Hydrogel-Delivered GM-CSF Overcomes Nonresponsiveness to Hepatitis B Vaccine through the Recruitment and Activation of Dendritic Cells

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More than a third of the world’s population has been infected with hepatitis B virus (HBV), and more than 350 million are chronic carriers, of whom 15–40% are at risk for developing HBV-associated liver diseases, including cirrhosis and hepatocellular carcinoma (1, 2). Vaccination with the hepatitis B surface Ag (HBsAg) derived from the plasma of HBV carriers or produced by rDNA technology is the main strategy for the effective control of the infection and viral transmission (3, 4). The benefit of the HBsAg vaccine is demonstrated by the fact that the universal HBV vaccination program in infants successfully reduced the rate of HBV chronic carriers from ∼5–10% of normal vaccine recipients (8) and ∼40–50% of patients on maintenance hemodialysis with depressed immune responses (9, 10) do not respond well to current HBsAg vaccines. The mechanisms underlying this hypo- or nonresponsiveness to HBsAg are not fully defined, but evidence from genetic studies indicates a close association between different HLA-DR alleles and specific low responsiveness in different ethnic populations (11). Consistent with the important role of HLA-DR molecules in stimulating the proliferation and activation of CD4⁺ Th cells, PBMCs from HBV vaccine nonresponders failed to proliferate in vitro against HBsAg, whereas those from responders with high anti-hepatitis B surface (HBs) titers showed a strong proliferative response to HBsAg (12–16). These results strongly suggest that hypo- or nonresponsiveness to HBsAg vaccine is mainly due to the lack of Th cell responses in the vaccinees, implying that strategies that can induce strong T cell proliferative responses might help to overcome nonresponsiveness to HBsAg.

GM-CSF is an important hematopoietic growth factor and immune modulator (17, 18) and has potent activity in stimulating the proliferation and maturation of dendritic cells (DCs) (19), which are crucial in inducing T cell-mediated immune responses. As a result, GM-CSF, in the form of a recombinant protein or a gene product encoded by plasmid DNA or viral vectors, has been codelivered as an adjuvant and has proved effective in enhancing immune responses to a number of vaccines (20, 21). In terms of HBV vaccines, systemic administration of rGM-CSF was investigated as an adjuvant in healthy nonresponders (22) and in hemodialysis patients (23–29). However, in these studies, GM-CSF showed no effect or only moderately increased the seroconversion rate and anti-HBs titers. It is known that the local expression of GM-CSF at the site of immune interaction is important for its function, as demonstrated in our previous study showing that a tumor Ag can be converted to a strong Ag and induce protective immunity only when it is covalently linked to GM-CSF (30).
hypothesized that a vaccine-delivery system that can colocalize GM-CSF and HBsAg in vivo might greatly improve the adjuvant activity of GM-CSF, leading to high anti-HBs Th cell and Ab titers in nonresponders.

To achieve local and sustained release of GM-CSF at the site of immune interaction, we used a thermosensitive, biodegradable copolymer (monomethoxy poly(ethylene glycol)-co-poly(lactic-co-glycolic acid [mPEG-PLGA] hydrogel)) to codevolve HBsAg and GM-CSF proteins. This novel diblock copolymer is biodegradable and forms an aqueous solution under 15°C, a characteristic convenient for Ag loading, but it becomes an insoluble gel at body temperature, which allows the slow, continuous release of entrapped proteins over a period of time (31). This new formulation of HBsAg vaccine was tested in a number of wild-type mice and the H-2-congenic strain B10.M (H2^d haplotype), which is known to be nonresponsive to HBsAg immunization (32), for its effectiveness in enhancing anti-HBs immune responses.

Materials and Methods

Animals

Female BALB/c, C57BL/6, and C3H/HeN mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan), and ICR mice were purchased from BioLASCO Taiwan (Il'an, Taiwan). B10.M mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained as a small breeding colony in our own animal facility. All animals were housed in a specific pathogen-free environment in the animal facility of the Institute of Biomedical Sciences, Academia Sinica. All experimental procedures complied with the regulations of the Academia Sinica Institutional Animal Care and Use Committee and the Council of Agriculture Guidebook for the Care and Use of Laboratory Animals.

Immunization

The thermosensitive mPEG<sub>5500</sub>-PLGA<sub>10,000</sub> diblock copolymer, composed of monomethoxy(polyethylene glycol)-copoly(lactic-co-glycolic acid), was developed and provided by the Industrial Technology Research Institute (31). Immediately before immunization, rHBsAg, produced in the yeast Pichia pastoris <sup>1</sup>, and mPEG-PLGA<sub>10,000</sub> hydrogel (31) to codeliver HBsAg and GM-CSF proteins. This novel diblock copolymer is biodegradable and forms an aqueous solution under 15°C, a characteristic convenient for Ag loading, but it becomes an insoluble gel at body temperature, which allows the slow, continuous release of entrapped proteins over a period of time (31). This new formulation of HBsAg vaccine was tested in a number of wild-type mice and the H-2-congenic strain B10.M (H2^d haplotype), which is known to be nonresponsive to HBsAg immunization (32), for its effectiveness in enhancing anti-HBs immune responses.

Statistics

The nonparametric Mann–Whitney U test was used for analyses of anti-HBs Abs. The unpaired Student t test was used for other analyses. Findings were regarded as significant if the two-tailed p value was <0.05.

Results

Hydrogel-formulated hepatitis B surface Ag plus GM-CSF vaccine enhances HBsAg-specific humoral and cellular immune responses

Hydrogel can be formulated with Ags and cytokines into a stable vaccine-delivery system capable of the sustained release of Ags and cytokines in vivo. To examine whether the sustained release of HBsAg and GM-CSF could enhance the Ag-specific immune responses, BALB/c mice were injected once s.c. with 2 μg of HBsAg in different formulations with or without hydrogel, and sera were collected from each group 4 wk later to measure HBsAg-specific Ab titers. As shown in Fig. 1A, mice immunized with HBsAg alone had low titers of anti-HBs Ab (14 ± 13 U/ml). Codelivery of GM-CSF with HBsAg (HBsAg+GM group) increased the anti-HBs titers 2-fold (28 ± 10 U/ml), whereas the hydrogel-delivered HBsAg (Gel/HBs group) produced 6-fold more measured on an ELISA reader. Concentrations of anti-HBs Abs in serum samples were estimated from a standard curve generated using a standard serum pooled from four mice given i.p. injections of 2 μg HBsAg with CFA (Life Technologies, Carlsbad, CA), and the results were expressed as arbitrary units per milliliter (U/ml; 1 U = 50% maximum OD).

Lymphocyte-proliferation assay

HBsAg-specific T cell proliferation was determined as described previously (34). Briefly, spleen cells were harvested from mice in each group and pooled for the assay. One hundred microliters of 2 × 10<sup>6</sup>/ml splenocytes in complete DMEM, supplemented with 5% heat-inactivated FCS, 2 μM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^-5 M 2-ME, was added in triplicate to each well of 96-well round-bottom plates, followed by the addition of HBsAg at a final concentration of 3 or 1 μg/ml for experiments in BALB/c mice and 10 μg/ml for B10.M mice. BSA (30 μg/ml) served as a negative control Ag, and Con A (2 μg/ml; both from Sigma-Aldrich) served as a positive mitogenic control. Control wells received cells only. After 4 d in culture, the cells were assayed with [3H]thymidine (1 μCi/well) for 18 h and then the incorporated radioactivity was measured on a TopCount microplate scintillation and luminescence counter (PerkinElmer, Wellesley, MA). The stimulation index was calculated as the mean cpm of the stimulated wells divided by the mean cpm of the control wells.

DC migration assays

For DC migration assays in C57BL/6 mice, mice were injected s.c. in the hind legs daily for 2 d with hydrogel containing saline or 5.4 μg GM-CSF. Bone marrow-derived DCs (BM-DCs) were generated, as previously described (35), and incubated for 10 min at 37°C in PBS containing 5% FBS and 5 μM CFSE (Molecular Probes, Eugene, OR). Then, CFSE-labeled BM-DCs were injected into both hind footpads (1 × 10<sup>6</sup> per footpad) 6 h after the second hydrogel injection, followed by collection of the draining popliteal lymph node (LN) samples 24 and 48 h after cell transfer. Then, single-cell suspensions were prepared from each sample and stained with PE-conjugated anti-CD11c mAb (clone HL3; BD Pharmingen, San Diego, CA), and the number of CD11c<sup>+</sup> cells in each sample was analyzed using FACS Calibur and CellQuest software (BD Biosciences, Franklin Lakes, NJ). For DC-migration assays and surface-marker analysis in B10.M mice, mice were injected s.c. in the hind legs daily for 2 d with hydrogel containing saline, 2 μg HBsAg, or 2 μg HBsAg plus 5.4 μg GM-CSF. The draining popliteal LN samples were harvested 24 and 48 h after the second hydrogel injection, and the number of CD11c<sup>+</sup> cells in each sample was analyzed, as described above. To determine the surface markers of migrating DCs, the cells were stained with PE-conjugated anti-CD11c mAb and FITC-conjugated anti-MHC class II mAb (clone H-2K<sup>d</sup>/I-E<sup>d</sup>), or biotinylated anti-CD40 (clone 3/23), anti-CD80 (clone 16-10A1), and anti-CD86 (clone GL1) mAbs or appropriate isotype-control Abs, followed by incubation with streptavidin-allophycocyanin. All mAbs, with the exception of the anti-MHC class II mAb (BioLegend, San Diego, CA), were purchased from BD Biosciences.

Statistics

The nonparametric Mann–Whitney U test was used for analyses of anti-HBs Abs. The unpaired Student t test was used for other analyses. Findings were regarded as significant if the two-tailed p value was <0.05.
the H-B-V AXII vaccine (182 m mice immunized with the Gel/HBs+GM vaccine produced much aluminum hydroxide as adjuvant. As shown in Fig. 1, hydrogel-formulated hepatitis B surface Ag plus GM-CSF (Gel/HBs+GM) vaccine enhances the production of anti-HBs Abs in mice. A, Groups of BALB/c mice (n = 5) were immunized once s.c. with HBsAg in saline (HBs), HBsAg plus GM-CSF in saline (HBs+GM), Gel/HBs, Gel/HBs+GM, or hydrogel-formulated HBsAg and hydrogel-formulated GM-CSF at two separate sites (Gel/HBs+Gel/GM). The dose of HBsAg used in all groups was 2 µg per mouse and that of GM-CSF, when used, was 5.4 µg per mouse. B, Groups of BALB/c mice were immunized once s.c. with commercial H-B-V AXII vaccine at the dose of 200 µl (2 µg) or with Gel/HBs+GM, as described above. C, Groups of C3H, C57BL/6, and ICR mice were immunized once s.c. with HBs+GM or Gel/HBs+GM, as described above. For all experiments, serum samples were collected from each group 4 wk after immunization, and the concentration of anti-HBs Abs was determined by ELISA using a standard curve generated from serially diluted control anti-HBs Ab, as described in Materials and Methods. The data are mean ± SD for five animals from one representative experiment. Each experiment was performed two or three times.

anti-HBs Abs (84 ± 69 U/ml) than the HBs group. The greatest increase was obtained using the hydrogel-formulated HBsAg+GM-CSF vaccine (Gel/HBs+GM group), which resulted in a marked increase in anti-HBs titer to 773 ± 227 U/ml; this was 56-, 27-, and 9-fold greater, respectively, than those obtained in the HBs (p = 0.009), HBs+GM (p = 0.009), and Gel/HBs (p = 0.009) groups. The adjuvant activity of GM-CSF was lost when Gel/HBs and hydrogel-formulated GM-CSF (Gel/GM) were injected at two separate sites (Gel/HBs + Gel/GM group), which resulted in a much lower anti-HBs titer (45 ± 6 U/ml) than in the Gel/HBs+GM group (p = 0.03; Fig. 1A), highlighting the importance of local GM-CSF activity in promoting immune responses. The hydrogel-formulated hepatitis B surface Ag plus GM-CSF (Gel/HBs+GM) vaccine was also compared with a commercially available HBsAg vaccine H-B-VAXII, which was formulated with aluminum hydroxide as adjuvant. As shown in Fig. 1B, BALB/c mice immunized with the Gel/HBs+GM vaccine produced much greater anti-HBs titers (1040 ± 660 U/ml) than those injected with the H-B-VAXII vaccine (182 ± 63 U/ml; p = 0.01). To confirm that the Ab-enhancement effect was not restricted to BALB/c mice (haplotype H-2b), two other inbred mouse strains (C57BL/6 [haplotype H-2b] and C3H/HeN [haplotype H-2d]) and one outbred strain (ICR) were immunized with the same dose of HBsAg and GM-CSF delivered with or without hydrogel. As shown in Fig. 1C, the Gel/HBs+GM vaccine resulted in a significant increase in anti-HBs titers in all strains tested, with a 4-fold increase in titer in C3H/HeN mice (p = 0.03), a 34-fold increase in C57BL/6 mice (p = 0.02), and a 6-fold increase in ICR mice (p = 0.04) compared with the same strain immunized with a simple mixture of HBs+GM.

We next examined whether the Gel/HBs+GM vaccine could enhance T cell immune responses. Groups of BALB/c mice were given two s.c. injections, at a 2-wk interval, with 2 µg of HBsAg in the different vaccine formulations described above. Two weeks after the second immunization, splenocytes were examined for proliferation in response to specific (HBsAg) and nonspecific (BSA) Ag stimulation. The results are shown in Table I. Splenic lymphocytes derived from the HBs group demonstrated a dose-dependent proliferative response to increasing amounts of HBsAg, with an average peak stimulation index of ~2.5 when the HBsAg concentration was 3 µg/ml. Compared with the HBs group, immunization with HBs+GM or Gel/HBs resulted in slightly greater cellular proliferation, with an average peak stimulation index (HBsAg at 3 µg/ml) of 6.5 and 6.1, respectively. However, the greatest increase in T cell proliferation was seen in mice immunized with Gel/HBs+GM, which had an average peak stimulation index (HBsAg at 3 µg/ml) of 31.0. The T cell responses were specific for HBsAg, because none of the mice in the different groups responded to the control protein BSA, even at a much greater concentration (30 µg/ml).

FIGURE 1. Gel/HBs+GM vaccine enhances the production of anti-HBs Abs in mice. A, Groups of BALB/c mice (n = 5) were immunized once s.c. with HBsAg in saline (HBs), HBsAg plus GM-CSF in saline (HBs+GM), Gel/HBs, Gel/HBs+GM, or hydrogel-formulated HBsAg and hydrogel-formulated GM-CSF at two separate sites (Gel/HBs+Gel/GM). The dose of HBsAg used in all groups was 2 µg per mouse and that of GM-CSF, when used, was 5.4 µg per mouse. B, Groups of BALB/c mice were immunized once s.c. with commercial H-B-V AXII vaccine at the dose of 200 µl (2 µg) or with Gel/HBs+GM, as described above. C, Groups of C3H, C57BL/6, and ICR mice were immunized once s.c. with HBs+GM or Gel/HBs+GM, as described above. For all experiments, serum samples were collected from each group 4 wk after immunization, and the concentration of anti-HBs Abs was determined by ELISA using a standard curve generated from serially diluted control anti-HBs Ab, as described in Materials and Methods. The data are mean ± SD for five animals from one representative experiment. Each experiment was performed two or three times.

BALB/c mice were immunized s.c. with various HBsAg vaccines, as described in the legend for Fig. 1, and boosted 4 wk later with the same vaccine. Two wk after the booster immunization, splenocytes pooled from three immunized mice from each group were examined for HBs-specific proliferative responses, as described in Materials and Methods. BSA served as a negative control Ag. Data are from one representative experiment of three and are presented as the mean stimulation index for triplicate wells ± SD.

<p>| Table I. Gel/HBs+GM vaccine enhances HBs-specific T cell-proliferative responses |
|-------------------------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Ag</th>
<th>3 µg/ml</th>
<th>1 µg/ml</th>
<th>BSA (30 µg/ml)</th>
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<tr>
<td>HBs</td>
<td>2.5 ± 0.6</td>
<td>1.4 ± 0.4</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>HBs+GM</td>
<td>6.5 ± 1.9</td>
<td>4.0 ± 3.2</td>
<td>1.3 ± 1.1</td>
</tr>
<tr>
<td>Gel/HBs</td>
<td>6.1 ± 3.8</td>
<td>2.3 ± 0.1</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Gel/HBs+GM</td>
<td>31.0 ± 3.5</td>
<td>17.8 ± 4.4</td>
<td>11.1 ± 0.1</td>
</tr>
</tbody>
</table>
B10.M mice immunized with the Gel/HBs+GM vaccine developed a significant T cell-proliferative response to HBsAg (average stimulation index of 11.0) but not the control BSA protein (average stimulation index of 1.0). Immunization with H-B-VAXII produced a low, but significant, HBsAg-specific T cell response (average stimulation index of 3.3), whereas HBs alone or HBs+GM did not induce a detectable T cell-proliferative response to HBs.

Gel/GM increases the number and activation status of DCs

We next investigated the cellular and molecular mechanisms of the adjuvant activity of the Gel/HBs+GM vaccine. We focused on DCs because they are unique among APCs in being able to activate naive T cells (36) and because GM-CSF potently stimulates the maturation and activation of DCs (19). We first examined whether administration of GM-CSF increased the migration of DCs to the draining LNs (365 ± 3 cells on day 2) than the Gel/saline group (8 ± 1 cells on day 1, p = 0.01; 9 ± 3 cells on day 2, p = 0.02), as well as significantly more endogenous DCs in the draining LNs (365 ± 24 cells versus 127 ± 46 cells on day 1, p = 0.002; 149 ± 32 cells versus 60 ± 24 cells on day 2, p = 0.03).

In another experiment, we investigated the effect of injection of combined GM-CSF and HBsAg at the same site on the migration and maturation of endogenous DCs in the nonresponder B10.M mice. Groups of B10.M mice were injected in both hind legs daily for 2 d with Gel/HBs+GM, Gel/BSA, or Gel/saline; 24 and 48 h after the second injection, the draining LN cells were collected and analyzed by flow cytometry for CD11c+ cells and for their expression of costimulatory molecules. As in responder C57BL/6 mice, injection of Gel/HBs+GM resulted in significantly more CD11c+ DCs in the draining LNs compared with Gel/HBs or Gel/saline treatment. Fig. 4A shows a representative result on day 1; after cell transfer. Fig. 3A shows representative results obtained on day 1 after BM-DC transfer, which show that injection of Gel/GM increased the number of transferred BM-DCs and the number of endogenous DCs by -3-fold each compared with control animals injected with Gel/saline. The absolute number of transferred BM-DCs and endogenous DCs, expressed as the number of CD11c+CFSE+ cells and CD11c+CFSE- cells per 10,000 LN cells, in each group on days 1 and 2 are shown in Fig. 3B and 3C. Mice injected with Gel/GM had significantly more transferred BM-DCs in the draining LNs (22 ± 7 cells on day 1 and 28 ± 12 cells on day 2) than the Gel/saline group (8 ± 1 cells on day 1, p = 0.01; 9 ± 3 cells on day 2, p = 0.02), as well as significantly more endogenous DCs in the draining LNs (365 ± 24 cells versus 127 ± 46 cells on day 1, p = 0.002; 149 ± 32 cells versus 60 ± 24 cells on day 2, p = 0.03).

In another experiment, we investigated the effect of injection of combined GM-CSF and HBsAg at the same site on the migration and maturation of endogenous DCs in the nonresponder B10.M mice. Groups of B10.M mice were injected in both hind legs daily for 2 d with Gel/HBs+GM, Gel/BSA, or Gel/saline; 24 and 48 h after the second injection, the draining LN cells were collected and analyzed by flow cytometry for CD11c+ cells and for their expression of costimulatory molecules. As in responder C57BL/6 mice, injection of Gel/HBs+GM resulted in significantly more CD11c+ DCs in the draining LNs compared with Gel/HBs or Gel/saline treatment. Fig. 4A shows a representative result on day 1;
the summarized results are shown in Fig. 4B. Mice immunized with Gel/HBs+GM had consistently more CD11c+ DCs (313 ± 628 on day 1; 345 ± 616 on day 2) in the draining LNs than those treated with Gel/HBs (185 ± 626 on day 1, p = 0.01; 190 ± 620 on day 2, p = 0.001). Compared with the saline group, Gel/HBs also slightly increased the number of CD11c+ DCs, but the difference was not statistically significant (Fig. 4B). Importantly, we also found that Gel/HBs+GM greatly enhanced the maturation of the recruited DCs, as shown by flow-cytometric analysis of levels of surface expression of MHC class II and costimulatory molecules on gated CD11c+ cells from the draining LNs in each group. Fig. 4C shows representative results on day 1 after treatment. Compared with the Gel/saline group, the Gel/HBs+GM group showed an increased percentage of MHC class II+, CD40+, CD80+, and CD86+ cells, whereas the Gel/HBs group showed only a slight increase in the number of CD86+ cells. Moreover, DCs from Gel/HBs+GM-treated mice displayed an increased mean fluorescence intensity (MFI) for MHC class II and all three costimulatory molecules, indicating that they were present in a status with higher Ag presentation ability. Compared to the Gel/saline control, Gel/HBs+GM increased the MFI by 1.9-fold for MHC class II, 2.3-fold for CD40, 2.1-fold for CD80, and 3.8-fold for CD86 (Fig. 4D). Gel/HBs had little effect on the MFI for MHC class II, CD40, and CD80, but it significantly increased the MFI for CD86 (1.7-fold). Collectively, these data demonstrate that more CD11c+ DCs with a more mature phenotype were recruited to the draining LNs in mice treated with Gel/GM-CSF vaccines.

Discussion

The current HBsAg-based HBV vaccine has been available since the early 1980s and has been demonstrated to be highly effective with an outstanding safety record (3, 4). However, ~5–10% of healthy subjects with certain HLA class II alleles, such as DRB1*03, DRB1*07, DQB1*02, and DPB1*1101 (37–40), as well as patients with a variety of chronic diseases (4) fail to develop an adequate anti-HBs response after the standard three-dose vaccination. These hypo- and nonresponder individuals remain at risk for HBV infection; thus, there is a need for a more immunogenic vaccine for protection. Previous work using inbred congeneric mouse strains that are genetically identical, except in the MHC region, showed that the immune response to HBsAg is strongly influenced by the MHC-linked genes and that B10.M mice, with the H-2d haplotype, are not responsive to HBsAg (32). Because of the similarity in the MHC-
linked response to HBsAg, mice provide an excellent animal model and were used in this study to evaluate a new HBV vaccine with improved immunogenicity.

Several previous clinical studies attempted to use GM-CSF as a bolus injection to improve the efficacy of HBsAg vaccine in healthy nonresponders (22) and in hemodialysis patients (23–29). However, in general, the improvement in vaccine efficacy was not impressive, which might be due to the short serum half-life of GM-CSF (41) and the low local GM-CSF concentration at the point where the immune response was taking place. To achieve sustained release of GM-CSF and HBsAg at the same location, we used the biodegradable and thermosensitive mPEG-PLGA hydrogel system as a matrix to encapsulate and deliver these two vaccine components. We demonstrated that, in HBsAg-responder mouse strains (BALB/c, C57BL/6, C3H/HeN, and ICR), the new Gel/HBs+GM vaccine elicited much greater anti-HBs titers than HBsAg in the free form (Fig. 1A) or adsorbed to alum (H-B-VAXII) (Fig. 1B).

Similar to the results of a previous report (42), we also observed that Gel/HBs increased anti-HBs responses (Fig. 1A) but to a much lower level than that induced by the Gel/HBs+GM vaccine, suggesting the importance of GM-CSF adjuvant activity. Importantly, the Gel/HBs+GM vaccine also generated significant levels of anti-HBs Abs in normally nonresponder B10.M mice (Fig. 2A), suggesting that the MHC-linked nonresponsiveness to HBsAg can be overcome by a vaccine formulation that can provide a sustained release of HBsAg and a strong adjuvant.

Our data also demonstrate the importance of the sustained release and colocalization of GM-CSF and HBsAg for the cytokine to exert its adjuvant effect, because the adjuvant activity of GM-CSF was largely lost when HBsAg and GM-CSF were administered without hydrogel or when Gel/HBsAg and Gel/GM were injected at two separate sites (Fig. 1A). Similar polymer- or gel-formulated GM-CSF had been used to enhance the vaccine efficacy of tumor peptides or Ags (43, 44), supporting the general application of this approach to improve the immunogenicity of low-immunogenic proteins. In addition to enhancing vaccine efficacy, an advantage of local GM-CSF delivery is that it avoids potential side effects associated with systemic administration of GM-CSF, such as the induction of histologically abnormal livers and spleens accompanied by an increased number and activation state of inflammatory cells (45, 46), as well as the generation of CD11b+Gr-1+ myeloid suppressor cells (47–49), which could inhibit T cell responses and ultimately decrease vaccine efficacy.

It is known that the primary protective immune response induced by rHBsAg vaccine is provided by anti-HBs Abs, and the successful production of anti-HBs Abs is critically dependent upon T cells. In the hypo- and nonresponder individuals, lack of response to HBsAg has been attributed to a number of mechanisms, including a defect in the generation of primary HBsAg-specific T cells (12–16) or deficient B cell repertoires (50, 51). The fact that, following Gel/HBs+GM vaccination, nonresponder B10.M mice were able to produce significant levels of anti-HBs Abs (Fig. 2A), with a titer comparable to that produced in the responder mouse strains (Fig. 1), rules out the possibility that a lack of HBsAg-specific B cells is the major cause of HBsAg nonresponsiveness. In contrast, there is substantial evidence that, in the nonresponders, standard HBsAg vaccines fail to stimulate Th cell responses (12–16), which is supported by our observation that two doses of the commercial H-B-VAXII vaccine (Fig. 2B) only induced weak HBsAg-specific T cell-proliferative responses in nonresponder B10.M mice. Significantly, this defect in T cell responses to HBsAg in B10.M mice was overcome by the Gel/HBs+GM vaccine, which induced a strong T cell-proliferative response to HBsAg (Fig. 2B), indicating successful production of anti-HBs Abs, strongly supporting the important role of T cells in breaking HBsAg nonresponsiveness.

GM-CSF has been extensively studied as an adjuvant for vaccination (20, 21). GM-CSF mediates its adjuvant effect by stimulating the differentiation and activation of APCs (19), including DCs, which is the major cell type involved in priming naive T cells. Our results showed that local injection of responder mice with Gel/GM greatly increased the migration of adoptively transferred BM-DCs (Fig. 3A, 3B) and endogenous DCs (Fig. 3A, 3C) to the draining LNs. Similarly, in the context of HBsAg vaccination, nonresponder B10.M mice receiving Gel/HBs+GM vaccine consistently recruited more CD11c+ DCs to the draining LNs than mice treated with Gel/saline (hydrogel alone) or Gel/ HBs (Fig. 4A, 4B). Importantly, Gel/HBs+GM vaccination also resulted in upregulation of MHC class II and costimulatory molecules (CD40, CD80, and CD86) on DCs (Fig. 4C, 4D), which can greatly increase their Ag presentation and interaction with naive T cells. Gel/HBs did not significantly increase the number of DCs in the draining LNs and only slightly enhanced CD86 expression, with no effect on CD40, CD80, or MHC class II expression (Fig. 4D), reflecting the importance of GM-CSF adjuvant activity in the Gel/HBs+GM vaccine. Our data suggest that more CD11c+ DCs with a more mature phenotype were recruited to the draining LNs in the mice treated with the Gel/HBs+GM vaccine, leading to increased HBs-specific Th cell responses (Fig. 2B), which subsequently provided help to B cells to produce anti-HBs Abs in the nonresponder B10.M mice. Because of technical difficulties, we were not able to isolate a sufficient number of DCs from the draining LNs of Gel/HBs+GM-treated B10.M mice to directly prove that their DCs became more potent in presenting HBs to Th cells. However, this mechanism is supported by a previous report that increasing MHC class II expression on DCs from HBV transgenic mice helped to overcome anti-HBs nonresponsiveness in an in vitro culture experiment (52).

Several strategies were reported to overcome nonresponsiveness to HBsAg. Administration of additional or greater vaccine doses can increase anti-HBs titers in some low responders, but this usually fails in nonresponders (53). HBsAg-pulsed DCs, a cell-based vaccine, was reported to induce anti-HBs Abs in HBsAg vaccine nonresponders (54). The pre-S–containing HBsAg (PreS/ S) vaccine, with addition of the pre-S1 and pre-S2 sequences to the S protein, helped to circumvent immune nonresponsiveness to HBsAg in B10.M mice (55, 56) and in several human clinical trials (57–59). Studies using overlapping peptides revealed that the pre-S region was important for eliciting T cell responses and, thus, help to improve the immunogenicity of the PreS/S vaccine (60). However, the enhanced immunogenicity was only observed for mammalian cell-produced PreS/S vaccines (57), not for yeast-derived PreS/S vaccines (58, 59), suggesting that glycosylation might also play a role in the improved immunogenicity. The expected high cost of the mammalian-produced vaccine will limit its clinical application. In contrast, our approach using hydrogel and GM-CSF can be used with the standard yeast-derived HBsAg, without the need for including additional Ag components in vaccine preparations. With the simplicity of vaccine formulation and the proven track records for safe drug delivery of the PEG/PLGA-based hydrogels (61), our hydrogel-formulated GM-CSF vaccine represents a simple and effective method for generating next-generation HBV vaccines with improved immunogenicity to induce anti-HBs Abs in poor and nonresponders, as well as to reduce the number of doses required for generating long-term HBV protection. A similar hydrogel-formulated GM-CSF method might also be applied to generate vaccines against other infectious and malignant diseases.
Disclosures

The authors have no financial conflicts of interest.

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