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Nanodecoy system: A novel approach to design hepatitis B vaccine for immunopotentiation

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Abstract

The progress toward subunit vaccines has been limited by their poor immunogenicity and limited stability. To enhance the immune response, subunit vaccines universally require improved adjuvants and delivery vehicles. In the present study, we propose the use of ceramic core based nanodecoy systems for effective immunization, which seems to exhibit a broad range of surface properties. Nanodecoy systems were prepared by self-assembling of hydroxyapatite core and cellobiose and finally the hepatitis B surface antigen (HBsAg) was adsorbed over the preformed nanodecoy systems. HBsAg loaded nanodecoy systems were characterized for size, shape and antigen loading efficiency. The effect of processing steps on the stability and integrity of HBsAg was assessed by in vitro antigenicity and SDS-PAGE experiments. Nanodecoy preparations were nanometric in size range and almost spherical in shape. SDS-PAGE studies confirmed the integrity of HBsAg protein in the formulation. Vaccine efficacy was determined in female Balb/c mice and results indicated that specific anti-HBsAg antibody titers in mice receiving nanodecoy system were more efficient than the conventional adjuvant alum followed by subcutaneous immunization. Studies also indicated that nanodecoy formulations could elicit combined Th1 and Th2 immune response. It is inferred that nanodecoy systems are a class of novel carriers and hold potential as an alternative adjuvant in vaccine technology.

Keywords: Hydroxyapatite; Calcium phosphate; Nanodecoy; Antigen delivery; Hepatitis B vaccine

1. Introduction

Hepatitis B virus (HBV) infection causes acute as well as chronic necroinflammatory liver disease and many HBV carriers eventually develop serious complications such as liver cirrhosis or hepatocellular carcinoma. The prospects for control of infection and disease depend on the availability of safe, effective and affordable vaccines. Cellular and humoral immune responses to HBV antigens are believed to play an essential role in the elimination of virus by the host. It is well established that the humoral immune response to HBV envelop antigens lead to protection against infection. In contrast, cellular immune response against HBV has been shown to be one of the most important factors contributing to virus elimination from infected hepatocytes and may play an important role in the pathogenesis of severity of hepatitis and the subsequent development of chronic liver disease (Chisari and Ferrari, 1995; Vyas et al., 2005).

Adjuvants play a pivotal role in vaccination, especially when the vaccine antigen itself possesses a weak immunogenicity (Audibert and Lise, 1993; Gupta and Siber, 1995; O'Hagan, 2001). Although a number of candidate adjuvants have been reported and examined in preclinical studies, at present, aluminum adjuvant is the only adjuvant that is clinically in use (O'Hagan, 2001). However, alum induces a poor cell mediated immune response and also causes local irritation such as erythema, subcutaneous nodules, content hypersensitivity and granulomas inflammation. Thus, the development of more safe and effective adjuvant is necessitated (Gupta and Siber, 1995).

Recently, nanodecoy system has shown promise as an adjuvant for various antigens including HIV and Epstein-Barr virus (Kossovsky et al., 1991; Kossovosky et al., 1993; Kossovsky et al., 1995a). Nanodecoy systems are ceramic core based biocompatible and biodegradable nanocarriers, which seem to exhibit a broad range of surface properties with a verisimilitude between them and live virus. Nanodecoy systems are utilized as an anti-

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gen/protein delivery system due to the presence of hydrophilic polyhydroxyl oligomeric film of carbohydrate on the outer surface of hydroxyapatite core, which could preserve the protein conformational state (Kossovosky et al., 1996; Arakawa and Timsheff, 1992). One of the features that distinguishes nanodecoy from other adjuvant(s) is that they do not form aggregates, surface coated polyhydroxyl oligomers maintain the nanometer scale architecture, which appears to be central to their ability to produce both humoral and cellular immune response (Kossovsky et al., 1995b).

In the present study the immunoadjuvant effect of hydroxyapatite (HA) core based nanodecoy systems was investigated using hepatitis B surface antigen (HBsAg). The nanodecoy systems containing HBsAg were prepared by self-assembling of HA and cellobiose and subsequent coated with HBsAg. The prepared systems were characterized for size, shape, entrapment efficiency and in process protein stability studies. The specific immunological responses elicited by the prepared systems were evaluated by subcutaneous administration.

2. Materials and methods

2.1. Materials

The $(NH_4)_2{\cdot}HPO_4$, $Ca(NO_3)_2{\cdot}4H_2O$ and cellobiose were obtained from Himedia (Himedia, India). Recombinant hepatitis B surface antigen was obtained from Panacea Biotech Ltd. (Lalru Punjab, India) as a gift sample. AUZYME monoclonal diagnostic kit and anti-HBsAg ELISA kit (AUSAB) was obtained from Abbott (Abbott Laboratory, Chicago, IL, USA).

2.2. Preparation of hydroxyapatite core

Hydroxy apatite core was prepared by self-precipitation method in synthetic body fluid (SBF) as previously reported (Tas, 2000). 0.174 M of $Ca(NO_3)_2{\cdot}4H_2O$ was first prepared in SBF at 37 °C followed by addition of 5.0 ml of ammonia–SBF mixture (1:2, v/v). Then, solution of 0.1561 M $(NH_4)_2{\cdot}HPO_4$ in SBF was added at 4 ml/min from the burette under continuous vigorous stirring. The opaque solution so obtained was further stirred for 2 h using mechanical stirrer (Remi, India). During whole process, the temperature of the reaction was maintained at 37 °C using a thermostated water bath. The precipitate was recovered from the supernatant by centrifugal filtration at 15,000 rpm for 10 min and washed with deionized water. The filtrate was dried at 90 °C overnight and the powder recovered from the primary drying step was mildly grounded for 20 min using a mortar and pestle and calcined at 800 °C for 6 h at 10 °C/min heating rate in a platinum crucibles.

2.3. Characterization of hydroxyapatite core

2.3.1. FTIR of hydroxyapatite

Fourier transformed infrared spectrophotometry was used for structure analysis of ceramic core. The KBr sample disk was prepared using 1% (w/w) of hydroxy apatite powder and compressed and dried at 100 °C. Infrared spectra were recorded in the wave number range of 4000–400 cm^{-1} (resolution 4.0 cm^{-1}) using FTIR spectrophotometer (Perkin-Elmer-1600, USA).

2.3.2. XRD analysis of hydroxyapatite

Hydroxyapatite ceramic core were exposed to Cu Kα radiation (45 kV × 40 mA) in a wide-angle X-ray diffractometer (Philips XRD-6000). The instrument was operated in the step-scan mode in increments of 0.03° 2θ. The angular range was 5–40° 2θ, and counts were accumulated for 1 s at each step.

2.4. Preparation of nanodecoy systems

Nanodecoy systems were prepared by the method reported previously with slight modifications (Kossovsky et al., 1991). Briefly, hydroxyapatite weighing approximately 1.5 mg was initially suspended in 3.0 ml of 29.2 mM cellobiose solution in a dust-free glass vial by liberal vortexing. The resultant suspension was sonicated for 10 min at a frequency of approximately 20 kHz at 25 °C using a probe sonicator (Soniweld, India). The dispersion was clarified by microcentrifugation at 10,000 rpm for 20 min. The remaining pellets were then discarded and the mixture was lyophilized overnight. Unabsorbed cellobiose was removed by microcentrifugation against 20 ml of 25 mM phosphate reaction buffer (pH 7.4). Dispersed further 5 mg of sugar coated particles with HBsAg solution (10 μg/ml in PBS pH 7.4) and kept at 4 °C over night. HBsAg antigen loaded nanodecoy systems were washed three times with deionized water by using centrifugation at 10,000 rpm for 30 min and stored at 4 °C until used.

2.5. Characterization of nanodecoy systems

2.5.1. Size and shape

The morphological examination of prepared systems was performed using a transmission electron microscope (Philips CM-10, Netherlands) following the negative staining of phosphotungstic acid solution (2%, w/v). The mean particle size and size distribution of prepared nanodecoy systems were determined by a photon correlation spectroscopy using a Autosizer II C apparatus (Malvern Instruments Co., UK).

2.5.2. Characterization of polyhydroxy oligomer coating

Sugar content was determined by the anthrone method (Hedge and Hofreiter, 1962) using glucose as a standard.

2.5.3. Zeta potential

The adsorption of sugar on the surface of hydroxyapatite was confirmed by studying zeta potential using Malvern Zetasizer 3000 HS (Malvern Instruments Co., UK).

2.5.4. Antigen loading efficiency

The percentage antigen loading efficiency of nanodecoy systems were determined as reported previously (Lewis, 1996). Briefly, accurately weighed antigen loaded nanodecoy formulations were suspended in Triton-X 100 (0.01%, w/v) and incubated in a wrist shaker for 1 h. The samples were centrifuged at

14,000 rpm for 15 min and antigen concentration in the supernatant was determined using micro BCA method while setting a blank of unloaded nanodecoy formulation.

2.5.5. In process stability studies

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the stability and integrity of HBsAg during the preparation of nanodecoy systems (Laemmli, 1970; Jaganathan et al., 2004). HBsAg was extracted by dissolving the nanodecoy systems in 2 ml of 5% (w/v) SDS in 0.1N HCl solution. The extracted antigen was concentrated and loaded onto a 3.5% stacking gel and subjected to electrophoresis on a 12% separating gel at 200 V (BioRad, Hercules, CA) until the Coomassie dye stained protein band reached the gel bottom.

The in vitro antigenicity of the HBsAg loaded nanodecoy systems was also determined during formulation process by EIA/protein ratio determination (Shi et al., 2002). The in vitro antigenicity of HBsAg was measured with an enzyme immunoassay kit (AUZYME monoclonal diagnostic kit, Abbott Laboratories, USA) following the manufacturer instructions. Plain HBsAg solution (PBS, pH 7.4) incubated at 4 °C was used as a control. All EIA/protein ratio values are the average of six measurements.

2.6. Immunization studies

2.6.1. Animals and inoculations

Female Balb/c mice aged 6–8 weeks, weighing 15–20 g were used for in vivo studies. Animals were housed in groups of five (six mice in each experimental group) with free access to food and water. The Institutional Animals Ethical Committee of Dr. Harisingh Gour University approved the study. The studies were carried out according to the guidelines of the Council for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India. One control group (group 1) and four dose groups (six rats per group) were used in the study. Each dose group was subcutaneously immunized with equivalent dose of 10 μg of soluble HBsAg (group 2), alum adsorbed HBsAg (group 3), HBsAg loaded hydroxy apatite core (group 4), HBsAg loaded nanodecoy systems (group 5). These groups were boosted with the same formulations on day 14. Serum samples were collected from retro-orbital plexus of the mice at days 1, 14, 28, 42, and 56 and assayed for anti-HBsAg antibodies using solid phase enzyme linked immunoassay kit (AUSAB, Abbott Laboratory, USA) following the manufacturer's instructions.

2.6.2. Analysis of humoral and cellular immune responses

Specific antibodies to HBsAg were analyzed by commercially available HBsAg ELISA (Abbott Laboratory, USA). End point titers were expressed as the log 10 of the reciprocal of last dilution, which gave an optical density (OD) at 450 nm above the OD of negative controls. Endogenous levels of IL-2 and IFN-γ in mouse spleen homogenates were determined using two separate ELISA kits (Amersham Biosciences, USA) following the instructions given by the manufacturer. Spleen homogenates were prepared by the method reported previously with slight modifications (Nakane et al., 1992). Briefly, spleens were weighed and homogenized in ice-cold PBS containing 1% CHAPS (Sigma, USA) and 10% (w/v) homogenates were prepared using tissue homogenizer (York, India). Homogenates were incubated on ice-bath for 1–2 h at temperature below 0 °C so that insoluble matters settled down. Supernatant was centrifuged at 2000 g for 20 min and the clear supernatant was used for cytokines estimation by sandwich ELISA.

2.7. Statistical analysis

Analysis of antibody titers was performed on logarithmically transformed data and the data is presented as standard deviation (S.D.). Student's *t*-test was used to compare mean values of different groups. Multiple comparisons were assessed using one-way analysis of variance (ANOVA) followed by post hoc analysis using Dunnet test. Statistical significance was considered at $P < 0.05$.

3. Results and discussion

3.1. Preparation and characterization of hydroxy apatite core

In the present study, hydroxyapatite core was prepared and used for the preparation of nanodecoy systems. HA particles were prepared by self-precipitation method under controlled process parameters, were smaller in size and crystalline in nature. HA particles with different morphologies can be obtained by this process, which has been extensively studied and reported elsewhere (Luo and Nieh, 1996). Photo correlation spectroscopy also confirmed the nanometric architecture of prepared nanodecoy systems. Both transmission electron microscopy and photon correlation spectroscopy results revealed the nanodecoy ranged from 50 to 150 nm with a small micro-size fraction (<6%).

The synthesis of hydroxyapatite ceramic core was characterized by X-ray diffraction patterns as shown in Fig. 1. Characteristic intense absorption peaks at 31–32, 49–50, 25–27 (2θ angle) indicated the crystalline behavior of HA. The XRD pattern of prepared sintered hydroxyapatite ceramic core complied with the standard HA core (Li et al., 1997).

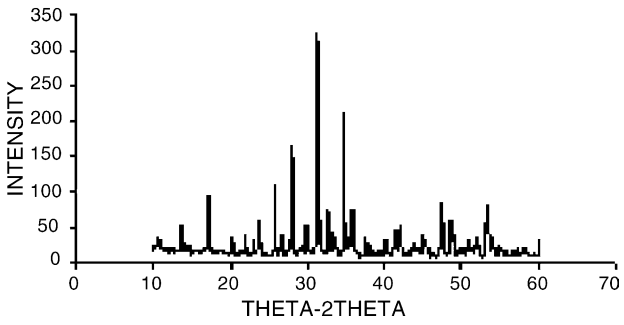

Fig. 1. X ray diffraction pattern of hydroxyapatite core particles obtained from mixture of calcium nitrate and di-ammonium hydrogen phosphate in SBF.

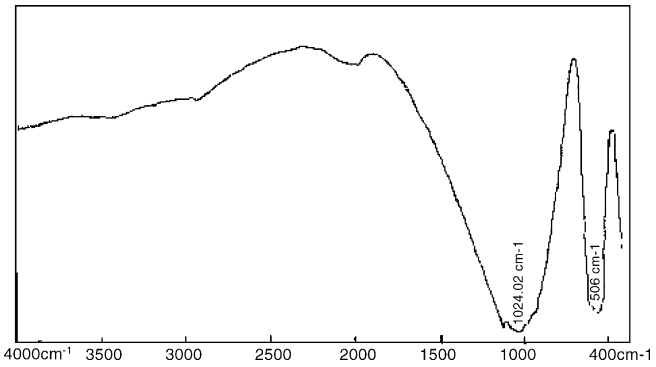

Fig. 2. FTIR spectra of hydroxyapatite core particles obtained from mixture of calcium nitrate and di-ammonium hydrogen phosphate in SBF.

The prepared HA core was also analyzed by FTIR spectroscopy. The bands for PO_4^{3-} of the calcined powder observed at 507, 604, 945, 964, 1024, and 1184 cm^{-1}, whereas the medium sharp peak at 633, 2910 and 3570 cm^{-1} was due to the OH^- bending deformation (Fig. 2). The FTIR spectrum of prepared HA core was in accordance of the previously findings (Tas, 2000).

The morphology of the HBsAg loaded hydroxyapatite core is shown in TEM image in Fig. 3A. The microstructure of HA core revealed that the particles were spherical and dense enough with rough surfaces.

3.2. Preparation and characterization of nanodecoy systems

Nanodecoy formulations were also characterized for the size, shape, and antigen loading efficiency. TEM image of nanodecoy systems indicated that the systems were smaller in size, spherical and elongated in shape (Fig. 3B). Coating of cellobiose on the surface of the hydroxyapatite core was determined by anthrone method (Hedge and Hofreiter, 1962), which suggests that hydroxyapatite core adsorbed 0.254% of cellobiose. The zeta potential of the hydroxyapatite ceramic core was 2.34 ± 0.15 that was reduced to -3.86 ± 0.38 after coating of cellobiose, which suggests the coating of sugar layer on ceramic core. The zeta potential of HBsAg loaded cellobiose coated hydroxyapatite core (nanodecoy) was -7.23 ± 0.42, which further suggests the surfacial association of antigen. Antigen loading of the nanodecoy systems was found almost 2.5 times lower in magnitude than the plain hydroxyapatite core without cellobiose coating (Table 1).

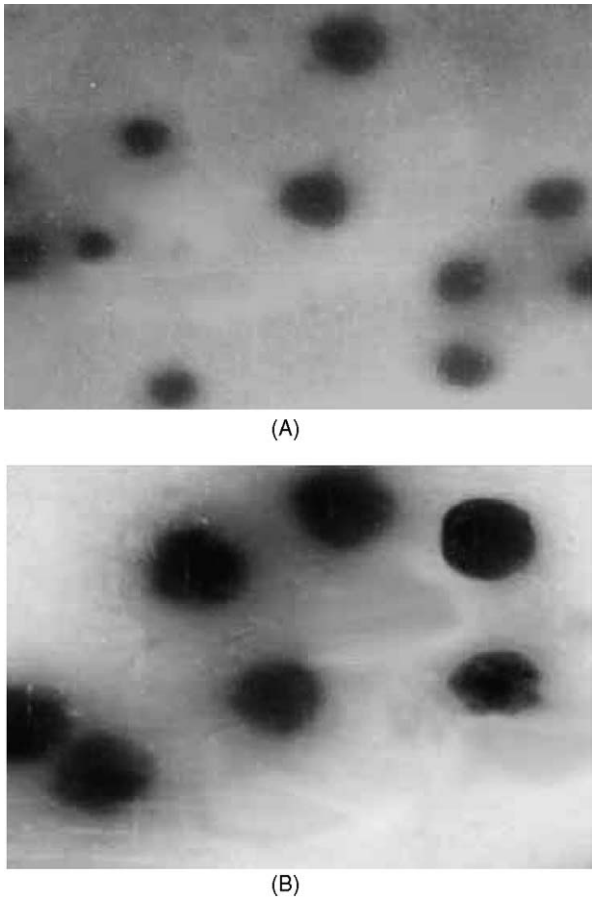

Fig. 3. TEM image of antigen-loaded formulation of: (A) plain hydroxyapatite ceramic core and (B) cellobiose coated hydroxyapatite ceramic core (nanodecoy).

3.3. In process stability studies

The ultimate stability of a protein containing preparations can often be a function of the physical and chemical conditions to which it was exposed during processing. Therefore, primary aim of the in vitro stability studies was to determine the structural integrity of antigen/protein during the preparation of nanodecoy systems by SDS-PAGE as well as by in vitro antigenicity assay.

In SDS-PAGE experiments, HBsAg protein appeared as a single band in all the formulations in the molecular weight region of ~24 kDa (Fig. 4) that corresponds to the molecular weight of plain HBsAg antigen.

In vitro antigenicity of HBsAg loaded nanodecoy systems was determined as a ratio of EIA and protein concentration

Table 1
General characterization of hydroxyapatite core based formulations

Formulations	Zeta potential (mV)	Antigen loading efficiency (%)
Hydroxyapatite core (HA core)[a]	+2.34 ± 0.15	–
Hydroxyapatite core coated with HBsAg (HBsAg–HA core)	−6.37 ± 0.48	49.52 ± 2.3
Hydroxyapatite core coated with cellobiose[a]	−3.86 ± 0.38	–
HBsAg loaded nanodecoy	−7.23 ± 0.42	20.57 ± 1.8

Data are shown as mean ± S.D. ($n = 3$).
[a] Without antigen.

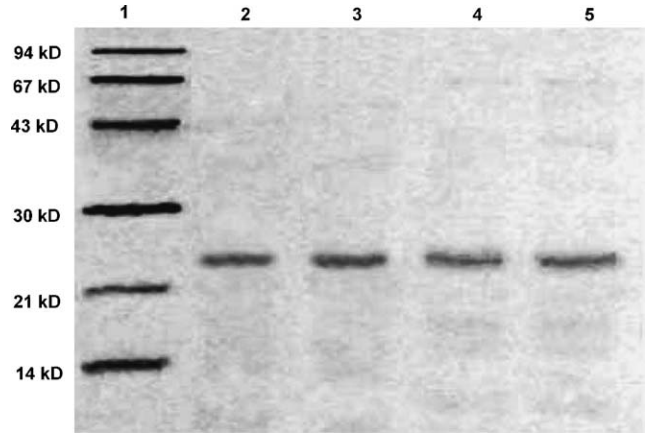

Fig. 4. SDS-PAGE analysis of nanodecoy formulations. Left to right—Lane 1: protein marker; lane 2: plain antigen; lane 3: alum adsorbed HBsAg; lane 4: HA core; lane 5: antigen-loaded nanodecoy.

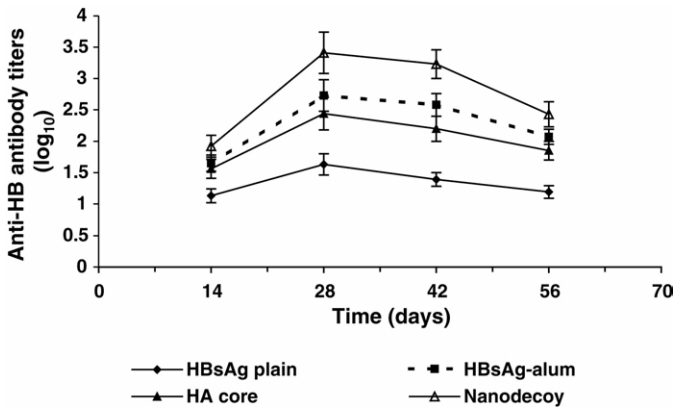

Fig. 6. Serum anti-HBsAg–antibody response of different HBsAg formulations ($n = 6$) following subcutaneous administration in Balb/c mice. Animals were immunized subcutaneously followed by a booster dose after 2 weeks with the same formulations. Nanodecoy showed significant differences in antibody response from control ($P < 0.05$).

and shown in Fig. 5. The antigenicity of nanodecoy formulations was almost equivalent to the HBsAg coated HA core and control antigenic solution incubated at 4 °C. The antigenicity was >95% in magnitude for all the formulations. The data suggest that there was not any deleterious effect on the HBsAg antigenicity and integrity during the preparation of nanodecoy formulations.

3.4. In vivo immunogenicity

The antibody titer is evident of fair adjuvanticity of nanodecoy formulations. In the immunization studies, the nanodecoy formulations (cellobiose coated and uncoated) were administered subcutaneously in the mice of each groups followed by boosting on day 14th. The specific anti-HBsAg antibody titer from the nanodecoy immunized animals (group 5) was found significantly higher ($P < 0.05$) than the alum group (group 3) as compared at day 28th (Fig. 6). The antibody titer obtained from HA core loaded HBsAg formulation (group 4) was found to be least 'among' other. This suggests that hydroxy apatite core as such is a weaker adjuvant compared to its cellobiose-coated nanodecoy systems. The better in vivo immunogenicity of the nanodecoy systems could be attributed to excellent antigen presentation of antigens to the immunocompetent cells. The presence of poly hydroxyl oligomer groups on the epitaxial surface of the nanodecoy signifies the adjuvant properties of nanodecoy. Further, the chemical nature of hydroxyapatite may dually be responsible for the intracellular targeting and processing and presentation of antigen.

Endogenous cytokine levels (IL-2 and IFN-γ) were estimated in spleen homogenate after 21 days following subcutaneous administration of different nanodecoy formulations (Figs. 7 and 8). A significant ($P < 0.05$) higher level of both IL-2 and IFN-γ was observed in mice immunized with HBsAg loaded HA core and nanodecoy systems than those of alum adsorbed HBsAg vaccinated group. Furthermore, a significant higher cytokines level was also observed than the intramuscular plain HBsAg vaccinated group and control. IFN-γ production is a well-known property of the cells after antigenic stimulation. These cytokines are Th1 dependent cytokines and their higher levels are evidenced for the strong cell mediated immune response that is equally important to eliminate virus from infected cells (Constant and Bottomly, 1997). It is

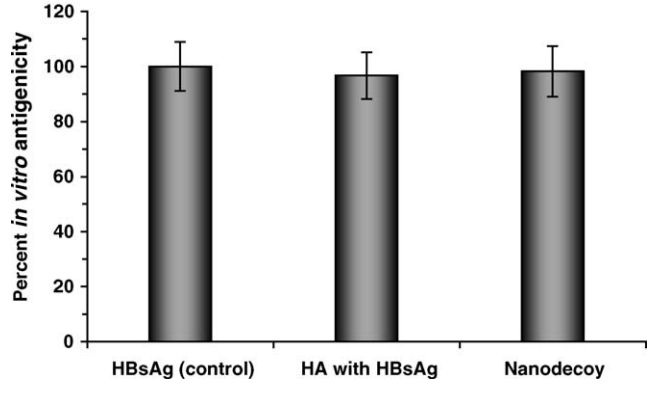

Fig. 5. In vitro antigenicity (percent ratio of the enzyme immunoassay response to protein concentration) of HBsAg loaded nanodecoy, hydroxyapatite core and control (plain HBsAg) formulations ($P > 0.05$) during preparation.

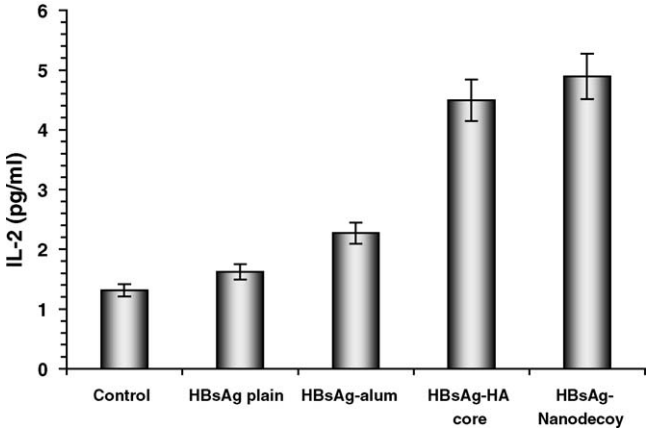

Fig. 7. Interleukin-2 level in spleen homogenate of mice immunized with different formulations after 21 days.

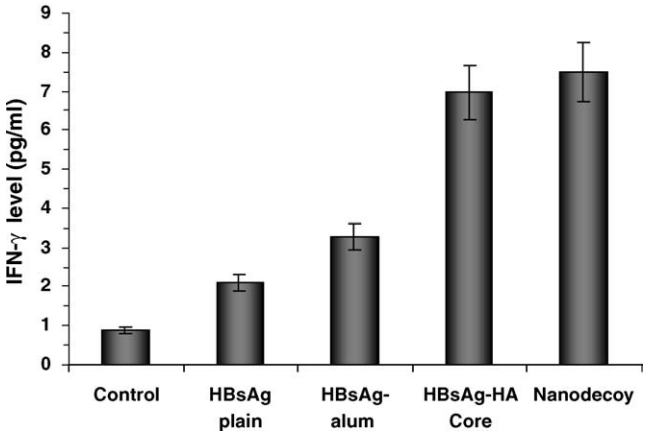

Fig. 8. Interferon-γ level in spleen homogenate of mice immunized with different formulations after 21days.

clear from the results that although conventional recombinant protein based vaccine elicited higher humoral response (antibody production) but it fails to elicit cell-mediated immune response, thus ineffective for treatment of chronic hepatitis B infection.

When mice are immunized with conventional adjuvant alum subcutaneously, the humoral type immune response, i.e. antibody response was more strong and effective than cellular type immune response, since the exogenous antigen is mainly presented by B cells through the MHC class II pathway to Th2 cells. In case of nanodecoy systems antigens are presented partly by the B cell through the MHC II pathway leading to a good antibody response, while part of the antigen is cleaved within the antigen presenting cells and presented through MHC class I pathway, leading to a Th1/CTL response (Kossovsky et al., 1995a,b). The generation of a dominant Th1 cytokine profile is important to facilitate eradication of HBV infection (Geissler et al., 1997; Davis, 2002). Thus nanodecoy system is effective in producing both antibody titer and Th1 cytokines in animals studied.

4. Conclusion

In conclusion, nanodecoy systems are useful nanosized carrier having superior surface characteristics; their ability to interact with the antigen presenting cells make them attractive system for vaccine applications. It is noteworthy that the nanodecoy systems performed anticipated adjuvanticity, hence could be successfully prepared and used as immunoadjuvants for proteineous antigen(s). Moreover, the present study suggests a enhanced humoral as well as cellular immune response but it needs an extensive work to prove the efficacy and safety of the nanodecoy systems before they can be accepted clinically as alternative vaccine delivery system(s).

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