Humoral Immune Responsiveness in Duck Hepatitis B Virus-Infected Ducks

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Immunofluorescence assays with fixed tissue sections were used to characterize antibody reactivity in sera obtained from duck hepatitis B virus-infected ducks. Under conditions of experimental infection, antibody to core antigen but not to surface antigen was detectable. A majority of the ducks infected at 8 days after hatching and a minority of those infected at 1 day after hatching showed a transient anti-core antigen humoral response; this response was stronger in the antibody-positive ducks infected on day 8 than in those infected on day 1. Antibody to core antigen was not detected in the sera of ducks congenitally infected with duck hepatitis B virus. Several of the infected ducks, but none of the uninfected ducks, exhibited autoantibody reactivity for α-islet-cell-associated antigen.

Most of the hepatocellular damage associated with hepatitis B virus infection has been ascribed to the immune response to viral antigen (9). Despite much clinical interest in this pathology, none of the several animal models of hepatitis B infection has been widely used for immunological analysis. We have, therefore, initiated a study of immune responsiveness in Pekin ducks infected with duck hepatitis B virus (DHBV).

Unlike other animal models of hepatitis B infection, the DHBV system may be used for a comparison of immune responsiveness under conditions of experimental infection (postnatal virus inoculation [6]) versus congenital infection (virus transmission from the mother [7], resulting in a high level of replication in early-stage embryos [4, 8]). Implicit in such a comparison is an assessment of the immunogenicity as well as the potential tolerogenicity of viral antigen. As an initial step in this analysis, this study focused on the humoral response to DHBV infection, examined at the level of serum antibody reactivity.

Twenty ducks inoculated intravenously with DHBV at 8 days after hatching and 21 uninoculated controls were maintained for 16 weeks and monitored at 2-week intervals for serum antibody reactivity to DHBV antigen-positive cells. All of the infected ducks became viremic by 2 to 4 weeks postinoculation, as indicated by a DNA hybridization assay for viremia (5). Test sera were screened for antibody in an immunofluorescence assay (described in the legend to Fig. 1) based on the use of fixed tissue sections. This form of assay was chosen because the preservation of tissue architecture facilitates testing of whether reactivity is directed to cell-associated antigen. Assays were carried out with sections prepared from pancreas tissue of either 2- to 3-week-old or 3- to 4-month-old congenitally DHBV-infected or uninfected ducks. Previous work has shown that numerous scattered pancreas acinar cells in young, congenitally DHBV-infected ducks and a majority of the α cells localized to α islets in older, congenitally infected ducks are positive for both viral core and surface antigens (1-3).

Antibody reactive to viral-antigen-positive acinar cells was detectable in sera from 16 of the 20 infected ducks but not in sera from the uninfected ducks (representative assays are shown in Fig. 1). Reactivity first appeared at 2 to 6 weeks postinoculation and generally persisted for 4 to 8 weeks before waning. Those sera that showed strong reactivity to viral-antigen-positive acinar cells also showed reactivity above background level to both viral-antigen-positive α-islet cells (Fig. 2) and viral-antigen-positive hepatocytes (data not shown). Reactivity to acinar cells or hepatocytes from uninfected ducks was not detected in sera from either infected or uninfected ducks (data not shown).

In tests of α-cell reactivity with sera from uninfected ducks, the large majority of cells in viral-antigen-positive α islets showed no fluorescence above the background level observed with the section as a whole (Fig. 2C and D). However, a minority of cells, comprising from 5 to 20% of the total number of cells in individual α islets, was fluorescent to levels considerably above background (Fig. 2C); as in all the assays of duck sera from uninfected ducks, fluorescence was scored with a fluorescein-specific barrier filter (because of the use of a fluorescein-labeled goat anti-chicken immunoglobulin serum). A similar percentage of fluorescent islet-associated cells was observed in assays of sera from uninfected ducks as tested with pancreas sections from age-matched uninfected ducks (data not shown). Most, if not all, of these cells were doubly fluorescent, scoring positively with either a rhodamine- or fluorescein-specific filter. Because islet tissue in pancreas sections that were unreacted with any sera showed a minority of autofluorescent cells as scored with either filter, the use of the rhodamine-specific filter served to enumerate autofluorescent cells in tissue reacted with fluorescein-labeled antisera. Since virtually all positive cells detected in pancreas tissue assayed with sera from uninfected ducks and with the fluorescein-labeled anti-immunoglobulin serum were doubly fluorescent, it seems likely that in these assays the fluorescence scored with the fluorescein-specific filter was due to autofluorescence rather than to antibody reactivity.

The double immunofluorescence assays (Fig. 1 and 2) did not in themselves distinguish between duck antibody recog-
FIG. 1. Serum antibody reactivity for viral-antigen-positive pancreas acinar cells. The two panels in each row show double immunofluorescence assays of the same field of exocrine pancreas cells from a 3-week-old congenitally DHBV-infected duck. (A and C) Serum from a 10-week-old DHBV-infected duck (infection at 8 days after hatching). (B and F) Rabbit anti-surface antigen serum. (D) Rabbit anti-core antigen serum. (E) Serum from a 10-week-old uninfected duck. Tissue sections were prepared as previously described (2). The following order was used for the addition of each reagent (30 to 40 µl) to an individual section: (i) the test duck serum (diluted 1:3 with phosphate-buffered saline), (ii) rabbit antiviral serum (diluted 1:40 with serum from an uninfected duck and further diluted to 1:200 with phosphate-buffered saline), (iii) fluorescein-labeled goat antibody directed to chicken immunoglobulin (the affinity-purified goat antibody preparation was purchased from Kirkegaard & Perry, Gaithersburg, Md., and used at a concentration of 200 µg/ml; its reactivity in the immunofluorescence assay was inhibited by a competing preparation of duck immunoglobulin), and (iv) a rhodamine-labeled immunoglobulin fraction of a goat anti-rabbit immunoglobulin serum (diluted 1:320 with phosphate-buffered saline). Bar equals 20 µm.

nition of viral antigen or recognition of nonviral antigen induced as a consequence of infection. Nevertheless, in terms of the relative levels of fluorescence associated with individual acinar cells (Fig. 1), the pattern of staining mediated by the duck antibody paralleled that mediated by core antigen-reactive but not by surface antigen-reactive rabbit antibody. These assays therefore suggested that much, if not all, of the reactivity in the positive duck sera was directed to viral core antigen.

To test this inference, preparations of DHBV surface antigen particles or immature viral cores were purified from serum or liver, respectively, as described previously (3), and were assayed for their capacity to compete with the reactivity of the positive duck sera. Whereas competition with surface antigen particles had no discernible effect, competition with immature cores reduced the level of cell-associated fluorescence to that of the background (representative assays with viral-antigen-positive acinar cells are shown in Fig. 3; data are not shown for assays with viral-antigen-positive α cells or hepatocytes). We therefore concluded that, as scored in the immunofluorescence assay, the specificity of the duck antibody was directed to viral core antigen. However, the possibility is not excluded that the absence of detectable antibody to viral surface antigen reflects the in vivo complexing of antibody rather than the lack of immune response.

Analysis was then extended to ducks experimentally infected with DHBV at 1 day after hatching or congenitally infected with DHBV. As tested with sera obtained up to 16 weeks after hatching, one or more blood samples from 13 of 30 ducks infected on day 1 exhibited reactivity for viral-antigen-positive pancreas acinar cells. By contrast, no blood samples from seven congenitally infected ducks exhibited such reactivity (data not shown), a result consistent with the premise of immune tolerance.

For 12 of the ducks infected on day 1 whose sera showed
FIG. 2. Serum antibody reactivity for viral-antigen-positive α cells. The two panels in each row show double immunofluorescence assays of the same field of pancreas from a 4-month-old congenitally DHBV-infected duck. (A) Serum from a 10-week-old DHBV-infected duck (infection at 8 days after hatching). (B and D) Rabbit anti-surface antigen serum. (C) Serum from a 10-week-old uninfected duck. The large structures resolved in panels B and D correspond to α islets, as shown by the reactivity of antiglucagon serum for these structures in adjacent sections. Bar equals 20 μm.

FIG. 3. Effect of competition with purified viral antigen on serum antibody reactivity for viral-antigen-positive pancreas acinar cells. The two panels in each row show double immunofluorescence assays of the same field of exocrine pancreas cells from a 3-week-old congenitally DHBV-infected duck. (A) Serum from a 10-week-old DHBV-infected duck (infection at 8 days after hatching) in competition with purified surface antigen particles (40 μg/ml). (B) Rabbit anti-surface antigen serum in competition with purified surface antigen particles. (C) Same serum as in panel A in competition with purified immature viral cores (20 μg/ml). (D) Rabbit anti-surface antigen serum in competition with purified immature viral cores. Bar equals 20 μm.
FIG. 4. Serum antibody reactivity for pancreas cells. The two panels in each row show double immunofluorescence assays of the same field of pancreas cells. (A, C, E, G, and I) Serum from the final blood sample (14 weeks postinfection) of a DHBV-infected duck (infection at 1 day after hatching). (B, D, F, and H) Rabbit anti-surface antigen serum and pancreas cells from a 4-month-old congenitally DHBV-infected duck (D), pancreas cells from a 4-month-old uninfected duck (α islet is shown) (F), and pancreas cells from the duck that was used to provide the test serum (α islet is shown) (H). (J) Rabbit antilucaglagon serum and pancreas cells from the duck that was used to provide the test serum. The antilucaglagon serum has previously been described (1) and, as used here, was diluted 1:5 with phosphate-buffered saline. Bar equals 20 μm.

demonstrable reactivity, antibody was detectable at 2 weeks postinfection, and for the remaining duck, antibody was detectable at 4 weeks postinfection. As with the ducks infected at 8 days after hatching, reactivity was blocked by competition with purified preparations of immature viral cores but not by competition with preparations of surface antigen particles. In addition to the lower percentage of antibody-positive ducks among the group infected at 1 day after hatching, two sets of observations indicated that the anti-core antigen humoral response was considerably weaker in these ducks than in the ducks infected on day 8: (i) a more rapid waning of antibody production (8 of the 12 ducks infected on day 1 that exhibited antibody reactivity at 2 weeks postinfection had lost detectable reactivity by 4 weeks postinfection) and (ii) a 2- to 3-fold-lower titer of serum antibody at 2 weeks postinfection (on the order of 1:3 for the antibody-positive ducks infected on day 1 versus 1:6 or 1:9 for the antibody-positive ducks infected on day 8).

Assays for α-islet cell reactivity were then carried out with sera obtained from the ducks infected on day 1 at times subsequent to the waning of detectable reactivity for viral-antigen-positive acinar cells. As tested with viral-antigen-positive islet tissue, most of these sera yielded a pattern of α-islet-associated fluorescence indistinguishable from that obtained with sera from uninfected ducks. However, sera obtained from one duck both at 12 weeks postinfection and when the duck was killed at 14 weeks postinfection exhibited reactivity to virtually all α-islet cells, including those not detectably viral antigen positive (Fig. 4A and B show results of the assay of the final blood sample from this duck). This serum did not exhibit detectable reactivity for viral-antigen-positive or -negative acinar cells (Fig. 4C and D) or hepatocytes (data not shown).

The recognition by this duck serum of viral-antigen-negative α-islet cells suggested that reactivity was directed to antigen expressed in uninfected α-islet cells. This possibility was confirmed in assays of the serum with pancreas tissue from age-matched uninfected ducks, with virtually all α-islet cells showing fluorescence above background level (Fig. 4E and F; positive fluorescence was detectable at serum dilutions of up to 1:81). The recognition of viral-antigen-negative α-islet cells in pancreas sections from the autologous duck (Fig. 4G and H) established that reactivity was mediated by an autoantibody. The reactivity of rabbit antilucaglagon serum for these target cells (Fig. 4I and J) served in turn to directly identify them as α cells. Under conditions of competition with a commercial preparation of bovine glucagon (1), the reactivity for α cells of the rabbit antilucaglagon serum but not of the autoreactive duck serum was blocked (data not shown).

Pancreas sections from the age-matched uninfected ducks were used to screen a large number of duck sera for a comparable reactivity. In addition to the duck described above, a second duck from the group of 30 ducks infected at 1 day after hatching exhibited serum antibody reactivity for DHBV-negative α cells; this reactivity was detected in the blood sample obtained at 14 weeks postinfection (serum antibody titer of 1:9) but not in earlier or subsequent samples. Antibody was also detected in the final sample obtained at 16 weeks after hatching from one of the 7 congenitally DHBV-infected ducks examined (antibody titer of 1:27) but in none of the samples obtained from the 20 ducks infected on day 8. All blood samples from uninfected ducks were antibody negative, with the test sample comprising the following three groups: (i) 21 uninoculated controls described above, which were monitored at 2-week intervals for up to 16 weeks after hatching; (ii) 30 ducks from the SPAFAS breeding flock, which were bled at 12, 16, and 20 weeks after hatching; and (iii) 60 ducks from the SPAFAS flock, ranging in age from 7 to 12 months, which were bled once.

The presence of autoantibody in a small percentage of the DHBV-infected ducks examined here and its absence in the uninfected ducks tested raises the possibility that DHBV
infection is a predisposing factor for the induction of α-cell autoreactivity. If this suggestion proves correct, the DHBV system may represent a useful model for the study of the induction of autoimmunity in the context of a hepadnavirus infection.

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LITERATURE CITED


