Specific immune responses induced by a multi-epitope antigen of hepatitis C virus in mice and rabbits

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Abstract Five highly conserved and immunogenic epitopes of hepatitis C virus (HCV) have been chosen to form a multi-epitope antigen gene and fused with β-galactosidase gene to express a hybrid GZ-PCX antigen, which could be specifically recognized by human HCV sera. High level of anti-GZ-PCX IgG has been induced when mice or rabbits were immunized with GZ-PCX antigen emulsificated with complete Freund's adjuvant or mixed with killed attenuated *Salmonella typhimurium* SL3261. The specific anti-GZ-PCX IgG reached a high titer of 10^{-6}, which remained for several months. Specific cytotoxic T lymphocyte (CTL) effects, delayed type hypersensitivity reaction (DTH) and proliferation of peripheral lymphocytes have been induced by GZ-PCX antigen or synthetic peptides. High level of anti-GZ-PCX sIgG has been detected in mice's intestinal washing fluids, which indicates that the antigen induced mucosal immunity as well as systematic immunity. The studies show that the HCV multi-epitope antigen induces high level of specific immune responses without obvious toxicity, which might be able to provide protectivity to any HCV genotypes and isolates.

Keywords: multi-epitope antigen, vaccine, immune response, hepatitis C virus (HCV).

Hepatitis C is one of the common infectious diseases, which lacks ideal treatment and prevention. B and T cell epitopes of HCV antigens might be helpful for the design of effective HCV vaccines. 5 highly conserved and immunogenic B and/or T cell epitopes corresponding to C, E1, NS3 and NS5 antigens of HCV and one foreign T cell epitope of tetanus toxin (TT) have been designed as a multi-epitope antigen. The synthetic multi-epitope antigen PCX, which encodes 89 amino acids[1], was fused with β-galactosidase (GZ) and highly expressed in *E.coli*[2]. The expressed product was used to immunize mice and rabbits. In the present study, the specific immunogenicity of the multi-epitope antigen was reported.

1 Materials and methods

(i) Cell line. BALB/c 3T3 cell was obtained from the Institute of Cell Biology of Chinese Academy of Sciences.

(ii) Agents. Horseradish peroxidase (HRP) labelled rabbit anti-mouse IgA, lipopolysaccharide (LPS) and bovine serum albumin (BSA) were ordered from Sigma; fetal bovine serum and IL-2 were the products of Sino-American Biotechnology Company; HRP-rabbit anti-human IgG, HRP-goat anti-rabbit IgG, HRP-rabbit anti-mouse IgG, ConA, PHA-M were purchased from Watson Biotechnology Company; GZ-PCX and GZ antigen were purified as described in ref. [2].

(iii) Isotope. ^{51}Cr and ^{3}H were ordered from Amersham Company.

(iv) Peptide synthesis. Peptide P2 (C:132—141 aa) and P6 (NS3: 1445—1453 aa) were synthesized by Cybersyn Inc. (New York, USA). P1 (C: 1—16 aa), P3 (NS5: 2781—2788 aa), P5(E1: 317—326 aa) and P4 (TT: IYSYFPSVD, 592—600 aa) were synthesized by Becks Inc. (Tokyo, Japan) and kindly donated by Prof. Kazunari K. Yokoyama of Tsukuba Life Science Center in Japan.

(v) Animals. ICR mice (4-week-old males and females, 17—21 g) and New Zealand rabbits (26-week-old males, 2.0—2.5 kg) were obtained from the Navy Medical Institute. Every group contained 5—10 mice or 4—7 rabbits.

(vi) Immunization. GZ-PCX or GZ antigen was dissolved in phosphate buffered saline (PBS), then emulsificated with complete Freund's adjuvant (Fa) or mixed with killed attenuated *Salmonella typhimurium* SL3261(St). Mice or rabbits were immunized subcutaneously at multiple sites every two weeks for 3 times, each with 100 μg or 1.0 mg of GZ-PCX Fa or GZ-PCX St. Blood samples were taken for serum collections from rabbit ear vein and mouse tail vein every two weeks after immunization.

(vii) Detection of specific antibodies. The procedures of ELISA were described in ref. [3]. Detection of anti-GZ-PCX IgG: GZ-PCX antigen was used as coated antigen, HRP-goat anti-rabbit or HRP-rabbit anti-mouse IgG was diluted to 1:10 000; detection of mouse anti-GZ-PCX IgA: GZ-PCX was used as coated antigen, HRP-rabbit anti-mouse IgA was diluted to 1:1 000; detection of mouse anti-GZ-PCX sIgG and sIgA in intestinal washing fluids: the intestinal washing fluids were used to react with GZ-PCX antigen, and HRP-rabbit anti-mouse IgG or IgA was used to combine with the antigen-antibody component; detection of anti-LPS IgG: LPS was used as coated antigen and HRP-goat anti-rabbit or HRP-rabbit anti-mouse IgG was added.

(viii) Detection of cellular immunity

(1) Murine CTL assay. Cytolytic activity of spleen cells *in vitro* was measured as previously described by using a 6-h assay ^{51}Cr-labeled targets[4,5]. To test antigen or peptide specific CTLs, ^{51}Cr-labelled BALB/c 3T3, as target cells, were mixed with 10 μmol/L antigen or peptide and cultured for 9 d. The ratio of effectors to target cells (E:T) was 100:1. The percentage of specificity of ^{51}Cr release was calculated as 100×[(experimental release−spontaneous release)/(maximum release−spontaneous release)]. Maximum release was determined from supernatants of cells that were lysed by 5% Triton X 100 and spontaneous release of ^{51}Cr was from target cells incubated without effector cells.

(2) ^{3}H-TdR incorporation assay. The assay of ^{3}H-TdR incorporation was previously described in ref. [3]. Lymphocytes from rabbits or mice from peripheral blood in 100 μL aliquots were each suspended in 0.9 mL RPMI-1640 medium containing 10 μmol/L antigen or peptide in 24-well plates. After cultivation at 37℃ for 72 h, 2 μL of ^{3}H-TdR was added to each tube and incubated further for another 16 h. Cells were harvested, and each sample received 40 μL of glacial acetic acid, washed by 5% trichloroacetic acid (TCA) and then dehydrated by ethanol. Radioactivity was measured by scintillation counting (Model LS6500, Beckmann, USA). The percentage of lymphoblast transformation was calculated by 100×(mean cpm with antigen)/(blank mean cpm).

(3) Delayed type hypersensitivity (DTH). This assay was carried out following the procedure described in ref. [3]. Footpad swelling of mice and diameter of swelling region of rabbits were measured 24 h after intradermal injection of 10 μg or 50 μg of antigen or peptide to each mouse or rabbit, respectively.

(ix) Safety. Mice were weighed individually during the first six weeks. Changes of their livers and spleens in weight and volume were determined.

2 Results

(i) Detection of anti-GZ-PCX IgG. Low level of anti-GZ-PCX antibody in mice was detected at week 2 and the titer of antibody reached the highest 10^{-6} at week 6, then decreased to about $1:10^4$ at week 16 (fig. 1). The titer of antibody of GZ-PCX Fa group (GZ-PCX emulsificated with complete Freund's adjuvant) is slightly higher than that of GZ Fa and GZ-PCX St (GZ-PCX mixed with killed attenuated *S. typhimurium* SL3261), but without significant difference. However, the killed attenuated *S. typhimurium* could serve as an efficient adjuvant in mice, although the titer of antibody is lower than that of complete Freund's adjuvant.

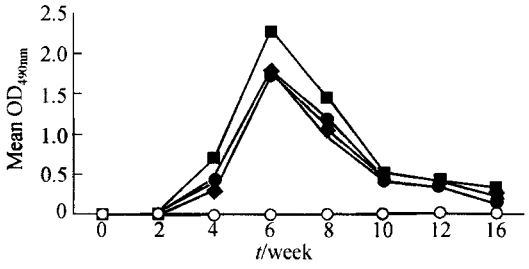

Fig. 1. Mouse anti-GZ-PCX IgG responses after immunization of GZ-PCX or GZ antigen mixed with complete Freund's adjuvant or killed attenuated *S. typhimurium* SL3261. ■, GZ-PCX Fa; ▲, GZ Fa; ◆, GZ-PCX St; ●, GZ St; ○, blank. GZ-PCX antigen was used to immunize ICR mice and it induced detectable anti-GZ-PCX antibodies from week 2. After the 3rd immunization, the titer boosted to the highest level of 10^{-6} at week 6, then reduced and kept stable from week 10. When the killed attenuated *S. typhimurium* SL3261 was used as an adjuvant, it induced almost the same immune responses as that of complete Freund's adjuvant. The control protein GZ could also induce high level of specific antibody reactions. Antisera were diluted to 1:6400.

In rabbits, the strongest anti-GZ-PCX IgG response of Fa group appeared at about week 8. The highest titer reached to about 10^{-5} both in GZ-PCX and GZ group and kept stable for several months (fig. 2), which was different from the titration curve of mice immunized with GZ-PCX antigen. For St group, the strongest response was observed at weeks 4—6, but decreased to only 1:800 at week 16, which was identical compared with Fa group ($P<0.001$). Thus, we conclude that St, as an adjuvant, induces distinctive immune response in mouse or rabbit.

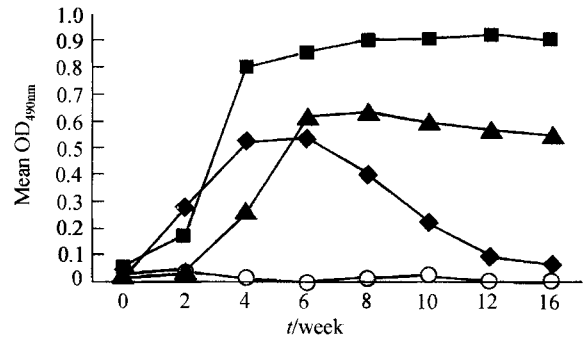

Fig. 2. Rabbit anti-GZ-PCX IgG responses after immunization of GZ-PCX or GZ protein mixed with complete Freund's adjuvant or killed attenuated *S. typhimurium*. ■, GZ-PCX Fa; ▲, GZ Fa; ◆, GZ-PCX St; ○, blank. GZ-PCX antigen was used to immunize New Zealand rabbits and it induced detectable anti-GZ-PCX antibodies from week 2 and after the 3rd immunization, the titer boosted to the highest level of 10^{-5} at week 8 and kept stable for several months. When the killed attenuated *S. typhimurium* SL3261 was used as an adjuvant, it induced much lower antibody reactions than that of complete Freund's adjuvant group. The control protein GZ also induced lower level of specific antibody reactions. Antisera were diluted to 1:6400.

(ii) Detection of anti-GZ-PCX IgA. Low level of anti-GZ-PCX IgA was detected in the sera of immunized mice, which showed significant difference between GZ-PCX St group and blank control ($P<0.01$), GZ St group and blank group ($P<0.05$). But GZ-PCX Fa group did not show obvious difference compared with GZ Fa group or normal blank group ($P>0.05$).

(iii) Detection of anti-GZ-PCX sIgG and sIgA. High level of anti-GZ-PCX sIgG was detected in mouse intestinal washing fluids at week 9 after the immunization of GZ-PCX antigen. There was significant difference between GZ-PCX or GZ group and blank ($P<0.001$), between GZ-PCX Fa group and GZ Fa group ($P<0.01$). But sIgA did not show any difference among all of the immunized groups and blank. Since mucosal secreted antibody is significant to immune response, the high level of sIgG in mice immunized with GZ-PCX may be able to play an important role in protecting against HCV infection.

(iv) Detection of anti-LPS IgG. Anti-LPS IgG response was examined at week 8 after immunization. In rabbits, the highest titer was detected in GZ-PCX St group, and there was a significant difference between the group and blank control ($P<0.01$), but GZ-PCX Fa induced weak anti-LPS antibody response ($P<0.05$). In mice, only GZ-PCX St and GZ St group induced specific anti-LPS IgG. The fact that anti-LPS was also significantly produced in the case of *S. typhimurium* as an adjuvant indicated that the vaccine candidate may be able to serve as a bivalent vaccine, probably specific for both HCV and *Salmonella*.

(v) Results of CTL responses. Specific lysis of target cells by CTLs against GZ-PCX Fa or GZ-PCX St was significantly induced by either GZ-PCX antigen or P2, P6 peptides, and the greatest specific lysis was shown to be 48.5%. There was an obvious difference between vaccine group and blank ($P<0.001$) or GZ group ($P<0.01$). The results indicated that the immunized mice produced CTLs specific to GZ-PCX antigen and the epitopes (fig. 3).

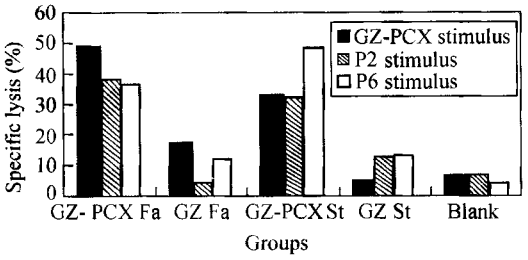

Fig. 3. CTL responses in mice after immunization induced by GZ-PCX antigen and P2, P6 peptides at week 10. The mice were killed and the spleen cells were used to culture and stimulated by GZ-PCX antigen and P2, P6 peptides for several days. The results showed that the GZ-PCX antigen and P2 and P6 peptides induced specific cytotoxic lysis of the effective spleen cells to target mouse BALB/c 3T3 cells, the highest lysis rates were 48.5% in GZ-PCX immunized groups which were much higher than those of GZ or blank groups. There was not obvious difference between complete Freund's adjuvant and killed *S. typhimurium* group.

(vi) Results of lymphoblast. When the peripheral lymphocytes of mice immunized by GZ-PCX antigen were stimulated with GZ-PCX antigen or P6 peptide as well as phytohemagglutinin (PHA) at week 9 after immunization, the proliferation rates were about 2 to 5 times higher than those of blank control, but the responses against P2 showed less efficient. P6 peptide induced obvious proliferation of lymphocytes in GZ-PCX group but not in GZ group, indicating that P6 was a specific and strong epitope in GZ-PCX antigen (fig. 4).

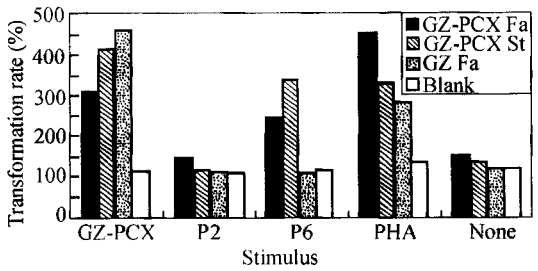

Fig. 4. Rabbit lymphocytes proliferation responses induced by antigens and peptides at week 9 after immunization. The rabbits' peripheral lymphocytes were cultured and stimulated by GZ-PCX, PHA , P2 or P6 peptides. When the cells were simulated by GZ-PCX antigen or PHA, significant lymphocytes proliferation responses were induced in both GZ-PCX and GZ groups; when simulated by P6 peptide, only GZ-PCX immunized mice induced high level of proliferation responses; when simulated by P2 peptide, there was no significant difference among all groups.

At month 5, the antigenic transformation activities of all 6 peptides as stimuli were examined (fig. 5). 4 peptides, P5, P6, P4 and P2, specifically stimulated the proliferation of peripheral lymphocytes

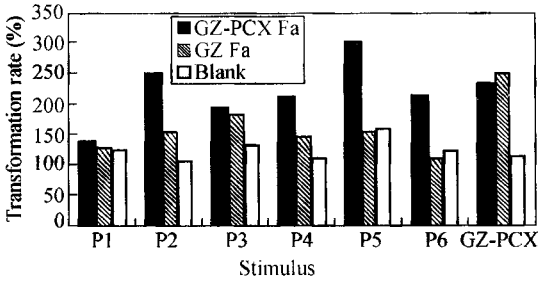

Fig. 5. Rabbit lymphocyte proliferation responses induced by 6 peptides and GZ-PCX antigen at month 5 after immunization. At month 5, all peptides and GZ-PCX antigen were used as stimuli to induce lymphocyte proliferation responses. The results showed that GZ-PCX, P5, P6 and P2 induced significant responses; P4 and P3 only slightly raised, but P1 did not show any stimulation responses compared with GZ immunized group and blank. The results showed that cellular immunity of the immunized rabbits kept for more than several months.

($P<0.01$ or $P<0.05$), the proliferation activity of P3 was slightly higher than those of GZ and blank control, but P1 did not show its efficacy.

(vii) Results of DTH. DTH responses were also examined by using GZ-PCX antigen to inject mice intracutaneously. At week 4, vaccine and vector groups, except blank group, induced obvious DTH reactions. At week 9, DTH reactions were more obvious (table 1). In rabbits, the DTH reactions appeared at week 4 and kept stable 6 months after immunization (table 2). At week 12, GZ, GZ-PCX, P5 and P6 induced strong DTH responses, and the other 4 peptides had moderate reactions. However, peptides such as P2, P3 and P5 cross-reacted between GZ-PCX and GZ group, although the reaction between them was still significantly different ($P<0.05$, fig. 6).

Table 1 DTH reaction in mice induced by GZ-PCX at week 9

Group	Number	++	+	±	−
GZ-PCX Fa	7	7			
GZ Fa	5	2	3		
GZ-PCX St	5	4	1		
GZ St	5	5			
Blank	5				5

Table 2 DTH reaction in rabbits induced by GZ-PCX at week 4 and month 6

Group	Number	Week 4/mm	Month 6/mm
GZ-PCX Fa	7	25.4	12.0
GZ Fa	5	14.6	10.0
GZ-PCX St	5	12.0	ND
Blank	4	0	0

ND, not done.

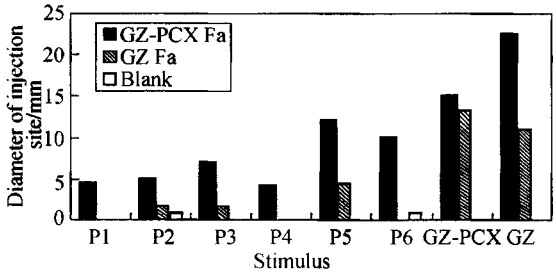

Fig. 6. DTH responses induced by peptides, GZ-PCX or GZ antigen in rabbits after immunization at week 12. After the peptides, GZ-PCX and GZ antigens were used as stimuli to induce DTH reactions in rabbits, the highest responses were induced by GZ-PCX and GZ antigens. P5 and P6 peptides also induced significant responses compared with other peptides. There were slight cross-reactions among GZ-PCX, GZ antigen immunized groups and blank group when induced by P2, P3, P5 or P6 peptide.

(viii) Safety. The body weights of the mice immunized with GZ-PCX Fa were normal compared with those of control mice. The quantity of livers and spleens of mice and rabbits did not show significant difference after immunization at week 12 (data not shown). No mice or rabbits died during the experiment. These indicated the safety of GZ-PCX antigen for immunization.

3 Discussion

Chiefly due to the hypervariable property and less knowledge about HCV immune mechanism, there is no effective HCV vaccine produced till now. The high variability of HCV results in difficulties of the researches of HCV vaccines. HCV vaccines of particular HCV type generated by PCR amplification might not be effective to other types or subtypes. Therefore, an alternative was used to design a multi-epitope of HCV antigen which covered every HCV type and subtype. In the present research, the selected epitopes were conserved and immunogenic, and the hybrid antigen might be able to induce strong immune responses against HCV infection of many types and subtypes. The design of multi-epitope antigen was based on the researches described as follows[1].

Most of the researches based on the B cell epitopes of core antigen showed that the N-terminus of this antigen was the best domain for inducing specific antibodies. Siemoneit et al. analyzed the human B cell response to a peptide fragment from amino acid residues 314-330 (EP3) covering the central

conserved sequence of this domain, 32% RIBA-confirmed donors displayed a significant antibody response to EP3. Other researchers show that the sequence RMAWDMMMNW (317—326 aa) was a B cell epitope which could be recognized by anti-HCV antibody. Shirai et al. identified highly conserved antigenic sites in HCV core recognized by both murine and human CTL. The CTLs from two HLA-A2-positive patients with acute and chronic hepatitis C recognized a 9-residue fragment of E1 (DLMGYIPLV, 132—140 aa) of the peptide presented by HLA-A2 and containing an HLA-A2-binding motif, extending only 1 residue beyond the murine epitope, which indicated that the conserved T cell epitope may become a valuable component of an HCV vaccine against a broad range of HCV isolates.

Erickson et al. used overlapping synthetic peptides to define specific $CD8^+$ T cell lines with identical specificities for an NS3 epitope generated from one chronically infected animal at weeks 16 and 28 postinfection. They found one T cell epitope named 189.2E (TGDFDSVID, 1445—1453 aa) induced high level of cytotoxic lysis. Weiner et al.[4] demonstrated that CTLs directed against this conserved epitope persisted in the liver of a chronically infected chimpanzee for at least 2 years after infection, but these CTLs did not recognize the HCV quasi-species presented in the plasma of this animal at week 16 postinfection or at later time points due to an aspartic acid to glutamic acid (D-E) substitution at amino acid position 1449 in all HCV genomes. The results of this study strongly support the concept that CTL responses can be selected for variant viruses with an enhanced ability to persist in a host and have important implications for the design of vaccines against HCV.

Lin et al. analyzed 151 partial or complete nucleotide sequences of HCV genome and 159 partial or complete amino acid sequences, searching conserved amino acid sequences and found 25 T-cell epitopes candidate according to the criteria of absolute conservation of amino acid sequence, together with characteristic sequence motifs, amphipathic helical structure, or both. A conserved peptide sequence ELITSCSS (2781—2788 aa) in NS5 was identified as a T cell epitope.

The purified GZ-PCX antigen could be specifically recognized by human anti-HCV sera from Shanghai, Beijing, Guangzhou and USA, and the positive rate was similar to that of anti-HCV EIA-2 commercial kit. It showed the possibility to design a multi-epitope antigen as the coated antigen to detect anti-HCV antibodies. But there are still some positive samples diagnosed by EIA-2 Kit which only showed faint positive detected by GZ-PCX antigen. The reasons might be due to the low affinity of GZ-PCX to the antibodies of some sera.

High level of anti-GZ-PCX IgG was induced after the hybrid antigen was used to immunize mice and rabbits and the titer of specific antibodies kept stable for several months in rabbits. Specific sIgA and/or sIgG against HCV antigen were detected in the secreted fluids of intestinal lavage in mice, showing the value of this vaccine candidate in inducing mucosal immunity. The dynamic analysis of body weights of mice showed that GZ-PCX antigen was safe without obvious toxicity.

The results showed that the multi-epitope antigen GZ-PCX might be able to serve as an effect vaccine candidate. More advanced work about protectivity experiments in Tree Shrews and monkey are under way now.

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