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Hepatitis B Vaccination Induces Mucosal Antibody Responses in the Female Genital Tract, Indicating Potential Mechanisms of Protection Against Infection

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Abstract: Vaccines against hepatitis B virus confer effective protection. Enzyme-linked immunosorbent assay was developed to test for specific antibodies in female genital tract secretions. Anti–hepatitis B IgG and IgA were detected in the cervicovaginal secretions of women after hepatitis B vaccination, indicating a potential genital tract role for neutralizing antibodies against sexually transmitted hepatitis B virus.

Hepatitis B virus (HBV) is a prevalent blood-borne and sexually transmitted pathogen. Current HBV prevention relies on second-generation yeast-derived recombinant DNA vaccines targeting the conserved immunodominant "*a*" region of the hepatitis B surface antigen (HBsAg) small protein. These vaccines confer rapid protection in most recipients via neutralizing anti-HBsAg antibody induction (anti-HBs)[1] and long-lived cellular memory responses.[2] Despite the success of these vaccines, concerns remain regarding nonresponders, mother-to-child transmission, escape mutants, and suboptimal vaccine antigenicity, highlighting the need for ongoing HBV vaccine research.[3]

Hepatitis B immune globulin activity in postexposure and posttransplant prophylaxis of HBV infection has led to the convention that the mechanism of action of HBV antibodies in preventing infection is systemic.[4] Little work has been performed investigating the potential role of neutralizing antibodies at mucosal levels in relation to sexually transmitted HBV. Our aim was to investigate the genital mucosal antibody response in women vaccinated AQ2 with Engerix-B (GSK, London, United Kingdom), the most widely used HBV vaccine in the United Kingdom. Evidence of anti-HBs in cervicovaginal secretions could indicate that HBV vaccine-induced responses may also be protective at the level of the genital mucosa in the context of sexually transmitted HBV infection.

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This study was approved by the York Research Ethics Committee (REC reference: 06/Q1108/100).
Contributors: C.J.N.L. acquired funding and conceived the study. C.C. consented participants and collected the samples. R.W., C.C., and J.M. performed the enzyme-linked immunosorbent assays and with input from J.M.F. and S.J.S. analyzed data. S.J.S., J.M.F., R.W., and C.J.N.L. led on manuscript preparation. All authors approved the final version.
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MATERIALS AND METHODS

Healthy, asymptomatic women aged 18 to 45 years were recruited from 2 sexual health clinics (York and Harrogate, Yorkshire, United Kingdom) after gaining informed consent. Serum was obtained from 17 volunteers (7 previously vaccinated intramuscularly, 10 unvaccinated). Of these, 5 vaccinated and 7 unvaccinated women provided cervical and vaginal mucosal samples. Two further volunteers (1 vaccinated, 1 unvaccinated) provided mucosal samples, bringing the total mucosal samples available to 6 vaccinated and 8 unvaccinated.

Endocervical and vaginal wall secretion sampling and storage was undertaken as previously described[5] using Weck-Cel surgical spears (Medtronic, Minneapolis, MN); secretion dilution factors were calculated according to Rohan et al.[6] Calculations for blood contamination in mucosal samples were undertaken based on methods of Kozlowski et al.[7] Mucosal samples were not obtained during menstruation to reduce blood contamination. Serum samples were centrifuged at room temperature for 10 minutes at 1500*g*; the serum portion was stored at −20°C.

Enzyme-Linked Immunosorbent Assays for HBsAg-Specific IgG and IgA in Serum, and Cervical and Vaginal Secretions

Indirect enzyme-linked immunosorbent assays (ELISAs) for IgG and IgA anti-HBs were developed for sera and mucosal samples by testing different experimental parameters: coating (assay) buffer, pH, concentration of antigen, blocking buffer, and sample concentration using checkerboard titrations with samples of known HBV vaccination status. A serum sample from an HBV-vaccinated individual with a known titer of total anti-HBs antibody (Architect; Abbott Laboratories, Abbott Park, IL) was used to create a 4-point standard curve and assigned units per milliliter from which sample values could be interpolated. Washing steps were undertaken using an automated plate washer (Scanwasher 400; Cox Scientific, Northants, United Kingdom) containing phosphate-buffered saline + 0.05% Tween 20 (PBST; Sigma-Aldrich, Poole, United Kingdom).

The ELISA established after development was undertaken as follows with all incubations at 37°C for 1 hour until substrate addition: Greiner Bio-One 96-well medium-binding microplates (Jencons, Leighton Buzzard, United Kingdom) were coated 1:1 with 50 μL of 10 μg/mL recombinant HBsAg (Engerix-B) in 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ (carbonate buffer) with assay

buffer blanks and then incubated. Unbound material was washed off with PBST, and plates were blocked with 10% fetal bovine serum in PBST. Plates were washed and the sample was added in triplicate. Samples were washed and peroxidase conjugate–linked Fc-specific IgG and IgA was added (Sigma-Aldrich). Plates were washed, and 3,3′5,5 tetramethylbenzidene substrate (KPL Inc, Gaithersburg, MD) was added and incubated for 5 minutes at room temperature; reaction was stopped by the addition of stop solution (KPL Inc). Absorbance was measured immediately after stop solution addition at 450 nm on a Bio-Rad 550 plate reader (Bio-Rad, Watford, United Kingdom). Triplicate sample optical densities (ODs) were averaged, and final OD was gained by subtracting buffer blank OD. Units per milliliter of anti-HBs in samples was interpolated from the standard curve and corrected for dilution factors.

Data were analyzed and plotted graphically (GraphPad PRISM v. 6.0, GraphPad Software Inc, La Jolla, CA). Comparisons in antibody levels between groups were performed using the Mann-Whitney *U* test. The relationship between serum and mucosal HBsAg-specific IgA and IgG levels was analyzed using the Spearman rank correlation coefficient.

RESULTS

Characteristics of the Study Cohorts

Vaccinated women were 26 to 42 years of age (median, 33 years), and unvaccinated women were 20 to 37 years of age (median, 26 years). There were missing data in the recording of contraceptive status, but of the 12 women, 4 were using combined oral contraception, 3 were using progesterone only, 2 were using the Mirena, and 3 were using barrier methods, with methods recorded in both cohorts.

Concentrations of specific IgG and IgA anti-HBs detected in serum, cervical, and vaginal samples are shown in Figure 1.

ELISA for HBsAg-Specific Serum IgA and IgG

Hepatitis B surface antigen–specific serum IgG levels in the vaccinated group (n = 7; median, 98.3 U/mL) were markedly greater than those in the unvaccinated group (n = 10; median, 7.95 U/mL; $P = 0.0002$). The vaccinated group's HBsAg-specific serum IgA levels (n = 7; median, 34.9 U/mL) were also significantly higher than those in the unvaccinated (n = 10; median, 9.8 U/mL; $P = 0.043$), but with a smaller disparity.

ELISA for HBsAg-Specific Mucosal IgA/IgG

Hepatitis B surface antigen–specific cervical IgG levels in the vaccinated group (n = 5; median, 18.4 U/mL) were greater than those in the unvaccinated (n = 7; median, 1.30 U/mL; $P = 0.0025$). The vaccinated group's HBsAg-specific cervical IgA levels (n = 6; median 6.33 U/mL) were also greater than those in the unvaccinated (n = 8; median, 1.75 U/mL; $P = 0.008$). There was no statistically significant difference in HBsAg-specific vaginal IgG in vaccinated (n = 5; median, 3.78 U/mL) compared with the unvaccinated (n = 8; median, 1.52 U/mL; $P = 0.09$), although HBsAg-specific vaginal IgA in vaccinated (n = 5; median, 7.39 U/mL) were greater than the unvaccinated (n = 8; median, 1.48 U/mL; $P = 0.006$).

Correlation Between HBsAg-Specific Serum and Mucosal IgA and IgG

A statistically significant correlation existed between serum and cervical anti-HBsAg IgG ($P = 0.033$), with cervical levels being 20.8% (95% confidence interval, 12.6%–40.4%) of those in serum. There was a nonsignificant correlative trend ($P = 0.06$) between serum and vaginal anti-HBsAg IgA, with vaginal levels being 6.6% (95% confidence interval, 0.9%–12.3%) of those recorded in serum. Cervical IgA and vaginal IgG were not significantly correlated with serum.

DISCUSSION

A novel ELISA was developed to test for HBsAg-specific IgG and IgA in female genital tract mucosal secretions. We found that anti-HBs IgG and IgA can be detected in the cervicovaginal secretions of women after intramuscular Engerix-B vaccination. Anti-HBs levels were greater in serum than in mucosal samples, and IgG levels were greater in cervical than vaginal samples.

Our study was limited by sample size, sample volumes, incomplete sample sets, and no definitive data for the participants' exact vaccination dates. We included extra samples in addition to the 12 matched pairs to demonstrate assay performance with every available sample in this small study. All correlations were performed on matched pairs only. We also consequently focused on measuring specific rather than type-specific anti-HBs. We consider this a reasonable approach because the women were on varying contraceptive methods and only 3 of 12 women for whom we have contraceptive data could be said to have had normal menstrual cycle, during which antibody levels will fluctuate.

Our data are comparable to that of Nardelli-Haefliger et al.,[8] who investigated the presence of antibodies against human papillomavirus (HPV) virus-like particles in cervical secretions in women vaccinated with an HPV virus-like particle vaccine. As they did, we showed a significant titer of vaccine-induced IgG antibodies in cervical secretions. Because of our smaller study, we could not show the cyclical variation in antibody levels in women with ovulatory cycles they demonstrated, but like them, we did observe a significant relationship between systemic and cervical specific IgG levels with a similar ratio, supporting transudation as the primary source of genital tract IgG.[8–10] The data also suggested a possible correlation between systemic and vaginal IgA anti-HBsAg. This is less well understood but could be due to a combination of factors including transudation from the systemic circulation[9] as well as local production[11] perhaps associated with plasma cell trafficking.[10]

The significance of secreted anti-HBs in the female genital tract is currently unknown. However, ~80% of hepatitis B vaccine–induced neutralizing antibodies are IgG1 subclass,[1] and 90% of cervicovaginal IgG antibodies are also IgG1 subclass.[9] In contrast to other mucosal surfaces, IgG is the dominant isoform in the cervix as opposed to IgA.[12] The mechanism of action of neutralizing antibodies in the female genital tract is well described in the context of HPV vaccination.[13] However, HPV is a purely epithelial pathogen with its target cells present in the anogenital mucosae, whereas HBV needs to undergo systemic dissemination to reach its target cells, including those in the liver. Hepatitis B virus infects a wide range of cell types in both acute and chronic infection, including lymphocytes, monocyte/macrophages, and neutrophils.[14,15] All these migratory hemopoietic cells express HSPGs, which act as an HBV receptor.[16] Overall, it seems likely that peripheral blood mononuclear cells ferry HBV from the anogenital tract to the liver, as they are a known transmission mechanism for mother-to-infant infection.[17]

We suggest that our data support a hypothesis that vaccine-induced anti-HBs antibodies, both secreted in the genital tract and systemic, act in concert with innate immune mechanisms to neutralize HBV, both at the portal of entry and on any migratory cells or in distal tissues before the HBV virion is able to mature[18] and successfully transit to its primary target, the hepatocyte. A larger study could explore these proposed mechanisms.

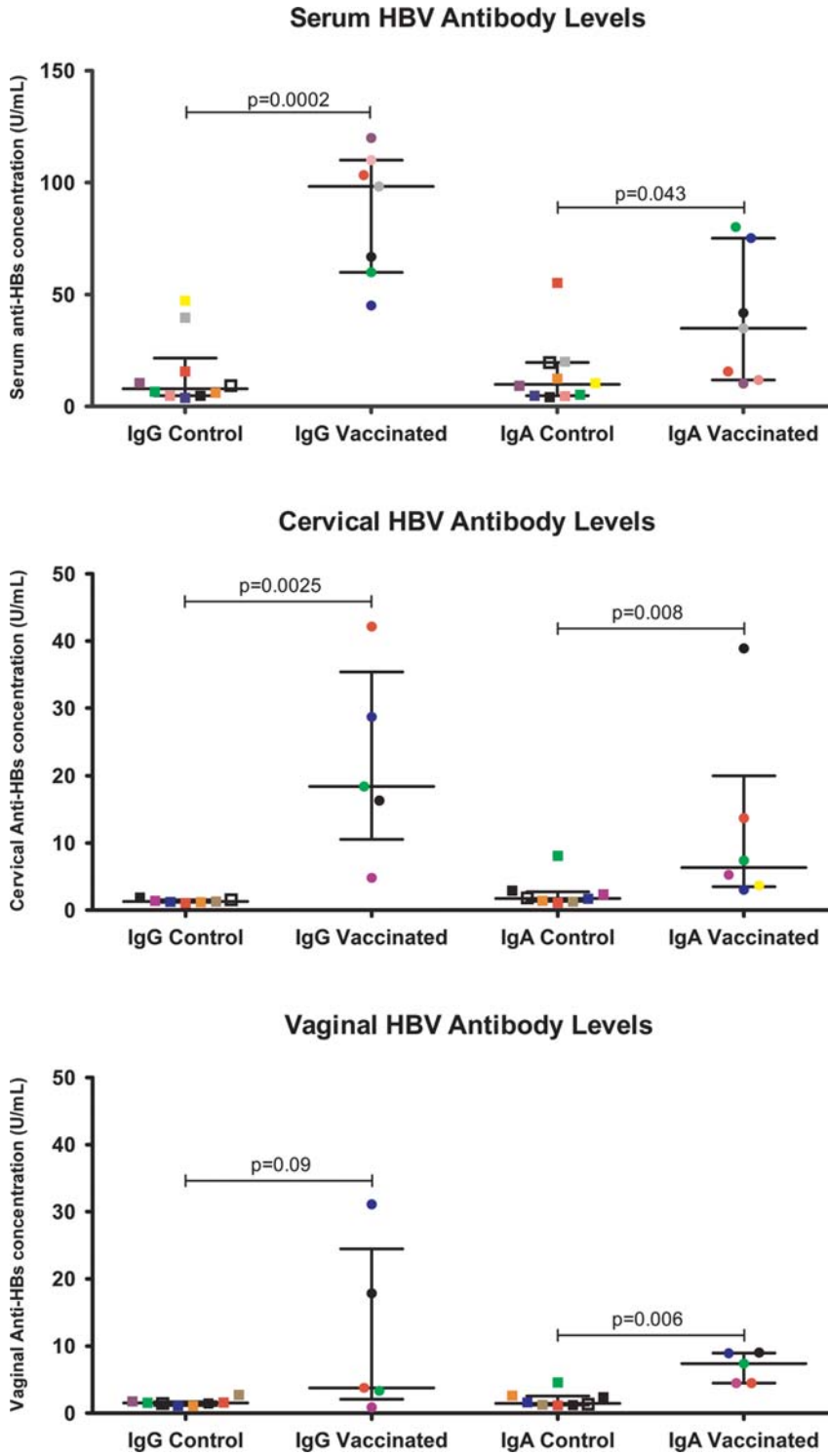

Fig 1 4/C

Figure 1. Concentration of anti-HBs in serum, cervical, and vaginal secretions. Enzyme-linked immunosorbent assays were performed to detect anti-HBs in the serum of control (n = 10) or vaccinated (n = 7) and cervical and vaginal secretions of control (n = 8*) or vaccinated (cervical, n = 6*; vaginal, n = 5) women. Data of individual participant are plotted as a separate matched color in the respective control (square symbols) or vaccinated (circular symbols) groups, with median detected concentration shown as the major horizontal line. Error bars show interquartile range. Statistical comparisons between data sets are indicated with corresponding *P* values. *One sample had insufficient volume available for all mucosal assays.

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