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Induction of Cytotoxic T Lymphocyte Response against the Core and *NS3* Genes of the Hepatitis C Virus in *Balb/c* Mice

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Hepatitis C virus (HCV) is a positive strand RNA virus of the Flaviviridae family and the major cause of post-transfusion non-A, non-B hepatitis. Vaccine development for HCV is essential but has been slowed by poor understanding of the type of immunity that naturally terminates HCV infection. The DNA-based immunization technique offers the potential advantage of including cellular immune responses against conserved internal proteins of a virus, as well as the generation of antibodies to viral surface proteins. Here, we demonstrate that cell lines expressing the HCV core and/or NS3 proteins can induce a specific CTL response in mice, and these results suggest a possibility that the HCV core and NS3 DNA can be used to induce CTL activity against the antigen in mice and can be further developed as a therapeutic and preventive DNA vaccine.

Hepatitis C virus (HCV) is a positive strand RNA virus of the Flaviviridae family and the major cause of post-transfusion non-A, non-B hepatitis (Levy et al., 1994). More than 50% of acutely infected individuals progress to a chronic carrier state that frequently results in cirrhosis. In addition, HCV infection is an independent risk factor for the development of hepatocellular carcinoma. Currently, interferon (INF) α alone or the two-drug combination of INFα and ribavirin is the only treatment, but its use is limited by low efficacy and high toxicity (Carithers and Emerson, 1997). Hence, alternative anti-HCV therapy is urgently needed to treat the growing population of patients. The induction of neutralizing antibodies of broad reactivity was hampered by several reasons; heterogeneous nature of HCV genome, high mutation rate, and hypervariable region in envelope proteins. Vaccine development is essential but has been slowed by poor understanding of the type of immunity that naturally terminates HCV infection (Sharara et al., 1996), which is rarely observed contemporaneously, and methods for thorough analysis of successful responses in protected individuals. To date, no one has been able to grow HCV reliably in laboratory cell cultures. This has slowed critical studies of everything from drugs to vaccines to the basic understanding of the viral life cycle. Thus far, a group of researchers have recently developed a new HCV culture system (Lohmann et al., 1999).

Recent studies suggest that $CD8^+$ cytotoxic T lymphocyte (CTL) are better correlated with protection against HCV infection than antibodies and imply that cell-mediated immune responses may have eliminated earlier infections and could now protect them from subsequent infections (Cooper et al., 1999). Cytotoxic T lymphocyte, which are believed to be central in the immunosurveillance of virally infected and transformed cells, can recognize intracellular proteins in the form of peptides presented by major histocompatibility complex (MHC) class I proteins (Townsend and Bodmer, 1989). Cellular and pathogenic proteins are proteolytically degraded to peptide fragments and transported to the endoplasmic reticulum where they bind to MHC class I molecules. These complexes are transported to the surface to be surveyed by the T cell receptor population. The ability of co-expressed MHC molecules to bind peptides of diverse sequences and the presence of T cells recognizing the peptide-bound MHC proteins will determine whether a CTL response to a specific pathogen can be induced in each individual. Protein antigens, therefore, can be used as tools for immunotherapy and immunoprevention. However, protein antigens provided exogenously can not be presented by MHC class I molecules. To circumvent this, a gene encoding the protein antigen has been delivered into cells directly so that the encoded protein is loaded on the MHC class I molecules.

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Several studies have shown that the DNA-based immunization technique offers the potential advantage of including cellular immune responses against conserved internal proteins of a virus, as well as the generation of antibodies to viral surface proteins (Donnelly et al., 1997; Tighe et al., 1998). Much knowledge has been accumulated showing that genetic immunization using plasmids expressing the HCV protein elicit strong CTL responses (Geissler et al., 1997; Encke et al., 1998; Large et al., 1999). Here, we demonstrate that cell lines expressing the HCV core and/or NS3 proteins can induce a specific CTL response in mice, and these results suggest a possibility that the HCV core and NS3 DNA can be used to induce CTL activity against antigen in mice and can be further developed as a therapeutic and preventive DNA vaccine.

Materials and Methods

Mice

Balb/c mice, 6-10 weeks old, were maintained in the Institute of Molecular Biology and Genetics, Seoul National University.

Cell lines

BNL CL.2 normal liver cell line (ATCC, Rockville, MD) and BNL 1NG A.2 transformed liver cell line (ATCC, Rockville, MD) were Balb/c mouse (H-2^d) origin. Both cell lines were maintained at 37℃ in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 100 units per ml of penicillin (Sigma), and 100 µg per ml of streptomycin (Sigma).

Construction of MFG retroviral vectors expressing HCV core and/or HCV NS3

MFG retroviral vector has moloney murine leukemia virus (Mo-MuLV) long terminal repeat (LTR) sequences which are used to generate both a full-length viral RNA for encapsidation into virus particles and a subgenomic mRNA for expression of inserted sequences. A 573 bp DNA fragment (HCV core) and a 1767 bp DNA fragment (HCV NS3) (Kim and Choe, 1997) were inserted between *Nco*I and *Bam*HI sites of the MFG retroviral vector, respectively, in such a way that the initiation codon of the inserted sequence was placed precisely at the position of the viral *env* initiation codon, and a minimal 3' nontranslated sequence was included in the insert. MFG retroviral vector sequence was inserted into the *Hind*III and *Eco*RI sites of pUC19. For the simultaneous expression of core and NS3 proteins, murine internal ribosomal entry site (IRES) was located between core and NS3 protein coding sequences (Fig. 1).

Introduction of the vectors into cells

To produce cell lines permanently expressing the HCV core and/or NS3 protein, each vector constructed and

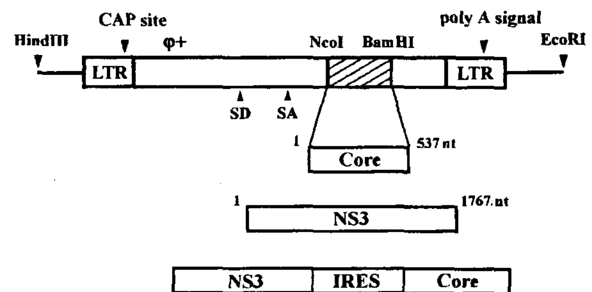

Fig. 1. DNA constructions for the expression of HCV core and/or NS3 proteins. A 573 bp DNA fragment of HCV core gene and a 1767 bp fragment of NS3 gene were inserted between the NcII and *Bam*HI sites of the MFG retroviral vector, respectively.

pSV2Neo^R containing a neo^R selection marker gene were co-transfected into the BNL CL.2 or BNL 1NG A.2 cell line by calcium phosphate precipitation method (Sambrook et al., 1989). The transfected cells were cultured with media containing 500 µg/ml of Geneticin (Gibco). The selected clones were named CL.2-core, CL.2-NS3#1, CL.2-NS3#2, A.2-core, A.2-NS3, and A.2-NS3core depending on the cell line used and genes transfected.

Western blot analysis

The selected clones as described above were analyzed for the presence of 21 KDa core protein or 65-70 KDa NS3 protein by Western blot analysis (Geissler et al., 1997). In brief, cell lysates were prepared in RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 50 mM Tris, 0.5% deoxycholate, and 1% SDS), separated by 15% SDS-PAGE for 21 KDa core protein or 10% SDS-PAGE for 65-70 KDa NS3 protein, and electrotransferred onto Protran membrane (Schleicher and Schuell). After blocking with 1% low fat dry milk, membranes were incubated with a anti-HCV core mAb or anti-HCV NS3 mAb, respectively, followed by detection of bound mAb with alkaline phosphatase-conjugated second antibody.

Cytotoxic T lymphocyte assay

To generate HCV core and/or NS3-specific CTL, Balb/C mice were immunized by intraperitoneally injecting with CL.2-core, CL.2-NS3#1, CL.2-NS3#2, A.2-core, A.2-NS3, or A.2-NS3 core cells (5X10^6 cells). For a CTL assay, the Jam test was employed as previously described (Matzinger, 1991; Kim et al., 1996). In brief, spleens were removed from mice after 2-6 wk of immunization and the spleen cells (4×10^6 cells) were cocultured with the same injected, but inactivated cell lines (2×10^6 cells) in 2 ml of RPMI-1640 (Gibco) containing 10% fetal bovine serum (Hyclone), 100 units/ml of penicillin (Sigma), and 100 µg/ml of streptomycin (Sigma). Each cell line was inactivated by treatment with mitomycin C (25 µg/ml) (Sigma) for 30 min just before the co-culture (Coligan et al., 1992). After 4 d of stimulation, the spleen cells were harvested and diluted

serially into a round bottom 96-well plate. As target cells, cells such as the injected cell lines were labeled with 5 µCi/ml [^{3}H] thymidine for 15-18 h, washed, and plated into wells (1×10^4 cells per well) containing graded numbers of stimulated sells. The plates with cells were incubated at 37℃ for 15-18 h and then harvested. Rather than collecting a standard volume of supernatant from each culture, which gives an estimate of the label released due to plasma membrane disintegration, the cells were harvested using a PHD Cell Harvester (Cambridge Technology) and vacuum aspiration onto glass fiber filters precisely as for cell proliferation assays. After counting the [^{3}H] thymidine remained on the filters, percentages of the specific killing was calculated as previously described (Matzinger, 1991; Kim et al., 1996).

Results

Expression of HCV core and NS3 proteins in mammalian cells

DNA sequences encoding HCV core and NS3 protein were inserted, respectively, between the *Nco*I and *Bam*HI sites of the MFG retroviral vector. For the simultaneous expression of core and NS3 proteins, murine internal ribosomal entry site (IRES) was inserted between the core and NS3 coding sequences (Fig. 1). Each expressing vector was transfected into mouse normal liver cell line (BNL CL.2) or mouse transformed liver cell line (BNL 1NG A.2), originated from the BNL CL.2, by the calcium phosphate precipitation method. The protein expression of these transfected cells was confirmed by Western blot analysis with antibodies recognizing each protein. As shown in Fig. 2, HCV core protein of approximately 21 KDa and HCV NS3 protein of about 65-70 KDa were detected. We have also confirmed that the bicistronic recombinant plasmids successfully led to synthesis of the two proteins. We choose the clones showing the highest expression HCV proteins by Western blotting, and used them for further studies. The selected clones were named CL.2-core, CL.2-NS3#1, CL.2-NS3#2, A.2-core, A.2-NS3, and A.2-NS3 core depending on the cell line used and DNA constructs transfected.

Induction of CTL responses against HCV core and NS3 transfectants in mice

We have investigated CTL response induced against syngeneic cells expressing the HCV core NS3. Cells of each selected clone were injected into syngeneic Balb/c mice (H-2^d). After 2 wk of immunization, spleen cells were collected from immunized mice and were stimulated *in vitro* with the same, but inactivated cells. CTL assay was carried out 4-5 d later using the same clone cells, non-transfectant cells, or mock-transfected cells as target cells. For example, the spleen cells which had been immunized with CL.2 NS3#1 cells were stimulated *in vitro* by CL.2-NS3#1. The CL.2-NS3#1 cells were used as target cells for the stimulated spleen cells. For negative controls, non-transfected BNL CL.2 cells and MFG vector transfected BNL CL.2 were used as target cells.

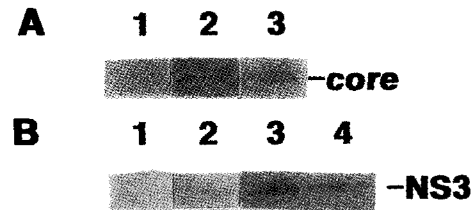

Fig. 2. Expression of HCV core and NS3 proteins in mouse liver cell transfectants. HCV core protein of 21 KDa and HCV NS3 protein of 65-70 KDa were detected by Western blot analysis using anti-HCV core mAb and anti-HCV NS3 mAb, respectively. In panel A, lane 1, BNL CL.2 cell not transfected; Lane 2, CL.2-core; Lane 3, A.2-NS3core. In panel B, lane 1, BNL CL.2 cell not transfected; Lane 2, CL.2-NS3#1; Lane 3, CL.2-NS3#2; Lane 4, A.2-NS3.

CTL activities against CL.2-NS3#1, CL.2-NS3#2, A.2-NS3 cells, but not against non-transfectant cells nor against mock-transfected cells, were induced (Fig. 3). At 66.8:1 of effector to the target ratio (E:T ratio), specific killing was shown to be about 74% in cells expressing A.2-NS3, but it was only 13.1% in the control. At the same E:T ratio, the lowest specific killing was 47.83% observed in CL.2-NS3#1. It was also a significant specific killing compared to the control. The difference of the two clones seemed to be due to the difference in the protein expression level. Comparing CL.2-NS3#1 with CL.2-NS3#2, the latter clone showing higher specific killing of 62.25% was shown to express more NS3 in the Western blot (Fig. 2 and 3). Therefore, the result shows the presence of CTL activity specific to the HCV NS3 gene product, but not to BNL CL.2 nor BNL 1NG A.2, nor MFG vector itself. Specific killing seemed to reach a plateau between 8.35:1 and

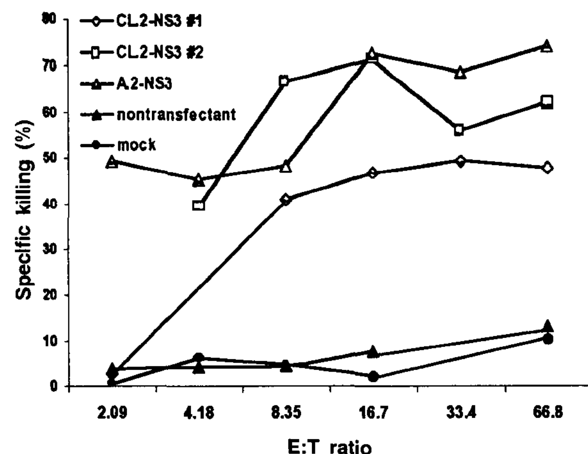

Fig. 3. Cytotoxic activity of the HCV NS3-specific CTL. Balb/c mice were immunized with the HCV NS3 gene-transfected cells and specific CTL responses were induced. Effector cells derived from the spleens of immunized mice were stimulated *in vitro* with the same, but inactivated cells. Target cells used are indicated in the figure. The Jam test was performed to measure CTL activity.

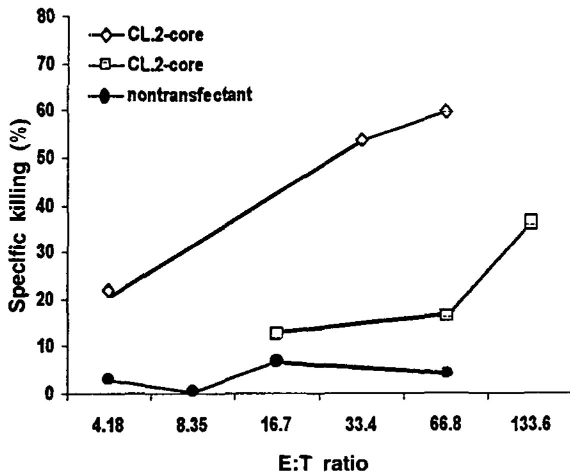

Fig. 4. Cytotoxic activity of the HCV core-specific CTL. Balb/c mice were immunized with the HCV core gene-transfected cells and specific CTL responses were induced. Effector cells derived from the spleens of immunized mice were stimulated in vitro with the same, but inactivated cells. Target cells used are indicated in the figure. The Jam test was performed to measure CTL activity.

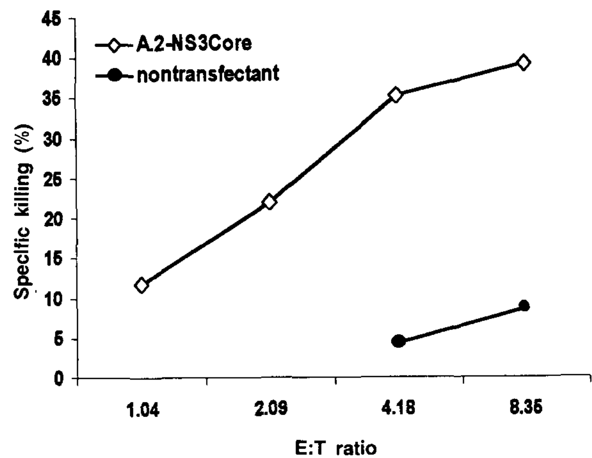

Fig. 5. Cytotoxic activity of the HCV NS3core-specific CTL. Balb/c mice were immunized with the HCV NS3 and core gene-transfected cells and specific CTL responses were induced. Effector cells derived from the spleens of immunized mice were stimulated in vitro with the same, but inactivated cells. Target cells used are indicated in the figure. The Jam test was performed to measure CTL activity.

16.7:1 in our experiment. The CTL data shows results from a single representative of triplicate experiments out of two independent experiments.

The experiment using CL.2-core showed a similar result as the one using CL.2-NS3 (Fig. 4). CTL activity against CL.2-core cells, but not against the control, was detected. The highest specific killing was 59.6% at the E:T ratio of 66.8.

To develop a more powerful immunogen of HCV, we attempted to induce CTL activity against two HCV gene products simultaneously using a cell line transfected with bicistronic recombinant vectors. Significant CTL activity against A.2-NS3 core cells was induced (Fig. 5). At 8.35:1 of E:T ratio, 39.22% of specific killing was observed compared to 8.6% of killing in the control. It is a similar or slightly low level of specific killing against the HCV core only or against the HCV NS3 only at the same E:T ratio (Fig. 3 and 4). Therefore, the level of CTL activity induced by the A.2-NS3core was not significantly enhanced compared to the one induced by A.2-NS3, even though the two experiments were performed separately.

Discussion

Hepatitis C virus is an emerging virus of great medical importance and almost always cause chronic infections. Vaccine development is therefore essential but has been slowed by poor understanding of the type of immunity that naturally terminates HCV infection (Sharara et al., 1996), which is rarely observed contemporaneously, and methods for the thorough analysis of successful responses in protected individuals. DNA vaccines represent a novel means of expressing antigens *in vivo* for the generation of both humoral and cellular immune responses. So, using DNA vaccines have some advantages compared to conventional protein vaccines (Donnelly et al., 1997; Tighe et al., 1998). Much knowledge has been accumulated showing that genetic immunization using plasmids expressing HCV protein elicit strong CTL responses (Geissler et al., 1997; Encke et al., 1998; Large et al., 1999). The most popular targets to date have been the core, nucleocapsid protein and NS3, the serine protease which is one of two viral enzymes that helps clip HCV's polyprotein into functional proteins.

Retroviral vectors are the current vehicle of choice for stable gene delivery and expression in target cells (Crystal, 1995). Moloney murine leukemia virus (MoMuLV) is widely used as a gene delivery vehicle in gene therapy. Between the two most popular types of retroviral vectors, MFG and LN-series, the former can derive higher gene expression than the latter.

In order to examine the possible development of DNA vaccine of HCV, we have cloned the HCV core and/or NS3 DNA into retroviral protein-expression vectors, MFG, and introduced them into mouse liver cell lines, BNL CL.2 and its transformant BNL 1NG A.2, originated from Balb/c mice. When Balb/c mice were immunized with the transfected cell lines, which had been confirmed for the expression of the HCV core or NS3 protein by Western blotting, specific CTL responses were induced. CTL response was not induced by non-transfected cells and by vehicle control (mock)-transfected cells. However, different target cells displayed different levels of specific killing. This phenomenon was probably due to different levels of expression of the HCV gene product as shown in Fig. 3.

We could not compare the absolute protein expression levels of all clones used in this study at the same time. The different protein expression levels of each clone may explain that co-expression of two HCV

proteins could not induce CTL activity more effectively than the expression of NS3 or the core protein alone. It was recently reported that the HCV core protein showed immunosuppressive activity in vaccinia virus-specific CTL responses (Large et al., 1999). It is not clear how the HCV core protein suppressed the CTL response to vaccinia virus. It may require other cellular or viral component(s) to display immunosuppressive activity in a cell. However, the core protein itself seems to be immunogenic to induce CTL activity since cellular and humoral immune responses to the hepatitis C virus core protein was observed using DNA-based vaccines together with cytokine-expressing plasmids (Geissler et al., 1997). Our CTL activities against HCV core or NS3 gene product were much higher than the recent reports from Wands's laboratory (Geissler et al., 1997; Encke et al., 1998). It may be because they injected naked DNA, whereas we injected transfected cell lines expressing HCV genes. They reported that at a E:T ratio of 100:1, about 25% of specific killing against HCV core gene and about 30% against HCV NS3 gene were observed. Our data showed at a E:T ratio of 66.8:1, about 60% specific killing against HCV core, and about 48-74% HCV NS3. These results imply that efficient expression of the injected DNA is very important to enhance CTL activity and also that efficient immunotherapy or immunoprevention requires not only a potent immunogen, but also a high level expression of a surface target antigen. Our results suggest a possibility that the HCV core and NS3 DNA can be used to induce CTL activity in mice and can be further developed as a therapeutic and preventive DNA vaccine.

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