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Formulation and Evaluation of a Novel Needle-Free Hepatitis B Surface Antigen Delivery System

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Abstract: Problem statement: Hepatitis B is a major public health problem, killing 1-2 million people annually, despite the introduction of an effective vaccine in 1982. Approximately 400 million persons worldwide have chronic hepatitis B. This is due to problems associated with vaccine delivery, stability and cost. Hence the present challenge in Vaccinology is to develop safer, cheaper and easy-to-deliver forms of vaccines. A novel needle-free oral vaccine will be an ideal tool to fight with this silent killer disease. **Approach:** Hence a study was conducted with the aim of formulating and evaluating an effective oral HBsAg vaccine. HBsAg-loaded microspheres were prepared by microencapsulation using albumin as polymer and sodium taurocholate as permeation enhancer. Bacitracin and aprotinin were incorporated as protease inhibitors. After *in vitro* studies, optimized drug-loaded microspheres were encapsulated in hard gelatin capsules which were further enteric coated with cellulose acetate phthalate. *In vivo* experiments were conducted in rabbits. Control group was administered with 20 µg of HBsAg intramuscularly and the test groups were given different formulations of oral vaccine as per the current vaccination schedule of 0, 1 and 6 months. Immunogenicity was compared with IM injected vaccine control group. **Results:** HBsAg microspheres with albumin as polymer and bacitracin as protease inhibitor induced immune response with specific antibodies and cell mediated immunity better than the formulation with aprotinin as protease inhibitor ($p = 0.047$). Vaccine-related adverse events were absent in all immunized rabbits. Basic haematological, biochemical and physical parameters were within normal limits. **Conclusion:** The stability exhibited by this vaccine at room temperature can be extremely useful to overcome the incomplete vaccine coverage due to cold chain requirement. Impressive large logistical advantages and elimination of blood-borne infections are other major benefits, which may have a great impact on global HBsAg immunization acceptance and compliance and on hepatitis B disease burden.

Key words: Hepatitis B, HBsAg, Needle-free, vaccine

INTRODUCTION

From birth, human beings have been exposed to a continuous stream of microorganisms that have the potential to harm their bodily processes. In the battle with microbial invaders, their body tries to defend itself by the immune system. So the primary function of the immune system is protection of the body. Immune system can be primed for this defense through immunization. The objective of immunization is to provide long-lasting immunologic protection against exposure to infectious agents. It is recognized as the most cost-effective method for controlling and eradicating microbial infections. In general, immunization programs have led to the elimination/or control of several different infectious diseases including small pox, polio, measles, rubella, haemophilus influenzae type B disease, pertussis, tetanus and diphtheria (Rosenthal and Zimmerman, 2006).

Despite the introduction of effective vaccines against a variety of microbial pathogens, many diseases remain unconquered even with today's state-of-the-art

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techniques. This may be attributable to issues associated with vaccine delivery methods, stability at room temperature and the high cost of all immunization regimen. For e.g., a very effective vaccine was available for hepatitis B infection since 1982 and yet this disease is a major public health problem even today (Borges *et al*., 2008). About 400 million people are chronically infected globally (Simani *et al*., 2009). Every year, 1-2 million deaths are reported particularly from cirrhosis and carcinoma of the liver caused by hepatitis B virus (Lai *et al*., 2003). Hepatocellular carcinoma ranks fifth in overall frequency (fifth in men and eighth in women) and fourth in annual mortality rate (Kew, 2002). So there is a need for improvement in the management of chronic hepatitis B virus infection and vaccination is likely to be the cheapest and potentially the most beneficial treatment.

At this juncture, there is an urgent need to further improve vaccine research. The present challenge in Vaccinology is to develop better, safer, cheaper, easier-to-administer forms of vaccines. Most traditional vaccines are not cost-effective as they require the cold chain. "Cold chain" refers to the materials, equipment and procedures required to maintain vaccines within a specific temperature range (often 2-8°C) from the time that they are manufactured until they are given to patients (Weir and Hatch, 2004). Cold chain is estimated to cost vaccine programs globally between 200 and 300 million dollars year (Das, 2004).

Currently, sterile manufacturing and trained personnel are indispensable for vaccine administration. The needles and syringes used for vaccination pose threats for environmental issues and blood-borne infections. Studies have shown that 20-80% of new hepatitis B infections are due to unsafe injections (Alam *et al*., 2007). Needle-phobia which is commonly seen in children and in some adults, is another factor to refrain from vaccination. Increasing concern over bioterrorism also favor needle free vaccine delivery research (Levine and Sztein, 2004). Hence development of a new generation of vaccines that can be effectively administered by simple, economic and practical immunization procedures is essential.

A novel needle-free oral vaccine will be an ideal solution to tackle the hepatitis B menace. Because of the many advantages of needle-free vaccine delivery, public health organizations like World Health organization, the Global Alliance for Vaccines and immunization and the Centers for Disease Control and Prevention support this novel idea. Oral vaccines have the advantages of ease of storage, dispensation and consumption. It has been recognized as the only economically feasible approach to mass vaccination (Giudice and Campbell, 2006). Impressive logistical advantages of orally administered vaccines were exemplified by two National vaccination Days in 1996, when 121 million Indian children were vaccinated against polio at 650,000 centers (Bloom and Widdus, 1998). The number of worldwide polio cases has declined 99.7% in 2004, largely due to the use of the Sabin oral polio vaccine (Giudice and Campbell, 2006). However it has been shown that it is very difficult to obtain a protective immune response following oral vaccination, the live-attenuated polio vaccines are being one of the few exceptions (Holmgren and Czerkinsky, 2005). For this reason, only few vaccines currently approved for human use are being administered orally.

Unfortunately the preparation of a single oral formulation is not an easy task. The most important obstacle related to the efficacy of oral immunization is the adverse environment of the gastrointestinal tract rich in acids and enzymes, which are capable of destroying the antigen. Still we have hopes to have an ideal oral formulation with developing key technologies in this 21st century. Last century has contributed many innovative delivery technologies. Some of those past and present technologies are microencapsulation (Cole *et al*., 2007), lipid nanoparticles (Gasco, 2007; Joshi and Muller, 2009), cell interior delivery (Tarrago-Trani and Storrie, 2007), ultrasound mediated drug delivery (Frenkel, 2008), DNA vaccine carriers (Hassett *et al*., 1999), thiomers (Bernkop-Schnurch, 2005), TLR agonists (Baldwin *et al*., 2009), plant-based vaccines (Howard, 2004), cell-based drug delivery (Pierige *et al*., 2008), lectin-mediated drug targeting (Bies *et al*., 2004), liposomes drug delivery (Huang, 2008).

The technique of microencapsulation is widely applied in pharmaceutical industries to obtain the controlled release of drug (Li *et al*., 2008). It promises an increase in shelf-life of a vaccine and offers the flexibility of controlled release kinetics of the administered drug. Microspheres have received a great deal of attention as antigen carriers for oral administration due to their reported ability to protect the antigen denaturation by bile salts, low p^H and high levels of degradative enzymes in the gut and intestine. While celebrating the success of oral polio drops in mass immunization programs, there is an urgent need for developing an effective oral vaccine against hepatitis B, which is a major killer disease in the developed and developing countries. This may overcome the shortcoming of compliance in the existing effective intramuscular HBsAg vaccine and make it a success story in the field of Vaccinology.

Hence this study was performed aiming to formulate a needle-free oral vaccine for hepatitis B infection and evaluate its effectiveness by comparing the immunogenicity of intramuscular and oral administered vaccine groups *in vivo*. Other major objective was stability studies to evaluate the effect of different storage conditions.

MATERIALS AND METHODS

Materials and suppliers: Bovine serum albumin and Aprotinin from Sigma-Aldrich Chem, Co. USA, Hepatitis B surface antigen from Shantha Biotech Pvt. Ltd. Hyderabad, Bacitracin from Hi-Media Laboratories Pvt. Ltd., HBsAg and anti HBs kits from Ranbaxy Laboratories, Interferon gamma and Interleukin 4 test kits from Ray Biotech Inc. All other chemicals and reagents were of analytical grade and purchased from commercial vendors.

Preparation of HBsAg-albumin microspheres: The method followed for the preparation of albumin microspheres was emulsion-solvent evaporation method as described by Thanoo *et al*. (1992). Albumin was dissolved in distilled water at room temperature and the aqueous solution of the drug (hepatitis B surface antigen) was incorporated into this solution using homogenizer. The oral adjuvants such as permeation enhancer sodium taurocholate and protease inhibitor such as aprotinin, bacitracin were incorporated and mixed uniformly using homogenizer. The dispersion was transferred into a beaker containing 100 mL liquid paraffin. Span-80 was used as emulsifying agent at a concentration of 2%. After homogeneous, stable emulsion was formed, Glutaraldehyde Saturated Toluene (GST) was added as cross-linking agent at varying concentrations at specified time intervals. This mixture was stirred at specified speed using a mechanical stirrer for 5 h. The suspension was centrifuged and microspheres were collected, washed with n-hexane until the oil phase was removed. Finally the microsphers were collected, vacuum dried for suitable time and stored in an airtight, amber colored container.

Detection and quantification of antibodies to HBsAg (anti HBs):

ELISA technique: Bioelisa anti-HBs is a direct immunoenzymatic method of the sandwich type. Samples to be analyzed were incubated in wells of a microplate coated with highly purified HBsAg. If a sample contains anti-HBs, it will bind specifically to the HBsAg in the well. After washing to remove the residual sample, HBsAg conjugate to peroxidase was added. After this second incubation and washing, an enzyme substrate containing a chromogen was added. This solution will develop a blue colour, if the sample contains anti-HBs. The blue color changes to yellow after blocking the reaction with sulphuric acid. The intensity of color is proportional to the concentration of anti-HBs present in the test preparation. The absorbance was taken at 450 nm in the ELISA reader. The concentration of antibodies in the sample was calculated by interpolation from the calibration curve.

Detection of Hepatitis B surface Antigen (HBsAg) by ELISA test: Bioelisa HBsAg color is a direct immunoenzymatic method of the sandwich type in which guinea pig anti-HBs antibodies coated to microplate wells act as the capture antibody and goat anti-HBs antibodies marked with peroxidase serve as conjugate antibodies. The samples to be analyzed were incubated in the antibody-coated wells. After washing to eliminate any unbound material, goat anti-HBs conjugate to peroxidase was added to the wells and allowed to react with the antigen-antibody complex formed in the first incubation. After a second incubation and subsequent washing, an enzyme substrate containing a chromogen was added. The substrate will develop a blue color if the sample is positive for HBsAg. Stop solution was added and the absorbance was taken at 450 nm in ELISA.

Detection of Interferon gamma (IFN γ) by ELISA method: After preparing all reagents, samples and standards as instructed in the test manual, the plate was incubated for 2.5 h at room temperature with standards and samples in the assigned wells. After discarding the solution and washing, biotinylated antibody was added to each well and incubated for 1 h at room temperature. Washing was repeated and streptavidin solution was added. The plate was incubated for 45 min at room temperature. Following washing, TMB one-step substrate reagent was added to each well. After 30 min of incubation at room temperature in the dark, stop solution was added and absorbance was taken at 450 nm immediately. Standard curve was plotted on log-log graph paper. The concentration of IFN γ in the test samples was calculated by interpolation from the calibration curve.

Detection of Interleukin 4 (IL 4) by ELISA method: Standards and samples were pipetted into the wells coated with antibody specific for IL 4. After incubation,

the wells were washed and biotinylated anti IL 4 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted to the wells. After incubation and washing TMB substrate solution was added. The plate was incubated for 30 min and stop solution was added. Absorbance was taken at 450 nm. Log-log paper was used to plot the standard curve and the concentration of IL 4 was obtained by interpolation.

Physicochemical characterization: The sizes of the microspheres were determined by light microscopy using calibrated eye piece micrometer. A total of about 200 microspheres were considered for size distribution. The entire data was analyzed for mean diameter, size range. To determine the entrapment efficiency (antigen content) and yield, a known quantity of microspheres was triturated and kept for 48 h under mechanical agitation in double distilled water. The resultant solution was centrifuged and supernatant solution was diluted suitably with double distilled water. The protein content was estimated. The drug content was expressed as the amount of drug encapsulated in a unit weight of microspheres. The drug content of each sample was determined in triplicate and results averaged. The yield of microspheres can be calculated by taking the total weight of the polymer, antigen, additives and cross linking agents against the total weight of the microspheres after drying.

***In vitro* drug release studies:** A known quantity of drug loaded microspheres was suspended in 10 mL PBS in 20 mL capacity stoppered vial which was placed in shaker waterbath maintained at 37±1°C at a speed setting of 25 cycles min^{-1}. The release study was carried out for a period of 36 h. Aliquots of samples were withdrawn at regular predetermined time intervals, filtered and analyzed for total protein content against appropriate blank.

***In vivo* evaluation:** *In vivo* evaluation was carried out for the antigen loaded microspheres. The animals (New Zealand White Rabbits) were obtained from Sri Venketeshwara Enterprices Bangalore. The experimental protocol for all the *in vivo* studies was approved by the Institutional Animal Ethics Committee (vide letter number-IAEC/KMC/06/2005-06) which is an approved body under the Council for the purpose of control and supervision of experiments on animals, Ministry of Social Justice and Empowerment, Government of India. The animals were maintained under controlled conditions of temperature and humidity. They were fed balanced diet. Water *ad libitum* was available always.

Antigen administration: Three groups of animals each having six rabbits (age range 6-12 months) were taken for the *in vivo* studies. One group was given intramuscular injection of 20 µg HBsAg which was designated as formulation 1. The enteric coated capsules containing antigen loaded microspheres equivalent to 40 µg HBsAg were administered to overnight fasting animals. Three doses of antigen were administered to each group of animals. One month after the first dose, second dose was given. Third dose was given after six months of first dose (vaccine schedule-0, 1 and 6 months). The antigen administration modalities with other details are given in Table 1.

Blood sample collection: Prior to the administration of antigens, 2 mL blood was drawn from the marginal vein of the rabbit ear and these samples were designated as 0T. After administering both HBsAg I/M injections and capsules, blood samples were withdrawn after 14 days and they were designated as 1T. After administering the second dose of both I/M and oral vaccines to the respective groups (one month after the first dose), blood samples were withdrawn on the 6th day and they were designated as 2T.

After administering the third dose of both vaccines to the respective groups (6 months after the first dose) blood samples were withdrawn on the 6th day and they were designated as 3T. After one year of initial vaccine delivery, blood samples were collected from all rabbits and designated as 4T (Table 2). The test parameters and the samples used are given in Table 3.

Table 1: Experimental protocol used in the *in vivo* studies in animals

Groups	Formulation code	Formulation	route of administration
1	I	20 µg HBsAg	I/M
2	II	40 µg HBsAg + Albumin + Bacitracin + ST	Oral
3	III	40 µg HBsAg + Albumin + Aprotinin + ST	Oral

I/M: Intramuscular; ST: Sodium Taurocholate

Table 2: Sample designation and description

Sample designation	Description
0T	Zero dose before vaccine administration
1T	14th day after first dose
2T	6th day after second dose
3T	6th day after third dose
4T	1 year after first dose

Table 3: Test parameters and sample designation

Test parameters	Sample designation
HBsAg	0T and 1T, all samples
Anti HBs	0T, 1T, 2T, 3T, 4T, all samples
IFN-γ, IL-4, HB, TLC, DLC, TP, A/G ratio	0T, 2T, all samples
Body weight	0T, 3T, all samples

HB: Haemoglobin; TLC: Total Leucocyte Count; DLC: Differential Leukocyte Count

Reactogenicity studies: To assess the reactogenicity of the vaccine formulations, all rabbits were physically examined on all vaccination days and further for 3 days following each vaccination. They were monitored for body temperature prior to each vaccination and for 3 consecutive days.

Stability studies: Optimized drug loaded microsphere formulations were sealed in 10 mL amber colored vials and maintained in different temperature and humidity conditions for a period of three months. At periodic time intervals, known amount of samples were withdrawn and analyzed for protein content. Drug loaded microspheres were encapsulated in hard gelatin capsule which was further enteric coated with cellulose acetate phthalate. The stability studies of these enteric coated capsules were also done for a period of one year. Finally, the capsules were analyzed for changes in morphological characters and *in vitro* release profiles.

RESULTS

Result (*in vitro* studies):
Physicochemical characterization: The mean diameter for albumin microspheres was found to be 23.16±3.84 μm. Entrapment efficiency was 61.45%

***In vitro* antigen release studies:**
Stability studies:
***In vitro* antigen release studies:**
Result (*in vivo* studies):

- HBsAg status: All samples were negative
- Anti HBs concentration
- Interferon gamma quantitation
- Interleukin 4 quantitation
- Haemoglobin estimation: Significant difference was not observed in any of the groups ($F = 1.845$; $p = 0.194$)
- Total Leucocyte count: Despite the statistically significant differences between the groups ($F = 8.393$; $p = 0.011$), clinical significance was not ruled out
- Differential Leucocyte count
  - Heterophils: Percentage of mean in 0T was 51±5 and 48±4 in 2T samples ($F = 9.671$; $p = 0.007$). Statistical significance was observed, but this is within the normal range of heterophils in rabbits (40-70%)
  - Lymphocytes: 0T and 2T mean values were 45 and 48 respectively ($F = 19.266$; $p = 0.001$). Normal range of lymphocytes in rabbits is 43-80%
  - Monocytes: Statistical and clinical significance was not observed ($F = 0.676$; $p = 0.424$)
  - Eosinophils: Pre and post vaccination results showed statistical significance ($F = 5.556$; $p = 0.032$). Since the values were in normal range (0-2%) clinical significance was not found
- Total protein, albumin, globulin and A/G ratio: The means observed for total protein in 0T was 6.5 g dL^{-1} and in 2T, it was 6.8 g dL^{-1} ($F = 39.286$; $p = <0.001$). No change in concentration was noted in the albumin levels ($F = 0.220$; $p = 0.646$). There was a significant change in the globulin level ($F = 19.41$; $p = 0.001$) and in the albumin/globulin ratio ($F = 6.612$; $p = 0.021$)
- Body weight: Body weight was taken for all animals prior to the first dose of vaccination and after the last dose of vaccine administration ($F = 808.962$; $p = <0.001$)
- Body temperature: All groups of animals showed normal body temperature
- Statistical analysis was done by students T-test both paired and independent

DISCUSSION

Hepatitis B is a vaccine preventable disease. The most effective way to prevent this infection is administration of hepatitis B vaccine. A comprehensive strategy to eliminate hepatitis B virus includes universal vaccination of infants at birth. Yet so far, we could not achieve the desirable result. We have to go miles in this field of exploring the effective HBsAg vaccine to overcome the roadblocks of compliance and coverage.

Most human pathogens enter the body through mucosal surfaces including respiratory, gastrointestinal and genitourinary tracts and colonize these sites causing diseases. More than 90% of all human infections begin at mucosal sites. Mucosal immunization is a more effective way of inducing immune responses at the mucosal surfaces than other administrative route such

as injection. However, with very few exceptions, all currently licensed human vaccines are administered by injection. Mucosal immunization provides several advantages including: induction of systemic immune responses, generation of protective immunity at mucosal sites distant from the immunization site, inexpensive, safe and easy administration and induction of slower age-related immune dysfunction than systemic responses (Lee *et al*. 2005).

The current strategy for the induction of a mucosal immune response involves the use of particulate delivery systems. Formulation of antigens into particulate carrier systems offers the potential of optimizing delivery to immune responsive sites and also protection of the antigen against proteolytic degradation in the gastrointestinal tract. Different carriers are employed for this purpose by different people. In our previous study we have used chitosan as the carrier (unpublished data). It has been found that chitosan is a good polymer for making HBsAg microspheres and inducing immune response.

Present study focused on albumin as the vaccine carrier. Albumin microspheres have been extensively investigated for drug targeting to various organs (e.g., lungs) and tissues because of their biodegradability, biocompatibility and ease of preparation (Sahin *et al*., 2002). To prevent the degradation of antigen, protease inhibitors like aprotinin and bacitracin were incorporated. Sodium taurocholate served as permeation enhancer. The emulsion solvent evaporation method was used to prepare the albumin microspheres. HBsAg loaded albumin microspheres exhibited good morphological characteristics with smooth boundary and mostly spherical ones were observed by light microscopy. However occasional deformed and discrete particles were seen. They were analyzed for mean diameter. The mean diameter for albumin microspheres was found to be 23.16±3.84 μm.

The emulsification coupled with solvent evaporation procedure resulted in a product yield varying from 60-75%. The method used for collection of the microspheric product would have considerable influence on the total yield of the product. The method followed in our experiment was centrifugation for collection of microspheres. The washing was accomplished by resuspending the pellet containing the encapsulated product and centrifugation again. This cycle repeated a number of times till a desirable product was obtained. Good drug loading was achieved for the different albumin formulations. Some of the antigen was lost to the external phase during preparation and recovery. The results also indicate that the entrapment efficiency of microspheres depends on the preparation conditions. Uniform and spherical albumin microspheres showed entrapment efficiency up to 61.45%

The release pattern observed from different formulations of albumin microspheres was biphasic, characterized by an initial burst effect followed by slow release. Around 15% of the drug was found to be released in the first 6 h. The *in vitro* antigen release from HBsAg loaded albumin microsphere Formulations A, B and C were given in Table 4 and Fig. 1. At the end of 36 h, the antigen release from Formulation A, B and C were 52.12, 52.68 and 52.34% respectively. This initial result could be due to the loosely bound or surface-embedded drug. The subsequent slow release may be because of the release medium being diffused into the polymer matrix, whereby drug may have diffused out of the microspheres. The results also showed that there is no significant difference in antigen release between different formulations of albumin microspheres. But a slight increase in antigen release is seen in case of Formulation-B.

Table 4: Cumulative percentage of drug release from formulation A, B and C

	Drug release (%)		
Time (h)	Formulation-A	Formulation-B	Formulation-C
0	0.00	0.00	0.00
1	4.63	4.62	4.54
3	7.46	7.34	7.22
6	14.92	15.98	15.04
9	18.48	18.12	18.24
12	24.63	25.68	25.14
15	30.75	31.34	31.00
18	35.78	35.06	35.81
24	41.24	42.16	41.17
27	45.38	46.64	45.42
30	48.46	49.28	49.27
33	51.16	51.12	51.04
36	52.12	52.68	52.34

A: Albumin + 40 μg HBsAg; B: Albumin + 40 μg HBsAg + Bacitracin + ST; C: Albumin + 40 μg HBsAg +Aprotinin + ST

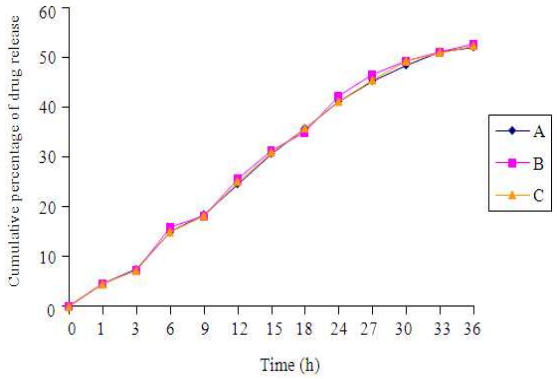

Fig. 1: Comparison of cumulative percentage of drug release from Formulation A, B and C

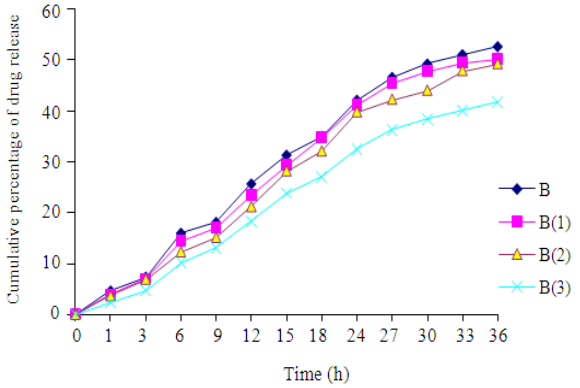

Fig. 2: Comparison of cumulative percentage drug release of Formulations-B, B(1), B(2) and B(3)

Table 5: Cumulative percentage of drug release of Formulation B, B(1), B(2) and B(3)

	Percentage of cumulative drug release			
Time (h)	Formulation B	Formulation B(1)	Formulation B(2)	Formulation B(3)
0	0.00	0.00	0.00	0.00
1	4.62	3.92	3.68	2.40
3	7.34	7.02	6.94	4.82
6	15.98	14.48	12.32	10.28
9	18.12	17.12	15.16	13.16
12	25.68	23.48	21.24	18.32
15	31.34	29.36	28.26	23.90
18	35.06	34.80	32.14	27.16
24	42.16	41.16	39.86	32.58
27	46.64	45.52	42.32	36.34
30	49.28	47.86	44.10	38.50
33	51.12	49.52	47.98	40.18
36	52.68	50.24	49.32	41.84

B: Albumin + 40 μg HBsAg + Bacitracin(SS) + ST; B(1): Formulation B at 4±2°C; B(2): Formulation B at RT; B(3): Formulation Bat 40±2°C/75±5%RH

Table 6: Anti HBs of Gp 1, 2 and 3 (mIU mL^{-1})

Gp	0T	1T	2T	3T	4T
1	0.73	1213.33	1455.50	1546.66	1547.33
2	0.68	42.26	48.13	49.08	48.93
3	0.70	18.21	20.36	21.43	20.18

Gp 1: Control (I/M); Gp 2: HBsAg, Albumin, Bacitracin, Sodium taurocholate; Gp 3: HBsAg, Albumin, Aprotinin, Sodium taurocholate

Estimation of protein content revealed that there was a decrease in the drug content of microspheres which were stored at 40±2°C/75±5% RH and no appreciable change in drug content of the formulations stored at RT and refrigerator. These results of *in vitro* release studies indicate that there was no appreciable change in drug release from formulation stored at RT and 4±2°C (Table 5 and Fig. 2). There was a difference between the above formulations and formulation stored at 40±2°C/75±5%RH.

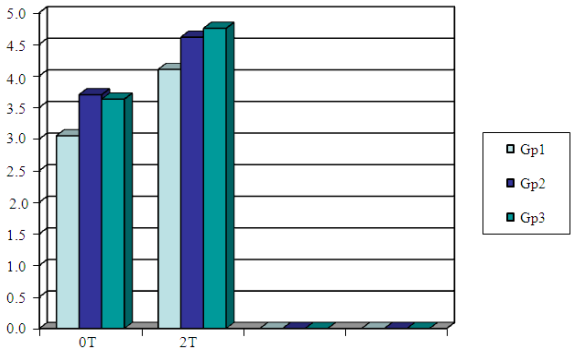

Fig. 3: Comparison of IFN γ concentration of 0T and 2T samples (pg mL^{-1})

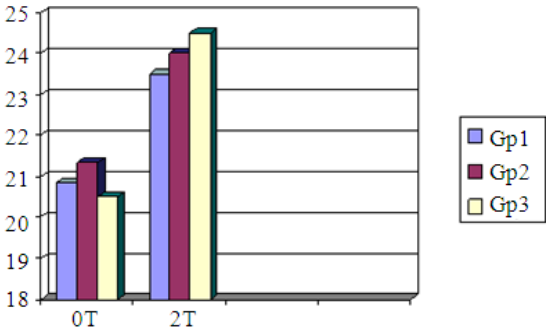

Fig. 4: Comparison of IL 4 concentration of 0T and 2T samples

Measurement of specific antibody production is a critical feature in vaccine design. Highest circulating antiHBs occurred in response to intramuscular injection. The antibody response was lower as compared with intramuscular injection, but in both formulations seroprotection (≥10 mIU mL^{-1}) was evident (Table 6). The formulation of vaccine with albumin, bacitracin and sodium taurocholate showed significant humoral immune response ($F = 5.112$; $p = 0.047$). Trials were conducted on HBsAg free animals for induction of immunity. The given antigen was utilized by them to stimulate immune response.

Since HBsAg is T cell-dependent glycoprotein, defects in the Th cell function, either Th1 or Th2 would result in failure of immune response to this antigen (Jafarzadeh and Shokri, 2003). Vaccination with HBsAg induces protective immunity through T cell dependent production of anti HBs antibody. In this study, both Th1 and Th2 responses have been investigated in all vaccinated groups. IFN γ secretion is a hall mark of Th1 lymphocytes. The measurement of IFN

γ production in response to specific antigen stimulation is frequently used in experimental studies to evaluate immune competence. In this study, the mean titre observed was 3.4 pg mL^{-1} in 0T (Fig. 3) and 4.4 pg mL^{-1} in 2T ($F = 118.262$; $p = <0.001$). IL 4 is secreted by Th2 cells, mast cells and a subset of NK cells. Secretion of IL 4 is a hallmark as well as an indicator of Th2 differentiation in T cells. We observed the mean titer of IL 4 in 0T as 20 pg mL^{-1} (Fig. 4) and 24 pg mL^{-1} in 2T samples ($F = 56.000$; $p = <0.001$). The production of Secretory Component (SC) is up regulated by Th1 (IFNγ) and Th2 (IL 4) cytokines. The SC is essential for the dimeric or polymeric forms of IgA to become secretory IgA, which is most important in mucosal immunity and oral immunization (Jafarzedeh and Shokri, 2003).

Safety is highly important in vaccine studies as vaccine is administered to healthy individuals and there is zero tolerance in this regard. In our study, vaccine-related adverse events were not reported in the animals. All groups showed normal body temperature (37-39.4°C) throughout the intervention period. Post vaccination reactogenicity was not observed. Body weight maintained as per the physiological response of the animals in the growing phase. All rabbits were healthy and active. Basic haematological and biochemical parameters were well within the normal range.

By analyzing all the results it can be concluded that between the two protease inhibitors, bacitracin showed a higher inhibitory activity than aprotinin. Sodium taurocholate due to its permeation enhancing effect has improved the permeation of antigen and this has also resulted in immunogenicity. It is evident from this study that HBsAg microspheres with albumin as polymer can induce seroconversion and seroprotection with specific antibodies and cell mediated immunity in oral immunization. Most traditional vaccines are not cost effective as they cannot be stored at room temperature and require the cold chain. The unsolved difficulties of maintaining the cold chain lead to ineffective delivery of actually effective vaccines. The stability exhibited by the microspheres for a period of one year at room temperature is remarkable. The thermolability of the present conventional vaccines can be overcome with the introduction of this type of oral immunization.

CONCLUSION

This study is a promising development in the arena of mucosal immunization and specially in oral immunization of HBsAg. This needle-free vaccination requires further more elaborate clinical studies for safety and efficacy. However 'vaccines without needles' is desired by all both young and old. This offers improved safety, better compliance and coverage, easier and fast painless vaccine delivery and cost reduction. There is no better substitute for mass vaccination than the oral mode of administration.

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