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Priming Hepatitis B Surface (HBsAg)- and Core Antigen (HBcAg)-Specific Immune Responses by Chimeric, HBcAg with a HBsAg '*a*' Determinant

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ABSTRACT

We developed an immunogen to stimulate multivalent immunity against hepatitis B surface antigen (HBsAg) and hepatitis B core antigens (HBcAg). Immune responses specific for both HBsAg and HBcAg play an important role in controlling the infection. HBsAg-specific antibodies mediate elimination of virions at an early stage of infection and prevent the spread of virus. The immunogen was constructed by inserting the immunodominant, antibody-binding '*a*' determinant (aa 111-149) of HBsAg (with or without a poly-glycine (PG) linker) into the e2 epitope of HBcAg. Only the constructs in which the HBsAg '*a*' determinant was inserted into HBcAg, flanked by PG linkers, expressed a chimeric protein in human embryonic kidney cells with HBsAg and HBcAg antigenicity. Both glycosylated and non-glycosylated forms of the chimeric protein were immunoprecipitated from cell lysate. Intramuscular DNA vaccination of mice with plasmids expressing chimeric HBcAg primed antibody responses against well-defined serologically-defined determinants of both, native HBcAg, and native HBsAg. In addition, $CD8^+$ T cell responses against HBcAg epitopes were primed by this chimeric HBV antigen. The e2 sequence of HBcAg can thus be used to present heterologous epitopes without loss of immunogenicity of the HBcAg protein. *Iran. Biomed. J. 10 (2): 61-68, 2006*

Keywords: HBV vaccine, Hybrid protein, Poly-glycine (PG) linker

INTRODUCTION

Despite the availability of a safe and efficacious vaccine, infection with hepatitis B virus (HBV) is still a common infectious disease with an estimated 450 million chronic HBV carriers worldwide [1]. Patients with chronic hepatitis B have a high risk of developing liver cirrhosis and hepatocellular carcinoma [2]. Immune responses specific for both hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) play an important role in controlling the infection. HBsAg-specific antibodies mediate elimination of virions at an early stage of infection and prevent the spread of virus [3], and HBcAg- and HBsAg-specific $CD4^+$ T cells 'help' humoral and cytotoxic T lymphocyte (CTL) immune response. Overwhelming evidence indicates that induction of a humoral immune response against the '*a*' determinant of HBsAg is necessary and sufficient for protection against HBV. However, clearance of the virus by post-exposure therapeutic vaccination involves cellular immune responses, in particular $CD8^+$ CTL responses [4, 5].

Particulate HBcAg elicits immune responses and provides T-cell 'help' for antibody responses to HBsAg. It has been used as a carrier to enhance the immunogenicity of heterologous epitopes [6]. HBcAg is a virus-like particle easy to work with because of its high-level expression and efficient particle formation in many expression systems, including bacteria. Appropriate target sites within the HBcAg sequence, in which epitope sequences of foreign antigens can be inserted, are therefore of interest. The N- and C-termini of HBcAg, as well as its major immunodominant region or tip of the spikes (e1 epitope aa 76-81) have been used to insert foreign epitopes derived from the VP2 protein of human rhinovirus type 2 [7, 8], the simian immunodeficiency virus envelope [9], or the V3 loop of HIV-I gp120 [10-12]. Insertion of 39 amino acids of the 'a' domain of HBsAg (positions 111-

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149) was the first successful attempt to mimic a conformational epitope on the surface of chimeric HBcAg particles [13] but there is a paucity of data concerning the use of e2 epitope. Studies with monoclonal antibodies revealed that sequence 127-133 occupies a superficial position on the native HBcAg particle. We therefore inserted the '*a*' determinant of HBsAg into this epitope in order to (i) make new chimeric particles; and (ii) to study their immunogenicity.

Genetic vaccination using plasmid DNA is a novel immunization technique that efficiently primes humoral and cellular immune response against a broad range of protein antigens. Research efforts to develop DNA vaccine products have progressed to the clinical evaluation of several vaccine candidates, including those for hepatitis B, herpes simplex-2, HIV-1, influenza and malaria [4, 14]. In this study, injection of a construct encoding chimeric HBcAg induced antibody response to both HBsAg and HBcAg, and primed HBcAg-specific CTL responses.

MATERIALS AND METHODS

Construction of chimeric HBc/'a' determinant gene. An *Xho*I (all enzymes were obtained from New England Biolab Company) site was introduced into the sequence of HBcAg by a nucleotide substitution (C to G) at position 389 (corresponding to amino acid 130 of the e2 epitope). This site was used for in-frame insertion of the fragment encoding the '*a*' determinant of HBsAg. The HBcAg sequences was derived from the *p*HBV320 plasmid that contains a full-length HBV genome (serotype ayw) cloned as a *BamH*1 fragment into *p*BR322 [15]. The HBcAg fragment aa 1-129 carrying *Nhe*I or *Xho*I sites at 5', or 3' ends, respectively, was amplified by PCR using the primers F01-HBc3 (5' AA*GCTAGC* ATGGACA TCGACCCTTATAAAGAATTTG 3') and R133-HBc2 (5' CTATAAG*CTC GAG*GAGTGCGAA TCC-3'). The PCR product was digested by *Nhe*I/*Xho*I and ligated into *Nhe*I/*Xho*I-digested *p*CI (Promega) to generate the intermediate plasmid *p*CI/Core 1-129. A Core fragment encoding aa 130-183 with a 5' *Xho*I and a 3' *Eco*RI was amplified by PCR using the primers F125-HBc1 (5' GGATTC GCACTC*CTCGAG*CTT ATAG 3') and R183-HBc8 (5'AA*GAATTC*CTAACATTGAGATTCCCG AGA TTGA GATC 3'). The PCR product was digested by *Xho*I/*Eco*RI and ligated to *p*CI/Core1-129 /*Eco*RI-*Xho*I. The plasmid designated *p*CI/Core-*Xho*I* has an *Xho*I site. The plasmid *p*CI/S encoding HBsAg (serotype ayw) was used as a template to amplify the '*a*' determinant (aa 111-149) of HBsAg with or without a poly-glycine (PG) linker by PCR using the primers: HBa1-forward (5'-CAAGGT*CTCGAGGA*CCAGGATCCTCAACTA CCAGCACG-3'); HBa2-reverse (5'AGCCCA*CT CGAG*GGGTGCAATTTCCGTCCGAAGGTTTG GT-3'); HBa3-forward (5'-CAAGGT*CTCGA GGAGGAGGAGGA*CCAGGATCCT CAACTAC CAGCACG-3'); HBa4-reverse (5'-AGCCCA*CT CG AG* GTCCTCCTCCTCCACAGG TGCAATTTCC GTCCGAAG-3'). The amplified products were purified, digested with *Xho*I, and cloned into pCI/Core-*Xho*I* digested with *Xho*I and dephosphorylated with alkaline phosphatase. The constructs are designated *p*CI/C-$S_{111-149}$ (without PG), and pCI/C-$S_{111-149}$PG (with PG linkers flanking the inserted sequence). Orientation of the inserts was checked by *Bam*HI, *Nhe*I/*Not*I and *Bgl*II digestions.

Transient expression of chimeric HBcAg. Expression of HBcAg, HBsAg and HBc/s chimeric proteins from the respective plasmid constructs was tested in transient transfection assays. Briefly, confluent human embryonic kidney (HEK) cells (10^6/ml) were transfected by the $CaPO_4$ method with 10 µg plasmid DNA of the vectors *p*CI/C (encoding HBcAg), *p*CI/S (encoding HBsAg), or *p*CI/C-$S_{111-149}$ and *p*CI/C-$S_{111-149}$ PG (encoding chimeric HBcAg/HBsAg with or without PG). Two days later, cells were labeled with 100 µCi ^{35}S-methionine (Amersham) in methionine-free RPMI 1640 medium supplemented with 10% FCS. Labeled cells were washed twice in PBS and extracted with lysis buffer (120 mM NaCl, 1% v/v aprotinin (Trasylol), 50 µM leupeptin, 10% immunoglobulin-free FCS, 0.5% nonident P-40 (NP-40), 10% glycerol, 50 mM Tris/HC1 (pH 8.0) for 30 min at 4°C. Extracts cleared by centrifugation (30 min, 20,000 ×g, 4°C) were subsequently incubated at 4°C for 2 h with protein A-Sepharose (Pharmacia, USA). The expressed proteins were precipitated with polyclonal rabbit anti-HBs or anti-HBc antisera and protein A-Sepharose at 4°C, and washed extensively in 0.5 M LiCl, 1% NP-40 and 0.1 M Tris/HC1 (pH 9.0). After two additional washes of the complexes in lX PBS, and one wash in 0.1X PBS, the HBsAg or HBcAg-containing immune complexes were recovered from protein A-Sepharose in 300 µl elution buffer (1.5% SDS, 5% 2-ME and 7 mM

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Tris/HC1, pH 6.8). Following incubation at 37°C for 30 min, SDS-denatured eluates were lyophilized and dissolved in 30 µl aqueous solution of 5% 2-ME, 10% glycerol and bromophenol blue. After boiling for 2 min, samples were analyzed by SDS-PAGE [16].

DNA immunization of mice. DNA was prepared from each plasmid by anion exchange column, and resuspended in endotoxin-free, sterile PBS. Groups of 5 female, anaesthetized C57BL/6 mice were injected with 100 µg plasmid DNA into the tibialis anterior muscles as described [17]. The muscle was pretreated 5 days before the DNA injection by an intramuscular injection of 50 µl 0.01 mM cardiotoxin (Latoxan, Rosans, France). Mice were boosted with same dose of plasmid DNA 4 and 8 weeks after the first injection. The sera of each mouse were obtained 3 weeks after each immunization.

Determination of serum antibody levels. Serum samples were repeatedly obtained from individual mice by tail bleedings 0, 4, 8 and 16 weeks post-injection. Antibodies against HBsAg were detected in mouse sera with the commercially available test IMxAUSAB (Abbott, USA). In this assay, microparticles coated with HBsAg were mixed with mouse serum samples to allow specific binding of anti-HBsAg antibodies. Microparticles coated with antigen-antibody complex were transferred and irreversibly bound to a glass fiber matrix. Biotinylated recombinant HBsAg was added and binding to the complex was traced by an alkaline phosphatase conjugated anti-biotin antibody followed by the addition of the substrate 4-methyl-lumbelliferyl-phosphate. Antibody levels were quantified using six standard sera (0-1000) mIU/ml). Values presented are calculated by multiplying the serum dilution with the measured antibody level (mIU/ml).

Antibodies against HBcAg were detected in mice sera using the CORE® anti-HBc kit (Abbott, USA). Concentration of anti-HBc was standardized against a reference standard of Paul-Erlich Institute and is shown as Paul-Erlich units (PEU)ml^{-1}.

Cytotoxicity assay. Single cell suspensions prepared from spleens of immunized mice were suspended in a MEM tissue culture medium supplemented with 10 mM HEPES buffer, 5×10^{-5} M 2-ME, antibiotics and 10% v/v FCS (Pan Systems, Germany). Responder cells (3×10^7) were co-cultured with 1.5×10^6 syngeneic, irradiated 20.000 rad) RBL5/C transfectants in 10 ml medium in upright 25 cm^2 tissue culture flasks in a humidified atmosphere and 7% CO_2 at 37°C [18]. After a 5-day co-culture, blast cells were harvested, washed and restimulated for a further 4 days. After 6 days of *in vitro* restimulation culture, serial dilutions of effector cells were cultured with 2×10^3 ^{51}Cr-labelled targets in 200 µl round bottom wells. Specific cytolytic activity of the cells was tested in 4 h ^{51}Cr-release assays against peptide pulsed or transfected targets, and compared with non-pulsed and non-transfected controls. After a 4-h incubation at 37°C, 100 µl supernatant fluids were collected for γ-radiation counting. The percentage specific release was calculated as [(experimental release - spontaneous release)/(total release - spontaneous release)] × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always less than 15% of the total counts. Data shown are the mean of triplicate cultures. The standard error of the mean (S.E.M) of triplicate data was always less than 15% of the mean [18].

RESULTS

Expression of chimeric HBcAg protein. The plasmid *p*CI/S encodes the small HBsAg (Fig. 1A) and the plasmid *p*CI/C encodes the non-chimeric HBcAg (Fig. 1B). The map of the two constructs encoding chimeric HBcAg particles is shown in Figure 1 C and D. Plasmid *p*CI/C-$S_{111-149}$ contains the '*a*' determinant of HBsAg inserted into HBcAg without PG (Fig. 1C). Plasmid *p*CI/C-$S_{111-149}$PG contains the same epitope inserted into HBcAg flanked at both ends by PG linkers (Fig. 1D). Expressions of hybrid HBcAg/HBsAg from the respective plasmids (Table 1) were tested in transient transfection assays using HEK cells. A protein was expressed in transfected HEK cells from plasmid *p*CI/C-$S_{111-149}$PG, but not plasmid *p*CI/C-$S_{111-149}$. This protein had both HBcAg and HBsAg antigenicity (Fig. 2A, lanes 2L; Fig. 2B,

Table 1. Plasmids constructed for expression of native and modified HBcAg.

Construct	Chimeric gene			
	Carrier		Insert	
	Length	*Insertion site*	*Length*	*Linker*
pCI/S	S_{1-226}	-	-	-
pCI/C	C_{1-183}	-	-	-
pCI/C-$S_{119-149}$	C_{1-183}	e2	$S_{111-149}$	-
pCI/C-S_{111-49}PG	C_{1-183}	e2	$S_{111-149}$	2 × 4 (gly)

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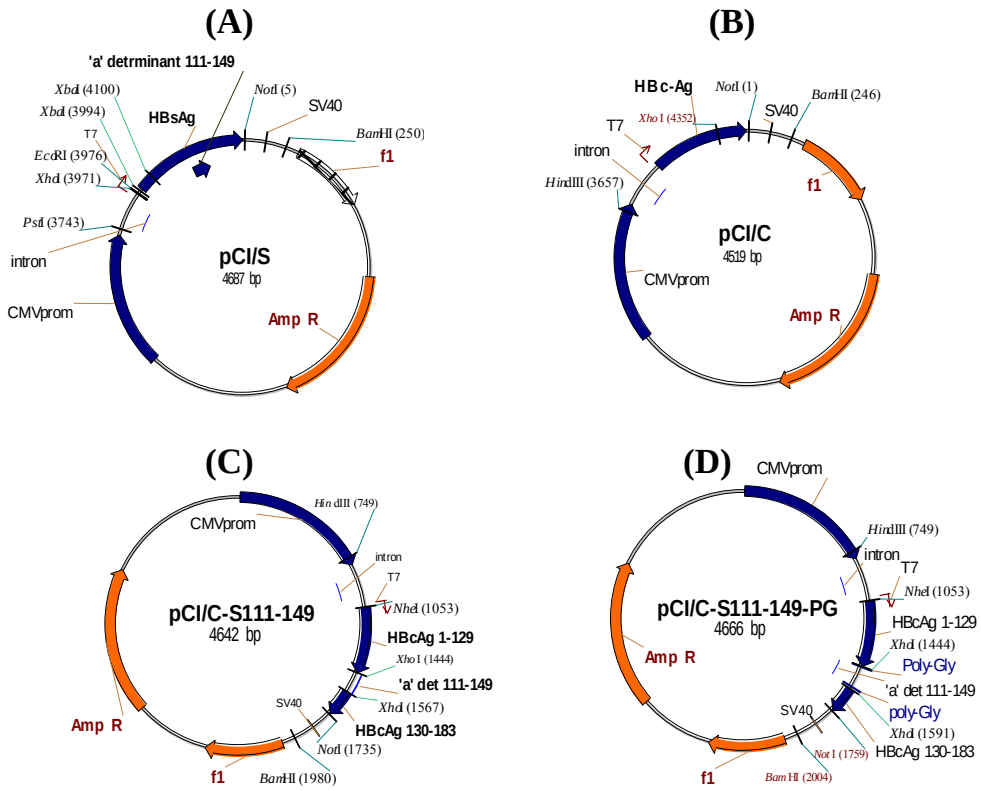

Fig 1. Schematic map of constructs expressed native HBsAg, HBcAg and chimeric HBc/sAg. **(A)**, pCI/S; **(B)**, pCI/C; **(C)**, pCI/C-$S_{111\text{-}149}$; **(D)**, pCI/$S_{111\text{-}149}$PG.

lanes 6L). Both glycosylated and non-glycosylated forms of the chimeric protein were immuno-precipitated from cell lysate by anti-HBsAg or anti-HBcAg antibodies. Expression levels of the chimeric HBcAg/HBsAg protein were comparable to expression levels of HBsAg from pCI/S, and HBcAg from pCI/C. A secreted form of the chimeric HBsAg/HBcAg protein was difficult to detect in immune-precipitation studies from supernatant conditioned by transfectants, although secretion of HBsAg from cells expressing pCI/S (but not of HBcAg from cells expressing pCI/C) was readily detectable (Fig. 2). The different and apparent molecular weights of non-chimeric HBcAg protein and chimeric HBcAg/HBsAg protein correlated well with the different length of the two proteins. Hence, chimeric HBcAg with immunogenic sequences of HBsAg can be produced if an appropriate insertion site is chosen, and if the insert is flanked by PG linker sequences.

The multispecific antibody response primed by HBcAg/HBsAg protein. We tested the immunogenicity of the chimeric protein by injecting pCI/C-$S_{111\text{-}149}$PG plasmid DNA into mice, and compared it to the immunogenicity of HBsAg (delivered by the pCI/S DNA vaccine), or HBcAg (delivered by the pCI/C DNA vaccine). In all three vector constructs, expression of HBsAg, HBcAg or hybrid HBcAg/HBsAg was driven by the same promoter sequences from the immediate early region of the human cytomegalovirus. Intra-muscular DNA vaccination with pCI/C-$S_{111\text{-}149}$PG led to the appearance of specific and rising anti-HBsAg (Fig. 3A) and anti-HBcAg (Fig. 3B) serum antibody titers after the second and third vaccine injection. The anti-HBsAg serum titers (expressed in mIU per ml) stimulated by vaccination with pCI/C-$S_{111\text{-}149}$PG were lower than those primed by vaccination with pCI/S and appeared later (Fig. 3A). But three weeks after the second DNA injection, 100% of the injected mice seroconverted to titers >10 mlU/ml, and many animals developed specific serum antibody titers >100 mIU/ml after the third injection. The variability in specific serum titers among individual, immunized and boosted mice was low. DNA vaccination with pCI/C-$S_{111\text{-}149}$PG led also to the appearance of specific and rising anti-HBcAg (Fig. 3B).

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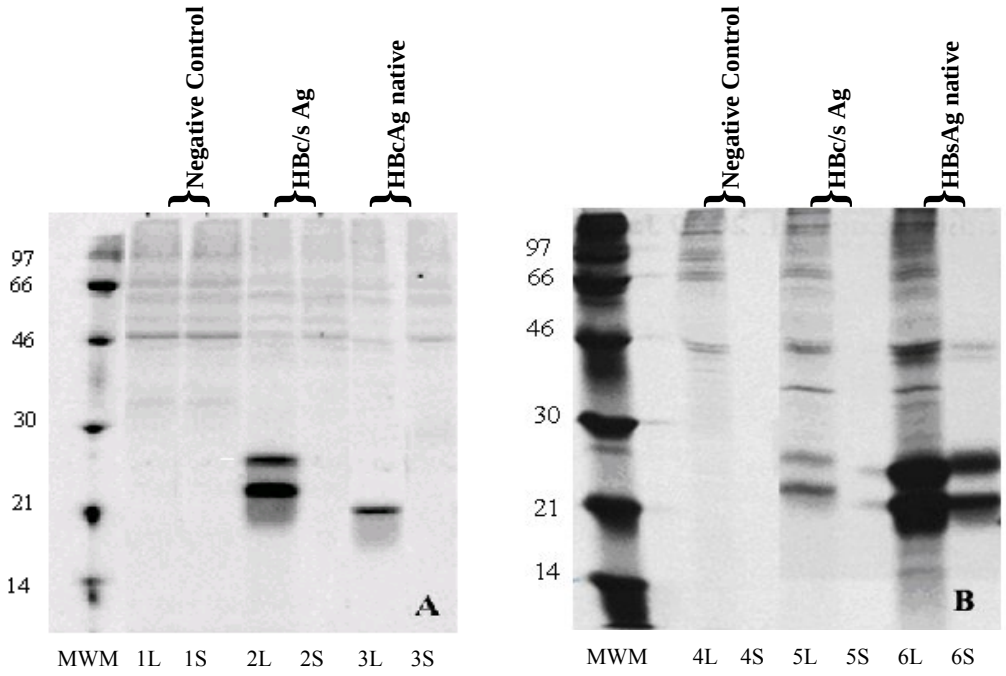

Fig. 2. Polyacrylamide gel electrophoresis of hybrid HBc-s proteins in the presence of sodium dodecyl sulfate. [^{35}S]methionine-labeled proteins from cellular lysate (L) and supernatants (S) of HEK cells were immunoprecipitated with antiserum to HBcAg **(A)**; Lanes: 1L and 1S, negative control (*p*CI); 2L and 2S, chimeric HBc-sAg (*p*CI/C-$S_{111-149}$); 3L and 3S, native HBcAg (positive control, *p*CI/C) and antiserum to HBsAg **(B)**; Lanes: 4L and 4S, negative control (*p*CI); 5L and 5S, chimeric HBc-sAg (*p*CI/C-$S_{111-149}$); 6L and 6S, native HBsAg (*p*CI/S); (L and S lysate and supernatant of infected cell culture).

Antibodies against HBcAg were also detected in sera of mice vaccinated with the pCI/C-$S_{111-149}$PG DNA vaccine using the HBcAb-2 steps competition kit (Equipar srl, Italy). Samples with OD_{450} nm values lower than the gray-zone are considered reactive for antibodies to HBcAg (>1 PEI U/ml). Mice injected with the pCI/C-$S_{111-149}$PG DNA vaccine produced antibodies specific for HBcAg. Anti-HBcAg serum antibodies were first detected at low levels (1-2 PEI U/ml) after the first DNA injection in some but not all mice, and continued to rise after the second (up to 3 PEI U/ml) and third (up to 7 PEI U/ml) vaccine injections. The specific serum antibody titers of mice vaccinated with either the pCI/C-$S_{111-149}$PG, or the pCI/C DNA vaccine were comparable.

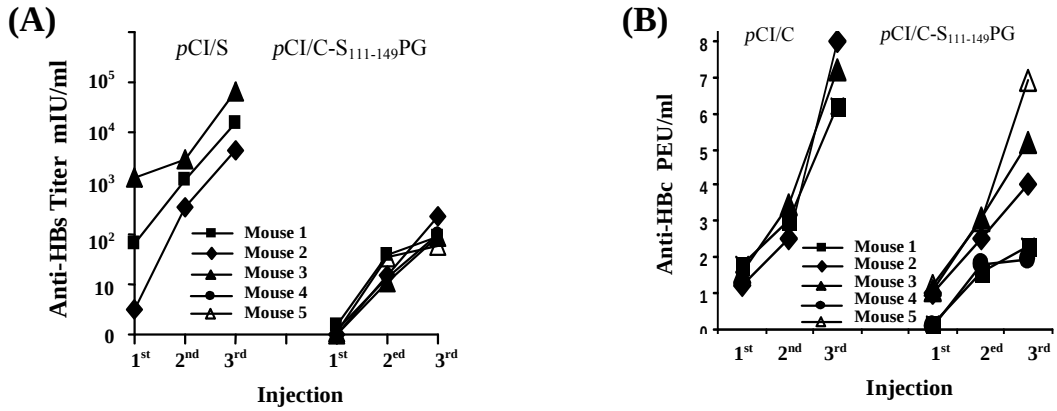

Fig. 3. Serum antibody response of DNA-vaccinated mice. Mice were immunized by i.m. injection of 100 μg DNA: pCI/C (encoding HBcAg), pCI/S (encoding HBsAg) and pCI/C-$S_{111-149}$PG (encoding HBcAg-$S_{111-149}$) plasmid DNA. Titers of anti-HBs or anti-HBc antibodies in sera of each mice obtained 3 weeks after each immunization. Inj, injection.

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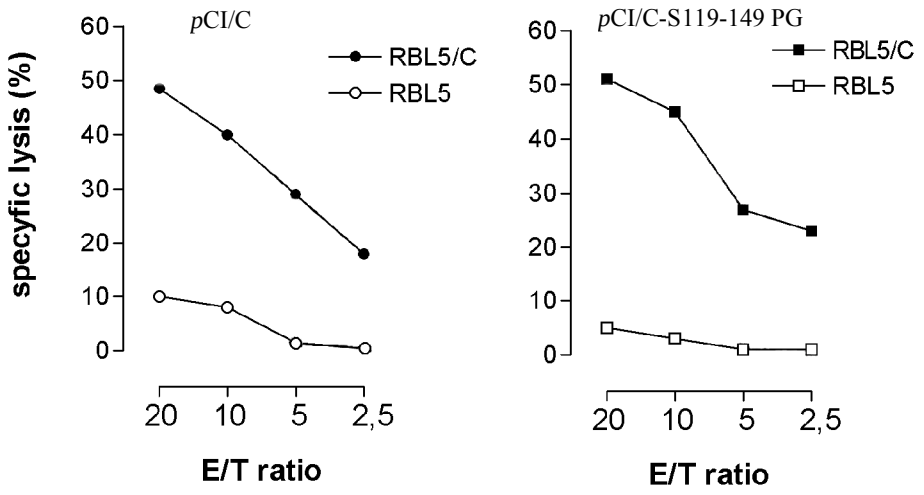

Fig. 4. The class I restricted T cell response of mice to native HBcAg and chimeric HBc-sAg following DNA immunization with respective plasmids. Mice were immunized by inoculation of 2 × 50 µg 'naked' plasmid DNA encoding native HBcAg and chimeric HBc-sAg into regenerating muscles. Three weeks post immunization, lymphoid cells were prepared from these mice and restimulated with syngeneic HBcAg transfected cells (RBL5/c). After a 5-day *in vitro* culture, effector cells were harvested and tested in a conventional assay against HBcAg expressing RBL5/C transfectant.

Priming MHC-I-restricted CTL by intramuscular injection of DNA encoding chimeric HBcAg. DNA vaccination with *p*CI/C efficiently primes CTL specific for HBcAg epitopes. This specific cytolytic reactivity of *in vivo* primed, *in vitro* restimulated CTL was detected in a 4 h ^{51}Cr-release assay using syngeneic transfectants as targets. We tested, if similar CTL can also be primed by the *p*CI/C-$S_{111-149}$PG DNA vaccine (Fig. 4). Specific CTL that recognized HBcAg epitopes were primed with comparable efficiency by a single intramuscular injection of either the *p*CI/C, or the *p*CI/C-$S_{111-149}$PG DNA vaccine. Hence, insertion of a HBsAg sequence into the HBcAg protein (in a site that does not destroy the CTL-defined epitope) does not affect its immunogenicity for $CD8^+$ T cells.

DISCUSSION

A new vaccination strategy is presented that induces multispecific, humoral and cellular immune responses to two different and major HBV antigens: *i.e.* HBsAg and HBcAg. The present study identifies the e2 (129-132 aa) region of HBcAg as a suitable insertion site for the '*a*' determinant of HBsAg. The data show that insertion of an antigenic sequence into this region does not affect the immunogenicity of the 'carrier', and also confers novel immunogenic properties to the chimeric protein. The successful expression of the insert did not affect the immunogenicity of HBcAg itself, contrary to results reported for the insertion of the same fragment into the e1 region of HBcAg [11, 19]. However, expression of the insert could only be detected by polyclonal antibodies binding either HBcAg, or HBsAg when the insert was flanked by PG linkers. When the HBsAg sequence is inserted into HBcAg without linkers, the chimeric protein may be very unstable. In contrast, the insertion of foreign epitope using PG linkers may support formation of HBcAg particle structure because the exposed loop is stabilized by anchoring both ends of the insert to the carrier moiety in a flexible way [20]. Also, the correct distance between the amino acid residues of a surface loop is important, as demonstrated for a virus-neutralizing epitope of HRV-14 inserted into the cowpea mosaic virus capsids [21, 22].

The amino acid residues 74-83 forming a 7 nm-long spike protruding from the surface of core particles may play a key role in the architecture of particulate, native HBcAg that determines its antigenicity and possibly its immunological properties [11]. It was therefore unexpected that insertion of the 'a' fragment into the e2 site without linkers resulted in the destruction of the native HBcAg structure. In contrast, insertion of the '*a*'

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determinant of HBsAg (111-149) into the e2 epitope using linkers maintained the antigenicity of HBcAg, although the immunogenicity of the 'a' determinant of HBsAg was lower than that of the native HBsAg particle.

Immunogenicity of HBcAg, HBsAg or chimeric HBcAg was determined by measuring antibody production in C57BL/6 mice after injection of expression plasmid DNA encoding HBcAg, HBsAg or chimeric HBcAg. All immunized mice mounted a strong response to the HBcAg component of the fusion protein, although this is a strictly intracellular antigen. The antibody response to the HBsAg '*a*' determinant was considerably lower than that exhibited against HBsAg. This response is expected as HBsAg is secreted but HBcAg is not. Following a boost vaccination, antibody titers of >10 mIU/ml were detectable in the sera of mice which is considered a seroconversion and represents a protective titer [23]. The HBsAg '*a*' determinant is a conformational epitope, and its immunogenicity is strongly dependent on its display on the surface and its correct 3-D folding. The reduced immunogenicity of the inserted fragment may thus result either from its decreased accessibility, and/or from its incorrect folding. These data resemble the results reported for the insertion of the same fragment into the e1 epitope of HBcAg [11], whereas the results reported for insertion of linear epitopes in e1 region resulted in no loss of immunogenicity [11].

Since antibodies against the '*a*' determinant are sufficient for protection against HBV infection, induction of $CD8^+$ T cell responses does not seem to be critical for a prophylactic vaccine against HBV. In contrast, $CD8^+$ CTL responses are important to control acute HBV infection, and their deficiency may lead to the establishment of chronic HBV infection. Specific reconstitution of $CD8^+$ T cell immunity to HBV is therefore a key requirement in developing an effective therapeutic vaccine against HBV [24]. An interesting feature of HBcAg as well as DNA vaccination is their ability to elicit a strong CTL response. In the present investigation, intramuscular injection of 100 μg plasmid DNA encoding the chimeric protein elicited a specific CTL response to HBcAg comparable to that of native HBcAg.

In conclusion, vaccination with a chimeric construct, in which the '*a*' determinant of HBsAg was inserted into the e2 region of HBcAg using poly-linkers, stimulated both humoral and CTL response against HBsAg and HBcAg in mice.

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