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Original Article

Immunopotentiation of Hepatitis B Vaccine Using Biodegradable Polymers as an Adjuvant

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BACKGROUND/PURPOSE: The purpose of this research was to develop an alternative adjuvant for hepatitis B vaccine (HBsAg) that elicits a long-lasting immune response after a single administration. In this study, the suitability of Poly (D, L)-lactide-co-glycolic acid (PLGA), Poly lactic acid (PLA) and Chitosan polymers as adjuvants for HBsAg were investigated.

METHODS: We used solvent evaporation and emulsion cross-linking techniques to encapsulate HBsAg into the different polymeric systems. The newly developed microparticles were evaluated for vaccine content, particle size distribution, *in vitro* release and immunogenicity.

RESULTS: HBsAg-encapsulated PLGA and PLA microparticles were smooth and spherical. However, Chitosan microparticles were homogeneous, and almost spherical, with rough surfaces. The vaccine loading and release patterns varied with the type of polymer used. In this study, Chitosan polymeric microparticles released antigen until day 63 post-injection; however, the release period of the PLGA and PLA systems was shorter. A significant increase in the level of antibodies to HBsAg was induced by all the polymeric microparticles.

CONCLUSION: The results indicate that Chitosan microparticles are a more efficient adjuvant for HBsAg than PLGA and PLA polymeric microparticles.

KEYWORDS: adjuvant, biodegradable polymers, hepatitis B vaccine, vaccine delivery system

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Introduction

The strategy of the World Health Organization is to develop efficient and inexpensive vaccines against various diseases. Traditionally, adjuvants have been used in vaccine formulations to augment the immune response, usually after a booster dose. Hepatitis B is an acute systemic infection, which constitutes a major health problem all over the world. The risk of infection may vary from

country to country. Approximately, 30% of world's population has serological evidence of hepatitis B infection.[1] Hepatitis B virus infection can lead to fulminant hepatic failure that threatens the life of affected patients.[2] A safe and effective vaccine against the hepatitis B virus has been available for nearly 20 years; however, it requires that at least three doses be administered at the appropriate time intervals soon after birth to elicit optimum immune responses. Currently, alum is the only adjuvant approved for human use and plays a major role in vaccine delivery, but alum is not a universal adjuvant because it is not suitable for small peptides. Furthermore, alum-type adjuvants induce cell-mediated immunity. The Global Alliance for Vaccine Initiative has suggested the use of a combined diphtheria-hepatitis B vaccine as a more efficient method of pediatric vaccine delivery. Unfortunately, the cost per dose of this combined vaccine is significantly higher than the separate diphtheria and hepatitis B vaccines.[3] Moreover, as diphtheria cannot be given before 6 weeks of age due to deprived immunogenicity, the combined diphtheria-hepatitis B vaccine cannot be given before 6 weeks of age either. Thus it is necessary to find an alternative adjuvant that elicits a long-lasting immune response after a single dose. Biodegradable polymers have been used as sutures and drug carriers because of their nontoxic nature and their adjustable biodegradable properties. Polymers chosen for parenteral administration should meet some of the requirements, e.g. being biodegradable, safe, drug compatible and permeable, stable *in vitro*, easy to process and inexpensive.

Biodegradable synthetic and natural polymers have been investigated for the controlled release of vaccines and drugs. Polymers in the form of microparticles are preferred for the controlled delivery of vaccines, which mimics both the primary and boosting dose of a conventional vaccine. Moreover, microspheres are also capable of protecting antigens from rapid destruction *in vivo*, allowing presentation of antigen in its native confirmation to various cells of the immune system.[4] The ability of future vaccines to provide maximum efficacy after a minimum dose is delivered safely and easily is of prime importance.[5] Based on these factors, the development of a single administration hepatitis B vaccine using a biodegradable polymeric system is an important advancement towards the betterment of human health care.

Methods

Materials

Poly (D, L)-lactide-co-glycolide acid (PLGA), with a lactide/glycolide ratio of 50/50 (MW = 50 kDa), and Poly lactic acid (PLA, MW = 2 kDa) polymers were purchased from Sigma Aldrich (St. Louis, MO, USA). Chitosan polymer (150 cps viscosity grade) and hepatitis B vaccine (alum-free HBsAg, 3 mg/mL solution) were provided by the Central Institute of Fisheries Technology, Cochin, India and Serum Institute of India, Pune, India, respectively. Extensive pre-formulation studies were carried out in order to encapsulate hepatitis B vaccine in the polymeric microparticles.

Preparation of hepatitis B vaccine-encapsulated PLGA/PLA microparticles

Briefly, PLGA or PLA polymer solutions were prepared by dissolving 100 mg of polymer in 1 mL of methylene chloride followed by emulsification with 200 µL of alum-free HBsAg aqueous solution (3 mg/mL) using a cyclomixer for 1 minute. The emulsion was sonicated at 15,000 rpm, dispersed in 2% w/v polyvinyl alcohol (PVA) solution and stirred for 6 hours to allow solvent evaporation. The resulting microparticles were collected by centrifugation at 10,000 ×*g* and were washed three times with phosphate buffered saline solution (pH 7.4) to remove free HBsAg. The collected microparticles were suspended in 50 mL of distilled water and immediately filtered through a membrane filter and dried in a vacuum desiccator.

Preparation of hepatitis B vaccine-encapsulated Chitosan microparticles

Briefly, 1% w/v solution of Chitosan (medium molecular weight with 75–85% deacetylation, 150 cps viscosity grade) was prepared in aqueous acetic acid (5% v/v). The solution was mixed with an emulsion containing sunflower oil, or liquid paraffin, to form a water-in-oil emulsion. To this emulsion, 1 mL of alum-free HBsAg aqueous solution (3 mg/mL) was added and stirred for half an hour. Finally, 1 mL of glutaraldehyde was added and the emulsion was stirred at 3,000 rpm for 1 hour. The emulsion was centrifuged at 15,000 rpm and the oil layer was removed. The remaining pellet was washed with organic solvents in order to remove excess oil. The collected microparticles were

suspended in 50 mL of distilled water and immediately filtered through a membrane filter and dried in a vacuum desiccator.

Physicochemical characterization

The morphological examination of microparticles was performed by scanning electron microscopy (SEM; JSM–T220, JEOL, Japan). Samples used for SEM were mounted on to metal stubs and coated with gold palladium to a thickness of 200–300 Å under reduced pressure. The size of the microparticles was determined using a microscope (Olympus BX 50, Tokyo, Japan) in which 100 particles were counted to calculate the average diameter. The magnitude of vaccine loading in the microparticles was determined by a previously established method with a slight modification.[6] The amount of HBsAg loaded into the PLGA and PLA microparticles was determined by dissolving 50 mg of microparticles in 1 mL of a 0.1 M NaOH/5% sodium dodecyl sulfate solution. The supernatant was centrifuged and analyzed for protein content using Lowry's method.[7] The stability of the vaccine-loaded and -unloaded PLGA microspheres was determined over a period of 8 weeks. The microspheres were exposed at 4°C, at room temperature, and at 50°C.

In vitro release study

Hepatitis B vaccine-encapsulated microparticles (100 mg) were suspended in 50 mL of phosphate buffer saline (154 mM, pH 7.4) containing Tween 80. The flask was then kept in shaker/incubator. At predetermined time intervals (Days 1, 2, 4, 7, 10, 14, 21, 28, 35, 42, 49, 56, 63 and 70) the antigenically active vaccine was analyzed by Enzyme Immune Assay for HBsAg (Diagnostic Products Corporation, Los Angeles, CA, USA). The quantity of HBsAg in the samples was calculated by comparing the standard curves.

Immunogenicity studies

Immunogenicity studies were carried out on healthy Wistar rats, weighing about 150–250 g. The animals were maintained and handled according to the standard guidelines of Institutional Animal Ethical Committee 290/CPCSEA/2006. The animals were divided into four groups and each group contains 6 rats. All the animals were injected intramuscularly at the thigh region with a quantity of 0.5 mL of vaccine formulation (2 μg i.e. one-tenth of the standard human dose). The various treatment groups are: Group 1, animals received hepatitis B vaccine-encapsulated Poly (D,L)-lactide-co-glycolic acid microspheres; Group 2, animals received hepatitis B vaccine-encapsulated PLA microspheres; Group 3, animals received hepatitis B vaccine-encapsulated Chitosan microspheres; and Group 4, animals received conventional alum-adsorbed hepatitis B vaccine. Blood samples were collected from the retro-orbital plexus using a capillary tube on Days 45 and 90. Sera were separated by centrifugation and stored at –20°C until assayed. The immunopotentiation was determined by measuring specific anti-hepatitis B antibodies using an enzyme-linked immunoabsorbent assay and an Immulite 2000 automated analyzer (Diagnostic Products Corporation). The immunoglobulin titers were measured using IgG and IgM assay kits (Turbox, Orion Diagnostica, Finland).

Results

Physicochemical characterization

The effect of two different concentrations of PVA on microparticle size, loading and stability are shown in Table 1. In both the PLGA and PLA polymer systems, the size of the microparticles were reduced, and the encapsulation

Table 1. Effect of two different concentrations of polyvinyl alcohol on microparticle formation

Polymeric system	PVA concentrations (% w/v)	Microparticle size (μm)	Loading of vaccine in microparticles (%)	Microparticle stability at 8 wk		
				4°C	Room temperature	50°C
PLGA	1.5	124.1±2.10	26.0±5.3	Stable	Less stable	Not stable
	2.0	122.1±1.60	30.0±2.1	Stable	Stable	Not stable
PLA	1.5	127.5±2.60	18.3±2.8	Stable	Less stable	Not stable
	2.0	94.0±1.90	36.1±1.3	Stable	Stable	Not stable

PVA = Polyvinyl alcohol; PLA = Poly lactic acid; PLGA = Poly (D,L)-lactide-co-glycolic acid.

Table 2. Effect of two oil phases on Chitosan polymeric system

Polymeric system	Oil phases	Microparticle size (μm)	Loading of vaccine in microparticles (%)	Microparticle stability at 8 wk		
				4°C	Room temperature	50°C
Chitosan	Sunflower	36.0±2.6	38.0±1.2	Stable	Stable	Not stable
	Liquid paraffin	88.0±1.2	26.0±2.6	Stable	Stable	Not stable

Table 3. Comparative immunogenicity study[a]

Group[b]	Anti-hepatitis B antibody (IU/L)		IgG (mg/dL)		IgA (mg/dL)		IgM (mg/dL)	
	Day 45	Day 90	Day 45	Day 90	Day 45	Day 90	Day 45	Day 90
1	10.2±0.6	11.3±0.3	793.6±2.4	791.3±1.3	77.8±2.3	78.8±2.6	85.0±1.2	85.3±1.8
2	10.1±0.2	11.2±0.1	793±1.7	789.6±2.3	75.1±1.7	77.3±2.1	80.0±1.6	85.3±1.4
3	11.8±0.1	12.4±0.1	805.3±4.8	809.0±3.5	83.0±1.4	80.6±2.4	81.6±2.0	85.3±1.4
4	10.1±0.6	10.9±1.3	793.6±1.7	789.6±2.3	75.1±1.7	77.3±2.1	80.0±1.6	85.3±1.4

[a]Data presented as mean±standard deviation ($n=6$; $p\leq 0.001$; Dunnet multiple comparison test); [b]group 1=animals received hepatitis B vaccine-encapsulated Poly (D,L)-lactide-co-glycolic acid microspheres, group 2=animals received hepatitis B vaccine-encapsulated Poly lactic acid microspheres, group 3=animals received hepatitis B vaccine-encapsulated Chitosan microspheres, group 4=animals received conventional alum-adsorbed hepatitis B vaccine.

efficiency and stability was increased when 2% w/v of PVA was used as the external phase. For the Chitosan polymer system, two different oil phases were used as the continuous phase to formulate the water-in-oil emulsion. The oil phases used were sunflower oil and liquid paraffin. The effect of the oil phases was determined in terms of microparticle size, vaccine loading and stability. It was clear that improved stability, loading and reduced micro-particle size were achieved when sunflower oil was used as the external phase (Table 2). The HBsAg-encapsulated PLGA and PLA microparticles, which prepared using the solvent evaporation method, were spherical with a smooth surface; however, the Chitosan microparticles had a rough surface and a more-or-less spherical shape.

In vitro release study

HBsAg release may be due to the degradation of the polymer latex leading to a swollen inner structure. A biphasic HBsAg release pattern was displayed in all the three microparticle system. Approximately 8–10% of encapsulated antigen was released from PLGA and PLA microparticles in the first 24 hours. Similarly, Chitosan microparticles also characterized by releasing 7–9% of antigen with in the first day of incubation. The amount of antigen released was significantly higher in the PLGA and PLA polymeric system during the first 4 days when compared to Chitosan polymeric microparticles. This may be due to various factors including surface morphology, particle size, viscosity grade, polymer composition and polymer molecular weight.

Immunogenicity studies

The results shown in Table 3 represent comparative studies of specific immunoglobulin levels on Days 45 and 90 (IgG, IgA and IgM). Dunnett's test was used to compare the immunoglobulin levels of the animals among different groups. The anti-HBsAg and immunoglobulin titers after immunization with HBsAg-loaded PLGA/PLA microparticles showed similar values on Days 45 and 90 compared with the conventional alum-adsorbed vaccine (group 4). This suggests that both the PLGA and PLA polymer microparticles elicited immune responses similar to

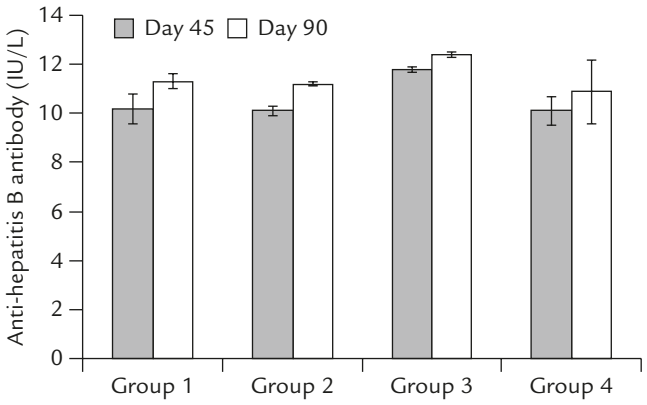

Figure. Anti-hepatitis B antibody titers on Days 45 and 90 post-vaccination. Group 1 = animals received hepatitis B vaccine-encapsulated Poly (D,L)-lactide-co-glycolic acid microspheres; Group 2 = animals received hepatitis B vaccine-encapsulated Poly lactic acid microspheres; Group 3 = animals received hepatitis B vaccine-encapsulated Chitosan microspheres, Group 4 = animals received conventional alum-adsorbed hepatitis B vaccine.

the conventional alum-adsorbed HBsAg. However, after a single-step immunization, the Chitosan microparticles (group 3) induced significantly higher immunoglobulin levels compared with the conventional alum-adsorbed (group 4), PLGA and PLA polymer microparticle systems (groups 1 and 2) up to Day 90 (Figure). These results show that the Chitosan microparticle system is a better adjuvant than either the PLGA or PLA systems.

Discussion

Vaccines are considered as the most successful medical interventions against infectious diseases. The development of new, more efficacious and easier to deliver vaccines has become an area of research that can certainly benefit from recent controlled release technology. In particular, the conversion of multiple dose vaccines into single dose vaccines represents an important advancement towards the betterment of human health care. Injectable biodegradable polymeric microparticles represent an exciting approach to control the release of vaccine, reduce the number of doses, and optimize the desired immune response. Microparticulate vaccine antigens can mimic the priming and boosting doses of vaccines.[8–11] Designing an effective vaccine against a particular pathogen involves certain key factors that target and trigger the immune response. Thus, the inclusion of "immunopotentiators", i.e. adjuvants that trigger early innate immune responses to aid in the generation of a robust and long-lasting adaptive immune response, is therapeutically very important. Therefore, adjuvants have traditionally been used in vaccine formulations to improve efficacy. The development of vaccine delivery systems prepared from biodegradable polymers has received considerable attention over the past two decades. Several research reports prove that polymeric microspheres can be considered as the next generation of adjuvants to replace aluminum salts for vaccine potentiation.[12] Vaccine research is often focused on the identification and application of novel antigens. The immune response to these antigens is routinely optimized by assessing the dose and the number of injections. Owing to advances in biotechnology, many vaccines are poorly immunogenic, and therefore require several boosters with a standard adjuvant. Currently, alum is the only adjuvant that is approved for clinical use. The use of alum-type adjuvants for immunization, however, has several disadvantages. They induce inflammation and stimulate the local production of granulomas. In addition, alum is not a universal adjuvant as it is not suitable for small peptides or recombinant proteins and cannot be frozen or lyophilized. Conventional alum-type vaccines require multiple recall injections at appropriately timed intervals in order to achieve long-lasting and optimal immune responses. However, it is very difficult, especially in developing countries, to maintain a high re-immunization rate in the case of multiple-administration immunization programs. Therefore, the development of more efficient and safe adjuvant/vaccine delivery systems, requiring only a single administration to obtain high and long lasting immune responses, is of primary importance.

In this study, our objective was to evaluate the suitability and potential of PLGA, PLA and Chitosan polymeric systems as adjuvants for hepatitis B vaccines that are easy to deliver and elicit a long-lasting immune response. Preformulation studies were performed to ascertain the variables of microparticle formation. The continuous phase was selected based on the polymers and techniques employed to formulate the microparticles. An external phase containing PVA is known to be a key factor that influences the size of polyester polymeric microparticles. The concentration of PVA influences both the formation and size of the microspheres. During the solvent evaporation process, there was a gradual decrease in the volume of PVA,

which leads to an increase in viscosity and particle aggregation. Therefore, larger amount of PVA were used during the micro-encapsulation process. Generally, the size of the microspheres dictates the length of the immune response. Larger microspheres have slower release rates and longer periods of immunogenicity.[13] Earlier reports have shown that the size of the microparticles influences the release rate of the antigen. It has been estimated that PLGA microparticles with particle sizes of 20–35 µm released about 50–90% of tetanus toxoid within the 1st day.[14] Similar types of antigen release were observed in our study to those in a report by Sanchez et al,[6] but the size of our PLGA and PLA polymeric microparticles are larger. In our study the size of PLGA and PLA microspheres was 120–126 µm (Table 1) displayed an initial burst release of 8–10% of encapsulated antigen. Almost 60% of antigen release was observed in both PLGA and PLA polymeric microspheres during the first 4 day of incubation. But the size of Chitosan microparticles was 36–39 µm displayed an intial burst release of 7–9% of antigen within 24 hours and sustained release was observed until 63rd day. This suggests that Chitosan microparticles are a better adjuvant than PLGA or PLA. We also undertook a comparative study of anti hepatitis B antibodies and immunoglobulin titers on Days 45 and 90 (Table 3). The required level of anti-hepatitis B antibodies to confer protection after vaccination is 10 IU/L. Therefore, it is clear that all three polymeric microparticulate systems provide the minimum protection level. However, Chitosan induced a better immune response, even on Day 90. In contrast with an earlier report,[11] our study proves that the entire immune system actively participated in the production of acquired immunity against the microparticles after a single-step immunization, especially when Chitosan microparticles were used.

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