Biodegradable nanoparticle delivery of a Th2-biased peptide for induction of Th1 immune responses

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Abstract

The type of immune response developed against the hepatitis B virus (HBV) is crucial in determining the outcome of the disease. The protective effects of vaccine-induced antibody responses against subsequent exposure to HBV are well-established. After the establishment of chronic HBV infection, cell-mediated immune response is curative while humoral response is detrimental. A therapeutic vaccine that could switch the type of response could lead to disease resolution. Hepatitis B core antigen $(HBcAg)_{129-140}$ has been identified as a Th2-biased peptide in $H-2^b$ mice when it is administered along with complete Freund's adjuvant (CFA). We formulated $HBcAg_{129-140}$ along with monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles. Naïve mice immunized with the nanoparticle formulation developed a strong Th1-type response while mice immunized with the control formulation of CFA and peptide did not. We then primed mice with CFA and peptide to establish a Th2-type immune response before administering the nanoparticle formulation. Mice receiving the nanoparticle formulation being primed with CFA still developed a strong Th1-type response, while mice that received incomplete Freund's adjuvant and peptide instead of nanoparticles did not. The ability of PLGA nanoparticles to alter the type of immune response elicited by a peptide, even in the context of an ongoing immune response, makes PLGA nanoparticles a strong candidate for the formulation of therapeutic vaccines.

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Introduction

When an organism invades the body, the immune system can respond with two different types of response: cellular and humoral. Cellular responses are aided by T helper 1 (Th1) cells while T helper 2 (Th2) cells assist humoral responses (Patel et al 1988; Dong & Flavell 2000; Jankovic et al 2001). T helper subsets are identified by their production of cytokines: Th1 cells produce interferon-γ (IFN-γ), lymphotoxin (LT), and interleukin (IL)-2 while Th2 cells produce IL-4, -5, -6, and -10 (Mosmann et al 1986; Mosmann & Coffman 1989; Reiner 2001; O'Garra & Robinson 2004). In most cases, the response to a pathogen is a mixture of cellular and humoral, the balance of the two located on a continuum between the opposite responses. In certain disease states or after certain modes of immunization, the response can be polarized to one side or the other (Spellberg & Edwards 2001; Christen & von Herrath 2004; Murakami et al 2004; Stadecker et al 2004; Sun & Ran 2004). Each Th subset has the ability to down regulate the other type leading to a potentially self-sustaining reaction of one type (Fiorentino et al 1989).

Th1-type responses are protective against altered self-cells: cancerous cells or cells containing an intracellular pathogen (Graham et al 1994; Maeda & Shiraishi 1996; Moran et al 1996). Extracellular organisms are eradicated by Th2-type responses. An inappropriate response is detrimental while an appropriate response is curative. This has been shown in Leishmania (Heinzel et al 1991; Locksley & Scott 1991) and human immunodeficiency virus (HIV) infections (Salk et al 1993). Due to the self-sustaining nature of the Th1 and Th2 responses, external assistance may be required to switch an established immune response to a more beneficial one. In an in-vitro system using human T cells it has been shown that polarized Th2 cells can become Th0 or Th1 cells under certain circumstances, opening up investigations into therapeutically useful ways to alter an ongoing immune response in-vivo (Kalinski et al 2000).

Hepatitis B is a disease where the type of immune response developed by the patient is crucial in determining disease outcome. There are 350–400 million carriers of the hepatitis B virus (HBV) worldwide and there is no effective therapy available. Chronic infection with HBV is prevalent in many parts of Asia and can lead to cirrhosis and hepatocellular carcinoma (Beasley et al 1981). After an acute HBV infection, some patients clear the virus effectively and recover, while others develop chronic disease. It has been shown that patients with chronic disease and hepatocellular carcinoma have an ongoing antibody response, indicative of a Th2-type response, while patients with self-limiting disease develop a cellular immune response with decreased antibody production consistent with a Th1-type response (Penna et al 1991; Milich 1997). If we could switch the immune response in patients from Th2-type to Th1-type, we may be able to alter the clinical outcome of the HBV infection. A therapeutic vaccine that could accomplish this switch in the type of immune response could lead to disease resolution.

Many factors are involved in the selection of the dominant Th response, such as the type of the antigen presenting cell, the affinity of the particular peptide for major histocompatibility complex (MHC) class II molecule, peptide dose, the strength of T cell receptor signalling, the costimulatory signals, and the cytokine microenvironment of antigen presentation (Constant & Bottomly 1997; Szabo et al 2003; Mowen & Glimcher 2004). Among these, the host factors such as genetic background, age, and MHC-peptide affinity are immutable. Factors that can be influenced by the antigen delivery parameters, such as dose of antigen, the type of antigen presenting cells, the costimulatory signals and the cytokine milieu of antigen presentation are more easily amenable to manipulation to achieve the desired immune responses. Vaccine delivery systems capable of selective antigen delivery to professional antigen presenting cells (e.g. dendritic cells) (Samuel et al 1998; Newman et al 2002; Diwan et al 2003; Elamanchili et al 2004; Waeckerle-Men & Groettrup 2005; Coester et al 2006) may be tailored to achieve modulation of several of these factors and manipulation of the immune responses.

Hepatitis B core antigen (HBcAg) is an important candidate for the design of therapeutic vaccines for chronic hepatitis B infection. A synthetic peptide containing a T helper epitope in mice and man, HBcAg residues 129–140, has been shown to generate a Th2-type response, based on cytokine profile and antibody isotype, in mice of the H-2^b haplotype after immunization with a control formulation of peptide emulsified with complete Freund's adjuvant (CFA) (Milich et al 1995). Studies with congenic mice differing only in their MHC haplotypes demonstrated that the type of response generated was MHC dependent (Milich et al 1995). The way in which an antigen is delivered can affect the type of immune response generated against the antigen (Guan et al 1998; Newman et al 1998a; Samuel et al 1998). HBcAg$_{129-140}$ is a therapeutically relevant antigen that allows us to study how formulation of the peptide can be used to alter the response to the antigen.

Poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles have several features that make them an attractive vaccine delivery system. PLGA is a biodegradable polymer currently approved for use in man and is suitable for the formulation of recombinant proteins (Ma et al 1998a, b), synthetic peptides (Newman et al 1998a, b), and plasmid DNA (Wang et al 1999). Delivery of antigen in PLGA nanoparticles can induce humoral (Ma et al 1998a; Newman et al 1998a), T helper (Ma et al 1998a; Newman et al 1998b; Wang et al 1999; Waeckerle-Men et al 2006), and cytotoxic T lymphocyte (Moore & Croxatto 1988; Nixon et al 1996; Partidos et al 1996a, b; Audran et al 2003) responses in-vivo. Finally, formulation characteristics can be tailored for optimal immune activation.

In this study we have examined whether a known Th2-biased peptide could be formulated to give a Th1 response. In addition, we have investigated the ability of a PLGA nanoparticle formulation to elicit a Th1-type response after the immune system had been imprinted to give a Th2-type response.

Materials and Methods

Preparation of PLGA nanoparticles

PLGA nanoparticles were prepared using a water/oil/water solvent evaporation technique (Ogawa et al 1988). Briefly, 100 μL 2 mg mL^{-1} solution HBcAg$_{129-140}$ (synthesized and provided by Dr D. S. Wishart's laboratory, University of Alberta, Edmonton, AB, Canada) was emulsified in 300 μL chloroform (Fisher Scientific, Nepean, ON, Canada) containing 100 mg PLGA (BPI, Birmingham, AL, USA), polymer (lactic acid:glycolic acid ratio 50:50; MW 6000) and 200 μL 2 mg mL^{-1} solution monophosphoryl lipid A (MPLA) (Biomira Inc, Edmonton, AB, Canada), using a microtip sonicator (Model XL2010, Heat Systems INC., Farmingdale, NY, USA). The resulting primary emulsion was added into 2 mL 9% w/v polyvinyl alcohol (PVA) (87–89% hydrolysed, MW 31–50 000; Aldrich Chemical Co. Inc., Milwaukee, WI, USA) and was further sonicated to form the secondary emulsion. This emulsion was added dropwise to 8 mL 9% w/v PVA and stirred for 3 h to evaporate the chloroform. The nanoparticles were then collected by ultra-centrifugation (40 000 g, 10 min at 20°C), washed twice with distilled water (20 mL) to remove residual PVA, resuspended in 5 mL distilled water, and freeze-dried for three days. Following freeze-drying, 50 mg nanoparticles were collected. Nanoparticles were stored at −20°C until needed.

Nanoparticle size determination

Nanoparticle size was determined by dynamic light scattering (Zetasizer 3000 HS, Malvern Instruments Ltd., San Bernadino, CA, USA). Five milligrams of nanoparticles were suspended in 1 mL PBS. Size was measured at 25°C.

Analysis of encapsulation efficiency

For determination of the encapsulation efficiency of the peptide, 10 mg nanoparticles were dissolved in 1 mL acetonitrile (Caledon Laboratories Ltd, Georgetown, ON, Canada) and

vortexed for 10 min. The sample was centrifuged for 10 min at $14\,000\,g$. The supernatant was discarded and the pellet was dissolved in $500\,\mu L$ distilled water. The peptide concentration in the sample was determined by comparing the UV absorbance of the peptide with a standard curve generated under the same conditions. The sample ($200\,\mu L$) was injected into a Waters 625LC HPLC (Waters, Mississauga, ON, Canada) using a Gilson 234 Autoinjector (Mandel Scientific Co., Guelph, ON, Canada). The HPLC was equipped with a C_{18} reverse phase column ($8 \times 100\,mm$) and a UV detector (Waters 486) set at 210 nm. The mobile phases employed were A, comprising 10% acetonitrile (Fisher Scientific) and 0.1% trifluoroacetic acid (Sigma, St Louis, MO, USA) in water, and B, comprising 70% acetonitrile and 0.085% trifluoroacetic acid in water. The peptide was eluted using a gradient of 9–50% B over 18 min. The peptide extracted from the nanoparticles was shown to have identical HPLC elution profile as that of the original peptide. The encapsulation efficiency was calculated using the formula: quantity peptide recovered in nanoparticles $\times$ 100/total quantity of peptide added. The peptide loading was calculated from the weight of the nanoparticles and the amount of peptide incorporated. Encapsulation efficiency of MPLA was determined by HPLC analysis (Chong et al 2005).

Immunization of mice

All animal experiments were performed after approval of the protocol by the University of Alberta Animal Policy and Welfare Committee and in accordance with Canadian Council on Animal Care regulations. Female C57BL/6 (H-2[b]) mice (8–12 weeks; Charles River Laboratories, Quebec, Canada) were used for the immune response studies. For the investigation of immune responses in naïve mice, there were two groups of six mice each. Group 1 mice were immunized on day 0 with 5 mg nanoparticles containing $3.5\,\mu g$ HBcAg$_{129-140}$ and $\sim 20\,\mu g$ MPLA in $200\,\mu L$ PBS and two weeks later received a booster with the same formulation. Group 2 mice (control) were immunized on day 0 with an emulsion of $100\,\mu L$ CFA (Sigma) and $100\,\mu L$ HBcAg$_{129-140}$ ($2\,mg\,mL^{-1}$) solution and received a booster two weeks later of $200\,\mu L$ incomplete Freund's adjuvant (IFA) (Sigma) and the peptide. For the investigation of immune responses in mice whose immune systems were imprinted for a Th2 response, on day 0 twelve mice were immunized with an emulsion of $100\,\mu L$ CFA + $100\,\mu L$ HBcAg$_{129-140}$ ($2\,mg\,mL^{-1}$). Two weeks later, six control mice were immunized with an emulsion of IFA and peptide, while six test mice were immunized with 5 mg nanoparticles containing $3.5\,\mu g$ HBcAg$_{129-140}$ and $20\,\mu g$ MPLA in PBS. All immunizations were via subcutaneous injection in the flank. Higher doses of peptide were used in the formulations with CFA and IFA because the free peptides in these formulations degrade quickly in-vivo.

T cell proliferation

The magnitude and specificity of the immune responses elicited by immunization were determined using a T cell proliferation assay. In this assay, the recall responses of T cells isolated from immunized mice were measured in-vitro against relevant and irrelevant peptide antigens.

Ten days after the second immunization, mice were killed by CO_2 asphyxiation and cervical dislocation. The inguinal lymph nodes were removed, the lymph node cells isolated and placed in RPMI-10 (RPMI 1640 medium (Gibco BRL, Burlington, ON, Canada) supplemented with 1 mL L-glutamine (Gibco BRL), 50 U penicillin (Gibco BRL), $50\,\mu g\,mL^{-1}$ streptomycin (Gibco BRL), $75\,\mu g\,mL^{-1}$ gentamicin (Gibco BRL), 10 mM HEPES (Gibco BRL), and 10% foetal bovine serum (Gibco BRL)). Lymph node cells were passed through nylon wool (type 200 L, Robbins Scientific, Sunnyvale, CA, USA) columns to enrich for T cells (Tham & Studdert 1985). The T cells were eluted off of the columns using warm RPMI-10. The purified T cells were then centrifuged at $200\,g$ for 7 min and resuspended at 1×10^7 cells mL^{-1} in RPMI-10.

In 96-well, flat-bottom microtitre plates (Costar, Cambridge, MA, USA), the T cells were plated at a concentration of 3×10^5 cells/well. Recall antigens were added to the wells at a concentration of $25\,\mu M$. Antigen presenting cells (APC) were added to the wells at a concentration of 1×10^6 cells/well. The recall antigens included one irrelevant peptide and two relevant peptides: MUC1 peptide (Biomira Inc.) with the sequence TAPPAHGVTSAPDTRPAPGST APP; the immunizing antigen HBcAg$_{129-140}$ with the sequence PPAYRPPNAPIL; and HBcAg$_{126-140}$ with the sequence IRTPPAYRPPNAPIL (core peptides were prepared by Dr D. S. Wishart's laboratory, University of Alberta, Edmonton, AB, Canada). In addition to the wells containing recall antigens, there were also wells used to measure background proliferation containing T cells and APC without any antigen, and positive control wells set up with T cells, APC, and $2\,\mu g$/well concanavalin A (Sigma) in place of a recall antigen. All wells were set up in triplicate.

APC were obtained from the spleens of unimmunized syngeneic mice. Spleens were removed and the cells collected in RPMI-10. Spleen cells were irradiated with 3000 rad in a ^{137}Cs irradiator. Irradiated spleen cells were washed thoroughly with RMPI-10 media, collected by centrifugation at $200\,g$ for 7 min, and resuspended in RPMI-10 at a concentration of 1×10^7 cells mL^{-1} before being added to the 96-well plates.

After 72-h incubation at 37°C/5% CO_2, $1\,\mu Ci$ [^3H]thymidine (Amersham Canada, Oakville, Ontario, Canada) in $50\,\mu L$ RPMI-10 was added to each well. After 24-h incubation, the cells were harvested onto a filter mat using a Mach III Harvester 96 and the filter was read in a Microbeta Trilux reader (Wallac, Turku, Finland). T cell proliferation was measured as the amount of incorporated radioactivity in counts min^{-1}. The proliferation of different groups was compared using their stimulation indexes (SI). SI is equal to the counts min^{-1} T cells incubated with APC and antigen/counts min^{-1} T cells incubated with APC without antigen.

Cytokine analysis

Culture setup was the same as for the T cell proliferation. Supernatants were collected from the wells after 24-, 48-, or

72-h incubation and frozen at $-20°C$ until they were analysed. Levels of IFN-γ and IL-4 in the supernatants were determined by sandwich ELISA. Briefly, 96-well microtitre plates (NUNC Maxisorp Immunoplates, Gibco-BRL, Burlington, Ontario, Canada) were coated with $50 \mu L$/well of a primary antibody: the primary antibody against IFN-γ (R46.A2; Biomira Inc.) was diluted to $1.5 \mu g\,mL^{-1}$ with PBS; the primary antibody against IL-4 (11B11; Biomira Inc.) was diluted to $0.5 \mu g\,mL^{-1}$. Plates were incubated for 30 min at $37°C$ and washed once with TPBS (PBS containing 0.05% v/v Tween 20 (Sigma)). A set of standards was prepared for each cytokine. Recombinant IFN-γ (Biomira Inc.) was diluted in RPMI-10 to concentrations from 5000 to $156\,pg\,mL^{-1}$. Recombinant IL-4 (Biomira Inc.) was diluted with RPMI-10 from 1000 to $25\,pg\,mL^{-1}$. Each plate received duplicate wells of the appropriate set of standards. Standards were added at $50 \mu L$/well. Negative control wells received $50 \mu L$ RMPI-10 media. In the first assay, undiluted supernatants were added at $50 \mu L$/well. Some of the supernatants had to be reanalysed after being diluted (10–50 fold) with RPMI-10 because the readings were above the upper limit of detection of the plate reader. Plates were incubated for 45 min at $37°C$ and were washed twice with TPBS. Biotinylated secondary antibodies were added at $50 \mu L$/well: the secondary antibody against IFN-γ (XMG1.2, Biomira Inc.) was diluted to $0.05 \mu g\,mL^{-1}$ with a solution of 1% bovine serum albumin (BSA) (Sigma) in TPBS; the secondary antibody against IL-4 (BVD6.24G2, Biomira Inc.) was diluted to $0.2 \mu g\,mL^{-1}$. Plates were incubated for 45 min at $37°C$ and then washed three times with TPBS. Peroxidase-conjugated streptavidin (Jackson Immuno-research lab Inc., West Grove, PA, USA) was diluted to $200\,ng\,mL^{-1}$ in 1% BSA in TPBS and $50 \mu L$ was added to each well. Plates were incubated for 30 min and then washed four times with TPBS. Peroxidase solution (KPL, Gaithersburg, MA, USA) was combined in equal parts with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate (KPL) and $100 \mu L$ of the mixture was added to each well. After 2 min, an optical density (OD) reading was taken at 650 nm using a microplate reader (Powerwave 340, Bio-Tek Instruments Inc., Winoski, VT, USA). Once the OD reading reached 0.8, $100 \mu L$ 1 M phosphoric acid (BDH Inc., Toronto, ON, Canada) was added to each well and an OD reading was performed at 450 nm.

Statistical analysis

The results are expressed as the mean $\pm$ s.d. for each group of mice. The significance of difference among groups was analysed by one- or two-way analysis of variance followed by the Student–Newman–Keuls post hoc test for multiple comparisons. Before executing the analysis of variance, data were tested for normality and equal variance. If any of those tests failed, data were compared using a Kruskal–Wallis one-way analysis of variance on ranks. A value of $P < 0.05$ was set for the significant difference among groups. The statistical analysis was performed with SigmaStat software (Jandel Scientific, San Rafael, CA, USA).

Results and Discussion

Characterization of HBcAg$_{129-140}$ nanoparticles

Nanoparticles containing HBcAg$_{129-140}$ and MPLA measured 510 ± 147 nm in diameter. Based on the HPLC analysis, the encapsulation efficiency of the peptide was determined as 17.5% and the polymer recovery as dry nanoparticles was 50%. The peptide concentration was $0.7 \mu g$ HBcAg$_{129-140}\,mg^{-1}$ (dry weight) PLGA nanoparticles. This corresponded to a peptide loading of 0.07%, w/w, of the polymer. Greater than 90% peptide was retained in the nanoparticles after 24-h incubation of the nanoparticles in PBS at $37°C$. The encapsulation efficiency of MPLA in nanoparticles was approximately 45%, which corresponded to a concentration of $1.5 \mu g$ MPLA$\,mg^{-1}$ (dry weight) PLGA.
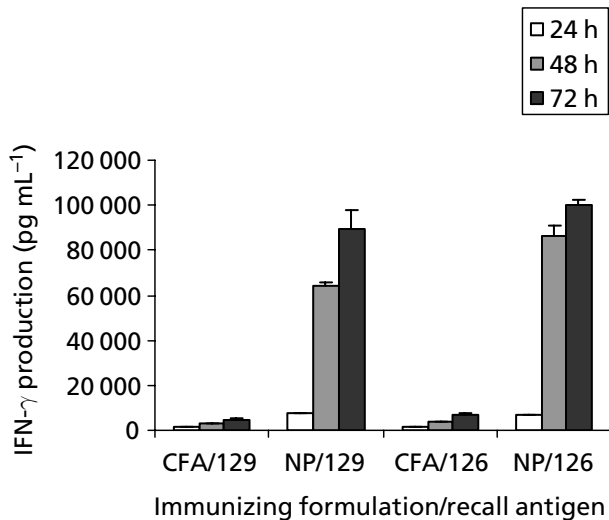
Characterization of immune response after immunization of naïve mice with nanoparticles containing HBcAg$_{129-140}$ and MPLA

To determine whether changing the formulation of a therapeutically relevant viral peptide could alter the type of immune response generated against the peptide, we formulated a peptide from the hepatitis B viral core antigen plus an adjuvant, MPLA, in PLGA nanoparticles. HBcAg$_{129-140}$ is a T helper epitope in C57BL/6 mice that has been identified as causing a Th2-type response in mice of the H-2^b haplotype when they are immunized with the peptide plus CFA (Milich et al 1995). After immunization with the nanoparticle and CFA formulations, we measured the antigen-specific T cell proliferation in-vitro using two HBcAg peptides as the relevant recall antigens and an MUC1 peptide (Newman et al 1998b; Samuel et al 1998) as the irrelevant recall antigen. The HBcAg peptides induced strong T cell proliferation, whereas the irrelevant MUC1 peptide induced little or no proliferation beyond the background proliferation in the wells without any added antigen, demonstrating the antigen-specificity of the response. A two-way analysis of variance showed a significant antigen-specific proliferation in both groups of mice treated with either CFA nanoparticle formulation (Figure 1) ($P < 0.0001$). A Student–Newman–Keuls post hoc test revealed that there was no significant difference in the amount of proliferation between the mice that were immunized with nanoparticles and the mice that received the CFA formulation ($P > 0.05$).

To characterize the type of immune response, supernatants from cell cultures were collected and analysed for IFN-γ and IL-4 content by ELISA. IL-4 concentrations were in all cases below the detection limit of the assay (data not shown). T cells from mice immunized with nanoparticles containing HBcAg$_{129-140}$ and MPLA produced significantly greater amounts of IFN-γ than T cells from mice immunized with CFA and peptide (Figure 2). A one-way analysis of variance test showed that this difference was statistically significant at all time points ($P < 0.0001$).
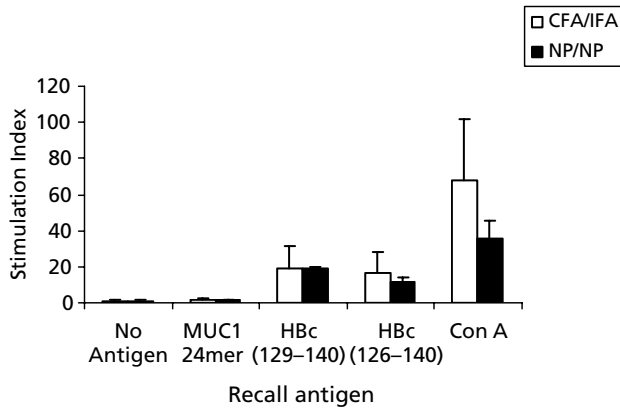
Figure 1 T cell proliferation after immunization of naïve mice. Naïve mice were immunized with HBcAg$_{129-140}$ either emulsified with CFA or encapsulated along with MPLA in PLGA nanoparticles. The proliferative response of T cells from immunized mice to a panel of recall antigens was measured by [^{3}H]thymidine incorporation. Stimulation Index (SI) = counts min^{-1} test group/ background counts min^{-1} wells without antigen. The background counts min^{-1} for the CFA group was 383.33 ± 119.63. The background counts min^{-1} for the nanoparticle group was 2819.33 ± 816. Con A, concanavalin A.
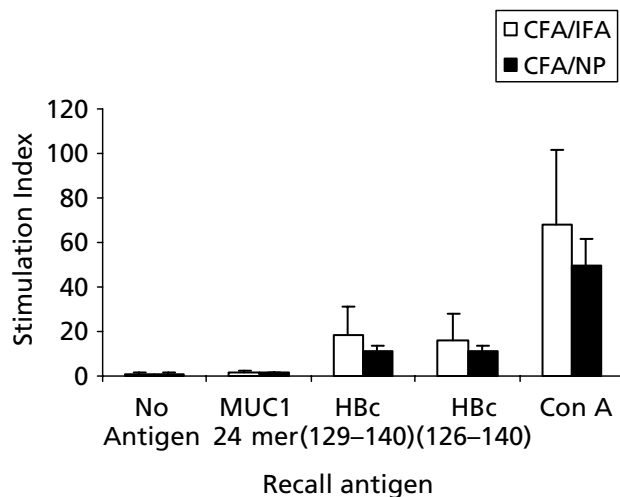
As reported in the literature, immunization with the control formulation of a CFA/HBcAg$_{129-140}$ emulsion did not generate a Th1 response even though CFA is capable of inducing both Th1 and Th2 immune responses, often with bias towards a Th1-type response. By formulating a therapeutic peptide that failed to give a Th1-type response, even in CFA, into nanoparticles along with the adjuvant MPLA, we demonstrated that with appropriate antigen delivery, it was possible to elicit a Th1-type response to the peptide in C57BL/6 mice. This was an important finding since a Th1-type response against the hepatitis B virus is believed to be necessary for recovery from infection.

Characterization of the immune response in mice imprinted for a Th2-biased response after immunization with nanoparticles containing HBcAg$_{129-140}$ and MPLA

The results described in the previous section were useful but not entirely therapeutically relevant. Although it was helpful to demonstrate that the formulation of a peptide could alter the type of immune response generated by immunization with the peptide, the experiment discussed was performed in naïve mice in the absence of an ongoing immune response. This lack of an ongoing immune response is in direct contrast to the therapeutic situation where a patient has an ongoing Th2-type response against the virus. An effective therapeutic vaccine against the hepatitis B virus must be able to switch an ongoing Th2-type response to a Th1-type response. To examine the potential of a PLGA nanoparticle formulation of HBcAg$_{129-140}$ to alter the type of immune response after the response has been established, we first immunized mice with the control formulation CFA + HBcAg$_{129-140}$ to imprint their immune systems with a non-Th1-type immune response. Two weeks later, mice in the test group were immunized with nanoparticles containing HBcAg$_{129-140}$ and MPLA, while mice in the control group were immunized with an IFA/HBcAg$_{129-140}$ emulsion. We measured and characterized the immune responses seen in the mice.

The antigen-specific T cell proliferative response to recall antigens in-vitro was measured as in the above experiment. Cells from mice who received the nanoparticle formulation as the second immunization and cells from mice that received the IFA/HBcAg$_{129-140}$ emulsion as the second injection proliferated significantly in response to the relevant recall antigens (Figure 3) (analysis of variance; $P < 0.0001$). There was no significant difference between the groups with respect to the amount of T cell proliferation or the extent of the immune response (Student–Newman–Keuls post hoc test; $P > 0.05$).

Again we examined the production of IFN-γ and IL-4 by the T cells in-vitro to characterize the type of immune response seen. Levels of IL-4 were below the limits of detection of the assay (data not shown). Despite the similar levels of T cell response seen in the two groups of mice, T cells from mice that received the nanoparticle formulation after being imprinted for a Th2-type response produced significantly more antigen-specific IFN-γ than mice who received a booster immunization with the IFA/

Figure 2 Interferon-γ (IFN-γ) production after immunization of naïve mice. Mice were immunized with HBcAg (129–140) either emulsified with CFA or encapsulated among with MPLA in PLGA nanoparticles. The production of IFN-γ by T cells from immunized mice in response to recall antigen was measured by ELISA. Values (pg mL^{-1}) were calculated using a standard curve of known quantities. Immunizing formulation: CFA = complete Freund's adjuvant and peptide, NP = nanoparticle formulation of peptide. Recall antigen: 129 = HBcAg$_{129-140}$, 126 = HBcAg$_{126-140}$. The background production of IFN-γ for the CFA group was 678 ± 145 (24 h), 279.26 ± 26.74 (48 h), and 1193 ± 135.57 pg mL^{-1} (72 h). The background production of IFN-γ for the nanoparticle group was 900 ± 68.13 (24 h), 456.1 ± 25.54 (48 h), and 1285 ± 178.63 (72 h).

Figure 3 T cell proliferation after immunization of primed mice. Mice were primed with $HBcAg_{129-140}$ and CFA. Mice were then treated with $HBcAg_{129-140}$ either emulsified with IFA or encapsulated along with MPLA in PLGA nanoparticles. The proliferative response of T cells from immunized mice to a panel of recall antigens was measured by [^{3}H]thymidine incorporation. Stimulation Index (SI) = counts min^{-1} test group/background counts min^{-1} wells without antigen. The background counts min^{-1} for the CFA/IFA group was 383.33 ± 119.63. The background counts min^{-1} for the CFA/NP group was 2974 ± 962.9. Con A, concanavalin A.
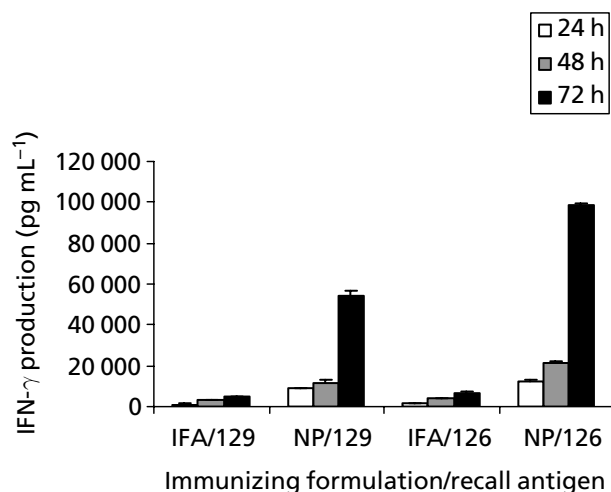
Figure 4 Interferon-γ (IFN-γ) production after immunization of primed mice. Mice were primed with $HBcAg_{129-140}$ and CFA. Mice were then treated with $HBcAg_{129-140}$ either emulsified with IFA or encapsulated along with MPLA in PLGA nanoparticles. The production of IFN-γ by T cells from immunized mice in response to recall antigen was measured by ELISA. Values (pg mL^{-1}) were calculated using a standard curve of known quantities. Immunizing formulation: IFA = incomplete Freund's adjuvant and peptide, NP = nanoparticle formulation of peptide. Recall antigen: 129 = $HBcAg_{129-140}$, 126 = $HBcAg_{126-140}$. The background production of IFN-γ for the IFA group was 678 ± 145 (24 h), 279.26 ± 26.74 (48 h), and 1193 ± 135.57 pg mL^{-1} (72 h). The background production of IFN-γ for the nanoparticle group was 906.71 ± 150 (24 h), 711.02 ± 62.06 (48 h), and 718.37 ± 29.81 pg mL^{-1} (72 h).

$HBcAg_{129-140}$ emulsion (Figure 4) (analysis of variance; $P < 0.0001$). Both groups of mice established a strong T cell response to the hepatitis B core peptide, but only mice immunized with the nanoparticle formulation established the Th1-type response that was necessary for recovery from hepatitis B chronic infection. The ability of the nanoparticle formulation to alter the type of immune response generated against a Th2-biased peptide in an immune system that had not been primed for a Th1-type response was a significant finding for the development of therapeutic vaccines.

The change that we made to the conventional immunization protocol gave rise to a more therapeutically relevant design. Therapeutic vaccines are not administered to healthy, naïve patients; they are administered to patients who are combating an infection and who have an ongoing immune response against the pathogen. Whether the aim of the therapeutic vaccine is to alter the type of response or to alter the epitopes that the patients' immune systems focus on, it is crucial to study the T cell response in an environment as similar to the therapeutic situation as possible. For diseases such as hepatitis B, where an animal model is not readily available and the immune response in animals does not necessarily parallel the situation seen in man with chronic infection, establishing an ongoing Th2-type immune response in mice before testing therapeutic vaccine formulations allows the immune response generated by the vaccine to be characterized and the potential effectiveness of the vaccine to be evaluated in a situation closer to the therapeutic situation than the traditional immunization protocol for T cell proliferation.

We have examined whether a nanoparticle formulation of a peptide from the hepatitis B core antigen, reported in the literature as eliciting a Th2-type response when mice of the H-2^b haplotype were immunized with a control formulation (Milich et al 1995), could alter the immune response to the peptide. We demonstrated that immunization of H-2^b mice with a formulation of $HBcAg_{129-140}$ in PLGA nanoparticles did not change the extent of the T cell response seen, as measured by antigen-specific recall proliferation ex-vivo as compared with immunization with a CFA/$HBcAg_{129-140}$ emulsion, but did change the type of response to a strong Th1-type response as indicated by high levels of IFN-γ production. A further study was performed to examine whether the nanoparticle formulation of the peptide could alter an ongoing immune response, in an attempt to mimic the situation of a chronic hepatitis B patient with an established Th2-type response. We found that mice that were imprinted for a non-Th1-type response through immunization with a CFA/$HBcAg_{129-140}$ emulsion could generate a strong Th1-type response after immunization with nanoparticles containing $HBcAg_{129-140}$ and MPLA. In contrast to earlier experiments with this peptide, we were unable to demonstrate the presence of IL-4 in our assay system; however, the relative lack of IFN-γ production in the control group, combined with the dramatic increase of IFN-γ production achieved in both naïve mice and mice primed to give a non-Th1-type response, allowed us to state that the nanoparticle formulation could elicit a Th1-type response, even

in the presence of an ongoing non-Th1-type response. Since we were unable to detect IL-4 production by T cells isolated from mice immunized with CFA/IFA formulation, additional studies are required to characterize the exact nature of T helper immune responses in these mice. Quantification of other Th2 cytokines such as IL-5 and IL-10, as well as antibody responses (ratio of IgG1:IgG2a/2b) induced by immunization with the whole protein after priming with a peptide formulation would be required to demonstrate conclusively a Th2 response.

There are several possible mechanisms for this ability of the nanoparticle formulation to elicit a Th1-type response. It has been shown that dendritic cells transfected with IL-12 could switch a Th2-type response to a Th1-type response (Kalinski et al 2000). MPLA was capable of causing the production of IL-12 by dendritic cells (Ismaili et al 2002) through activation of the toll-like receptor-4 (TLR-4) ligand pathway (Martin et al 2003). The co-delivery of the peptide antigen and the adjuvant to the same dendritic cells enhanced the effect on the immune system; it allowed the peptide antigen to be presented to the T cells by an antigen-presenting cell that was producing IL-12 and that was capable of stimulating a Th1-type response. Soluble forms of antigen delivery could not achieve this co-delivery as effectively as the particulate delivery. Previously, we have shown that nanoparticle delivery of peptide antigens without incorporation of any adjuvant produced low levels of T cell activation, with barely detectable levels of IFN-γ, whereas a formulation incorporating MPLA with peptide showed strong T cell activation with high levels of IFN-γ secretion (Newman et al 1998b). Our recent studies also showed that to produce strong Th1 immune responses, the MPLA needed to be incorporated in the nanoparticle, since formulations made by simple mixing of the nanoparticle containing a peptide antigen with MPLA (at comparable doses) did not result in stronger T cell responses beyond that achieved by the nanoparticles containing peptide alone (Elamanchili et al unpublished data). The co-delivery approach with antigens and adjuvants in the same particle may be of broad applicability in vaccine design. MPLA may be replaced by other TLR ligands (Takeda et al 2003; Pasare & Medzhitov 2004; Netea et al 2005) including CpG oligonucleotides (Diwan et al 2002, 2004).

Although the immunological mechanisms mediating HBV clearance in the acute self-limited infection and its persistence in chronic infection have not been completely characterized, the current knowledge supports the view that cytotoxic T lymphocytes (CTLs) and Th1 cells are key mediators in viral clearance (Milich 1997; Lohr et al 1998; Xing et al 2001; Jiang et al 2002). Th1/Th2 balance significantly influences the outcome during HBV infection. A Th1-type response against the hepatitis B virus was curative (Penna et al 1991). Patients who developed a Th2-type response against the virus developed chronic infection, often leading to cirrhosis, hepatocellular carcinoma, and death (Penna et al 1991). Further, IFN-γ, a Th1 cytokine, is recognized as an important non-cytolytic mediator of HBV control and eradication (Hodgson &

Michalak 2001; Ren et al 2003; Szkaradkiewicz et al 2003; Tang et al 2005). Therefore, therapeutic vaccines for chronic hepatitis B virus should be designed to bias the immune response in the patient towards a Th1-type response, with augmentation of IFN-γ production, in the presence of an ongoing immune response. The ability of PLGA nanoparticles to alter the type of immune response elicited by a peptide, even in the context of an ongoing non-Th1 immune response, makes it a strong candidate for the formulation of therapeutic vaccines.

Conclusions

The results have shown that a nanoparticulate delivery system, capable of co-delivery of an antigen and an immunomodulator, would be able to induce a Th1 immune response, even when the antigen by itself had a tendency to give a non-Th1 response. More importantly, our results suggested that this approach may be used to induce a strong Th1 response even when there is an already established non-Th1 mode of immune response, possibly with a Th2 bias. These findings have important implications for the design of therapeutic vaccines for chronic viral infections and cancer, where the Th1/Th2 balance may profoundly influence the therapeutic outcome.

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