{51}Cr-release assay with the corresponding peptide-specific CTL lines, P04 and P54. (●), % specific lysis values of peptide-pulsed cells; (○), values for cells not loaded with peptide. E:T ratio, effector-to-target cell ratio.

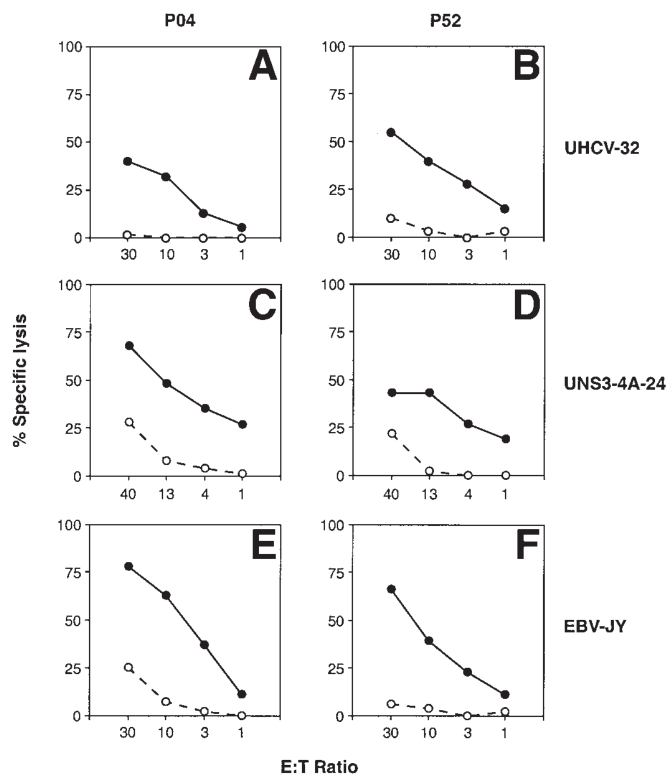

FIG. 2. HLA-A2–restricted HCV-specific human CTL lines recognize HCV proteins endogenously processed in U-2 OS–derived HCV inducible cell lines. (A) and (B) UHCV-32 cells or (C) and (D) UNS3-4A-24 cells were cultured in the presence (○) or absence (●) of tetracycline and analyzed by a 4-hour ^{51}Cr-release assay with the HCV aa 1073-1081–specific CTL lines, P04 and P52. (E) and (F) EBV-JY cells were infected with the recombinant vaccinia virus, SC59 NNRd, which allows expression of HCV aa 364-1619 (●) or with the WR wild-type control vaccinia virus (○) and analyzed by a 4-hour ^{51}Cr-release assay with the HCV aa 1073-1081–specific CTL lines, P04 and P52. E:T ratio, effector-to-target cell ratio.

ence of tetracycline.[18,19] In the experiment shown in Fig. 2, UHCV-32 and UNS3-4A-24 cells were cultured for 48 to 72 hours in the presence or absence of tetracycline, followed by a ^{51}Cr-release assay with P04 or P52 CTL lines as effector cells. EBV-JY cells infected with the recombinant vaccinia virus SC59 NNRd, which allows expression of aa 364-1619 (E2 through most of NS3) derived from the HCV-1 strain,[28] served as controls. As shown in Fig. 2, both UHCV-32 and UNS3-4A-24 cells cultured in the absence of tetracycline, *i.e.*, expressing NS3 in the context of the entire HCV polyprotein and the NS3-4A complex, respectively, were efficiently lysed by NS3-specific human CTL. Cells cultured in the presence of tetracycline, however, were not lysed. These data indicate that the HCV aa 1073-1081 CTL epitope was efficiently generated and demonstrate the tight control of HCV protein expression in tetracycline-regulated cell lines.

HCV Proteins Do Not Affect MHC Class I Cell-Surface Expression. The ability of U-2 OS–derived inducible cell lines to efficiently process and present HCV antigens via MHC class I allowed us to examine whether the expression of HCV proteins interferes with MHC class I antigen processing and presentation as a potential mechanism of immune evasion. In this context, a frequently observed mechanism is down-regulation of MHC class I expression.[13,14] Therefore, we first examined the influence of HCV proteins on MHC class I cell-surface expression. UHCV-11 and -32 cells were cultured for 36 hours in the presence or absence of tetracycline, stained with monoclonal antibodies against HLA-A2, HLA-B12, and HLA-B44, and analyzed by flow cytometry. As shown in Fig. 3, no difference in the expression of these MHC class I molecules was found in UHCV cells that inducibly express all HCV structural and nonstructural proteins in the biologically relevant context of the entire HCV polyprotein.

These findings were substantiated by experiments, in which UNS3-4A-24 cells cultured in the presence or absence of tetracycline were exogenously loaded with a synthetic peptide corresponding to HCV aa 131-140 (ADLMGYIPLV), a previously identified CTL epitope in the core region.[25] Based

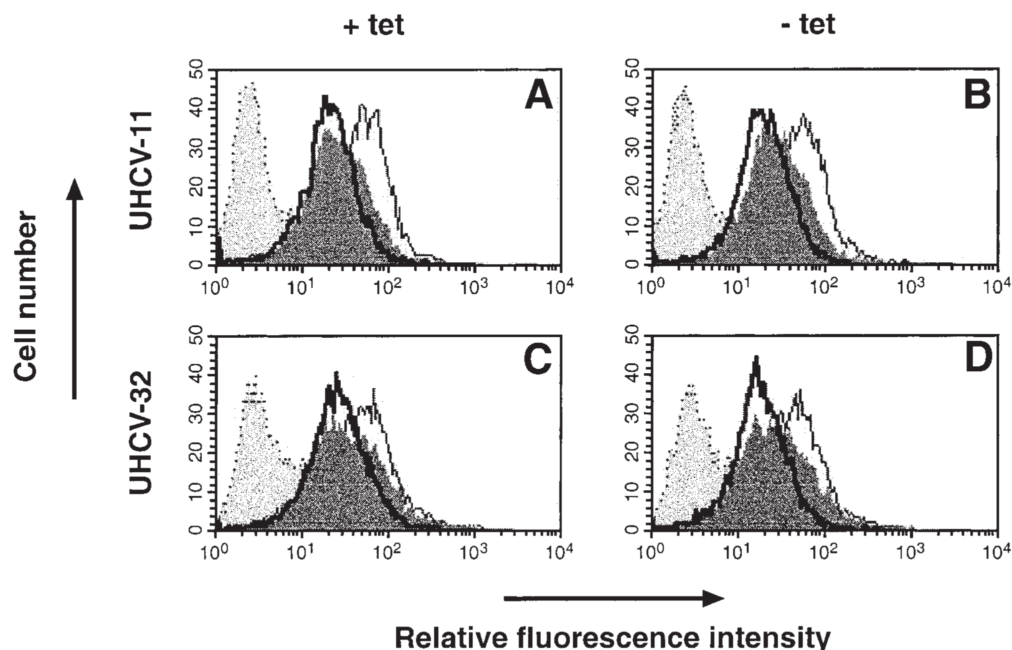

FIG. 3. HCV proteins do not affect MHC class I cell-surface expression. UHCV-11 and -32 cells were cultured for 36 hours in the presence (A and C) or absence (B and D) of tetracycline, stained with monoclonal antibodies against HLA-A2 (*white* histograms with *thin black lines*), HLA-B12 (*dark-gray* histograms), and HLA-B44 (*white* histograms with *thick black lines*), and analyzed by flow cytometry. *Light-gray* histograms with *dotted lines* represent negative isotype controls. Measurements were performed with a Becton Dickinson FACS-SCAN flowcytometer and Cellquest software.

on ^{51}Cr-release assays with HLA-A2–restricted, core peptide–specific CTL lines, no difference in the lysis of peptide-pulsed cells was found in these experiments (data not shown). Similarly, when cell lines inducibly expressing the HCV core protein were cultured in the presence or absence of tetracycline and then loaded with the HCV aa 1073-1081 peptide, there was no difference in lysis by the NS3 peptide–specific CTL lines, P04, and P52 (data not shown). These experiments clearly indicate that the HCV core and NS3-4A proteins do not interfere with functional expression of MHC class I molecules on the surface of U-2 OS–derived inducible cell lines.

HCV Proteins Do Not Interfere With Intracellular Proteasome Activity. Endogenously synthesized antigens are processed by the proteasome, a multicatalytic protease complex that is present in the cytosol and the nucleus.[30] Interference of viruses with this step of MHC class I processing has been described.[13,14] We therefore examined the effect of HCV protein expression on proteasome activity. Intracellular proteasome activity was measured by incubation of UHCV cells cultured in the presence or absence of tetracycline with MeOSuc-GLF-AMC, a membrane-permeant fluorogenic substrate of the chymotrypsin-like proteasome activity.[29] As shown in Fig. 4, this intracellular proteasome activity was not affected by the expression of HCV structural and nonstructural proteins in UHCV-11 and UHCV-32 cells.

As a control, we tested whether a limitation of proteasome activity and, as a consequence, of peptide supply for MHC class I molecules would lead to a down-regulation of class I cell-surface expression in U-2 OS–derived inducible cell lines. To this end, UHCV-11 and UHCV-32 cells were cultured for 16 hours in the presence of 20 or 50 μm of the specific proteasome inhibitor lactacystin, and subsequently, cell-surface expression of HLA-A2, HLA-B12, and HLA-B44 was assessed by flow cytometry. As expected, a profound, dose-dependent reduction in the cell-surface expression of HLA molecules was observed as a result of proteasome inhibition, indicating that a potential HCV-mediated interference with the generation, transport, or loading of class I ligands would have been detected if it had occurred (data not shown).

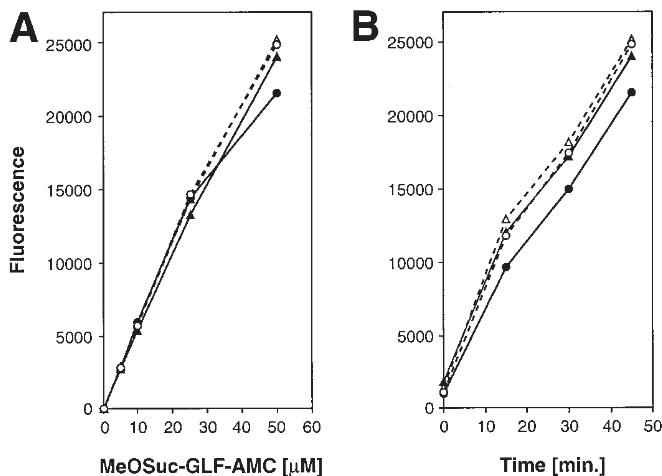

FIG. 4. HCV proteins do not interfere with intracellular chymotrypsin-like proteasome activity. UHCV-11 (○, ●) and -32 cells (△, ▲) were cultured for 36 hours in the presence (○, △) or absence (●, ▲) of tetracycline. Proteasome activity was measured with the fluorogenic peptide substrate, MeOSuc-GLF-AMC, as described.[29] (A) Cells were incubated for 45 minutes at the indicated concentrations of MeOSuc-GLF-AMC. (B) Cells were incubated for the indicated times in the presence of 50 μmol/L MeOSuc-GLF-AMC. Fluorescence was measured with a SPECTRFluorPLUS Fluorimeter (Tecan) using 360 nm as excitation and 465 nm as emission wavelengths. Data points represent mean values of triplicates with a standard deviation $<5\%$. No significant difference between the intracellular chymotrypsin-like proteasome activity of cells cultured in the presence or absence of tetracycline was observed in 3 independent experiments.

DISCUSSION

The mechanisms by which HCV evades the host immune response to cause persistent infection in the majority of patients are poorly understood. Various strategies of immune evasion have been proposed, including, among others, down-regulation of HLA gene expression, viral interference with antigen processing and presentation, inhibition of cellular signaling pathways, and the generation of viral humoral and cellular immune escape variants.[3,4] The lack of an efficient cell-culture system or a suitable small-animal model permissive for HCV infection and replication, however, has thus far limited the systematic investigation of these mechanisms.

Here, we demonstrate that U-2 OS human osteosarcoma–derived tetracycline-regulated cell lines can efficiently process and present endogenously synthesized HCV proteins via MHC class I and can serve as targets for HCV-specific HLA-A2–restricted human CTL. For use as CTL targets, these cells, which normally grow as monolayers, were cultured in non–tissue culture–treated petri dishes, where they remained non- or only slightly adherent. Under these experimental conditions, U-2 OS–derived inducible cell lines were specifically and efficiently lysed by peptide-specific human CTL lines. As compared with peptide-pulsed or vaccinia virus–infected EBV-immortalized B lymphoblastoid cells, specific lysis values observed in these cells were somewhat lower. This may, among other factors, be explained by lower MHC class I cell-surface expression on U-2 OS cells as compared with EBV-JY cells (data not illustrated), and by a certain degree of heterogeneity of protein expression levels inherent to the tetracycline-regulated gene expression system.[31,32] However, vaccinia virus–infected EBV-JY cells were nonviable after 24 hours, whereas the inducible cell lines continued to express the proteins for at least 7 to 10 days, at which time cells had to be passaged. The U-2 OS–derived inducible cell lines, therefore, represent a unique tool to analyze virus-host interactions in a steady-state setting and without interference of vaccinia virus gene products.

The ability of these cell lines to efficiently process and present HCV antigens via MHC class I allowed us to examine whether the expression of HCV proteins interfered with this central pathway as a potential mechanism of immune evasion. It must be emphasized that UHCV cells express HCV structural and nonstructural proteins in the context of the entire polyprotein. We believe that this is a particularly important feature, because interactions between viral proteins will influence their characteristics. In this regard, we have recently shown that the 54-aa polypeptide cofactor NS4A profoundly influenced the subcellular localization, stability, and *trans*-cleavage competence of the HCV NS3-4A complex.[19] These and other recent observations[33-35] demonstrate the importance of studying HCV proteins in the context of the entire polyprotein.

Down-regulation of MHC class I expression is a commonly observed mechanism of viral persistence.[13,14] However, no difference in the cell-surface expression of MHC class I molecules was found in two independent clones of UHCV cells

that inducibly express all HCV structural and nonstructural proteins. Therefore, under these experimental conditions, HCV does not appear to use this strategy. Another strategy, namely up-regulation of MHC class I antigens, is suspected to be used by other members of the *Flaviviridae* family to impair formation of memory.[36] Transient MHC class I up-regulation may, according to a model proposed by these authors, lead to autoimmunity, followed by down-regulation, resulting in reduced autoimmunity and virus-specific T-cell memory formation. Our data, however, do not indicate an increased expression and presentation of MHC class I antigens, and thus does not support this concept for HCV. We cannot, however, rule out more complex regulatory mechanisms acting via modulation of MHC class I antigen expression.

Another potential mechanism of immune evasion is interference of viral gene products with processing of endogenously synthesized antigens by the proteasome, a multicatalytic protease complex that is present in the cytosol and the nucleus.[30] In human cytomegalovirus–infected cells, for example, expression of the viral phosphoprotein pp65 inhibits the generation of virus-specific T-cell epitopes[37] and the EBV nuclear antigen-1 contains a Gly-Arg repeat that interferes with its proteasomal proteolysis.[38] We therefore examined whether HCV proteins interfere with proteasome activity. In this context, preliminary experiments performed with the proteasome inhibitor lactacystin indicated that the intracellular generation of the HCV aa 1073-1081 epitope is indeed proteasome-dependent (data not illustrated). Intracellular proteasome activity was measured by incubation of UHCV-11 and -32 cells cultured in the presence or absence of tetracycline with MeOSuc-GLF-AMC, a membrane-permeant fluorogenic substrate of the chymotrypsin-like proteasome activity.[29] In this experimental system, intracellular proteasome activity was not affected by the expression of HCV structural and nonstructural proteins. Taken together, our results indicate that the expression of HCV proteins does not affect MHC class I processing and presentation in UHCV and other U-2 OS–derived inducible cell lines.

In conclusion, based on experiments with HLA-A2–positive U-2 OS–derived tetracycline-regulated cell lines that efficiently process and present endogenously synthesized HCV proteins via MHC class I, we demonstrate that expression of HCV proteins does not interfere with functional MHC class I cell-surface expression and intracellular proteasome activity. Interactions of HCV with MHC class I processing and presentation, therefore, appear not to be central to viral immune evasion. Future studies will address whether CTL epitopes other than the HCV aa 1073-1081 epitope analyzed in this study will behave in the same way. Based on the data presented here, however, it is likely that HCV targets other cellular defense systems. In this context, interference of HCV proteins with interferon alfa–induced signaling through the Jak-STAT pathway[21] or with interferon effector functions[22,39-42] may be operative. Further studies using tetracycline-regulated cell lines as target cells for HCV-specific human CTL should allow for the identification of novel and genuinely immunodominant CTL epitopes and for the isolation of naturally processed MHC class I ligands. Such studies may ultimately lead to the development of novel immunotherapeutic strategies against HCV infection.

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