



Bioneedles as alternative delivery system for hepatitis B vaccine

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ARTICLE INFO

Article history:

Received 26 February 2010

Accepted 30 June 2010

Available online 8 July 2010

Keywords:

Hepatitis B surface antigen

Bioneedle

Freeze drying

Stability

Adjuvant

Formulation

ABSTRACT

An alternative vaccine delivery system for needle injections is the Bioneedle. Hepatitis B surface antigen (HBsAg) was formulated with Bioneedles. Three formulations were used: plain antigen, HBsAg adjuvated with aluminum hydroxide and HBsAg with LPS-derived *lpxL1*. Bioneedles with HBsAg-*lpxL1* were the most stable and the most immunogenic formulations. The conventional liquid alum adjuvated vaccine lost 40% of its antigenicity after 1 week at 50 °C whereas the HBsAg-*lpxL1* Bioneedles showed no significant decrease after 3 weeks at 50 °C. *In vivo* studies revealed that the HBsAg-*lpxL1* Bioneedle formulations induced comparable IgG titers as conventional liquid formulations after 2 immunizations, but higher IgG2a titers were found already after 1 immunization. The *in vivo* and *in vitro* studies showed that the Bioneedle is an attractive alternative for needle injections of HBsAg vaccines.

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1. Introduction

Most vaccines are delivered as a liquid formulation by using syringes and needles. Despite their common use, regular injections have several limitations such as the risk of needle stick injuries and the reuse of needles and syringes. Injections, although safe in the developed countries and relatively painless, may cause serious stress, fear and concern in children as well as parents [1–4]. This may lead to reduced participation in vaccination programs. Therefore, in Western countries only limited injections per immunization session are given. In e.g. The Netherlands the policy is to give children no more than two injections per session. Since children, until the age of nine years, already receive 14 injections in 7 sessions, the vaccination program is considered full. The further extension of the vaccination program leads to more injections or more complex antigen mixtures in the form of combination vaccines. The development of combination vaccines is difficult and expensive [5,6] due to possible immunological interference.

To address all the limitations of needle injections, research is being carried out on alternative delivery methods and alternative routes [7]. Alternative routes can be mucosal routes (oral, pulmonary, nasal) [8–11] or dermal [12–15]. These routes can be reached without needles; vaccines can be delivered via liquid formulations, functional foods, droplets, sprays, microneedles, or patches. For these routes lipid- and polymeric based nanoparticle and microparticle delivery systems can be used to protect the antigen and to enhance the delivery.

The conventional method for vaccine delivery is subcutaneous or intramuscular injection. Needle free approaches for subcutaneous and intramuscular delivery can be liquid jet injectors and powder jet injections [16,17]. Less common approaches are implants from biological material incorporating the vaccine. Examples are silicone implants [18], sugar-glass needle [19] and the Bioneedle [20].

Bioneedles are small hollow mini-implants fabricated from biodegradable polymers. These mini-implants are filled with liquid formulations and freeze dried. The freeze dried Bioneedles can be injected using compressed air. Once beneath the skin, subcutaneous or intramuscular, the biodegradable mini implant dissolves and the antigen is released, being reconstituted by the body fluids. It has been shown that Bioneedles can penetrate through the skin causing less external skin damage than with conventional needles [20]. A phase 1 clinical study in 18 volunteers (van de Wijdeven et al., publication in preparation), each receiving 2 Bioneedles subcutaneously in the arm, showed that the Bioneedles were safe and showed minimal adverse local and systemic effects. Previous research has shown that Bioneedles filled with tetanus toxoid induced a same antibody response in mice with four times less antigen [21]. The tetanus toxoid Bioneedles showed a better thermostability than the conventional liquid formulations. These stable formulations diminish the need for a cold chain during transport and for short term storage. With the Bioneedle a fast delivery procedure is possible without the risk of cross contamination, since there are no needles and syringes that can be re-used or cause needle stick injuries. No needles are perceived which is expected to lead to less stress and fear in children as well as in parents. For Western countries this is a big advantage over the conventional needles. For developing countries, the safe

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immunization practice with the Bioneedles and the lack of need for a cold chain are important advantages.

When developing a new delivery system for vaccines, the most important requirement is that the delivery via the new system is at least as effective as the conventional delivery system. Besides the advantages of needle free delivery systems, as described above, aspects such as improved immunogenicity by using adjuvants, dose reduction and higher stability of the formulation are all advantages that make the alternative even more attractive.

In this paper hepatitis B surface antigen has been formulated in Bioneedles and compared *in vitro* and *in vivo* with conventional injections. *LpxL1* has been compared to aluminum hydroxide as an adjuvant and the heat stability and dose reduction has been evaluated for liquid and Bioneedle formulations.

2. Materials and methods

Three sources of yeast derived, commercial GMP grade, hepatitis B surface antigen (HBsAg) bulk material have been used. In the text the three sources are referred to as manufacturer A, manufacturer B and manufacturer C.

Control vaccine: Engerix-B 20 µg/1 ml hepatitis B vaccine (adjuvated to Al(OH)₃) from GSK, Rixensart, Belgium.

Al(OH)₃, Alhydrogel 2% was obtained from Brenntag Biosector, Denmark.

lpxL1 LPS was obtained from ImsaVac, Bilthoven, The Netherlands.

Bioneedles were produced of thermoplastic starch according to Bioneedle Technologies Group's proprietary procedures using injection moulding. Bioneedles are 16 mm long and 1.2 mm wide. The cavity inside the Bioneedles has a volume of 4.0 to 4.5 µl.

2.1. Formulations

All formulations contained 2 µg HBsAg. The adjuvants used were either 50 µg Al(OH)₃ or 0.5 µg *lpxL1*. Formulations with only HBsAg (HepB), with HBsAg adsorbed to Al(OH)₃ (HepB + Al) or with HBsAg adjuvated to *lpxL1* (HepB + *lpxL1*) have been prepared. Liquid formulations (L) for subcutaneous injections were formulated in a volume of 500 µl. Liquid formulations for intramuscular injections were formulated in a volume of 100 µl. The freeze dried formulations (F) were formulated in 500 µl and freeze dried using a Leybold GT 4/6 freeze dryer. Bioneedles (B) were filled, by using a specially designed filling apparatus, with liquid formulations containing 5% w/w of D-trehalose di-hydrate (purchased from Biosynth, Switzerland) as a stabilizer. The filled Bioneedles were frozen on a plate at minus 45 °C. Subsequently the freeze dried Bioneedles were transported to a Leybold GT 4/6 freeze dryer with a shelf temperature of minus 45 °C and freeze dried. Freeze dried Bioneedles were individually stored in vials, closing these under ambient air and relative humidity, using rubber stoppers and alucaps.

2.2. Hepatitis B antigen ELISA

Bioneedles were dissolved by incubation in 1 ml water, adding 5 µl of a 120 kilonovo units/ml solution of 1,4- α -D-glucan glucano hydrolase (Novozymes, Denmark) and 5 µl of a 330 units/ml solution of pullulanase (Novozymes, Denmark) during 1 h at room temperature. A kilonovo unit is the amount of enzyme which degrades 4870 mg starch dry matter, Merck soluble amyllum, per hour under standard conditions.

For HBsAg quantification a commercial kit of Abbott, Murex HBsAg version 3, was used according to the manufacturers specifications. In short, the sample was pre-incubated in microwells (3 or 4 dilutions) pre-coated with a mixture of mouse monoclonals specific for different epitopes on the 'a' determinant of HBsAg. Affinity purified goat antibody to HBsAg conjugated to horseradish peroxidase was then added. After washing, to remove sample and unbound conjugate,

peroxidase activity is detected by addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate and hydrogen peroxide. The enzyme reaction was terminated with sulfuric acid. Substrate conversion was determined spectrophotometrically at 450 nm.

Each ELISA plate contained a reference HBsAg sample (liquid formulation stored at 4 °C) for calculation of the antigen concentration in the samples.

2.3. Circular dichroism

Liquid and reconstituted freeze dried HepB formulations, in the presence and absence of *lpxL1* were analyzed with a Chirascan™ Circular Dichroism Spectrometer (Applied Photophysics, UK). Liquid and freeze dried formulations were stored for 3 weeks at 4 °C, 37 °C, 50 °C and 60 °C prior to the measurements. Freeze dried formulations were reconstituted with water prior to the measurements. Far UV spectra were taken at 25 °C in the region of 200–260 nm. A protein concentration of 40 µg/ml was used in 1 mm Quartz cuvettes. The scan velocity was 0.1 nm/s with step lengths of 0.5 nm. The monochromator bandwidth was 1 nm. The CD spectra were corrected for buffer and smoothed with a smoothing factor of 5. Chirascan™ CDNN software was used to provide an estimate of the secondary structure. The software is based on algorithms designed for fitting CD spectra of proteins.

2.4. In vivo studies

All *in vivo* experiments were approved by the animal ethics committee of the NVI.

Prior to immunization, all animals were anesthetized with a mixture of 1-isoflurane, N₂ and O₂. In all experiments BalB/C mice obtained from Harlan (Horst, The Netherlands) of 10–14 g, were immunized via regular injection or with Bioneedles. Two weeks after primary immunization, blood was collected prior to a booster vaccination. Four weeks after the booster vaccination, all mice were sacrificed by bleeding.

Liquid formulations were either injected intramuscularly (I.M) in the rear leg, in a volume of 100 µl or subcutaneously (s.c.) in the groin, in a volume of 500 µl. Freeze dried formulations were reconstituted in 500 µl water prior to subcutaneous injection.

Bioneedles were implanted in mice by using a sterilized trocar with mandrin (1 in., 14 gauge). The procedure is identical to the application of electronic identification chips in animals, but instead of a chip, a Bioneedle is applied subcutaneously in the neck between the ears. In animals as small as mice, Bioneedles cannot be delivered intramuscularly.

Formulations used for the heat stability study were incubated for 3 weeks at either 4 °C, 37 °C 50 °C or 60 °C prior to immunization. For the dose response study, the hepatitis B doses used were 2 µg, 1 µg, 0.5 µg and 0.1 µg.

2.4.1. IgG, IgG1 and IgG2a ELISA

Polystyrene microtiter plates were coated with 0.2 µg/well HBsAg diluted in phosphate buffered saline (PBS), pH 7.2 (Gibco, Paisly, UK). The plates were incubated overnight at room temperature. The plates were washed with tap water containing 0.1% (w/v) Tween 80. Serum was added in series of three fold dilutions. All dilutions of serum were prepared in PBS containing 0.5% (w/v) BSA and 0.05% (w/v) Tween 80. After 2 h of incubation at 37 °C the plates were washed and goat anti mouse immunoglobulin conjugated to horse radish peroxidase (IgG-HRP, IgG1-HRP or IgG2a-HRP) from Southern Biotech, Birmingham, Ala, USA) was added in the concentration that was given by the manufacturer (1/5000). The conjugate was incubated for 1.5 h at 37 °C. After washing the plates, 100 µl TMB substrate solution (see above) was added to each well. The reaction was stopped after 10 min with 2 M H₂SO₄ and the absorbance was measured at 450 nm. The

titre was determined as the dilution factor at which the absorbance was 50% of the maximum absorbance (plateau value).

2.5. Statistics

Antibody titers are expressed as the mean \log_{10} titer of eight or ten independent observations plus the standard errors of the means. Antigenic recoveries are expressed as the mean of three independent observations, plus the mean standard errors of the means.

Statistical evaluations are done with a Student *t* test (one way ANOVA) by using a two tailed distribution and a two sample unequal variance.

3. Results

3.1. Liquid formulations vs. freeze dried formulations vs. Bioneedles

The commercial hepatitis B vaccine is a liquid vaccine of HBsAg adjuvated to $\text{Al}(\text{OH})_3$ and delivered intramuscularly (L[HepB + Al]_{IM}). Since Bioneedles are freeze dried formulations that can only be delivered subcutaneously in mice, the effect of freeze drying and the route of immunization on the IgG titer were evaluated with liquid vaccine formulations. The commercial available Engerix-B vaccine, an alum adjuvated vaccine, was used to evaluate the difference between subcutaneous and intramuscular injections. Intramuscular injections performed significantly better ($p = 0.003$) than subcutaneous injections.

To evaluate the effects of freeze drying, HBsAg was freeze dried, reconstituted in water and injected subcutaneously. The reconstituted freeze dried formulations induced comparable IgG titers as the liquid formulations injected subcutaneously but with liquid HepB formulations one non responder was found within the group. Liquid HepB formulations were tested several times and always showed one or two non responders after subcutaneous injection (Fig. 1).

3.2. Adjuvant

In a pilot experiment, Bioneedle formulations of HepB adjuvated with alum performed not as good (several non responders) as the conventional liquid vaccine delivered via the intramuscular route. To improve the Bioneedle formulations another adjuvant, the LPS derivative *lpxL1*, was evaluated as well as no adjuvant at all (Fig. 2). Mice were immunized with formulations containing no adjuvant, with aluminum hydroxide or with *lpxL1* as adjuvant.

With Bioneedle formulations containing alum comparable IgG titers were induced as with liquid formulations containing alum, (L[HepB + Al]_{sc}), but with non responders within the group. When *lpxL1* was used as an adjuvant or when no adjuvant was used at all, all mice responded in the group.

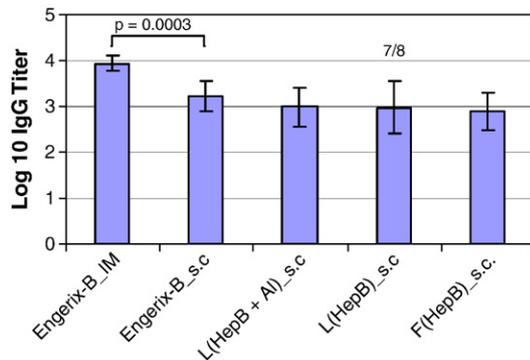


Fig. 1. IgG titers in mice ($n = 8$); L: Liquid formulations, F: freeze dried formulations, HepB: hepatitis B surface antigen of manufacturer C, Al: aluminum hydroxide, S.C: subcutaneous, I.M intramuscular. Engerix-B: commercial available vaccine from GSK.

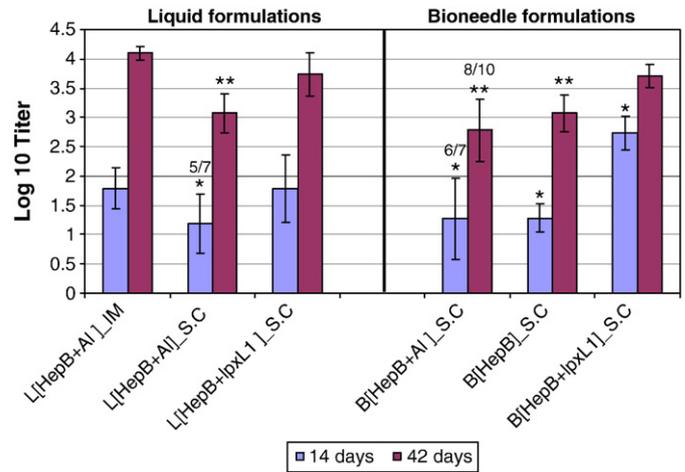


Fig. 2. IgG titers after one (14 days) and two immunizations (42 days). L: liquid formulation; B: Bioneedle formulation, HepB: HBsAg from manufacturer B, Al: $\text{Al}(\text{OH})_3$, S.C: subcutaneous, I.M: intramuscular. Asterisks (*) indicate titers that are significant different ($*p < 0.005$) from the liquid i.m. group for the titers after 14 days. Asterisks (**) indicate titers that are significant different ($*p < 0.005$) from the liquid i.m. group for the titers after 42 days. Titers are average titers of the responders. The lower number of animals after 14 days is due to insufficient serum from some animals to perform the ELISA test.

When *lpxL1* is present in either liquid or Bioneedle formulations, comparable IgG titers were induced as with the intramuscular conventional vaccine, L[HepB + Al]_{im}. After one vaccination (14 days) Bioneedle formulations with *lpxL1* showed the best immunogenicity results. All mice responded and the IgG titer was significantly ($p < 0.001$) higher as compared to the intramuscular L[HepB + Al]_{im} group. After 2 immunizations (42 days) the IgG responses were comparable to the responses in the L[HepB + Al]_{im} group.

The sera after 42 days were also analyzed for IgG1 and IgG2a antibody responses. Fig. 3 shows the IgG2a/IgG1 ratio. Formulations containing *lpxL1* showed significant higher IgG2a/IgG1 ratios as compared to the intramuscular group with alum. This was true for both Bioneedle formulations and liquid formulations. With Bioneedle formulations containing aluminum hydroxide no IgG2a could be detected.

3.3. Dose response

The dose response for conventional liquid formulations L[HepB + Al]_{im} was compared to the dose response of Bioneedle *lpxL1*-HBsAg formulations delivered subcutaneously. Fig. 4 shows that both the

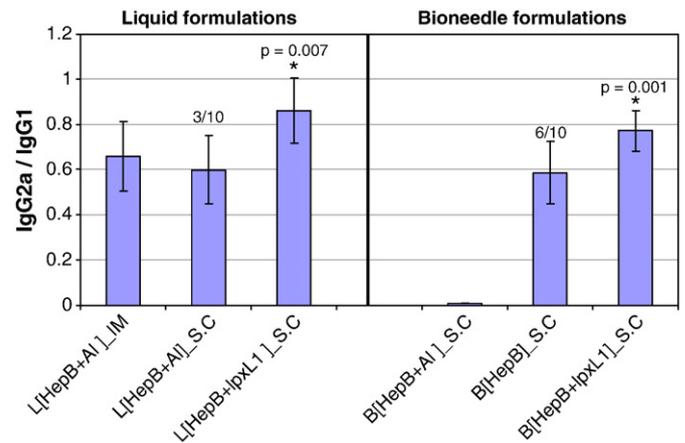


Fig. 3. IgG2a/IgG1 response after 42 days. L: liquid formulation; B: Bioneedle formulation, HepB: HBsAg from manufacturer B, Al: $\text{Al}(\text{OH})_3$, S.C: subcutaneous, I.M.: intramuscular. Asterisks (*) indicate titers that are significant different from the liquid i.m. group. Titers are average titers of the responders.

liquid formulations and Bioneedle formulations showed a plateau value for the IgG titer with 2, 1 and 0.5 μg HepB. For both formulations a significant decrease ($p < 0.005$) was only observed when formulations contained 0.1 μg HepB.

In order to discriminate between the subcutaneous and intramuscular routes, liquid formulations L[HepB + *lpxL1*] were injected both intramuscularly and subcutaneously (Fig. 5). Just like alum containing formulations (see Fig. 1), *lpxL1* formulations induced significant higher IgG titers after 42 days ($p = 0.012$) via intramuscular injections as compared to subcutaneous injections. Via the intramuscular route, *lpxL1* formulations (L[HepB + *lpxL1*]_{IM}) showed comparable immunogenicity to aluminum hydroxide formulations (L[HepB + Al]_{IM}). The L[HepB + *lpxL1*]_{sc} and B[HepB + *lpxL1*]_{sc} formulations showed in this experiment significant (p -values were respectively 0.0012 and 0.0011) lower IgG titers as compared to the L[HepB + Al]_{IM} formulations whereas in Fig. 2 comparable titers were induced with these formulations.

The IgG2a/IgG1 ratio for the liquid formulations containing aluminum hydroxide, L[HepB + Al]_{IM}, was significant lower than for all formulations containing *lpxL1*. All formulations containing *lpxL1* showed similar IgG2a/IgG1 ratio's irrespective of delivery route or of formulation (liquid or Bioneedle).

3.4. Heat stability of liquid formulations vs. Bioneedles

The stability of Bioneedle formulations without adjuvant and with *lpxL1* was determined. This was done *in vitro* with an antigen ELISA, to measure the recovery in antigenicity, and with circular dichroism for the α -helical content. *In vivo* studies were performed to measure the immunogenicity of heat treated samples.

3.4.1. Antigenicity of stressed antigen

Bioneedle formulations containing plain HepB or HepB + *lpxL1* and conventional liquid formulations of HepB adjuvated to alum were incubated at different temperatures. The antigenic recoveries are shown in Fig. 6.

The recoveries shown in Fig. 6 are average values of 3 experiments. In each experiment each formulation was incubated in duplicate or in triplicate at the different temperatures. After 1 and 3 weeks each sample

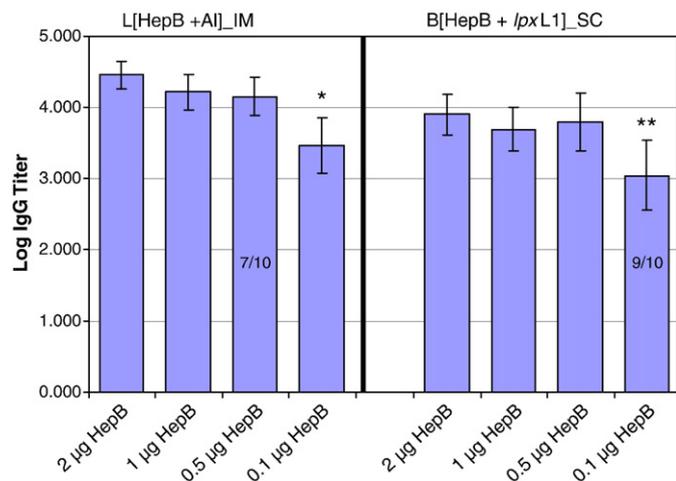


Fig. 4. Dose response. Antibody titers after 42 days (2 immunizations). Mice ($n = 10$) have been immunized with conventional formulations containing alum via i.m injections and with Bioneedle formulations (inserted subcutaneously) containing *lpxL1*. The dose of adjuvant is kept constant whereas the dose of antigen is variable (2, 1, 0.5 and 0.1 μg hepatitis B/dose). HepB: HBsAg from manufacturer A, Al: $\text{Al}(\text{OH})_3$, S.C: subcutaneous, I.M: intramuscular. Titers are averages of the responders. Asterisks (*) indicate titers within the L[HepB + Al] groups that are significant different from the L[2 μg HepB + Al]_{IM} group ($*p < 0.005$). Asterisks (**) indicate titers within the B[HepB + *lpxL1*] groups that are significant different from the B[2 μg HepB + *lpxL1*] group.

was measured in an antigen ELISA. The conventional liquid vaccine formulation L[HepB + Al] showed a significant decrease in antigenicity after 3 weeks at 37 °C. After 1 week at 60 °C a recovery of 40% was found and the recovery decreased to 20% after 3 weeks at 60 °C.

Bioneedle formulations containing only HBsAg retained all antigenicity after 3 weeks at 37 °C. A significant decrease ($p < 0.001$) was found after 3 weeks at 50 °C. After 1 week at 60 °C a recovery in antigenicity was found of 60%. Unlike the conventional formulation no further decrease in antigenicity was observed after incubation of 2 more weeks at 60 °C. Bioneedle formulations containing *lpxL1* showed excellent stability. After 3 weeks at 50 °C this formulation lost 10% of its antigenicity. Although a significant decrease in antigenicity was only found after 3 weeks at 60 °C a recovery in antigenicity of 60% was still found with the B(HepB + *lpxL1*) formulations whereas for liquid formulations (L[HepB + Al]) only 20% recovery was found.

3.4.2. Higher order structure of stressed antigen

Liquid formulations and freeze dried formulations of hepatitis B surface antigen in the presence and absence of *lpxL1* were stored for 3 weeks at 4 °C, 37 °C, 50 °C and 60 °C and the tertiary structure of the antigen was determined with circular dichroism.

As shown in Fig. 7, both liquid and freeze dried formulations of only HBsAg showed a decrease in α -helix content at higher temperatures. For liquid samples, L[HepB], a significant decrease ($p < 0.05$) in α -helix content was observed at 50 °C as compared to 4 °C, whereas for freeze dried samples, F[HepB], this significant decrease was only observed at 60 °C. When *lpxL1* was included in the formulations, no significant change in α -helix content was observed after 3 weeks storage at temperatures up to 60 °C. This was true for both liquid and freeze dried formulations. When liquid HBsAg was incubated overnight at 100 °C the protein denatured, resulting in a decrease of the α -helix content to only 25%.

3.4.3. Immunogenicity of stressed antigen

The immunogenicity and antigenicity of heat treated formulations is shown in Fig. 8. As shown in Fig. 6, a significant decrease in antigenicity was found for liquid formulations (54% recovery) and Bioneedle formulations of HepB (67% recovery) incubated for 3 weeks at 50 °C.

Both liquid formulations and Bioneedle formulations induced comparable IgG titers after heat treatment, regardless of antigenic recoveries. In the negative control group (overnight at 100 °C) which lost all antigenicity, immunogenicity also disappears. For both liquid formulations and for Bioneedle formulations a decrease in IgG2a was observed as compared to the conventional L[HepB + Al]_{im} group, although for the Bioneedle formulation this decrease was less evident ($p = 0.007$ for group L[HepB + Al]_{50 °C} and $p = 0.029$ for group B[HepB]_{50 °C}).

4. Discussion

Bioneedles have been tested *in vivo* in mice. The read out for the *in vivo* studies were antibody responses. For hepatitis B this is the correlate of protection [22]. In such small animals as mice, Bioneedles can only be delivered subcutaneously. Results have shown (see Figs. 1 and 5) that the subcutaneous delivery induces lower IgG titers than the intramuscular delivery. The study was designed to optimize Bioneedle formulations and to achieve at least comparable antibody responses as the conventional alum adjuvated vaccine.

Bioneedle formulations of conventional alum adjuvated hepatitis B (B[HepB + Al]) showed relatively low IgG titers and non responders. The lower performance of the freeze dried Bioneedle formulations with alum can probably be explained by the coagulation of aluminum hydroxide during freeze drying. The HBsAg is entrapped in the coagulated alum matrix and cannot be released resulting in a lower immunogenicity [23]. When HBsAg is freeze dried, reconstituted, and injected subcutaneously, comparable IgG titers (see Fig. 1) to liquid

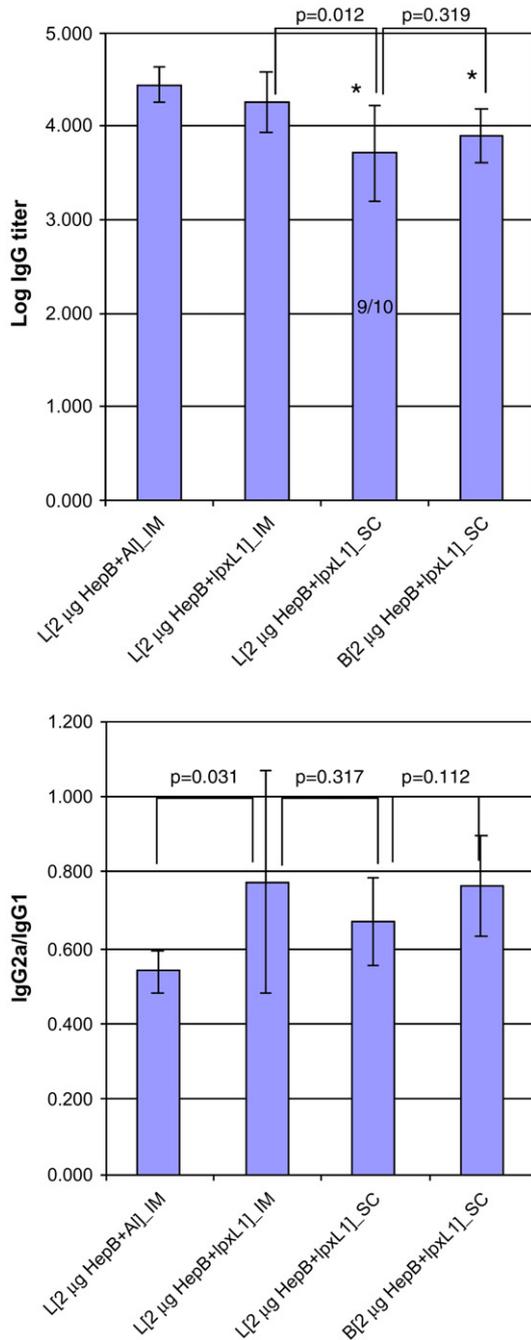


Fig. 5. IgG titers and IgG2a/IgG1 ratio. $N=10$. The effect of delivery route (intramuscular and subcutaneous), of the adjuvant (Alum or *lpXL1*) and of the formulation (liquid or Bioneedle) are compared. Titers are averages of the responders within a group. Asterisks (*) indicate titers that are significant different from the L[2 µg HepB + Al]_IM group. P -values between other groups are indicated in the graph. HepB: HBsAg from manufacturer A, Al: Al(OH)₃, S.C: subcutaneous, I.M: intramuscular.

alum adjuvated (L[HepB + Al]_SC) formulations were induced, showing that the freeze drying procedure has no effect on the immunogenicity of HBsAg. Freeze drying of HBsAg in Bioneedles, in the presence of trehalose, showed comparable immunogenicity results and therefore we conclude that HBsAg can be freeze dried in Bioneedles without loss of immunogenicity.

When *lpXL1* was included in the formulations, significantly higher IgG titers were obtained (see Fig. 2). With subcutaneous delivery of HepB + *lpXL1*, comparable titers were obtained as with conventional vaccine via the intramuscular group (L[HepB + Al]_IM). The high IgG titers are rather attributed to *lpXL1* as an adjuvant than to the

Bioneedles, since liquid vaccine containing *lpXL1*, injected subcutaneously, also performed with high IgG titers. These results were not confirmed in the dose response *in vivo* study (Figs. 4 and 5). Here, both liquid and Bioneedle formulations with *lpXL1* induced significant lower IgG titers than with the conventional alum adjuvated intramuscular vaccine. In the two *in vivo* experiments (adjuvant study and dose response study) different HBsAg sources have been used. In the adjuvant study (Fig. 2) HBsAg of manufacturer B was used whereas in the dose response study (Figs. 4 and 5), HBsAg of manufacturer A was used. Three formulations, L[HepB + Al]_im, L[HepB + *lpXL1*]_sc and B[HepB + *lpXL1*]_s.c, have been prepared with both HBsAg sources and are present in both *in vivo* studies. The formulations with *lpXL1*, L[HepB + *lpXL1*]_sc and B[HepB + *lpXL1*]_s.c, showed the same titers in both experiments. Differences were only observed in the groups receiving the conventional alum adjuvated vaccine via the intramuscular route. Apparently the alum adjuvated HBsAg of manufacturer A performed, via the regular i.m. route, superior to the alum adjuvated HBsAg of manufacturer B. This may explain the difference in the significant decrease in the dose response study and the non significant decrease in the adjuvant study of *lpXL1* formulations. The higher IgG response of HBsAg of manufacturer A via the intramuscular route is confirmed in the immunogenicity study with stressed antigen.

The IgG2a/IgG1 ratio was higher for the *lpXL1* formulations than for all other formulations. The high IgG2a production with the *lpXL1* formulations indicates a Th1 skewed response. This is in agreement with earlier findings, showing Th1 directed responses by LPS-derived adjuvants [24–26]. The value of this superior Th1 directed immune response is not fully clear. Clinical trials are needed to find out

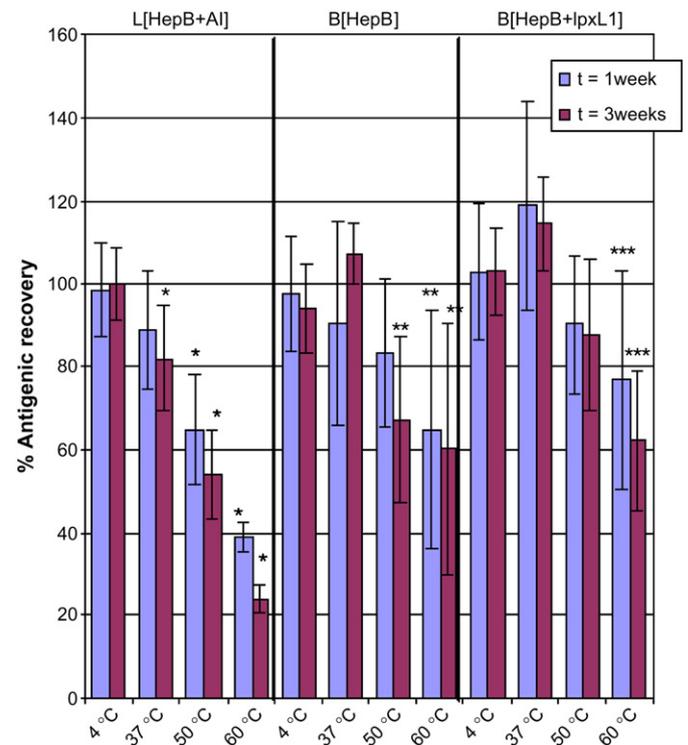


Fig. 6. Stability of Bioneedle formulations of HepB after 1 week and 3 weeks incubation at 4 °C, 37 °C, 50 °C and 60 °C as measured with an antigen ELISA. L: liquid formulation, B: Bioneedle formulation, HepB: HBsAg, Al: Aluminum hydroxide. Asterisks (*) represents recoveries of L[HepB + Al] formulations that are significant lower than L[HepB + Al]_4 °C ($P < 0.001$ as determined by the Student t test). Asterisks (**) represents recoveries of B[HepB] formulations that are significant lower than B[HepB]_4 °C ($P < 0.001$ as determined by the Student t test). Asterisks (***) represents recoveries of B[HepB + *lpXL1*] formulations that are significant lower than B[HepB + *lpXL1*]_4 °C ($P < 0.001$ as determined by the Student t test).

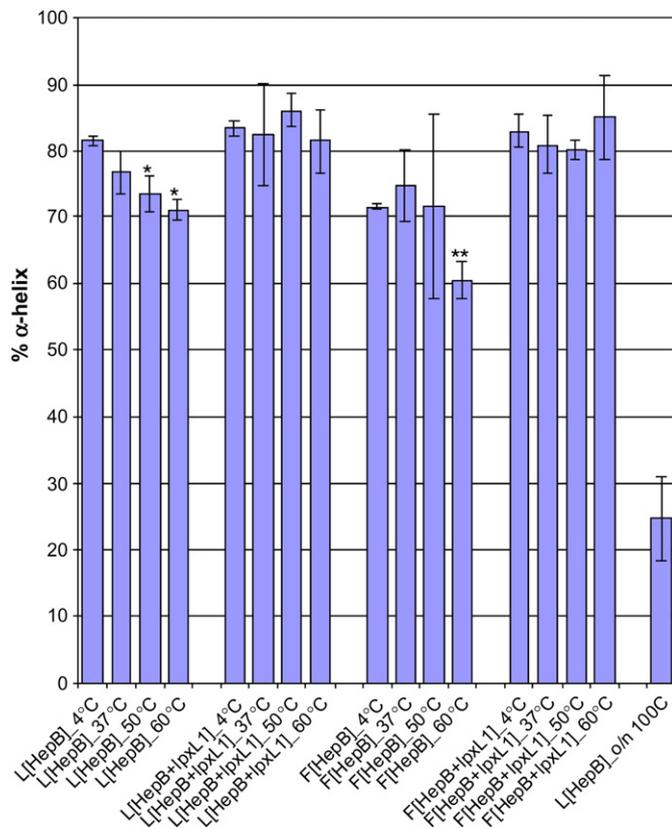


Fig. 7. Circular dichroism: α -helix content of formulations incubated for 3 weeks at different temperatures. L: liquid formulation, F: freeze dried formulations. Presented values are the average of 3 independent samples. Asterisks (*) represent liquid formulations with a significant lower α -helix content as compared to the liquid HepB formulation at 4 °C. Asterisks (**) represent freeze dried formulations with a significant lower α -helix content as compared to the freeze dried HepB formulation at 4 °C.

whether this Th1 skewed response will also be found in humans and whether this results in better protection.

Hepatitis B vaccines are known to be rather heat stable. Different studies with heat treated hepatitis B vaccines show that the vaccine is potent for up to 4 years at temperatures of 8 °C, for months at 25 °C, for weeks at 37 °C and for days at 45 °C [27]. Studies in endemic countries showed that hepatitis B vaccines, given by midwives to babies, are safe and effective whether stored cold or outside the cold chain [28–30]. Nevertheless there is no recommendation to store hepatitis B vaccine entirely outside the cold chain yet. Because of freeze drying, Bioneedle based vaccines have an improved thermostability as compared to the liquid form. Bioneedle formulations of HepB (B[HepB]) are stable for at least 3 weeks at 37 °C whereas liquid formulations show a significant decrease under the same conditions. When *lpXL1* is included, the Bioneedle formulations can be stored for 3 weeks at 50 °C without significant loss in antigenicity. The exact mechanism of stabilization of *lpXL1* is not clear but *lpXL1* may prevent HBsAg particle disintegration, perhaps by interaction with the lipid core of the HBsAg particles. This was indicated by size exclusion chromatographic analysis and is subject to further investigation.

Gaining approval for new adjuvants is time consuming due to extensive (pre) clinical studies. Nevertheless the approval of MPL, also a lipid A derivative, in HPV vaccines shows that the introduction of new adjuvants is possible.

Fig. 6 shows that there is no correlation between immunogenicity and antigenicity. Formulations with significant lower antigenicity all showed comparable IgG titers as the formulations with conserved antigenicity upon heat exposure. However, the negative control group (L[HepB + Al]_{100°C}), which has been incubated at 100 °C, shows a restored link between antigenicity and immunogenicity; both are zero.

It may be that the accuracy in the animal studies is too low in order to establish a reliable correlation. It is often difficult to demonstrate a relation between *in vitro* and *in vivo* assays. Parallel testing of many batches is usually required, which is beyond the scope of this study.

Gómez-Gutiérrez et al. [31] showed that antigenic activity of the surface antigen was abolished at 60 °C corresponding to a decrease in helical content. Circular dichroism (CD) results (see Fig. 7) indicate indeed a decrease in helical content of liquid formulations at temperatures higher than 50 °C. The analytical characterization of Bioneedle content was technically not possible. Instead, circular dichroism has been performed with freeze dried HBsAg samples in vials. The helical content of surface antigen in freeze dried samples was better conserved upon heat exposure. In Bioneedle formulations, HBsAg is also freeze dried. Therefore the conformation of HBsAg in Bioneedle formulations is probably better conserved. This higher antigenic stability of freeze dried HBsAg, as shown with the Bioneedle formulations, is in accordance with the results of Diminsky et al. [32]. They showed that hepatitis B liquid formulations (without alum) retained their antigenic stability after 6 months storage at 4 °C, but lost 80% of their antigenicity after 3 months at 37 °C. Freeze dried samples in contrary were stable for 6 months at 37 °C without loss in antigenicity.

With *lpXL1* in the formulation, no decrease in helical content was observed upon exposure to 60 °C. Apparently not only freeze drying, but also the presence of *lpXL1*, plays a role in conserving the helical content upon heat exposure.

The study shows that hepatitis B was successfully formulated with Bioneedles. Bioneedles with *lpXL1*, when delivered subcutaneously, induce higher IgG titers after one vaccination than conventional liquid

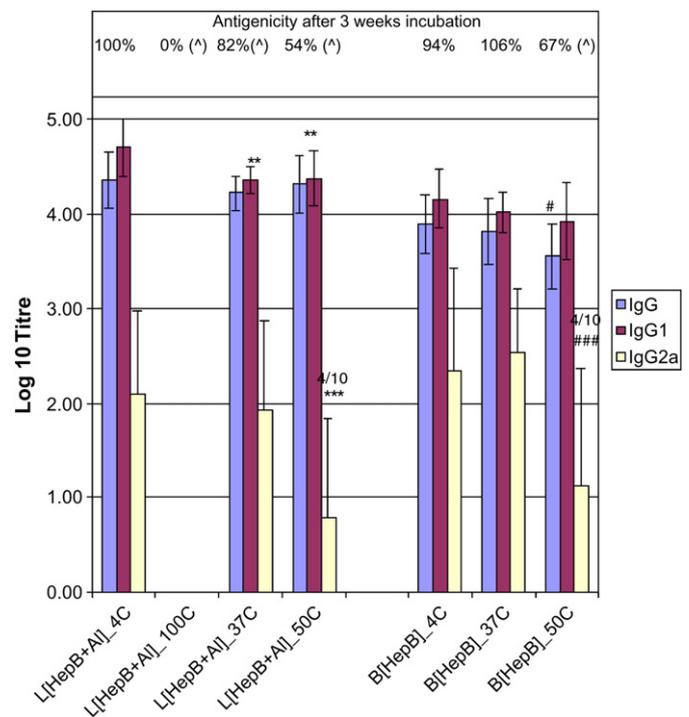


Fig. 8. Immune response in mice ($n=10$) after 2 vaccinations with heat treated formulations. L: liquid formulation; B: Bioneedle formulation; HepB: HBsAg (manufacturer A); Al: (Al(OH)₃). Liquid formulations were incubated for 4 h at 100 °C, 3 weeks at 4 °C, 37 °C or 50 °C prior to intramuscular injection. Bioneedle formulations were incubated for 3 weeks 4 °C, 37 °C or 50 °C prior to subcutaneous insertion. Titers are average titers of all 10 mice (responders and non responders). Antigenicity recovery as determined with an ELISA is indicated above. (^) antigenicity recovery that are significant different from the same formulation stored 3 weeks at 4 °C. (*) Significant differences in IgG (*), IgG1 (**) and IgG2a (***) within the liquid formulations as compared to L[HepB + Al]_{4°C}. (#) Significant differences in IgG (#), IgG1(##) and IgG2a (###) within the Bioneedle formulations as compared to B[HepB]_{4°C}. Differences are significant when $p < 0.05$ as determined with a Student *t* test.

alum adjuvanted vaccine delivered intramuscularly. After a booster vaccination comparable IgG titers are induced as with the conventional liquid vaccine but with higher IgG2a/IgG1 ratios. Bioneedles with *lpxL1* also showed much better heat stability than the conventional liquid vaccine. Although these advantages are mainly attributed to *lpxL1*, the Bioneedle has the additional advantages that it is a fast delivery system with no perceived sharps and with no sharp waste after delivery.

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