BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, June 2013. Vol. **10**(1), 415-418

Assessment of Immune Response to Hepatitis B Vaccine: Comparison of Novel Polycaprolactone Blend Microparticles Based Hepatitis B Vaccine and Alum Based Vaccine

Vivek Shrivastava[1-2] and U.K. Jain[1]*

[1]Bhopal Institute of Technology & Science-Pharmacy, Bhojpur Road, Bangrasia, Bhopal, India.
[2]Research Scholar Jawaharlal Technological University, Kukatpally, Hyderabad, India.

(Received: 25 March 2013; accepted: 20 May 2013)

The study reports on the development of a novel hepatitis b vaccine prepared by physically mixing smaller microparticles (8-12 μm; blend PCL-PLGA; containing equivalent to 10μg HBsAg) with larger microparticles (12-20 μm; blend PCL-PLA; entrapping 10μg HBsAg). The vaccine was reconstituted with alum adsorbed HBsAg (equivalent to 10μg HBsAg) and was injected in BALB/c mice subcutaneously as single dose (Total 30 μg HBsAg). The generated immune responses in mice was investigated by Enzyme Linked Immunosorbate Assay (ELISA) technique and was compared with the response generated with alum based vaccine (control group) injected three times at 0, 4, 24 weeks in mice (Total 30 μg HBsAg). The peak antibody titer (4.6491 ± 1.20) generated by vaccine formulation (test group) after single injection were comparable to the control group (4.9099 ± 0.78). The titers sustained in both test & Control groups during 25 week study period. However, at the end of 25th week the titers were slightly higher for control group. The study has shown the feasibility of blend polymeric microparticles as vaccine carrier.

Key words: HBsAg (Hepatitis B Surface Antigen); PLGA; PCL; ELISA; Immune Response.

Hepatitis B, the inflammatory disease of the liver cells is caused by infection with hepatitis B virus. Over 300 million people worldwide are infected with the virus, and more than 1 million of them die every year as a result of liver failure or hepatocellular carcinoma (HCC). The clinical features of the disease include acute (short term and clinically apparent) hepatitis B and chronic (long- term and mostly unapparent) disease. The only way to stop the spread of this virus is vaccination. The marketed hepatitis B vaccine consists of purified, recombinant HBsAg antigen (24 KD, lipoprotein, expressed in yeast) adsorbed on to aluminium adjuvant (1, 2 & 3). HBsAg is a poor immunogen and needs booster with adjuvants like alum for generation of protective immunity[1,2,4]. Primary vaccination consists of three intramuscular doses of vaccine at 0, 1, 6 months The hepatitis B vaccine should be stored between +2 °C and +8 °C & the vaccine is freeze-sensitive. In spite of the presence of an effective vaccine, the immunization coverage is poor. The reasons for this being high drop out rates due to repeated immunization schedules. Individuals who take first prick often do not come for further prick. Secondly due to lack of infrastructural facilities, frequent power cuts & unawareness in the third world countries, maintenance of cold chain is often difficult & accidental exposure of vaccine to temperatures outside cold chain harbors risk of suboptimal dose administration. Thus a single dose vaccine will abolish these concerns & this motivated us to prepare single dose vaccine by combining initial & booster dose of vaccine in one injection (1,2). Unlike the alum adsorbed antigen, the release of

* To whom all correspondence should be addressed.
Tel.: +919826075570;
E-mail: ukjain65@gmail.com

antigen from microparticles is slow and the duration of release can be tailored from week to months by using suitable polymer of appropriate molecular weight. Several recent studies have demonstrated the possible benefits of single dose vaccine in developing countries where there is a very high drop out rates & high rates mortality due to infectious diseases.

MATERIALS

HBsAg bulk (2.24 mg/ml) & Poly å Caprolactone (PCL; Mol Wt 42000, Sigma Aldrich) was kindly provided by Dr K S Jaganathan, Shantha Biotech Pvt Ltd, Hyderabad (A.P.). PLGA (Poly lactic co glycolic acid) & PLA (Poly lactic acid) were also from Sigma Aldrich. All the other entire chemicals used were of analytical grade.

METHODS

Preparation of blend PCL-PLGA & blend PCL-PLA microparticles

The preparation process was optimized initially to produce small (8-12 µm) & Larger (12-20 µm) size microparticles of blend polymers, suitable for vaccine design. Two kind of microparticles were prepared; small microparticles (blend PLGA-PCL [1:1] microparticles), which were supposed to prime of immune system. Large size microparticles made of high molecular weight polymer (PLA-PCL blend [1:1]). The role of large particles was to sustain release of HBsAg, once priming has been done by smaller particles. All the prepared microparticles were characterized for surface morphology, particle size, antigen load & in vitro release profile (6, 7 & 8). Microparticles were prepared by a modified double emulsification solvent evaporation technique described earlier (5). Briefly 3ml solution of polymer blend in desired concentration was dissolved in dichloromethane & was emulsified with 1 ml of antigen (2.24 mg/ml, HBsAg) by sonication for 10 sec. Resulting primary emulsion (W/O) was added drop wise to 25 ml PVA solution (5 % w/v) in distilled water under vigorous stirring to get double emulsion. The stirring was sustained for 1 hour & afterwards the multiple emulsions was transferred on to a magnetic stirrer & was set aside for overnight stirring at room temperature to evaporate methylene chloride. The resulting microparticles were collected by centrifugation at 8000 rpm for 15 minutes, washed thrice with distilled water & dried. For preparing PCL-PLGA microparticle both the polymers were blended in 1: 1 ratio and the concentration of polymer used was 3% w/v, while for fabrication PCL-PLA particles both the polymers were blended physically in 1: 1 ratio & total concentration used for preparation process was 5% w/v.

In Vivo Studies

Study protocol

The protocol for *in vivo* studies was approved by Animal Ethical Committee of Bhopal Institute of Technology & Science -Pharmacy. (Reg. No. 1316/ac/09/CPCSEA). All the ethical guidelines regarding animals use in the research protocol were followed carefully.

Animals

BALB/c Mice, 6 weeks old about 18-22 grams in weight were enrolled for the study. 24 mice were divided into three groups of eight mice each. Test group received microparticle based vaccine (30 µg) as single dose. Control group received alum adsorbed HBsAg (10 µg), which was injected three times at 0, 1 & 6 months (total, 30 µg). Negative control group did not received any vaccine.

Immunization studies

Antibody responses in immunized animals were monitored using a indirect ELISA. Microtiter plates were coated with 100 µl/well of HBsAg (10 ìg/ml) in PBS (pH 7.4) and incubated overnight at 4°C. The plates were washed three times with PBS-Tween 20 (0.05%, v/v) (PBST) and blocked with PBS-BSA (3% w/v) for 2 h at 37ºC, followed by washing with PBS-T. The serum/ body fluids were serially diluted with PBS and 100 µl of each sample was added to each well of coated ELISA plates. The plates were incubated for 1 h at room temperature and washed three times with PBST. 100 µl of goat-anti-mice-HRPO conjugate diluted in PBS-T was added into each well. The plates were covered and after incubation for 1 h at room temperature washing was repeated. Finally, hundred microleters of O-phenyl diamine (OPD) in citrate-phosphate buffer (pH 4.5) together with hydrogen peroxide was added to each well and incubated for twenty minutes at room temperature. Reaction was stopped by adding 5N H2SO4 (50 µl/ well) and the absorbance was measured at 492 nm.

Blood from each mouse was collected at the end of 5th, 7th & 25th week from retro orbital plexus. It was allowed to clot and by centrifugation serum was collected. End point titers were expressed as the log of the reciprocal of the last dilution, which gave an optical density (OD) at 492 nm above the OD of negative control (non immunized sera, NMS).

Statistical analysis

Analysis of antibody titers was performed on logarithmically transformed data and the data were presented along with standard deviations (S.D.). Multiple comparisons were made using one way analysis of variance (ANOVA) followed by post hoc analysis using Dunnet test. Statistical significance was considered at $P<0.05$

RESULTS

The size of PCL-PLGA blend microparticle produced was 10.37±0.09 µm & the surface was smooth with spherical morphology as evident by Scanning electron microscopy (SEM). The loading was 10.7 µg/mg of polymer. The size of PCL-PLA blend microparticle produced was found to be 18.75±1.5 µm & the surface was smooth with spherical morphology as evident by Scanning electron microscopy (SEM). The loading determined was 6.5 µg/mg of polymer. In PCL-PLGA blend microparticles, 30.4% burst release of HBsAg was witnessed and thereafter the release rate rises gradually. The release rate becomes almost constant after day 28. PCL-PLA blend microparticles displayed 94.8% release over 42 day study period. The data are in agreement with previous work (10, 11 &12).

The peak immune response generated in test group injected with single injection of PCL blend microparticles based vaccine formulation (total dose 30 µg) were comparable with Control

Table 1. Primary immune response observed after injection of vaccine formulation prepared from PCL blend microparticles & alum based vaccine in BALB/c Mice

S.	Week(s)	(Test)		(Control)	
No	After Initial Injection	Geometric Mean of Log Dilution at which the Absorbance is more than NMS	Standard Deviation (S.D.)	Geometric Mean of Log Dilution at which the Absorbance is more than NMS	Standard Deviation (S.D.)
1	5	3.0837	0.88	3.7058	0.78
2	7	4.6491	1.20	4.9099	1.82
3	25	2.8281	0.42	3.5552	1.4931

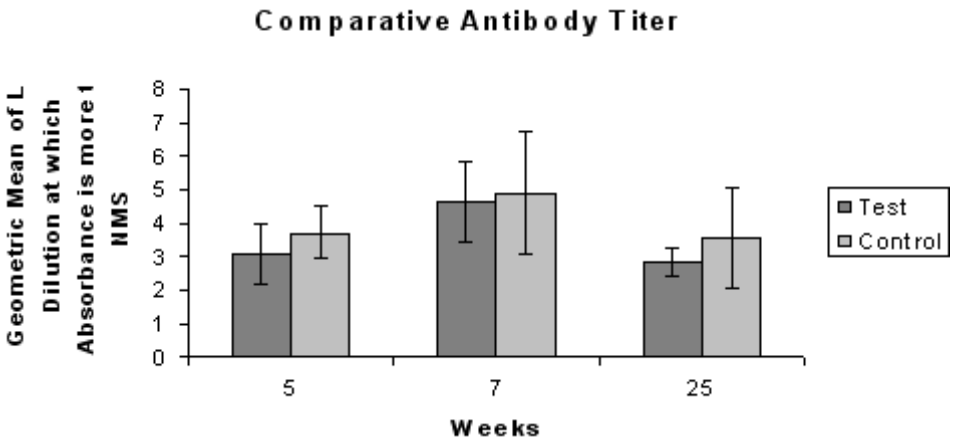

Fig. 1. Comparative antibody titers generated in BALB/c mice

group injected with three injections of alum adsorbed HBsAg (total dose 30 µg). The antibody titer sustained till the end of 25 week study period , though the titers were lower than control group.

DISCUSSION

Hepatitis B is the inflammatory disease of the liver cells infecting over 300 million peoples worldwide. Although effective vaccine is available yet the immunization coverage is poor & this has resulted in spread of disease. The repeated immunization schedule, ineffective infrastructural facilities, unawareness among people & poor cold chain maintenance has resulted in underutilization of vaccine. A single dose vaccine can eliminate these concerns and improve immunization coverage. PLGA (Poly Lactic Co Glycolic Acid) has been widely used as alternate vaccine delivery carrier for the development of single dose vaccine. The generations of acidic microenvironment pH around antigen inside devise due to release of lactic & glycolic acid during antigen release has limited the use of this polymer for acid sensitive antigen like HBsAg. Polycaprolactone has also shown promising results as vaccine delivery system. It has slow degradation therefore do not generate acidic microenvironment unlike PLGA & and but it exist in rubbery state during physiological temperatures. Thus blending PCL with PLGA or PLA can combine advantages of both polymers in one delivery system and at the same time it will circumvent the problem of acid generation during the course of polymer erosion (9, 10 & 13). This motivated us to prepare a vaccine formulation with physically mixing smaller microparticles (8-12 µm; blend PCL-PLGA) with larger microparticles (12-20 µm; blend PCL-PLA) to prepare vaccine against hepatitis B. The peak titer generated by vaccine formulation was comparable to control. This shows the ability of vaccine formulation to prime the immune system. A good *in vitro-in vivo* correlation was seen in test group till week 7, but onwards it was not thoroughly established probably due to fibrous encapsulation of microparticles *in-vivo* and further antigen load was not released.

CONCLUSION

The study has shown the potential of blend Polycaprolactone microparticles as alternative vaccine delivery system. Further work is needed to consolidate the potential benefits of this delivery carrier for vaccine innovation.

ACKNOWLEDGEMENTS

The author is thankful to Dr K S Jaganathan, Shantha Biotechnics Ltd, Hyderabad for providing HBsAg antigen and to Dr Yogesh Katare for technical guidance given for performing ELISA.

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