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A New Class of Therapeutic Vaccines Produced in Insect Cells for the Treatment of Chronic Viral Infections

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Chronic viral infections are major healthcare issues. Hepatitis B virus (HBV), hepatitis C virus (HCV), human papilloma virus (HPV), and human immunodeficiency virus (HIV) are prime examples of viruses which cause chronic infections. HBV is a member of a family of DNA viruses that primarily infect liver cells which can lead to acute or chronic liver disease. Approximately 3–5% of adults and 90% of infants infected by HBV become chronic carriers of the virus. More than 350 million people worldwide are chronically infected by HBV. Chronic HBV carriers have a significant probability of developing cirrhosis of the liver and hepatocellular carcinoma. There are effective prophylactic vaccines for HBV infection, but these have proven ineffective against chronic infections. Antiviral agents are effective in controlling the viremia, but the emergence of mutant strains of the virus is a cause for concern.[1–11]

In addition, although antivirals can reduce the replication of the virus, host immune help is necessary for the elimination of the infection. The finding that spontaneous resolution of HBV infection is the result of strong multiepitopic T-cell responses and that in chronic HBV infection, these responses are either weak or totally absent, suggest that an immunotherapeutic vaccine may be an effective treatment for chronic HBV infection. A therapeutic vaccine must be able to elicit host immune responses against viral antigens to which the host immune system is tolerant. This involves the delivery of antigens to antigen-presenting cells (APCs), especially dendritic cells (DCs), for appropriate antigen processing and presentation which results in a broad immune response and clearance of the virus infection. Therapeutic vaccines for the treatment of chronic viral infections are unmet medical needs.[12–19]

Chimigen™ vaccines are a new class of recombinant fusion proteins which incorporate functional domains of relevant antigens and the Fc fragment of a "xenotypic" monoclonal antibody (MAb). This bifunctional molecule is designed to target the viral antigen to DCs which hitherto had failed to elicit immune responses against the viral infection. The chimeric fusion protein is recognized as "foreign," ultimately resulting in the production of functional T-cells to break tolerance to the viral antigens. The vaccine has two domains: an immune-response domain (IRD) that contains the recombinant viral antigen, and a target-binding domain (TBD), which contains an Fc fragment of a xenotypic monoclonal antibody. The monomeric form of HBV Chimigen vaccine fusion protein can be schematically represented as shown in Figure 1.

Chimigen vaccine technology possesses several unique features in its design and function. The chimeric design favors the formation of antibody-like structures that facilitate uptake through specific receptors, resulting in the appropriate antigen presentation to T-cells. Receptor-mediated uptake augments the processing and presentation via both major histocompatibility complex (MHC) class I and II pathways, resulting in a broad immune response against the antigen.[20]

Processing through the proteasomal pathway and the presentation of T-cell epitopes complexed with MHC class

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I result in a cytotoxic T lymphocyte (CTL) response, whereas processing via the endosomal pathway and peptide presentation by MHC class II produces a humoral response. The TBD mediates the binding of the Chimigen vaccine to specific APC receptors such as Fcγ receptors. The binding of the molecule to Fcγ receptors on immature DCs would be expected to result in the processing of the antigen, mostly through a class I pathway. The xenotypic nature of the TBD and the linker peptides of varying lengths incorporated in different regions of the fusion protein make the molecule more immunogenic, allowing the molecule to be recognized as "foreign" by the host immune system of a chronic HBV carrier.[18]

Because the recombinant proteins are produced in insect cells, the glycosylation differs from mammalian cells. These proteins are highly immunogenic and may help generate immune responses against the tolerated viral antigen in a chronically infected host. Mannose/pauci-mannose glycosylation introduced in insect cells also permits the targeting of mannose receptors on APCs for uptake. As a result of this unique glycosylation, Chimigen vaccines would be internalized by the APCs through mannose receptors (CD206) or other C-type lectin receptors in addition to Fcγ receptors. The uptake via specific receptors, processing through the endosomal and proteasomal pathways, and presentation on both classes of MHCs would be expected to result in a broad immune response capable of eliminating the virus-infected cells. The generation of a CTL response is critical to clear virus-infected cells.[20-24]

Here we report on the design, expression, characterization, and immunological evaluation of an HBV Chimigen protein for possible use as a therapeutic vaccine for the treatment of chronic HBV infection.

Materials and Methods

Cloning, Expression, and Purification

The IRD and TBD components of the HBV Chimigen vaccine were cloned into the plasmid pFastBac HTa (Invitrogen, Carlsbad, CA) for expression in insect cells. This plasmid also encodes for an N-terminal 6xHis tag, which facilitates purification. The TBD, which contains sequences from murine IgG1 C_H1-Hinge-C_H2-C_H3, was generated by PCR of the cDNA of the murine hybridoma 2C12 (ViRexx) to generate pFastBacHTa-TBD. The HBV antigen fragments were generated by PCR and cloned into pFastBacHTa-TBD to produce pFastBacHTa IRD-TBD.

Recombinant baculoviruses were generated using the "Bac-to-Bac" expression system (Invitrogen). The pFastBacHTa IRD-TBD plasmids were transformed into *E. coli* DH10Bac cells to generate recombinant bacmids. The bacmids were isolated using standard plasmid isolation protocols and were transfected with CellFectin (Invitrogen) into Sf9 insect cells. The supernatant from the cells, which contains recombinant baculovirus, was collected following 72 hour incubation at 27° C.

Expression of recombinant proteins was accomplished by infecting Sf9 insect cell cultures with the recombinant baculovirus at an optimized multiplicity of infection (MOI) and time in shaker culture flasks using ESF921 media (Expression Systems, Woodland, CA). Infected cells were harvested by centrifugation at 2,800 x g for 10 minutes and cell pellets were used for purification.

The expressed protein was purified under denaturing conditions in the presence of 6 M guanidine hydrochloride and 8 M urea. The protein was purified using Ni-NTA affinity chromatography (Ni-NTA Superflow, Qiagen, Hilden, Germany), followed by ion-exchange chromatography on DEAE-Sepharose (Amersham Biosciences, Piscataway, NJ).

6xHis-rTEV Protease Site-HBV Antigen-Linker Peptide-Part C_H1-C_H2-C_H3-Peptide

[IRD] [TBD]

Through intermolecular sulfhydryl bridge formation, the protein can form a dimer molecule.

Figure 1. Chimigen vaccine fusion protein.

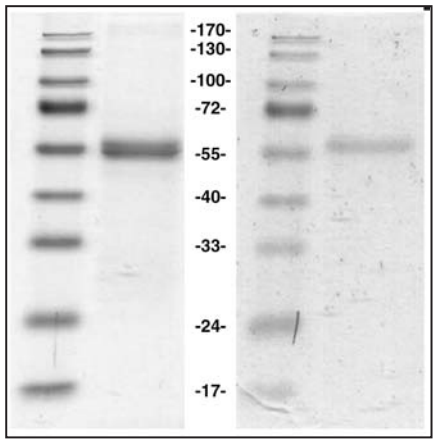

Figure 2. Coomassie blue and glyco-staining of HBV Chimigen vaccine. Purified proteins were subjected to 12% SDS-PAGE and subsequently stained with Coomassie Brilliant Blue R-250 (left) or by the GLYCO-PRO kit (right).

Biochemical Characterization

The concentration of protein was estimated by Micro BCA assay (Pierce, Rockford, IL) and purity assessed by SDS-PAGE. Antibodies specific to different regions of the protein were used for Western blots in order to establish the identity and intactness of the protein. The antibodies used were: anti-6xHis (Clontech, Palo Alto, CA), anti-PreS (Virogen, Watertown, MA) and anti-murine Fc (Sigma, St. Louis, MO). The bound antibodies were detected using ECL Western Blotting Detection Reagent (Amersham Biosciences, Baie d'Urfé, Québec, Canada).

For the qualitative detection of glycosylation of the purified protein, the protein was separated by SDS-PAGE (12% acrylamide), and stained using the GLYCO-PRO kit (Sigma).

Immunological Characterization

Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque-1077 (Sigma) density-gradient centrifugation of a leukapheresis preparation obtained from healthy non-HBV vaccinated donors with the HLA-A2 haplotype. Adherent PBMCs (monocytes) were cultured in AIM V media (Invitrogen) with 2.5% donor-matched

serum and 1000 IU/ml each of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN) to induce differentiation in immature DCs.

Immature DCs obtained after 24 hours of culture were used for binding assays. Binding of Chimigen vaccine was determined by incubating cells with various concentrations of the protein for 60 minutes at 4° C in Dulbecco's phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (PBSB). For the binding inhibition experiments, the cells were incubated for 1 hour at 4° C with receptor-blocking antibodies before the addition of Chimigen vaccine. These included the MAbs MOPC-21 (mouse IgG1 isotype), anti-CD32, and anti-CD206 (BD Biosciences, San Diego, CA), as well as murine Fcγ fragments (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were then washed with PBSB and incubated for 20 minutes with biotinylated anti-mouse IgG MAb (BD Biosciences) or biotinylated goat anti-mouse antibody (Invitrogen). Subsequently, cells were washed, incubated for 20 minutes with SA-PE-Cy5 (BD Biosciences), washed again, and resuspended in PBSB with 2% paraformaldehyde (PF). Data was acquired and analyzed with a FACSCalibur flow cytometer fitted with CellQuest Pro software (BD Biosciences).

For evaluation of binding by confocal microscopy, immature DCs were plated onto chamber slides and pulsed with antigen at 4° C for 1 hour. The cells were washed, and the bound Chimigen vaccine detected using a goat anti-mouse (H+L) IgG conjugated to FITC (Southern Biotech, Birmingham, AL). The cells were fixed in 4% PF, washed, and mounted in SlowFade Light mounting medium (Invitrogen). Fluorescence was assessed by confocal microscopy (Zeiss LSM 510 confocal microscope).

The immune response of naïve T-cells to antigen presented by DCs was measured using an *ex vivo* antigen presentation assay. T-cell function and specificity were assessed by determination of the percentage of intracellular interferon-gamma (IFN-γ)$^+$ and tetra-

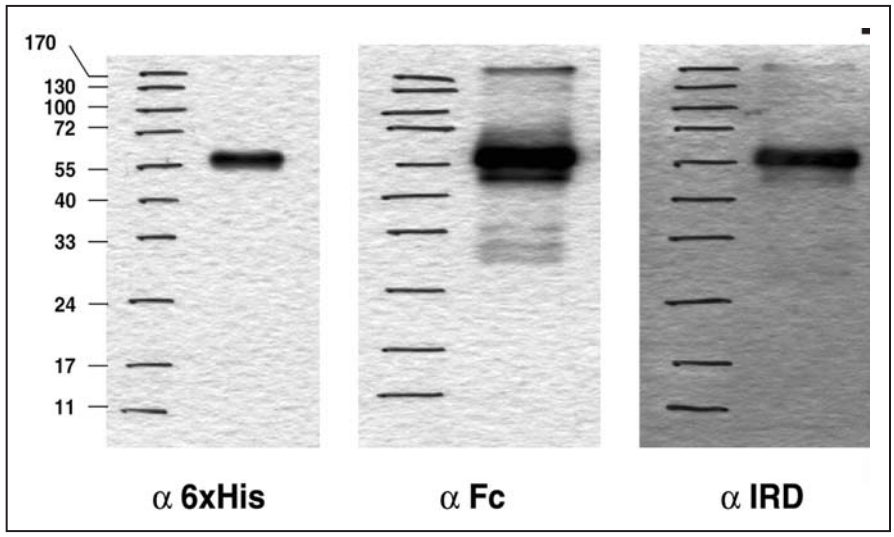

Figure 3. Western blots of purified HBV Chimigen vaccine. Purified protein was subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was performed using anti-6xHis, anti-PreS, and anti-Fc antibodies, which recognize the N-terminal 6xHis tag, HBV antigen, and murine Fc regions of the fusion protein, respectively. Detection of bound antibodies was performed by ECL chemiluminescence detection reagent.

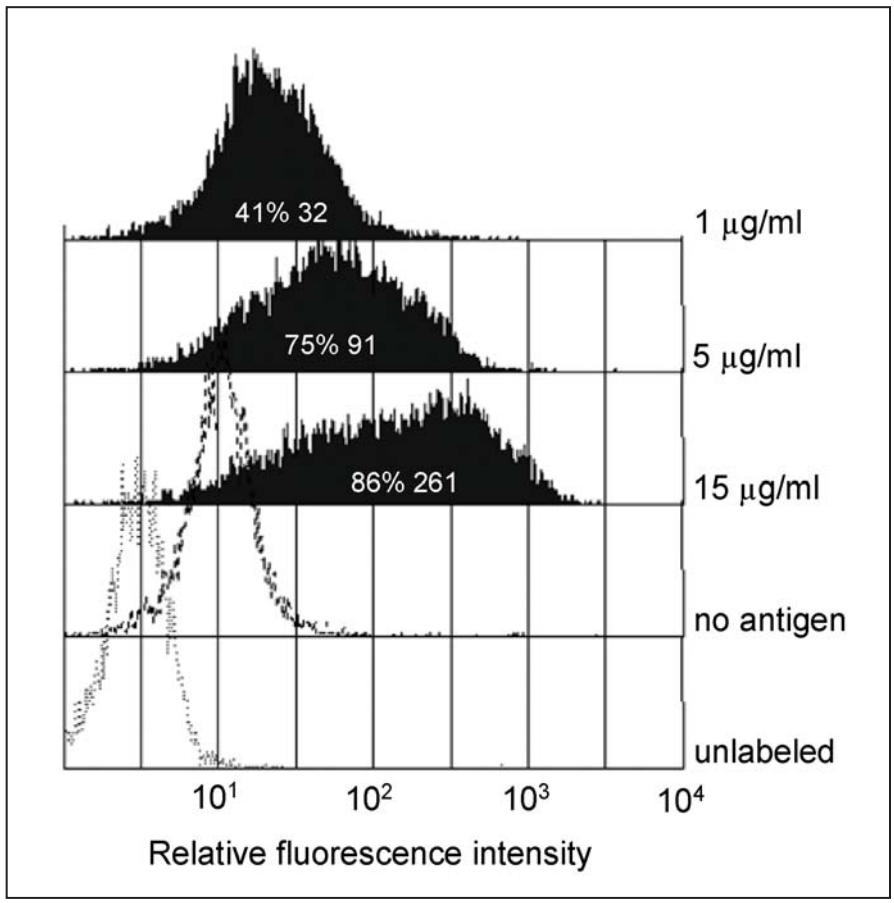

Figure 4. Binding of HBV Chimigen vaccine to immature DCs detected by flow cytometry. PBMC-derived immature DCs (24 hour culture) were incubated for 1 hour at 4° C with different concentrations of Chimigen vaccine. HBV Chimigen vaccine was detected using biotinylated goat anti-mouse IgG and SA-PE-Cy5. The percentage of cells binding the vaccine and MFI of the binding are shown.

mer+ cells, respectively. Immature DCs are loaded with HBV Chimigen vaccine, differentiated into mature DCs, and cultured with autologous naïve T-cells. T-cell IFN-γ production and tetramer binding were determined after two additional stimulations with antigen-loaded mature DCs.

Immature DC were prepared as outlined above and loaded with the antigen for 24 hours. The cells were differentiated to mature DCs by culturing for 24 hours with either PGE_2 (1 μM), interleukin 1 beta (IL-1β; 10 ng/ml), and 10 ng/ml tumor necrosis factor alpha (TNF-α), or 20 μg/ml polyIC (Sigma) and the following cytokines (R&D Systems): IL-1β (10 ng/ml), TNF-α (10 ng/ml), IL-6 (10 ng/ml), IFN-α (1000 U/ml), and IFN-γ (1000 IU/ml). The resulting antigen-loaded mature DCs were cultured along with T-cells which were isolated from PBMCs using a T-cell negative selection kit (Dynal Biotech, Oslo, Norway) according to the manufacturer's procedure. After four days of T-cell culture with the antigen-loaded mature DCs, IL-2 (R&D Systems) was added (10 IU/ml) and the cells cultured for three days. T-cells were then harvested and cultured for seven days with antigen-loaded mature DCs produced as outlined above. Subsequently, the T-cells were harvested and stimulated a third time with antigen-loaded mature DCs. T-cells were harvested after six hours of culture for determination of intracellular IFN-γ, and after five days of culture for measurement of tetramer binding. For determination of intracellular IFN-γ, T-cells were labeled with anti-CD3-FITC and anti-CD8-PE-Cy5 (BD Biosciences), washed, fixed, and permeabilized. The cells were then incubated with anti-IFN-γ-PE MAb (BD Biosciences) for 30 minutes at 4° C, washed, and resuspended in 2% PF in PBS for flow cytometric analysis. For tetramer analysis, the cells were labeled with anti-CD3-FITC, anti-CD8-PE-Cy5, and HBV antigen/HLA-A*0201 PE-conjugated tetramers (Beckman Coulter, San Diego, CA) for a 30-minute incubation at room temperature. Subsequently, the cells were washed and resuspended in 2% PF in PBS. Typically, data for greater than 80,000 cells was acquired using the FACSCalibur.

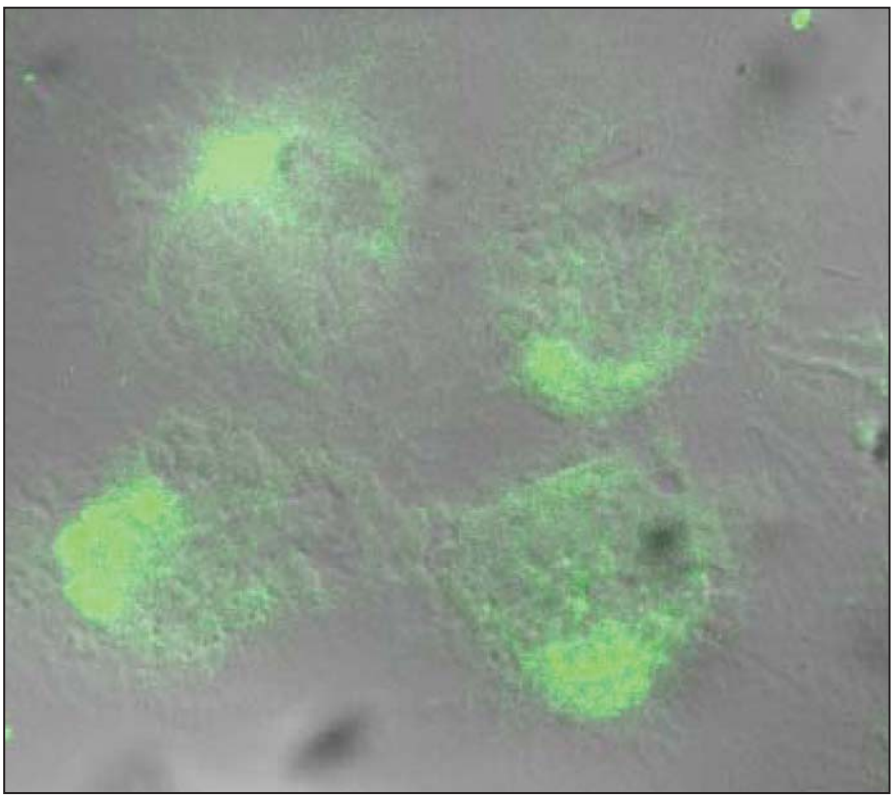

Figure 5. Binding of HBV Chimigen vaccine to immature DCs detected by confocal microscopy. Immature DCs (24 hour culture) were pulsed with HBV Chimigen vaccine for one hour at 4° C, fixed with PF, stained with FITC-conjugated goat anti-mouse IgG, and imaged by confocal microscopy. Chimigen vaccine (green) has a localized distribution on the surface of DCs.

Results

HBV Chimigen Vaccine Has Been Expressed and Purified

HBV Chimigen vaccine expressed in Sf9 cells was purified under denaturing conditions. The purified proteins were separated on 12% SDS-PAGE gels and stained with Coomassie brilliant blue R-250 to visualize the protein, and with the GLYCO-PRO glycoprotein detection kit to visualize the glycosylation. The protein appears to be a doublet in the Coomassie blue-stained gel with a molecular weight in the range of 53–58 kDa (Figure 2, left panel), possibly resulting from the different levels of glycosylation (Figure 2, right panel).

In order to verify the intactness of the purified protein, Western blot analysis using antibodies against different domains of the protein was performed. Following the separation by 12% SDS-PAGE, the purified protein was electroblotted onto nitrocellulose membranes (Amersham Biosciences). Three antibodies, each specific to a different region of the fusion protein, were used for this purpose: anti-6xHis-HRP-conjugated MAb detected the N-terminus, anti-PreS MAb/goat anti-mouse IgG (Fab specific)-HRP detected the HBV antigen, and goat anti-mouse IgG (Fc-specific)-HRP detected the Fc region in the fusion protein. All three antibodies recognized the purified protein, suggesting that protein had been purified intact (Figure 3). This finding was further verified by N-terminal sequencing and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy (data not presented).

Specific Receptors on DCs Bind HBV Chimigen Vaccine

Immature DCs were used to investigate the binding of HBV Chimigen vaccine. Immature DCs (day 1 culture) were incubated with varying concentrations of HBV Chimigen vaccine (1–15 μg/ml) for one hour at 4° C. The

binding was detected with biotinylated anti-mouse IgG and SA-PE-Cy5. The percentage of cells binding the vaccine (% positive cells) and the relative amount of bound vaccine, expressed as mean fluorescence intensity (MFI), were determined by flow cytometry. There was a dose-dependent binding of the protein to immature DCs (Figure 4). The results suggest that HBV Chimigen vaccine binds very effectively, and at high levels, to immature DCs, confirming one of the predicted properties of the protein.

Confocal microscopy also was used to study the binding of HBV Chimigen vaccine to the immature DCs. Immature DCs were pulsed with the vaccine for one hour at 4° C and labeled with FITC-conjugated goat anti-mouse IgG. The results indicate that HBV Chimigen vaccine (green) is localized on the surface of immature DCs (Figure 5).

The TBD region of the fusion protein encompasses ligands for the receptors CD32 (FcγRII), as well as C-type lectin receptors such as CD206, on immature DCs. In order to identify the receptors on immature DCs which are involved in the binding of the protein, immature DCs were incubated with HBV Chimigen vaccine in the presence of anti-CD32 and anti-CD206 (blocking MAbs), or with murine IgG Fc fragments. The binding was detected using either an anti-mouse IgG or anti-HBV MAb. The relative amount of bound HBV Chimigen vaccine (MFI) was determined by flow cytometry. The presence of anti-CD32, anti-CD206, or murine Fcγ fragments resulted in a decrease in the binding of the vaccine (Figure 6), suggesting that CD32 and CD206 are involved in the binding of the protein to DCs.

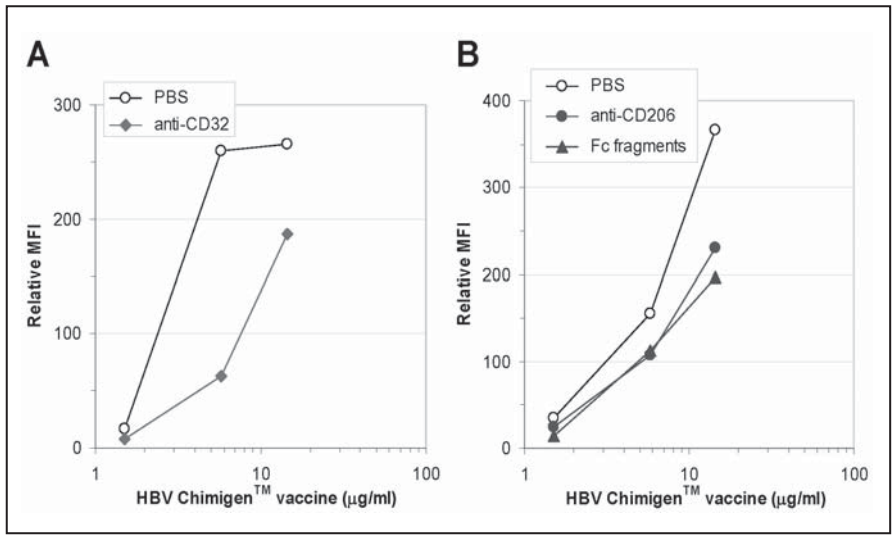

Figure 6. Inhibition of HBV Chimigen vaccine binding to DCs by anti-CD32, anti-CD206 or Fc fragments (IgG). Immature DCs were incubated for one hour at 4° C with (A) anti-CD32 MAb (50 µg/ml), (B) anti-CD206 (100 µg/ml), or murine Fc fragments (200 µg/ml) before incubation with HBV Chimigen vaccine (1 µg/ml) for one hour at 4° C. Chimigen vaccine was detected using either (A) biotinylated anti-mouse IgG mAb or (B) biotinylated anti-HBV preS MAb followed by SA-PE-Cy5.

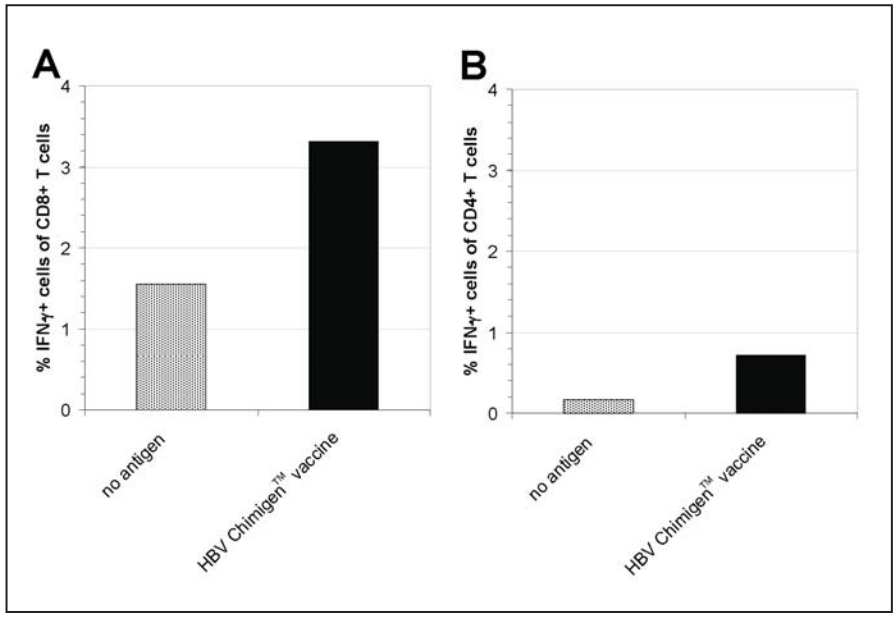

Figure 7. Intracellular IFN-γ production in $CD8^+$ and $CD4^+$ T cells following stimulation with HBV Chimigen vaccine-loaded DCs. Naïve T-cells were stimulated three times with DCs loaded without antigen or with HBV Chimigen vaccine (2.5 µg/ml). T-cells were harvested six hours following the third stimulation and the degree of IFN-γ production in (A) $CD8^+$ and (B) $CD4^+$ T-cells was assessed by intracellular cytokine labeling with detection by flow cytometry.

Increased IFN-γ Production by T-Cells

The function of T-cells following three stimulations with HBV Chimigen vaccine-loaded DCs was quantified by assessing IFN-γ production. The population of T-cells which produce IFN-γ following stimulation with antigen-loaded mature DCs was quantified by intracellular cytokine labelling using an anti-IFN-γ MAb. The percentage of $CD8^+$ T-cells expressing IFN-γ was higher in T-cells stimulated with vaccine-loaded DCs, compared to the no-antigen control (Figure 7A). Similarly, there was a small increase in the percentage of $CD4^+$ T-cells expressing IFN-γ, compared to the control (Figure 7B).

Expansion of HBV Antigen-Specific T-Cells

The specificity of the cellular immune response was evaluated using HBV epitope-specific MHC class I iTAg

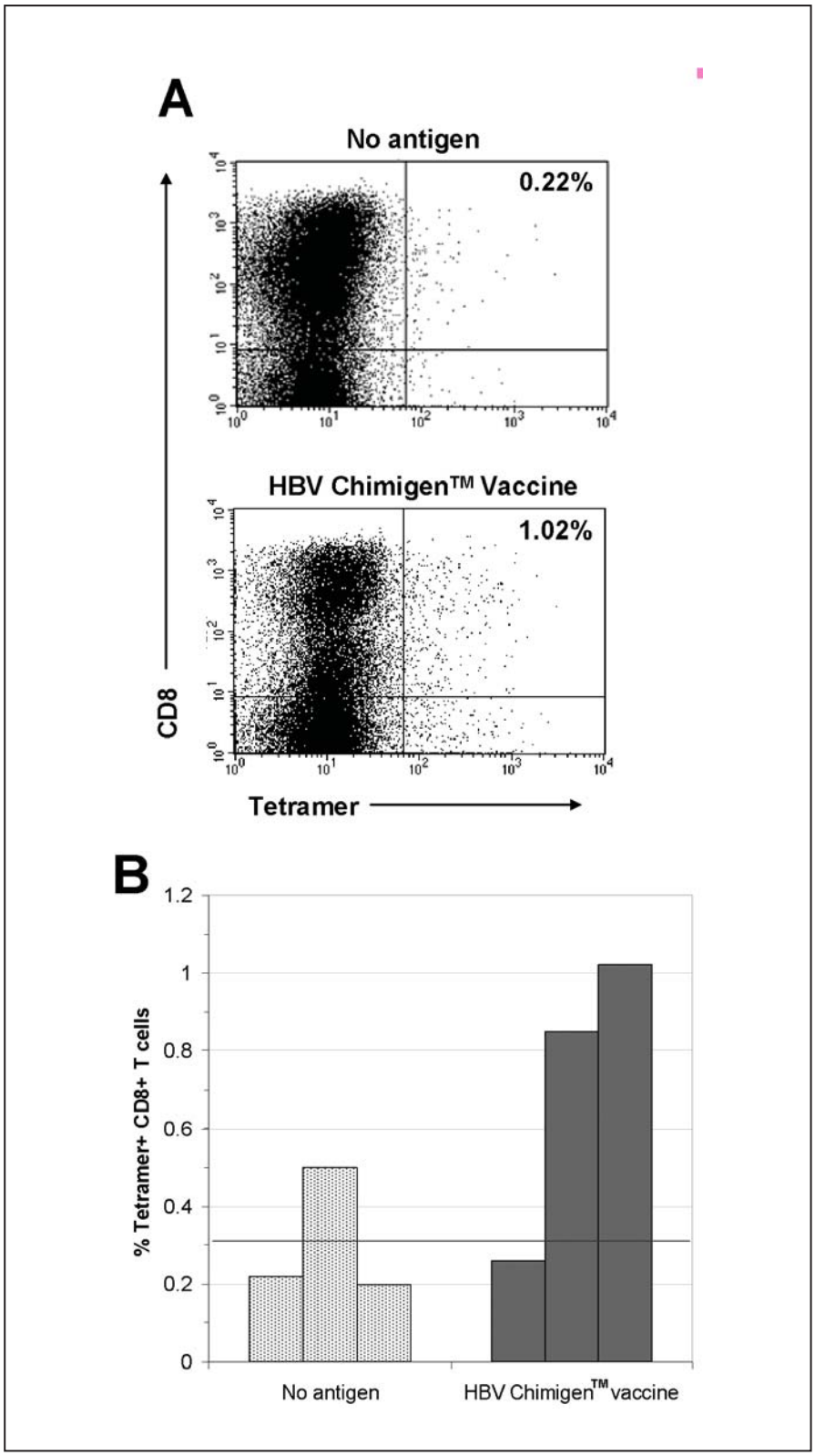

Figure 8. The percentage of HBV antigen tetramer-positive T-cells following antigen presentation by HBV Chimigen vaccine-loaded DCs. Naïve T-cells were stimulated three times with DCs loaded with buffer or with 2.5 µg/ml of HBV Chimigen vaccine. Following the third stimulation, T-cells were harvested and HBV-specific T-cells were assessed by tetramer labeling and detected by flow cytometry. (A) Dot plot profile of tetramer versus CD8 labeling, and (B) graphical representation of tetramer-positive cells of each of three wells in buffer versus HBV Chimigen vaccine group.

tetramers which can identify the T-cells that are able to recognize HBV epitopes in the context of HLA-A2 antigen presentation.

The tetramer assay was performed five days after the third stimulation of T-cells with antigen-loaded DCs. Cells were labeled with HBV epitope tetramer PE-conjugate; together with anti-CD3 and anti-CD8 MAbs. The Chimigen vaccine-stimulated T-cells showed a marked increase in the percentage of $CD8^+$ T-cells that were HBV epitope tetramer-positive as compared to the no-antigen control group (Figure 8). Thus, HBV Chimigen vaccine presentation by DCs results in the expansion of naïve $CD8^+$ T-cells which are specific for HBV epitopes.

Discussion

Active immunotherapy is a major unmet medical need in the area of chronic viral diseases. Although antiviral therapy is useful in controlling viremia, a strong, virus-specific, multiepitopic $CD8^+$ and $CD4^+$ T-cell host immune response is necessary for the elimination of viral infection. ViRexx's Chimigen vaccine platform is a novel approach to targeting DCs for antigen processing and presentation in order to generate a broad immune response against a viral antigen. Breaking tolerance to the antigens in chronic viral infections has been a major hurdle in the development of therapeutic vaccines. A chimeric fusion protein combining the recombinant antigen with a xenotypic MAb fragment, as well as the post-translational modifications introduced by the insect cells, enable the recognition of the antigen as "foreign" by the host immune system, thus breaking tolerance to the viral antigen. [13]

Our results from flow cytometric analysis and confocal microscopy demonstrate that the HBV Chimigen vaccine binds to DCs. This binding was shown to involve CD32 (Fcγ RII), likely through the Fc region of the molecule, and CD206, likely due to the presence of mannose glycosylation of the protein produced in insect cells. Other C-type lectin receptors besides CD206 may also be involved in the binding because of

the mannose glycosylation. Antigen-presenting cells such as DCs process antigens differently depending on the localization of the antigen. Exogenous antigens are processed via the endosomal pathway resulting in class II presentation, whereas intracellular antigens get processed via the proteasomal pathway and result in class I presentation. Chimigen vaccine, following receptor-mediated uptake, is expected to be predominantly presented via class I presentation, resulting in a cellular response, critical in clearing chronic viral infection. Antigen presentation via class II is also possible, resulting in a humoral response, which may augment the CTL response in the elimination of virus infection.[18,24,25]

In an antigen-presentation assay using HBV Chimigen vaccine loaded-DCs and autologous T-cells, the protein stimulated the expansion of IFN-γ producing T-cells with specificity for HBV antigen, suggesting that the DCs bind, internalize, process, and present the protein. The resultant generation of IFN-γ^+ T-cells in both the $CD4^+$ and $CD8^+$ population suggests that the Chimigen vaccine is processed in both the class I and class II pathways. In addition, tetramer assays using HBV peptide-HLA-A2 tetramers confirmed the specificity of $CD8^+$ T-cells to HBV antigen. These results show strong potential for the development of the fusion protein as a therapeutic vaccine for the treatment of chronic HBV infection.

A therapeutic vaccine against chronic HBV infection should show good immunological potential, and at the same time demonstrate a therapeutic effect in patients. Earlier studies reported vaccines with the ability to prime HBV-specific $CD8^+$ T-cells in healthy volunteers, but these vaccines failed to have any significant therapeutic effect in HBV-infected patients. Breaking tolerance to the HBV antigens in chronic carriers is a major hurdle in the development of therapeutic vaccines, in the light of a recent study, which analyzed the population of $CD8^+$ T-cells in patients with chronic HBV infection.[26,27]

It was noted that these responses were "highly heterogeneous" and are "differentially regulated," with the possible deletion/tolerance of HBV-specific T-cells emphasizing the complex nature of chronic HBV infection. The design of ViRexx's HBV Chimigen vaccine has incorporated several special features in order to address some of the problems observed in previous studies. The proof of the therapeutic efficacy of the vaccine will come from clinical trials using the HBV Chimigen vaccine to induce functional T-cell responses in chronically HBV-infected patients.[28]

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