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Reference


DOI: 10.1111/j.1574-695X.2001.tb01577.x
PMID: 11335145
Priming of immune responses to hepatitis B surface antigen in young mice immunized in the presence of maternally derived antibodies

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Received 4 December 2000; received in revised form 16 February 2001; accepted 21 February 2001

Abstract

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Keywords: Immunization; CpG ODN; DNA vaccine; Maternally transferred antibody

1. Introduction

One of the greatest achievements in modern medical science has been the development of effective vaccines resulting in a remarkable reduction of many infectious diseases, including the complete eradication of smallpox [1]. Although most live attenuated, whole killed or subunit vaccines offer good protective immunity to adults, their success in immunizing infants is limited, probably due to the immature state of their immune system. Successful vaccination of newborns can be further hampered by the presence of maternally derived antibodies [2]. Although antibodies provided by the mother in utero or in colostrum/breast milk can protect against numerous viral and bacterial pathogens, such passive immunity only lasts for a short time, after which infants become vulnerable to infection. Therefore, it is important to develop vaccination strategies that are capable of inducing active immune responses in the presence of maternally derived antibodies. Several studies in animal models have shown that DNA vaccines can successfully prime immune responses in mice in the presence of maternal antibodies [3–5]. However, several other studies showed contrasting results [6,7]. Furthermore, results in human clinical trials with DNA vaccines have been disappointing with most failing to induce strong antibody responses [8–10]. A number of safety concerns must also be addressed before use of DNA vaccines in healthy infants.

Numerous studies have shown that oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN) have potent Th1-like adjuvant properties when used with a wide variety of antigens [11–19]. We have previously shown that hepatitis B surface antigen (HBsAg) administered with the adjuvant combination of alum and
CpG ODN can prime strong humoral and cell-mediated immunity in mice injected 3 days after birth [17]. Herein, we examine the potential of this vaccine to induce B cell and CTL priming in young mice in the presence of maternally derived antibodies, and compare it to a HBsAg-expressing DNA vaccine.

2. Materials and methods

2.1. Animals

Adult BALB/c mice were purchased from Charles River Laboratories (Montreal, QC, Canada) and housed at the animal care facility at Loeb Health Research Institute at Ottawa Hospital-Civic Site. Naive female mice or female mice with high antibody titers against HBsAg (anti-HBs) following immunization with HBsAg/alum/CpG [19] were bred onsite and checked daily for births. Immunization was carried out on groups of 5-12 pups aged 1, 3 or 7 days, with the date of birth being the day that the pups were first found. Pups were weaned and sexes separated at 3 weeks of age. Some pups (n = 25) were not immunized but were bled at weekly intervals for 12 weeks to determine the kinetics of decline of maternally derived anti-HBs.

2.2. Immunization of young mice

Mice were immunized with HBsAg at 1, 3 or 7 days of age using either the protein/adjuvant or DNA vaccine, both of which were administered by intramuscular (IM) injection. The total dose (see below) was administered in two equal injections of 10 μl each, given bilaterally into the posterior thigh muscles.

Subunit vaccines contained recombinant HBsAg (0.1 μg ml⁻¹, subtype ay, yeast-derived; Genzyme Diagnostics, San Carlos, CA, USA) at a final concentration of 0.05 mg ml⁻¹. The HBsAg was combined with alum (25 μg Al₃⁺ μg⁻¹ protein), CpG ODN (10 μg CpG ODN μg⁻¹ protein, using CpG ODN #1826 comprised of sequence 3’-TCCATGACGTTCTGACGTG-3’, made with a nucleoside resistant phosphorothioate backbone, Coley Pharmaceutical Group, Wellesley, MA, USA) or alum plus CpG ODN [19].

The DNA vaccine encoded the major hepatitis B virus envelope protein (S) of subtype ay under the control of the early immediate cytomegaloviral promoter and had a CpG-optimized backbone, as previously described [pMCG50-S] [20]. Plasmid DNA was purified on Qiagen DNA purification columns (Qiagen GmbH, Hilden, Germany), redissolved in 0.15 M NaCl and used at 5.0 mg ml⁻¹.

All mice were bled 4, 6, 8, 10 and 12 weeks post-immunization by retro-orbital puncture and plasma was recovered for enzyme-linked immunosorbent assay (ELISA). At 12 weeks post-immunization, the animals were injected IM with 1 μg HBsAg without adjuvant (‘challenge’), and then were bled 3 and 7 days later to evaluate anti-HBs responses. At 7 days post-challenge, mice were killed and spleens were removed. CTL activity was measured as described previously [21]. Briefly, splenocytes were re-stimulated for 5 days using mouse mastocytoma cells expressing HBsAg (p815-S). CTL activity was measured by ⁵¹Cr release assay. ⁵¹Cr-labeled p815-S cells were used as targets.

A group of mice (n = 25) with maternally derived anti-HBs antibodies was left unimmunized and bled at bi-weekly intervals up to 12 weeks of age to determine the kinetics of decline of maternally transferred antibodies. Subsequently, they were injected with 1 μg HBsAg without adjuvant and anti-HBs and CTL responses were evaluated 7 days later (‘challenge control’).

2.3. Evaluation of immune responses

Anti-HBs titers were determined by end-point dilution ELISA as previously described [19].Titers for total IgG, IgG1 and IgG2a isotypes were defined as the highest dilution that resulted in an absorbance (OD₄₅₀) value two times greater than that of the pre-immune plasma, with a cut-off value of 0.05. Data were reported as group geometric mean titers ± the standard errors of the mean (GMT ± S.E.M.) of values of individual samples, which themselves were the average of triplicate assays. Seroconversion was defined as a titer ≥ 100.

Antigen-specific CTL responses of individual animals were measured using splenocytes recovered from immunized animals at 7 days post-‘challenge’, as described previously [21].

2.4. Statistical analysis

Statistical analyses were performed using the InStat program (Graph PAD Software, San Diego, CA, USA). The statistical differences between groups were determined by Student’s t-test (for two groups) or by one-factor ANOVA followed by Tukey’s test (for three or more groups) on raw data (for CTL) or on transformed data (log₁₀, for antibody titers).

3. Results

3.1. Persistence of maternal antibodies

Twenty five pups born to four different mothers (with post-partum anti-HBs titers between 2.1 and 3.3×10⁴) were used to evaluate the rate of decline of maternally transferred anti-HBs antibodies. The pups had very high anti-HBs titers early in life; at 2 weeks of age these were 4-6 times higher than those in their mothers. The levels of maternally derived antibody declined rapidly over the first
4 weeks of life and then more slowly. They were almost undetectable by 11 weeks of age (Fig. 1).

3.2. CTL responses in young mice immunized with protein or DNA vaccines

HBsAg-specific CTL responses were assessed using splenocytes obtained from animals immunized in the presence of maternally derived anti-HBs antibodies (Fig. 2). All vaccine formulations tested were capable of inducing strong CTL responses in 7-day-old animals in spite of the presence of maternally transferred anti-HBs antibodies at the time of immunization. However, HBsAg/alum/CpG was capable of inducing CTL in animals injected 1 day after birth, and CTL activity in animals immunized at all ages (1, 3 or 7 days) was significantly higher than in control animals ($P < 0.05$) at all effector:target (E:T) ratios (100:1, 50:1 or 10:1, data not shown for 100:1 and 10:1 ratio). Animals immunized with DNA 3 and 7 days after birth, but not at day 1, had significantly higher CTL activity compared to controls at 100:1 and 50:1 E:T ratios ($P < 0.05$). Animals immunized with HBsAg/alum 3 days after birth also had significantly higher CTL activity compared to challenge controls at 100:1 and 50:1 E:T ratios. In contrast, animals immunized at 1, 3 or 7 days after birth with HBsAg/CpG did not have significant CTL responses.

3.3. Anti-HBs antibody responses in young mice immunized with protein or DNA vaccines

None of the mice immunized in the presence of maternal antibodies had a detectable anti-HBs response (titer $\geq 100$) at 12 weeks of age (a time at which the maternal antibodies had essentially disappeared) irrespective of the age at which they were vaccinated and the vaccine formulation used, including DNA vaccine. At this time, mice were 'challenged' with 1 $\mu$g HBsAg without adjuvant to determine whether there was an anamnestic response which would indicate priming by the early life immunization (Fig. 3, upper panel). According to our previous work, adult mice immunized with HBsAg alone require at least 4 weeks to seroconvert to titers $\geq 100$ [19]. Therefore, high titers seen in other groups at 7 days post-challenge indicate priming by the antigen formulation given early in life. None of the 'challenge control' mice that were first immunized at 12 weeks of age with 1 $\mu$g HBsAg developed a detectable anti-HBs response by 7 days.

Among mice immunized with DNA vaccine and HBsAg/alum/CpG alum 7 days after birth in the presence of maternally derived anti-HBs, seroconversion (anti-HBs titers $> 100$ upon challenge) reached 57% and 43%, respectively (Table 1). However, antibody titers remained
low (Fig. 3, upper panel) compared to immunization with the same vaccine formulations in the absence of anti-HBs antibodies of maternal origin, which led to 100% seroconversion and much higher overall anti-HBs titers (Fig. 3, lower panel).

In groups immunized in the presence of maternally derived anti-HBs antibody, plasma samples obtained 7 days post-challenge were assayed for IgG1 and IgG2a isotypes to determine the Th polarization of the response, a high IgG2a antibody titer being recognized as indicating a preferential Th1-type response in BALB/c mice. Isotypes were not determined for samples taken 1 or 3 days post-challenge or following immunization with HBsAg/CpG or HBsAg/alum at any age, since antibody titers were too low to give meaningful results. In control animals immunized at day 7 in the absence of maternally transferred anti-HBs antibodies, the antibody responses were either mixed (DNA immunization) or Th2-biased (HBsAg/alum, HBsAg/alum/CpG) (Fig. 4A). Interestingly, immunization in the presence of maternal antibodies led to a different pattern of anti-HBsAg isotypes. Indeed, IgG1 responses elicited by DNA or HBsAg/alum/CpG were inhibited to a much larger extent than IgG2a antibodies (Fig. 4B). This resulted in IgG2a/IgG1 ratios of 88

Table 1
Number of responders and % seroconversion at 12 weeks post-immunization of BALB/c mice with maternally derived anti-HBs antibodies

<table>
<thead>
<tr>
<th>Age at vaccination</th>
<th>No. responders/total number of animals (% seroconversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA vaccine</td>
<td>HBsAg+</td>
</tr>
<tr>
<td></td>
<td>Alum</td>
</tr>
<tr>
<td>Day 1</td>
<td>4/12 (33)</td>
</tr>
<tr>
<td>Day 3</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Day 7</td>
<td>4/7 (57)</td>
</tr>
</tbody>
</table>

Mice were immunized at days 1, 3 and 7 of age with different vaccine formulations.
(HBsAg/alum/CpG) or 7 (DNA vaccine), compared to ratios of 0.03 or 0.81 in mice respectively immunized with HBsAg/alum/CpG or DNA in the absence of maternal antibodies. Thus, maternal antibodies exerted significantly more inhibition of IgG1 than of IgG2a B cell priming.

4. Discussion

Although the inhibitory effect is reversible with the eventual decline of maternally derived antibodies, the period of reduced vaccine efficacy can last well beyond the period when maternally derived antibodies are at a sufficient level to protect the infants [2]. Immunization with live attenuated pathogens, DNA vaccines, or recombinant viral vectors can occasionally induce immune responses despite the presence of maternally derived antibodies [3–5,22–24]. This was postulated to result from the in vivo antigen synthesis in host cells that may result in direct cell-to-cell antigen presentation without interference by circulating antibody. However, other studies showed failure to induce good immunity using DNA vaccines, live attenuated vaccines or recombinant viral vectors when administered in the presence of maternally transferred antibodies. This inconsistency in results may be due to the presence of high levels of maternal antibodies in proportion to the amount of vaccine antigen used, age of the animal at time of immunization and differences in the specificity of antibodies of maternal origin and those induced by vaccines administered neonatally [25].

CpG ODN has been shown to have potent adjuvant effects with numerous antigens in adult mice [19], and with HBsAg in non-human primates [26] and humans [27]. Furthermore, CpG ODN has been shown to overcome hyporesponsiveness to HBsAg in young non-human primates [28]. CpG ODN has strong synergy with other adjuvants, including alum [29]. In very young mice, a HBsAg/alum/CpG vaccine induces stronger anti-HBs and CTL responses than a DNA vaccine expressing HBsAg [17], and alum/CpG was effective in inducing adult-like Th1 responses to a panel of vaccine antigens [30].

In the present study, we demonstrate that HBsAg/alum/CpG was as effective as a DNA vaccine in immunizing newborn mice in the presence of maternally derived antibodies against HBsAg. However, the strength of the antibody responses remained much weaker compared to mice immunized at the same age that had no maternally transferred anti-HBs antibodies. This study also demonstrated that even a HBsAg-encoding DNA vaccine could not escape interference from maternal antibodies, as indicated by poor B cell responses. This is in accordance with the current understanding that in vivo synthesized antigens have to be released from host cells produce the antigen to become available for binding by infant B cells, a process leading to their simultaneous exposure to maternal antibodies. It will be interesting to confirm that this inhibition is due to the unavailability of antigen synthesized in vivo by DNA vaccines to the B cells, as a result of the formation of antigen–antibody complexes, rather than to B cell anergy. In addition, it is interesting to note that some vaccine formulations (HBsAg/alum/CpG and DNA) although not very strong, do allow a certain degree of B cell priming. Therefore, it is likely that this response could be further enhanced by optimizing the antigen dose, adjuvant dose and the age of immunization (to allow maternal antibodies to deplete to a lower titer).

Even though the overall titers were low, and no statistical significance was achieved between groups due to high inter-group variation in antibody titers (likely due to differences in maternally transferred antibody levels amongst animals), the humoral response in mice immunized with HBsAg/alum/CpG was much more strongly Th1-biased (high proportion of IgG2a antibodies) than with the DNA vaccine. Unexpectedly, priming with HBsAg/alum/
CpG or DNA in the presence of maternal antibodies led to much better preserved IgG2a than IgG1 antibodies, compared to control 7-day-old mice immunized with no maternally transferred anti-HBs antibodies. In fact, there was no significant inhibition of post-challenge IgG2a antibodies following HBsAg/alum/CpG priming, compared to a > 3 log10 inhibition of IgG1 antibodies. This difference in the nature of the immune response could be due to the difference in antigen uptake by professional antigen presenting cells, i.e. distinct receptor-mediated uptake of antigen via Fc receptors of HBsAg/Ab IgG1 or IgG2a complexes in animals with maternally transferred anti-HBs antibodies vs. uptake of exogenous antigen in animals without maternally transferred anti-HBs antibodies. Another hypothesis is that the lower antigen dose available to the immune system in animals immunized in the presence of maternally derived anti-HBs antibodies would favor priming of IgG2a antibodies. Antigen dose has indeed been shown to play an important role in determining the nature of the immune response [31,32]. Last, this differential inhibition of IgG1 compared to IgG2a antibodies could reflect preferential binding of HBsAg epitopes by maternal IgG1 rather than IgG2a antibodies, resulting in preferential inhibition of IgG1 versus IgG2a cell priming. Future experiments will try to address this interesting issue.

In contrast to inhibition of B cell responses by maternal antibodies, there was strong induction of CTL responses by HBsAg/alum/CpG vaccine from a very early age (1-day-old) despite the presence of high maternally derived antibody titers. DNA vaccines were also capable of inducing strong CTL responses, albeit only in older animals (3-day-old). These results are consistent with previous studies which showed that high titers of maternally transferred antibodies which inhibited B cell responses did not affect T cell responses induced by four different measles vaccine formulations [33], or tetanus toxoid vaccine [2]. Furthermore, the fact that maternal antibodies do not inhibit T cell responses may explain the efficacy of the current vaccination strategy, which involves administering HBV vaccine together with high concentrations of anti-HBs immunoglobulins to infants born to HBV-infected mothers. Under these circumstances, cell-mediated immunity consisting of CTL and, as shown more recently, Th1-type cytokines are needed to protect the infants rather than antibody responses [34].

In conclusion, this study shows that maternal antibodies hinder the induction of effective B cell responses but not T cell responses to both DNA and protein vaccines against HBsAg. However, some B cell priming was observed with HBsAg/alum/CpG and DNA vaccines. Further studies are needed to optimize the conditions (i.e. antigen dose, adjuvant dose, and maternal antibody titers at time of immunization) that would help overcome the interference of maternal antibodies.

Acknowledgements

We wish to thank Amanda Boyd and Lu Zhang for their excellent technical assistance. This work was supported by Coley Pharmaceutical Group Inc., and an operating grant from the Medical Research Council (Canada) to H.L.D., who is also the recipient of an Ontario Ministry of Health Career Scientist Award.

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