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A dendritic cell-targeted chimeric hepatitis B virus immunotherapeutic vaccine induces both cellular and humoral immune responses *in vivo*

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ABSTRACT

Chimigen® HBV Immunotherapeutic Vaccine (C-HBV), a recombinant chimeric fusion protein comprising hepatitis B virus (HBV) S1 and S2 surface antigen fragments, Core antigen and a murine monoclonal antibody heavy chain fragment (Fc), was designed and produced in Sf9 insect cells. C-HBV targets the host immune system through specific receptors present on dendritic cells (DCs) which facilitates antigen internalization, processing, and presentation on MHC class I and II to induce both cellular and humoral immune responses against HBV antigens. T cell responses, previously assessed by *ex vivo* antigen presentation assays using human peripheral blood mononuclear cell (PBMC)-derived DCs and T cells from uninfected and HBV chronic-infected donors, demonstrated that C-HBV was highly immunogenic. A vaccine dose response study was performed in sheep to analyze the immunogenicity of C-HBV *in vivo*. Sheep (n = 8/group) received three consecutive subcutaneous injections of each dose of C-HBV at four-week intervals. Analysis of serum antibody levels confirmed C-HBV induced a dose-dependent antibody response to C-HBV and S1/S2-Core. Kinetics of the S1/S2-Core specific antibody response was similar to hepatitis B surface antigen (HBsAg)-specific antibody responses induced by ENGERIX-B. Analysis of cell-mediated immune responses (CMI) confirmed C-HBV induced both dose-dependent S1/S2-Core-specific lymphocyte proliferative responses and IFN-γ secretion. These responses were stronger with blood lymphocytes than with cells isolated from the lymph node draining the vaccination site. No correlation was seen between antibody titers and CMI. The results confirm C-HBV is an effective delivery vehicle for the induction of T cell responses and may be an appropriate candidate for immunotherapy for chronic HBV infections.

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Introduction

Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. World Health Organization statistics show that more than 2 billion people have been infected by HBV, and among this population, about 257 million are estimated to be chronically infected with the virus.[1] People with chronic HBV infection are at risk for significant liver damage. Approximately 20–30% of chronically infected people develop cirrhosis of the liver and/or liver cancer over a 20–30-year period, resulting in more than 1 million deaths annually. Although prophylactic vaccines have reduced the number of newly acquired HBV infections, chronic HBV is still a major public health challenge.[2] The goal of HBV treatment is to achieve a "functional cure", defined as the clearance of HBsAg (hepatitis B surface antigen) and DNA from serum, normalization of liver alanine transaminase (ALT) and the presence of anti-HBsAg antibody, rather than a complete elimination of the virus from the host. Currently approved treatments include nucleoside analogs and interferon-α (IFN-α).[3] While nucleoside analogs are effective in reducing viral replication, life-long treatment is usually required and cure is rarely achieved.[4] IFN-α treatment achieves a cure in only 10–15% of patients but severe side effects can limit its use.[5] New strategies to increase the functional cure rate with short duration therapy and minimal side-effects represent major goals for improved treatment of chronic HBV infections.

It has been shown that both innate and adaptive immune responses are involved in viral clearance in patients with acute self-limited HBV infection.[6] Cellular immune responses, including cytotoxic T lymphocytes (CTLs) and CD4 T cell responses, to viral antigen were observed but these immune responses were weak or absent in chronic HBV carriers.[7] Enhancing HBV-specific immune responses in chronic HBV carriers may be the key to eliminating persistent infections. Immunotherapy strategies have been proposed which would be able to induce HBV-specific CD8 and CD4 T cell responses in chronic HBV carriers and eliminate persistent viral infections.[8,9] Therapeutic vaccines (immune therapies) have been in development to restore a functional adaptive immune response that achieves a high functional cure rate without causing harmful side-effects. However, therapeutic vaccines based on HBsAg, virus-like particles (VLPs) of HBsAg and hepatitis B virus core antigen (HBcAg), DNA and peptides have been tested in clinical trials with disappointing results.[10,11] An emerging strategy for immunotherapy development is to target HBV antigens to dendritic cells (DC) via specific receptors to re-activate the compromised immune responses in HBV carriers.

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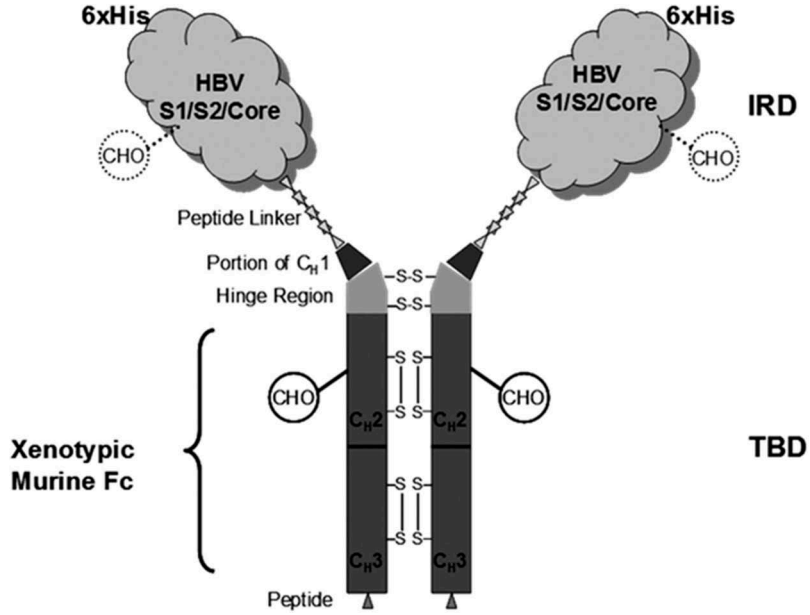

Figure 1. A schematic representation of the Chimigen® HBV Immunotherapy Vaccine (C-HBV).
The fusion protein comprises HBV S1 and S2 antigen fragments, HBV Core antigen and the Fc fragment of a xenotypic monoclonal antibody, along with N-terminal 6xHis Tag as an affinity tag for purification. Immune Response Domain (IRD), Target Binding Domain (TBD), CHO- denotes glycosylation.

Chimigen® HBV Immunotherapy Vaccine (C-HBV) contains two domains: an immune response domain (IRD) containing the antigens PreS1, PreS2 peptide fragments and the entire HBV Core; and the target binding domain (TBD) contains a xenotypic Fc fragment from mouse IgG_1 (Figure 1). The Fc portion of the molecule mediates vaccine binding to specific antigen-presenting cell (APC) receptors such as Fcγ receptors (CD16, CD32, CD64) which results in antigen processing and presentation by both the MHC class I and II pathways. The fusion protein was expressed in Sf9 insect cells which impart primitive non-mammalian glycosylation (terminal mannose, no sialic acid residue).[12,13] This glycosylation pattern also permits targeting of mannose receptors (CD206, CD209) on antigen-presenting cells (APC). Antigen uptake via these receptors, processing through the endosomal and proteasomal pathways, and presentation on both classes of MHCs without using an adjuvant, result in the induction of broad humoral and cell-mediated immune responses. Autologous therapies in which DCs are isolated from a patient, treated and re-infused back into the patient[14] have been attempted but are technically difficult and prohibitively expensive.[15] C-HBV can be injected subcutaneously or intramuscularly and will utilize the patient's own immune system to generate immune responses. C-HBV has been produced and evaluated both biochemically and immunologically.[16] Using blood samples from both un-infected and chronically HBV-infected donors to perform *ex vivo* antigen presentation assays, it has been shown that C-HBV can induce a variety of HBV-specific T cell responses. These responses included proliferation of CD4 and CD8 T cells, induction of Th1 cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and increased expression of cytotoxic T cell effector proteins granzyme B and perforin.[16]

The capability of C-HBV to induce HBV-specific immune responses *in vivo* was evaluated using sheep as a naïve animal model. Non-human primates (NHP) have classically been used as the large animal of choice for predicting human immune responses.[17,18] However, the use of NHP to evaluate vaccine immunogenicity is restricted by both cost and animal welfare concerns. The ideal NHP HBV model is the chimpanzee[19] but their use is currently not permitted. NHPs provide the most appropriate model when doing a final evaluation of a GMP vaccine preparation before clinical trials.[20] Sheep have provided an excellent model for studying many different aspects of immune system function, development, and the trafficking of effector cells that is of direct relevance to human immunology.[21] Further, as an outbred species, sheep provide an excellent model to evaluate the variability of immunogenicity for a vaccine that will be used in humans.[22,23,]

The aim of the present study was to evaluate if C-HBV was bound by sheep peripheral blood mononuclear cells (PBMCs) and then determine if C-HBV could induce both cellular and humoral immune responses. The specificity of the immune responses to individual components of the C-HBV fusion protein was also evaluated. We confirmed C-HBV was bound by sheep PBMCs and induced dose-dependent HBV-antigen specific cellular and humoral immune responses in the absence of an exogenous adjuvant.

Materials and methods

Animals

Animal experiments were performed following guidelines of the Canadian Council on Animal Care and all procedures were approved by the University of Saskatchewan Animal Care Committee (Protocol # 19992004). Forty 8-week old, cross-bred, male and female lambs were randomly assigned to five experimental groups (n = 8/group). The clinical veterinarian conducting the vaccine trial was blinded to treatments used in the groups.

C-HBV production

C-HBV was expressed in Sf9 insect cells and purified by Ni-chelation affinity chromatography under denaturing conditions as previously described.[16] Purified protein was formulated in a buffer containing 10 mM NaH_2PO_4, 150 mM NaCl, 0.05% Tween 20, pH 7.4 and stored at 4°C.

Comparison of binding of C-HBV to human and sheep myeloid cells

From a leukapheresis preparation from healthy humans with the HLA-A2 haplotype (Biological Specialty Corporation, 213-15-04), human PBMCs were obtained by Ficoll-Hypaque (Sigma-Aldrich, GE17-1440-02) density gradient centrifugation.[24] The PBMCs isolated from uninfected human donors or naïve sheep were cultured in 100 mm culture dishes (BD Biosciences, 353003) for 1 h at 37°C in AIM V media (Thermo Fisher Scientific, 12055–091) with 2.5% autologous heat inactivated plasma. Following culture, the non-adherent cells were removed and adherent cells harvested and seeded into 96 well v-bottom plates (Corning, 3894) at 2×10^5 cells/well. All remaining steps were performed at 4°C. Cells were incubated for 1 h with 1, 5, 20, 50 µg/mL C-HBV diluted with PBSB (Dulbecco's phosphate buffered saline, Thermo Fisher Scientific, 14190–250 containing 0.1% (w/v) bovine serum albumin (BSA)). Protein binding was detected by incubating cells with rat anti-mouse IgG_1-biotin (BD Biosciences, 553441) in PBSB for 20 min, followed by streptavidin phycoerythrin cyanine-5 (SA-PE-Cy5;BD Biosciences, 554062) for 20 min. Cells were re-suspended in PBSB containing 2% paraformaldehyde (PF) and surface binding of C-HBV assessed by flow cytometry.

Flow cytometry data acquisition and analysis

Cells were analyzed with a FACSCalibur fitted with CellQuest Pro acquisition and analysis software (BD Biosciences, 342974). An acquisition gate excluding dead cells or debris was defined by using the forward scatter (FSC) and side scatter (SSC) profiles and ≥20,000 events were acquired for each sample. The relative mean fluorescent intensity (MFI) was determined as MFI of the test sample – MFI of the control sample.

Injections

All injections were subcutaneous on the right side of the neck. The experimental groups were:

(A) Control: Naïve animals injected with Dulbecco's phosphate buffered saline (Thermo Fisher Scientific, 14190-250)
(B) ENGERIX-B: Animals injected with 20 µg of an HBsAg subunit vaccine
(ENGERIX-B; GSK Canada, ATC Code: J07BC01) at each immunization
(C) Low dose C-HBV: Animals injected with 5 µg C-HBV at each injection
(D) Medium dose C-HBV: Animals injected with 20 µg C-HBV at each injection

Table 1. Experimental schedule and sample collection/analysis.

Exper. week	Vaccination	Serum collection	PBMC/LN collection	Comments
D-1				Identify and assign animals to exp. groups
0	√	√(Naïve)		
2		√		
4	√	√		
6		√		
7				
8	√	√	√(PBMC)	Collect samples prior to vaccination
10		√		
12		√	√ (PBMC-D 83) √ (LN- D 85)	Terminate experiment

(E) High dose C-HBV: Animals injected with 50 µg C-HBV at each injection

Injections were performed at one-month intervals for a total of three injections without any added adjuvant (Table 1).

Blood sampling

At the designated time points (Table 1), 10 mL of blood was collected in serum-separating tubes (Vacutainer®STT™ tubes, BD, B367987). Serum was separated, aliquoted into duplicate 96-well Immulon plates (Thermo Fisher Scientific, 3455) and stored at −20°C until analyzed by ELISA. Fifty milliliter of blood was collected from the jugular vein with 0.1% EDTA and PBMCs were isolated using one-step 60% Percoll® (GE Healthcare, 17-0891-02) density gradients.[24] Cell suspensions were re-suspended in AIM V medium (supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, 12484-028)) plus antibiotics and antimycotics (Sigma-Aldrich, A5955) at a final concentration of 2×10^6 cells/mL for lymphocyte proliferative response (LPR) assays and 5×10^6 cells/mL for ELISPOT assays.

Superficial cervical lymph node (SCLN) collection

Sheep were euthanized on the last day of the experiment (d 85; week 12) and the right SCLN, which drained the vaccine injection site, was collected in ice-cold phosphate buffered saline. A single cell suspension was prepared by gently pressing 3 mm^2 pieces of LN tissue through the 40 µm mesh of Sterile Cell Strainers (Thermo Fisher Scientific, 352340). Cell suspensions were re-suspended in AIM V medium supplemented with 10% FBS plus antibiotics and antimycotics at a final cell concentration of 2×10^6 cells/mL for LPR assays or 5×10^6 cells/mL for the IFN-γ ELISPOT assays.

ELISAs for serum antibody titers

Polystyrene microtiter plates (Immulon 2, Thermo Fisher Scientific, 3455) were coated with either C-HBV, recombinant HBsAg or S1/S2-Core fusion protein diluted to a final concentration of 1.0 µg/mL in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ pH 9.6). A final volume of 100 µL was added to each well and plates were incubated at 4°C for 16 h. Plates were washed twice with Tris Buffered Saline + 0.005% Tween 20 (Sigma-Aldrich, SRE0031) and blocked with blocking

buffer (PBS containing 2% BSA) at room temperature for 1 h. Serum samples were serially diluted in blocking buffer, added into corresponding wells and incubated for 2 h at room temperature. Antibodies were detected with rabbit anti-sheep IgG (H + L) HRP labeled (Kirkegaard & Perry Laboratories, 5260–0057) diluted 1:3000. ELISAs were developed using 150 µg/mL ABTS substrate (Sigma-Aldrich, 10102946001) diluted in 0.055 M citric acid (pH 4.0) with 0.3% H_2O_2. The reaction was stopped after 30 min by the addition of 10% SDS. Optical density (OD) of the reaction product was measured at λ 405 nm using a λ 490 nm reference on a Microplate reader (Bio-Rad Laboratories, 1681000). A positive titer was defined as the reciprocal of the final serum dilution for which an OD reading was obtained that exceeded the mean + 2SD of the reading obtained with a negative serum sample.

LPR assay

Lymphocyte proliferation was determined using a modified LPR assay.[25] Proliferation induced by C-HBV and S1/S2-Core was assayed in 96-well U-bottom culture plates (Thermo Fisher Scientific, 168136) with 2×10^5 PBMCs or LN cells/well. Cells were cultured in AIM V supplemented with 10% FBS and 2×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, M3148). Triplicate cultures were stimulated for 72 h with either 5 µg/mL purified C-HBV or 3.3 µg/mL S1/S2-Core fusion protein. Cells were cultured in a final volume of 200 µL medium and pulsed with 0.4 µCi/mL [^{3}H] thymidine (GE Healthcare, TRK120) during the final 6 h of culture. Incorporation of [^{3}H] thymidine was determined using a liquid scintillation counter (Beckman Coulter LS6500, 8043-30-1194). Proliferative responses were calculated as specific counts per minute (CPM) by subtracting CPM for cells cultured in medium alone from CPM recorded when cells were cultured with either C-HBV or S1/S2-Core protein. Data are presented as the mean of triplicate cultures.

ELISPOT assay for IFN-γ-secreting cells

C-HBV-specific IFN-γ-secreting cells were detected using a modified ELISPOT assay.[26] Briefly, microtiter nitrocellulose filtration plates (Millipore, MSHAS4510) were coated with 2.5 µg/mL of anti-bovine IFN-γ monoclonal antibody (Bio-Rad, MCA2689GA). Cells (0.5×10^6 cells/well) were added to triplicate wells in a final volume of 200 µL of either media alone (AIM V media plus 2% FBS) or media containing either 5 µg/mL purified C-HBV or 3.3 µg/mL S1/S2-Core fusion protein. Following overnight incubation at 37°C, cells were lysed with distilled water and captured IFN-γ visualized with rabbit anti-bovine IFN-γ antisera (Rockland Immunochemicals, 201-401-C41) and biotinylated-goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific, 31820). Spots were counted with an inverted light microscope, averaged for triplicate cultures, and expressed as IFN-γ secreting cells/million cells. The number of C-HBV and S1/S2-Core specific IFN-γ-secreting cells was calculated as the difference between the mean number of spots/million cells from triplicate cultures stimulated with protein (C-HBV or S1/S2-Core) minus mean number of spots per million cells for triplicate cultures with media.

Statistical analysis

Standard descriptive statistics, including calculation of both group means and variance and group medians and ranges, were undertaken to verify and assess data distribution (GraphPad Prism 7.04; GraphPad Software Inc. GPS-1237593-L). Data were found to be normally distributed and comparisons within and among groups for antibody titer, LPR, and IFN-γ were analyzed using a repeated measures 2-way ANOVA with time and treatment as variables. When significant ($p < .05$) time or treatment-dependent differences were identified then values for individual treatment groups were compared at each time point using a Dunn's Multiple Comparison. Differences were considered significant with $p < .05$.

Results

Binding of C-HBV to human and sheep PBMCs

To evaluate C-HBV for immunogenicity in a sheep model, it was necessary to first confirm if C-HBV bound to sheep myeloid cells, including monocytes and DCs. To characterize C-HBV binding by sheep myeloid cells, plastic-adherent cells were harvested from PBMCs and incubated with C-HBV. Surface binding of C-HBV was determined by FACS and compared to human PBMCs. C-HBV bound to sheep myeloid cells in a concentration-dependent manner and the binding was comparable to that observed with plastic-adherent cells harvested from human PBMCs (Figure 2).

Humoral immune response to C-HBV

The production of anti-C-HBV antibody in sheep was measured every 2 weeks throughout the course of the study (Table 1). The analysis of C-HBV-specific antibody titers revealed control animals (Figure 3A and B) maintained low titers throughout the

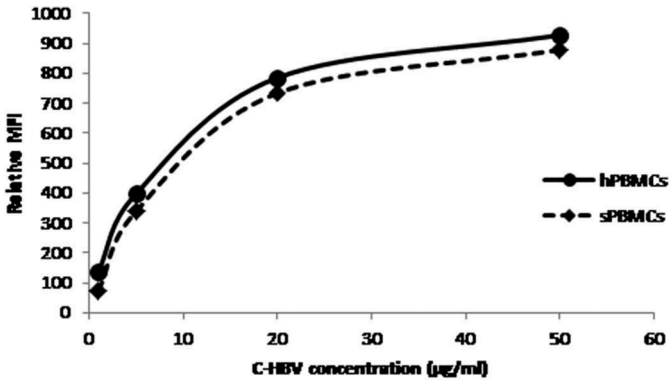

Figure 2. Comparison of the binding of C-HBV to human (hPBMCs) with sheep (sPBMCS) peripheral blood mononuclear cells.

PBMCs were isolated from either sheep or human blood and incubated for 1 h at 4°C with C-HBV (1–50 µg/mL). Following labeling with biotinylated anti-mouse IgG mAb and SA-PE-Cy5, bound protein was quantified by flow cytometry and expressed as the relative mean fluorescence intensity (MFI) of labeled cells.

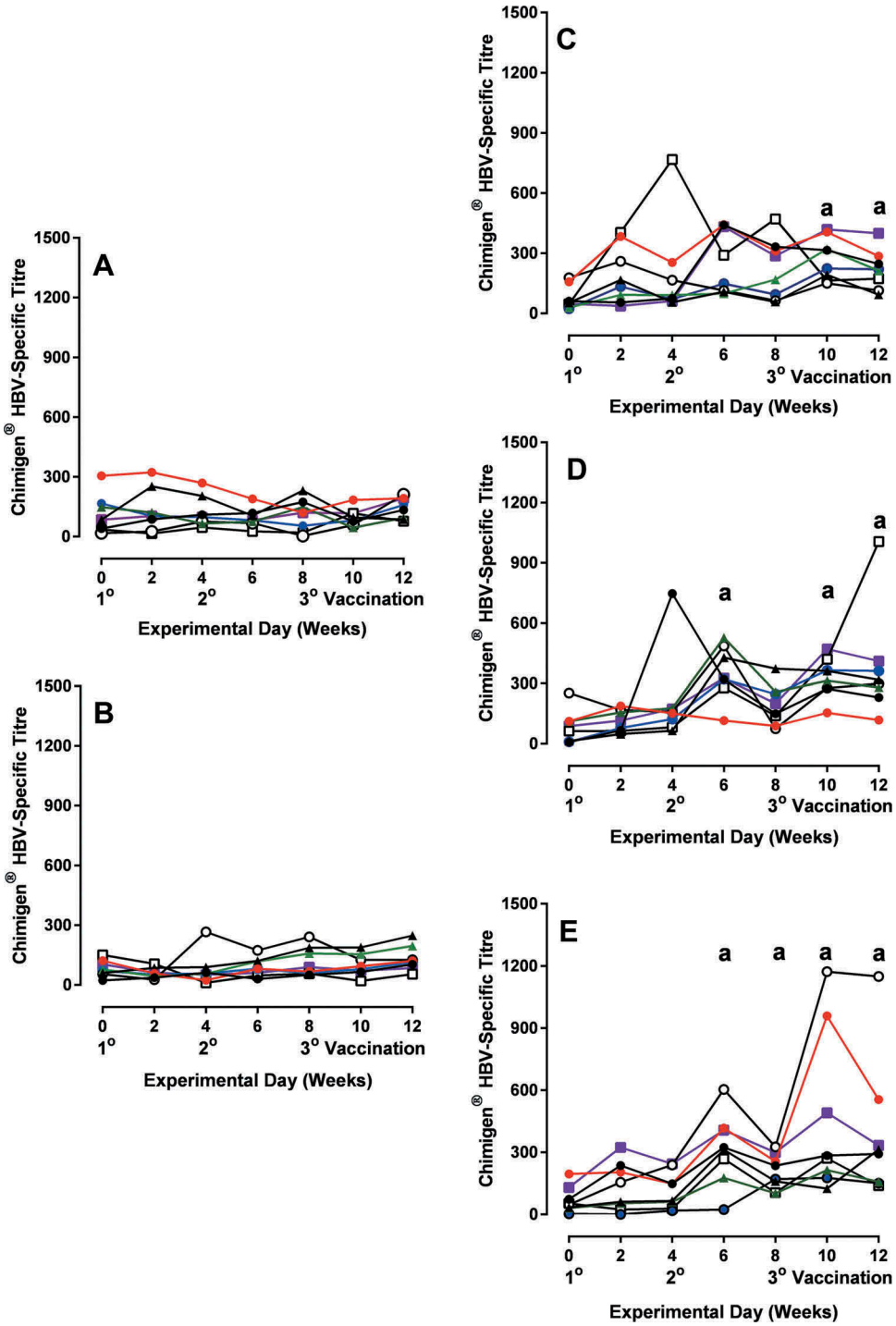

Figure 3. Chimigen® HBV-specific serum antibody titer in sheep following first, second and third injections.

Lambs (n = 8/group) were injected subcutaneously with one of the following formulation: Naïve Control (PBS; Panel A). ENGERIX-B (20 µg/dose; Panel B); C-HBV (5 µg/dose, Panel C; 20 µg/dose, Panel D; and 50 µg/dose, Panel E). Serum antibody titers were determined with a capture ELISA and wells were coated with C-HBV. Titers are expressed as the reciprocal of the serum dilution which gave an optical density greater than the negative cutoff value. Data presented are values for individual animals within each group. Antibody titers were analyzed as repeated measures within each group and compared relative to week 0 (pre-immunization) values. Significant increases in antibody titers, relative to pre-vaccination titers within the same group, are indicated (**a** = $p < .05$).

study, which provided a baseline comparison for the C-HBV ELISA. The commercially available, adjuvanted HBsAg prophylactic vaccine (ENGERIX-B) contains yeast-derived recombinant HBsAg and was used as a positive control for detection of HBsAg-specific antibodies. ENGERIX-B did not induce cross-reactive antibodies to C-HBV epitopes (Figure 3B). There was a significant rise in C-HBV-specific antibody titers observed following two subcutaneous (sc) injections with 20 and 50 µg doses of C-HBV. Following three sc injections, this antibody response was more prominent (Figure 3D and E). With the 20 µg dose, there was one high responder, one non-responder and six low-responders (Figure 3D), whereas with the 50 µg dose, all animals showed a significant increase in antibody production (Figure 3E). Among the eight animals in this group, three were

high responders and the remaining five were low responders. All animals in the high dose group showed an increase in the antibody response to C-HBV, relative to pre-vaccination titers, following the third immunization. At no time was there a significant dose-dependent difference when comparing antibody titers among the low, medium, and high dose C-HBV groups, although the lowest dose of C-HBV (5 μg) induced significant increases in antibody titers only after the third vaccination (Figure 3C). These results confirmed C-HBV was immunogenic in sheep in the absence of an adjuvant but the variability of individual animal responses within each group precluded identification of significant changes in antibody responses when comparing among vaccine groups.

The commercial HBV prophylactic vaccine (ENGERIX-B) is expected to induce high levels of anti-HBsAg in sheep.[27] The analysis of HBsAg-specific titers confirmed all sheep had low background titers prior to immunization and these low titers remained constant in the Control group (Figure 4A). There was a significant rise in HBsAg-specific antibody titers following the second ENGERIX-B immunization and elevated antibody titers persisted after the third immunization (Figure 4B). The ENGERIX-B group provided a positive control for antibody responses to HBsAg and revealed the kinetics of these responses in sheep. C-HBV does not contain HBsAg so, as expected, none of the groups injected with C-HBV developed a significant increase in HBsAg cross-reactive antibodies (Figure 4C, D and E). One animal in the high dose group (Figure 4E) had an increased HBsAg titer after the third injection. However, the increase in titer in this one animal reflects a difference of less than one serial dilution (three-fold dilutions) so an increased titer observed at this one time point may be explained by a small change in the level of nonspecific cross-reactive antibodies. As an outbred model, this response may reflect individual animal variation in immune responses to environmental antigens rather than a response to C-HBV.

HBV antigen-specific antibodies induced by C-HBV

The therapeutic potential of C-HBV is dependent on the induction of predominantly cellular responses, but humoral immune responses to the selected HBV antigens may also play a role in restricting the spread of virus among cells. Therefore, an ELISA was used to quantify S1/S2-Core-specific antibody responses (Figure 5). Low S1/S2-Core-specific antibody titers were present in all sheep prior to injection and titers remained unchanged in the control group throughout the experiment. This confirmed exposure to environmental antigens did not induce S1/S2-Core cross-reactive antibodies. There was a minor but not significant increase in S1/S2-Core-specific antibody titers in one animal following both the secondary and tertiary injections with ENGERIX-B (Figure 5B) for reasons that are unclear. There was a significant ($p < .05$) increase in S1/S2-Core-specific antibody titers in sheep injected with higher doses (20 and 50 μg) of C-HBV (Figure 5D and E) following the second and third injections, but no significant response in the 5 μg dose group (Figure 5C). Therefore, C-HBV induced an S1/S2-Core-specific antibody response and the specificity of these responses was confirmed by the absence of antibody cross-reactivity with recombinant HBsAg protein. Variability of individual animal S1/S2-Core-specific antibody responses within each group precluded identification of a significant dose-dependent antibody response.

HBV antigen-specific cell-mediated immune responses to C-HBV

Cell-mediated immune responses are a significant correlate of immune-mediated clearance of HBV infection, and this response was primarily a core antigen-specific T cell response.[28,29] Therefore, T cell responses to both C-HBV and the S1/S2-Core protein were assayed following the second and third injections. Following two injections, a significant, C-HBV-specific cell-mediated immune response was observed in PBMCs (Figure 6). Significant ($p < .05$) C-HBV (Figure 6A) and -S1/S2-Core protein (Figure 6B) specific lymphocyte proliferative responses (LPR) were observed with the 20 and 50 μg doses but not the 5 μg dose of C-HBV when compared to the Control group. There was not, however, a significant vaccine dose-dependent effect on the magnitude of the LPR, reflecting in part the variability in individual animal responses within each group. A significant ($p < .05$) IFN-γ secretion response to both C-HBV (Figure 6C) and S1/S2-Core (Figure 6D) was induced only by the highest dose of C-HBV when comparing responses to the Control group. A subset of animals within each C-HBV group displayed an increased frequency of IFN-γ secreting cells and again there was no significant difference in the magnitude of this response when comparing among the three vaccine groups. Following the third injection, there was again a significant ($p < .05$) increase in both C-HBV (Figure 7A) and S1/S2-Core protein-specific (Figure 7B) LPR in both the medium and high dose vaccine groups relative to the Control group but no significant dose-dependent effect when comparing among vaccine groups. Further, a significant ($p < .05$) increase in S1/S2-Core specific IFN-γ secreting cells (Figure 7D) was induced by all doses of C-HBV and this response was similar for all vaccine groups. Thus, C-HBV induced a strong cell-mediated immune response to the HBV antigens but the onset of this immune response following either two or three vaccinations was influenced by C-HBV dose.

C-HBV-specific cell-mediated immune responses in the lymph node (LN) draining the site of vaccine injection were also analyzed following the third injection (Figure 8). These analyses did not reveal a significant persistence of either C-HBV (Figure 8A) or S1/S2-Core (Figure 8B) LPR at 4 weeks following the third C-HBV injection but a significant ($p < .05$) S1/S2-Core protein-specific IFN-γ secreting cell response (Figure 8D) was observed with the highest C-HBV vaccine dose. Thus, the dose of C-HBV vaccine used may influence the duration of responses at the site of immune response induction. When comparing the cell-mediated immune responses in blood (Figure 7) and the draining LN (Figure 8) at 12 weeks, it was apparent that a significant ($p < .05$) increase in responses persisted longer in blood than the draining samples and this analysis also confirmed C-HBV induced a systemic cell-mediated immune response which is necessary to target HBV-infected hepatocytes.

A possible correlation between serum antibody responses and cell-mediated immune responses induced by C-HBV was investigated. A correlation between both aspects of the immune

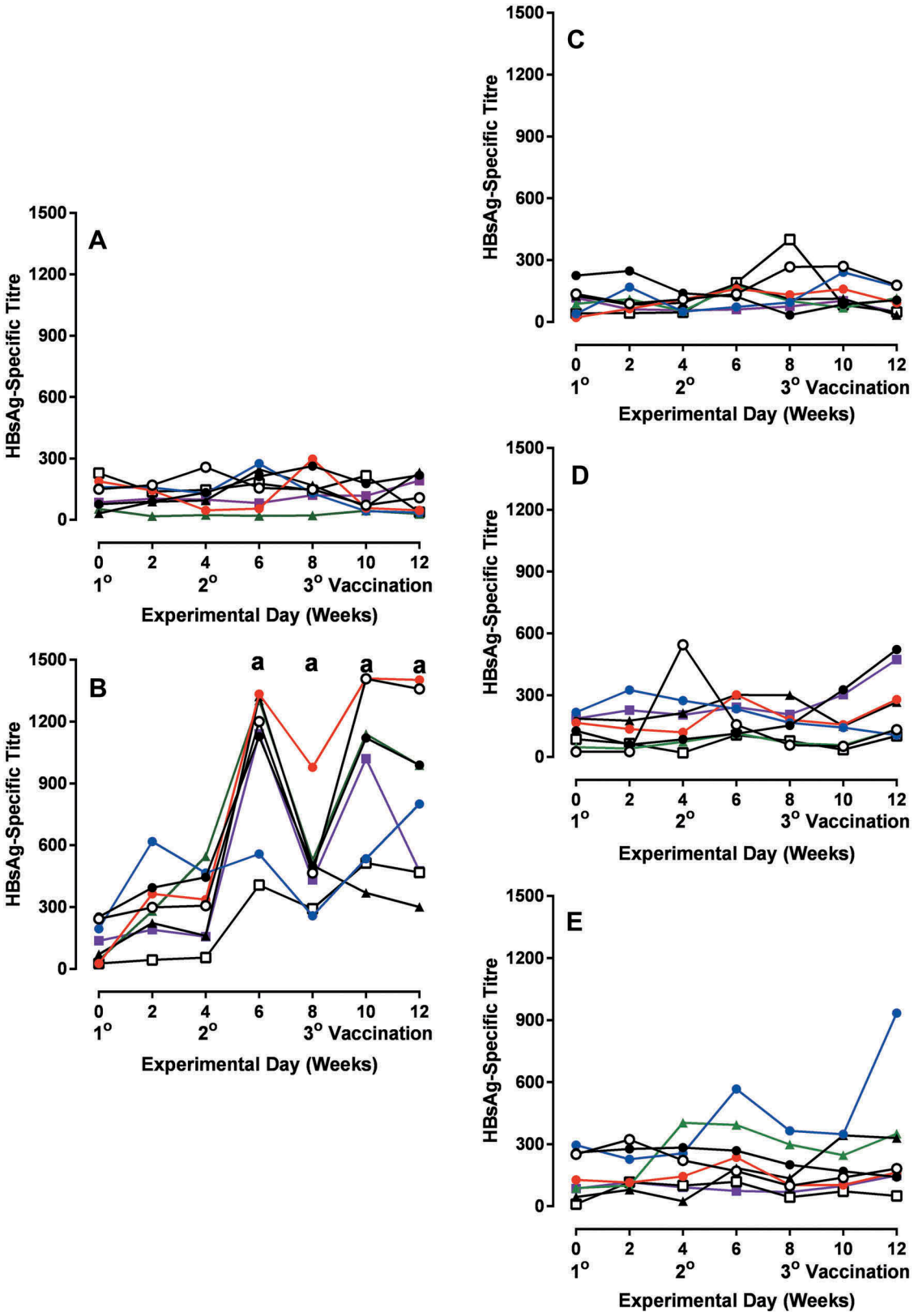

Figure 4. HBV surface antigen (HBsAg)-specific antibody titer in sheep following first, second and third injections of C-HBV.

Lambs (n = 8/group) were injected subcutaneously with one of following formulations: Naïve Control (PBS; Panel A); ENGERIX-B (20 µg/dose; Panel B); C-HBV (5 µg/dose, Panel C; 20 µg/dose, Panel D; and 50 µg/dose, Panel E). Serum antibody titers were determined with a capture ELISA and wells were coated with recombinant HBsAg protein. Titers are expressed as the reciprocal of the serum dilution which gave an optical density greater than the negative cutoff value. Data presented are values for individual animals within each group. Antibody titers were analyzed as repeated measures within each group and compared relative to week 0 (pre-immunization) values. Significant increases in antibody titers, relative to pre-vaccination titers within the same group, are indicated (**a** = $p < .05$).

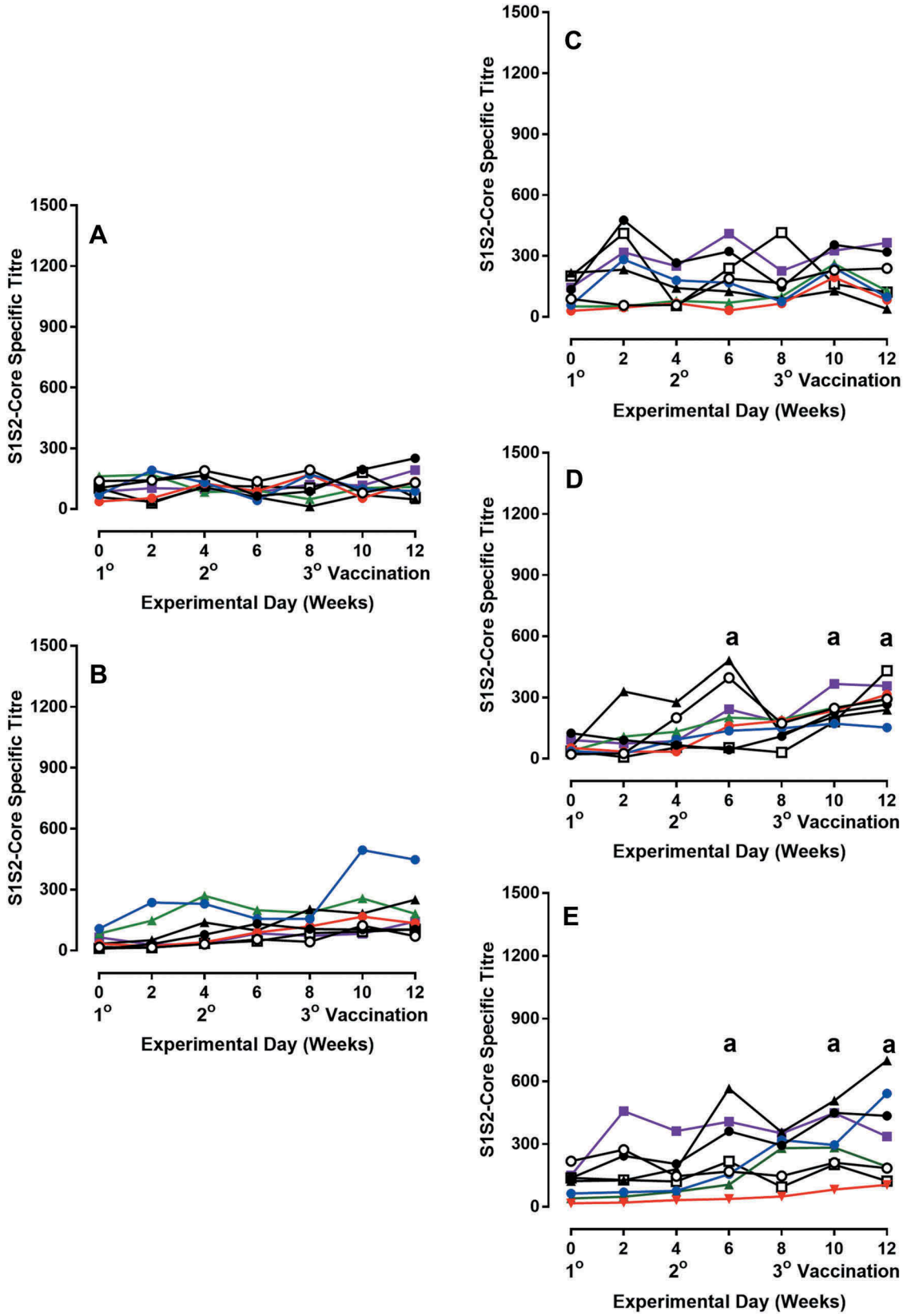

Figure 5. HBV S1/S2-Core antigen-specific antibody titer in sheep following first, second and third injections of C-HBV.

Lambs (n = 8/group) were injected subcutaneously with one of following formulations: Naïve Control (PBS; Panel A); ENGERIX-B (20 µg/dose; Panel B); C-HBV (5 µg/dose, Panel C; 20 µg/dose, Panel D; and 50 µg/dose, Panel E). Serum antibody titers were determined with a capture ELISA and wells were coated with recombinant S1/S2-Core protein. Titers are expressed as the reciprocal of the serum dilution which gave an optical density greater than the negative cutoff value. Data presented are values for individual animals within each group. Antibody titers were analyzed as repeated measures within each group and compared relative to week 0 (pre-immunization) values. Significant increases in antibody titers, relative to pre-vaccination titers within the same group, are indicated (**a** = $p < .05$).

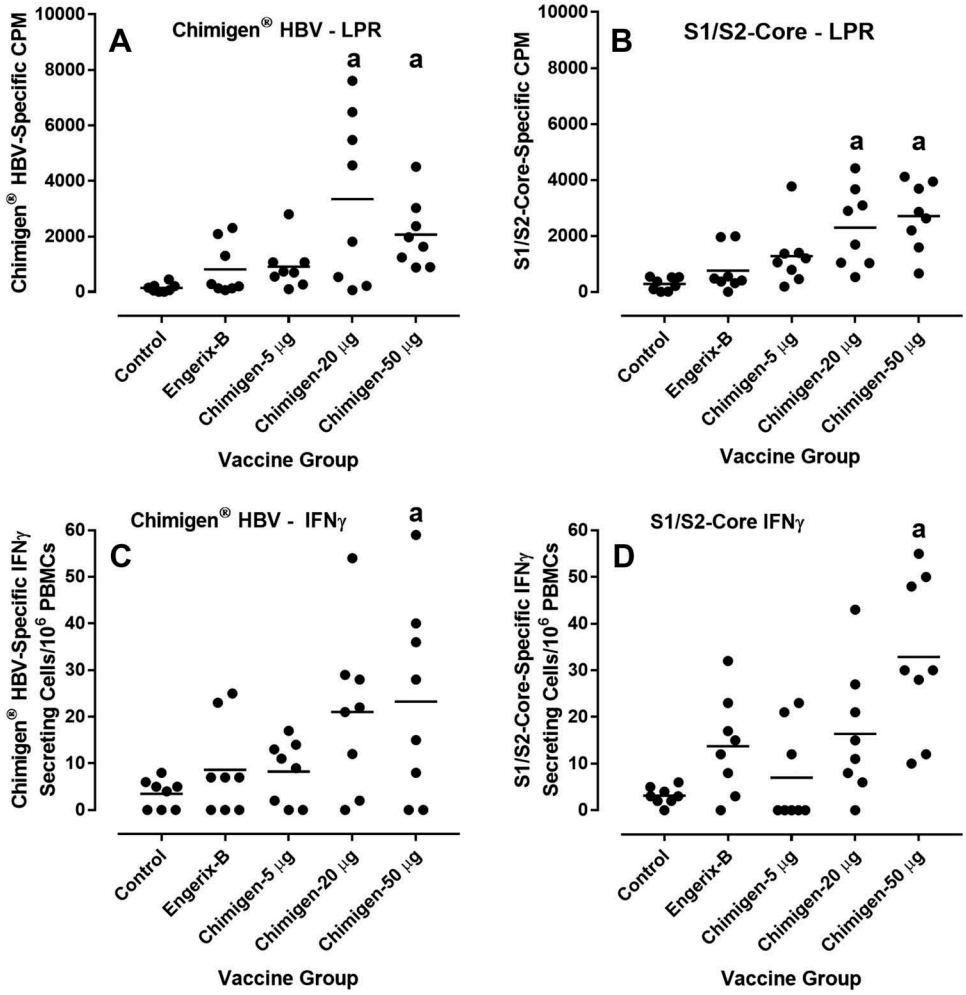

Figure 6. Chimigen® HBV and S1/S2-Core-specific LPR and IFN-γ secretion of sheep PBMC following the second C-HBV injection.

Lambs (n = 8/group) were injected subcutaneously with one of following formulations: Naïve Control (PBS); ENGERIX-B (20 µg/dose); Chimigen® HBV (5 µg/dose, 20 µg/dose, and 50 µg/dose). LPR were determined by [^{3}H]-thymidine incorporation (CPM) at 72 h following stimulation of PBMC with either 5 µg/mL Chimigen® HBV (Panel A) or 3.3 µg/mL S1/S2-Core protein (Panel B). Data presented are the mean value of triplicate assays and this value is presented for each animal within a group. An IFN-γ capture ELISPOT assay was used to enumerate the frequency of IFN-γ secreting cells at 24 h following stimulation of PBMCs with either 5 µg/mL Chimigen® HBV (Panel C) or 3.3 µg/mL S1/S2-Core protein (Panel D). Data presented are the mean value of triplicate assays and this value is presented for each animal within a group. Antigen-specific IFN-γ secreting cells were calculated by subtracting the number of IFN-γ spots in the absence of antigen from the number of IFN-γ spots enumerated in the presence of antigen. Significant increases in either CPM or IFN-γ secreting cells relative to the Naïve Control group are indicated (**a** = $p < .05$). There were no significant differences among the three Chimigen® HBV vaccine groups.

response might allow the use of a single immune parameter as a correlate of immune protection. Alternatively, the use of multiple immune response parameters may provide a more robust identification of high and low-responding individuals following vaccination. Regression analysis of S1/S2-Core-specific serum antibody titers and either LPR or IFN-γ secreting cell frequency following the secondary (8 weeks) and tertiary vaccination (12 weeks) was performed for both the high and medium dose C-HBV groups. At 8 weeks, no significant correlation was observed between S1/S2-Core-specific serum antibody titers and either LPR (p = .490; R^2 = 0.083) or the frequency of IFN-γ secreting cells in blood (p = .452; R^2 = 0.097) of the high dose (50 µg) group. Similarly, at 12 weeks, no significant correlation was observed between S1/S2-Core-specific antibody titers and either LPR (p = .838; R^2 = 0.007) or the frequency of IFN-γ secreting cells in the blood (p = .721; R^2 = 0.022) of the high dose group. Similar results were observed for the medium dose (20 µg) group following both the secondary and tertiary immunization (data not shown). Therefore, we conclude that no significant correlation exists between S1/S2-Core specific antibody responses and S1/S2-Core specific T cell responses as measured in the present study. Further, looking collectively at humoral and cell-mediated immune responses would not provide a more sensitive measure of an individual animal's capacity to respond to C-HBV.

Discussion

Current direct acting HBV antiviral agents are effective in inhibiting viral replication and limit HBV-associated disease progression.[4] The treatment is lifelong, rarely results in viral clearance and discontinuing treatment results in a rebound of the viremia. Immune intervention using interferon is effective only in a small subset of HBV carriers.[9] These treatments rarely achieve complete elimination of HBV. For a functional cure of chronic HBV infections, induction of HBV-specific T cell

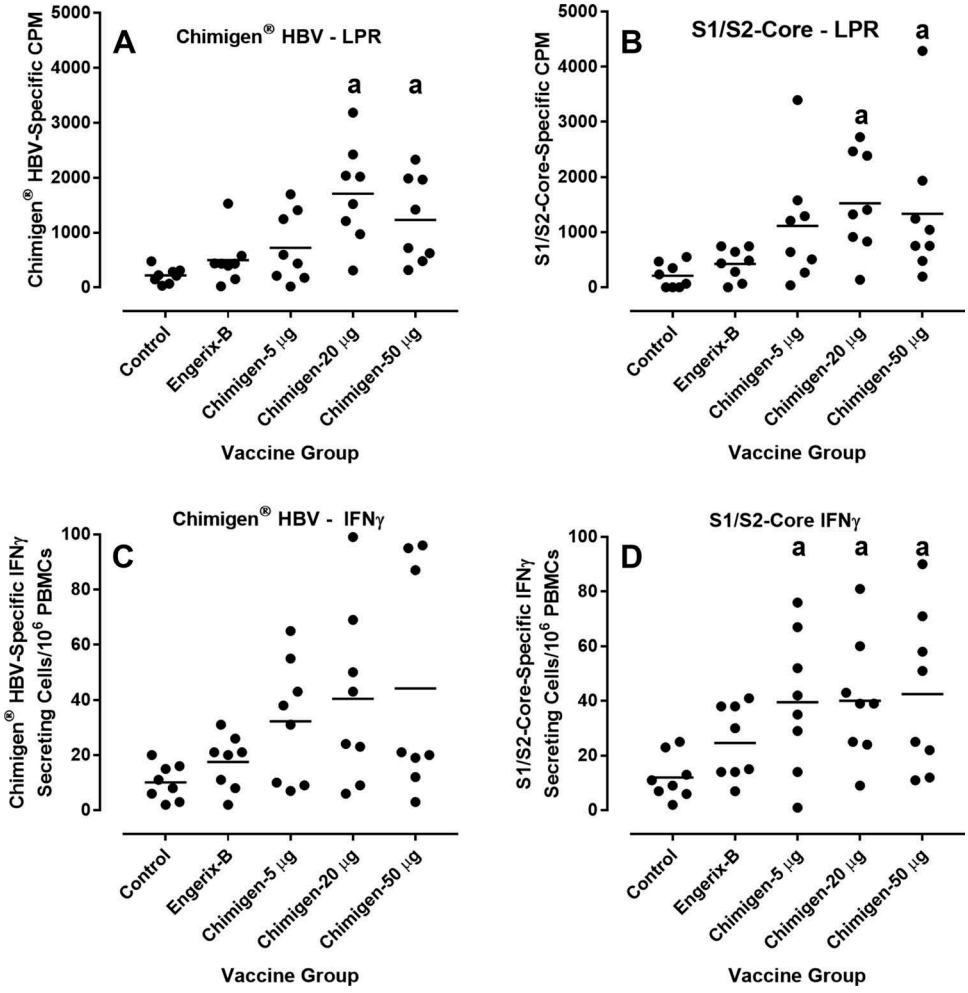

Figure 7. Chimigen® HBV and S1/S2-Core-specific LPR and IFN-γ secretion of sheep PBMC following the third C-HBV injection.

Lambs (n = 8/group) were injected subcutaneously with one of following formulations: Naïve Control (PBS); ENGERIX-B (20 µg/dose); Chimigen® HBV (5 µg/dose, 20 µg/dose, and 50 µg/dose). LPR were determined by [^{3}H]-thymidine incorporation (CPM) at 72 h following stimulation of PBMC with either 5 µg/mL Chimigen® HBV (Panel A) or 3.3 µg/mL S1/S2-Core protein (Panel B). Data presented are mean values of triplicate assays and this value is presented for each animal within a group. An IFN-γ capture ELISPOT assay was used to enumerate the frequency of IFN-γ secreting cell at 24 h following stimulation of PBMCs with either 5 µg/mL Chimigen® HBV (Panel C) or 3.3 µg/mL S1/S2-Core protein (Panel D). Data presented are the mean value of triplicate assays and this value is presented for each animal within a group. The number of antigen-specific IFN-γ secreting cells was calculated by subtracting the number of IFN-γ spots in the absence of antigen from the number of IFN-γ spots in the presence of antigen. Significant increases in either CPM or IFN-γ secreting cells relative to the Naïve Control group are indicated (**a** = $p < .05$). There were no significant differences among the three Chimigen® HBV vaccine groups.

activation is believed to be necessary which can be achieved through the use of immunotherapeutic agents. C-HBV is designed to elicit broad antigen-specific cellular and humoral immune responses to clear viral infections. C-HBV contains the antigen peptide fragments of PreS1 and PreS2, the entire HBV Core and a xenotypic Fc fragment from mouse IgG_1 (Figure 1).

Using PBMCs isolated from un-infected and chronic HBV-infected donors, in *ex vivo* antigen presentation assays, C-HBV was shown to bind to specific receptors on DCs and be internalized, processed and presented to T cells to elicit functional T cell responses (CD4+ and CD8+) specific to the selected antigens in C-HBV.[16] In the present study, we assessed the ability of C-HBV to induce both humoral and cell-mediated immune responses *in vivo*, using sheep as a naïve outbred animal model to confirm C-HBV immunogenicity and analyze the specificity of the immune responses. Although NHPs, especially the chimpanzee,[19] are valuable models for evaluating immunotherapy vaccines for chronic HBV, the cost is high and availability is limited. Sheep have been used for evaluating a broad range of immune functions[21] but there is substantial individual animal variation in immune responses.

C-HBV induced dose-dependent C-HBV-specific antibody and cell-mediated immune responses. The low (5 µg) dose group induced a low antibody response against C-HBV after the third injection whereas the number of responders, in addition to the number of high responders, increased with higher vaccine doses (20 µg and 50 µg) after the second and third injections. These responses were specific for the S1/S2-Core fusion protein portion of C-HBV. In LPR and cytokine ELISPOT assays, the responses displayed the same dose-dependent responses as observed for serum antibody responses. These responses were not observed with ENGERIX-B.

It is noteworthy that much of the LPR and IFN-γ responses seen with the C-HBV (left panels Figures 6–8) were specific for the S1/S2-Core portion of the vaccine (right panel). The magnitude of

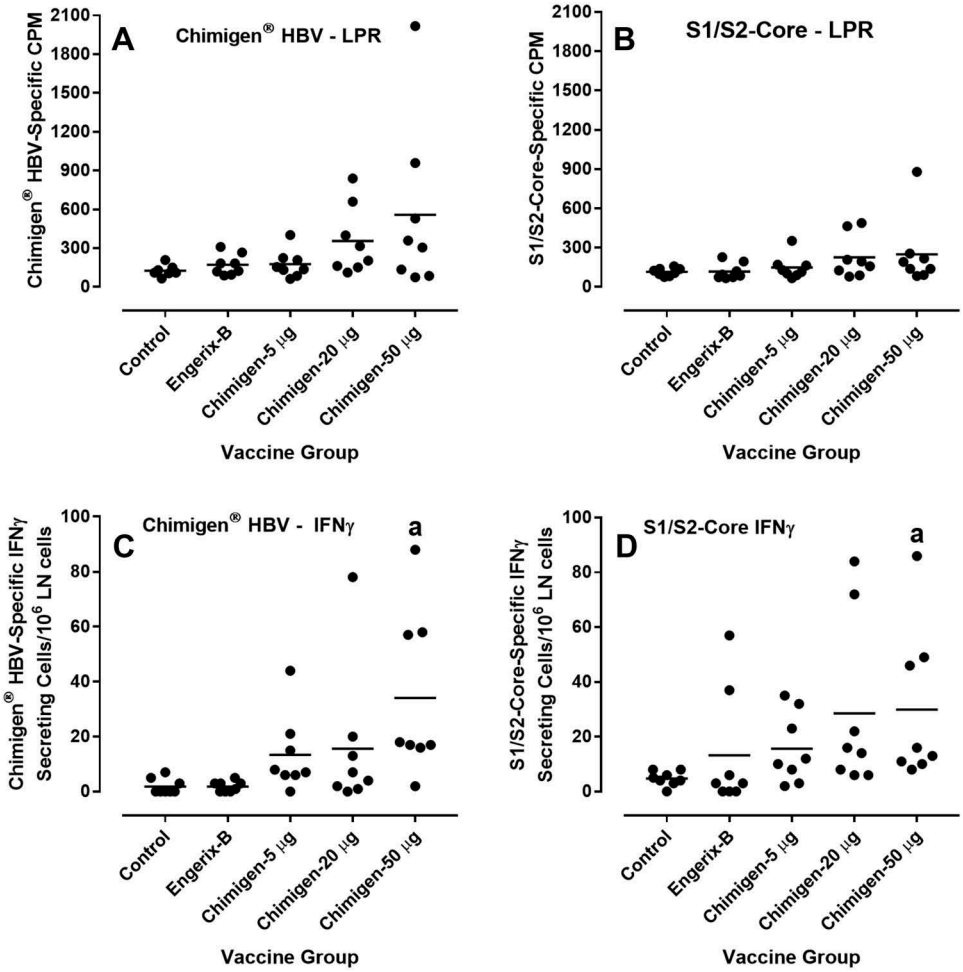

Figure 8. Chimigen® HBV and S1/S2-core-specific LPR and IFN-γ secretion in cells isolated from LNs draining the vaccine injection site.

Lambs (n = 8/group) were injected subcutaneously with one of following formulations: Naïve Control (PBS); ENGERIX-B (20 µg/dose); Chimigen® HBV (5 µg/dose, 20 µg/dose, and 50 µg/dose). Cells were isolated from LNs draining the vaccine injection site at 4 weeks after the third injection. LPR were determined by [^{3}H]-thymidine incorporation (CPM) at 72 h following stimulation of LN cells with either 5 µg/mL Chimigen® HBV (Panel A) or 3.3 µg/mL S1/S2-Core protein (Panel B). Data presented are mean value of triplicate assays and this value is presented for each animal within a group. An IFN-γ capture ELISPOT assay was used to enumerate the frequency of IFN-γ secreting cells at 24 h following stimulation of PBMCs with either 5 µg/mL Chimigen® HBV (Panel C) or 3.3 µg/mL S1/S2-Core protein (Panel D). Data presented are the mean value of triplicate assays and this value is presented for each animal within a group. The number of antigen-specific IFN-γ secreting cells was calculated by subtracting the number of IFN-γ spots in the absence of the antigen from the number of IFN-γ spots in the presence of antigen. Significant increases in either CPM or IFN-γ secreting cells relative to the Naïve Control group are indicated (**a** = $p < .05$). There were no significant differences among the three Chimigen® HBV vaccine groups.

responses seen in the draining lymph node (Figure 8), which is the immune induction site, was relatively low but an important observation is that higher levels of antigen-specific cells were observed in blood. This finding is important since HBV-specific effector cells must traffic through the blood to the site of infection in the liver. The absolute number of effector cells detected in blood at any one time may not be the critical factor but rather it may be important that a vaccine continues to generate a pool of effector cells over time. This would ensure that effector cells are available for sustained recruitment to the liver, ensuring a persistent infection can be cleared. Further, we enumerated the frequency of S1/S2-Core-specific IFN-γ secreting cells/million PBMCs so there is potentially a very large pool of effector cells circulating in the blood. For comparison, a 75 kg individual has 7% body weight as blood, which is a total volume of 5.25 L blood. Normal white blood cells (WBC) counts range from 4 to 10×10^9 WBCs/L and over 50% of WBCs are mononuclear cells (PBMCs were used in our ELISPOT assay). Therefore, using an estimate of at least 2–5 $\times 10^9$ mononuclear cells/L blood, we calculate a total population of 10.5 to 27.5 $\times 10^9$ mononuclear cells in the blood of a 75 kg individual. If there were 40 S1/S2-Core IFN-γ secreting cells/million PBMCs (average value in vaccine groups in Figure 7B left panel), this would represent a total of 420–1100 million S1/S2-Core-specific effector cells circulating in the blood of an immunized individual. This provides a very large effector population to be recruited to the target organ. Therefore, the magnitude of responses observed in the current study may be sufficient for an effective therapeutic vaccine. Chimigen® Technology predicts receptor-mediated uptake of the chimeric antigen and both class I and II antigen presentations.[16] The generation of cellular and humoral immune responses in the sheep confirmed the chimeric antigens were presented via the predicted mechanisms, suggesting that the fusion proteins were intact, although this would not speak to the conformation of the protein. Induction of HBV-specific T cell-mediated immune response is necessary for clearing chronic hepatitis B.[28-30] The current study confirmed the

induction of HBV-specific T cells, a necessary outcome for HBV immunotherapy. C-HBV also induced HBV-specific antibody responses which may indirectly support the resolution of a persistent HBV infection by assisting in the removal of both virus and viral antigens.

There are several new antiviral treatments currently in discovery and early development stage to improve the clinical outcome of chronic hepatitis B, which include direct acting antivirals, host targeting antivirals and immune modulators, including immunotherapy vaccines.[31] The need for immune system re-activation, although well-recognized as necessary for controlling and ideally clearing HBV infection,[28-30] and the development of an HBV therapeutic vaccine has not yet been successful. Since impaired immune responses are detected in chronic HBV-infected patients, a therapeutic vaccine must be able to re-educate the host immune system to recognize the virus and the viral antigens so cytotoxic T cells can eliminate infected cells and antibodies can bind circulating antigens and enhance their elimination.

Various approaches have been used to design and produce HBV immunotherapeutic vaccines.[10,31] These include protein/peptide-based, antibody-based, DNA and viral vector-based as well as cell-based therapies. Some have been unsuccessful, and others are at different stages of development. Chimigen® Technology is a new concept in vaccine and immunotherapy development. Chimigen® Molecules are recombinant proteins that incorporate functional elements of antigen(s) and a xenotypic antibody. This chimeric molecule is recognized by the host immune system as "foreign" after targeting antigen-presenting cells, especially dendritic cells (DCs), through specific receptors. Chimigen® Molecules can be processed through the proteasomal pathway and presented to T cells to elicit a cytotoxic T lymphocyte response. Furthermore, when processed through the endosomal pathway and presented to T helper cells (Th), the Th cells can activate B cells and elicit an antibody response.[32] In addition, antigen uptake by DCs may directly prime B cells.[33-37] This broad immune response can effectively break tolerance to persistent viral antigen and facilitate the clearance of the viral infection.

Targeted delivery of antigens to DCs have been a challenge, although several studies have shown that antigens targeted to DCs more efficiently generate immune responses.[38] Antibody-based therapies partially achieve this goal by binding to the respective antigen and the antigen-antibody complex entering the DCs via receptor mediated uptake. There are, however, several disadvantages to antibody-based therapies. They require antigen to be complexed *in situ* for efficient delivery to DCs. This requires high levels of circulating antigens and these levels may vary among individuals. The efficiency of uptake by the DCs also will depend on the nature of the complex. Aggregates of the complex could also be engulfed by scavenger cells and macrophages, resulting in a lack of antigen presentation.[39] Antigen-antibody complex that bind appropriate DC receptors result in antigen uptake, processing, and productive presentation, whereas aggregates taken up via macropinocytosis may enter lysozomes, being degraded with little antigen presentation.[39] Another problem is that if the antibody is in excess of antigen, T-cell responses are inhibited.[40] Almost all HBV therapeutic vaccines in development have used adjuvants. However, a recent study has shown that adjuvant-based vaccines can induce specific T cell responses to cancer cells but most of the antigen-specific T-cells were sequestered and deleted at the site of vaccination due to the persisting depot effect of the adjuvanted vaccine. This is one possible reason for the failure of these vaccines.[41] Chimigen® Vaccines are administered without an adjuvant. In light of this formulation, C-HBV has a greater potential to break tolerance and induce therapeutic immune responses to HBV infection. C-HBV, the lead candidate developed using Chimigen® Technology has shown promising results *ex vivo* and the predicted immune responses in a large animal (sheep), *in vivo*.

Although the present study provided the efficacy of C-HBV in generating antigen-specific cellular and humoral responses, there are limitations to this study. The major limitation is that sheep is not a model for HBV infection. Therefore, a comparison of the present results with previous vaccine candidates may not provide any meaningful conclusion. It is also not possible to evaluate the biological significance of the immune responses generated. Measuring variation in individual animal immune response is important when working in outbred populations such as humans. More substantial evidence for the safety, immunogenicity, and efficacy of C-HBV will become evident in future clinical trials. One other limitation is that sheep is an outbred species, much like humans and the genetic variability between individual animal is high. This results in variability in immune responses particularly when, as in the present study, group sizes were limited to eight animals. To estimate expected differences among treatment groups and determine the significance of these responses, the number of individuals needed in clinical trials need to be substantially larger and this remains to be established.

Conclusion

The results of the present study demonstrated that C-HBV, a dendritic cell receptor-targeting fusion protein of HBV antigens and a xenotypic antibody Fc fragment, can elicit both a humoral and cell-mediated immune responses in a large animal model. C-HBV induced the production of HBV-antigen specific antibodies and T cell proliferation and secretion of IFN-γ in PBMCs and LN cells. HBV antigen-specific T cells were induced in LNs draining the site of C-HBV injection, and were disseminated systemically in blood. This is critical for an immunotherapy targeting HBV-infected cells. This was achieved without the use of an adjuvant, making C-HBV a promising immunotherapy candidate for the treatment of chronic HBV infection.

Abbreviations

C-HBV	Chimigen® HBV Immunotherapeutic Vaccine
DC	dendritic cell
ELISA	enzyme linked immunosorbent assay
ELISPOT	enzyme linked immunospot assay
FACS	fluorescence activated cell sorting
GrB	granzyme B
HBV	hepatitis B virus
HBsAg	hepatitis B virus surface antigen
IFN-γ	interferon gamma
LPR	lymphocyte proliferative response
LN	lymph node
PBMC	peripheral blood mononuclear cell
Pfn	perforin

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Disclosure of potential conflicts of interest

The authors, RG, AM, BM, YES, QL and PG declare they have no conflicts of interest.

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