Primary Immunoglobulin Response of Herons to Infection with Venezuelan Encephalitis Virus

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Seven to nine days after inoculation with a replicating antigen, Venezuelan encephalitis virus, hemagglutination-inhibiting antibodies were present in plasmas of 18 of 20 black-crowned night herons (BCNH), 14 of 15 great egrets (GE), and 7 of 7 snowy egrets (SE). 19S (immunoglobulin M) preceded 7S (immunoglobulin G) antibodies in all but one bird of six GE, six SE, and six BCNH. 19S antibodies were detected for only 2 to 4 weeks post-inoculation. The induction period for both types of antibody was prolonged by 2 to 6 days as compared with earlier studies in gallinaceous birds using nonreplicating antigens. A marked delay in reaching peak titer of 7S antibodies was also observed. Hemagglutination inhibition tests were nearly as sensitive as neutralization tests for detecting 19S and early 7S antibodies. Size of virus inoculum did not measurably affect time of induction or titer of antibodies.

For epidemiological studies, data are needed regarding the appearance and disappearance of classes of immunoglobulins after infection with replicating agents such as arboviruses. For example, knowledge of the temporal sequence of development of specific immunoglobulins in wild birds might permit accurate timing of infection. Recent infections would imply that the virus was still cycling in the area or, in the case of migrating birds, that the bird had recently arrived from an area in which the virus was active.

The sequence and timing of the appearance of immunoglobulins in species of gallinaceous birds (chickens, turkeys, pheasants, and Chinese quail), usually inoculated with massive doses of nonreplicating antigens, have been established within the last years (2, 8, 12, 14, 15, 18–20). These antigens have included bovine and human serum albumin, keyhole limpet hemocyanin, mammalian erythrocytes, inactivated bacteria or viruses, bacterial components, and bacteriophage. Soluble antigens were shown to elicit an immunological response in chickens differing from that produced by particular antigens (8, 15). These studies utilized a replicating antigen, viz., Chlamydia sp., (14), Japanese encephalitis virus (JE) (13), and influenza virus (10). Although many workers have experimentally infected birds with arboviruses (3, 4, 11, 21) or Newcastle disease virus (5), their interest has been confined mainly to viremia levels and overall antibody development. One report (13) concerns transformation of immunoglobulins after two inoculations of a single adult fowl with large amounts of JE virus.

In the present study, we followed the immune response in wild-caught birds after inoculation with a small amount of a replicating arbovirus. Further, to facilitate epidemiological investigations, we explored the possibility of using the antibody response to estimate the time of onset of infections in samples from wild populations. Three species of herons (family Ardeidae) were chosen for these studies because they inhabit and nest in colonies in areas in which Venezuelan encephalitis (VE) virus is endemic, and are occasionally infected naturally with this agent (7).

MATERIALS AND METHODS

Virus. Strain 64A87 of VE virus, isolated from mosquitoes (Culex opisthopus) in Mexico, was used. This strain belongs to the enzootic (or sylvatic) subtype 1, variety E, found throughout Central America (22). Virus was inoculated (i) as the original mosquito suspension, (ii) after one passage in colonized Aedes aegypti mosquitoes injected intratracheally, or (iii) after one passage each in black-crowned night herons (Nycticorax nycticorax; BCNH) and in suckling mouse brains.

Experimental birds. The herons used in these experiments were great egret (formerly called American or common egret [Egretta alba]; GE), snowy egret (Egretta thula; SE), and BCNH. With the exception of five GE received from Florida, all birds were taken as nestlings from heron colonies in New Jersey or on Long Island, N.Y., under federal and state permits. Their ages were determined to within 2 to 3 days by external measurements and the stage of plumage...
development. Age at time of inoculation ranged from 8 to 20 days (30 to 35 days in those from Florida) in GE, 8 to 14 days in SE, and 6 to 26 days in BCNH. One GE was 1 year old when inoculated.

All birds were pre-bleed and then inoculated subcutaneously in the breast or thigh with 0.1 ml of a suspension containing 10⁶ to 10⁸ chicken embryo cell culture (CEC) plaque-forming units (PFU) of virus, with the exception of two BCNH that received 10⁵ PFU. For the detection of viremia, blood samples (0.8 to 2.0 ml) were taken on days 1 to 4 post-inoculation from the external jugular vein by using syringes containing 0.05 to 0.1 ml of heparin (2 U in isotonic saline solution). For antibody studies, 2.5 to 5.0 ml of blood was taken every second or third day between days 7 and 14, at least once a week to day 36, and monthly thereafter. Whole blood for virus studies was stored at -70 C.

**Titration of whole blood and plasma.** Titration of blood samples for quantitative determination of virus (expressed as negative log₁₀ CEC) PFU per milliliter of blood were carried out in CEC with agar overlay prepared and used as described elsewhere (17). Initial antibody assays were performed on day 7 plasmas, thereby allowing time for the clearance of replicating antigens. The hemagglutination inhibition (HI) tests were performed by microtechniques previously described (6). Plasma samples to be tested for HI antibody were extracted twice with acetone to remove nonspecific inhibitory lipids prevalent in bird blood and adsorbed once with 0.025 ml of packed goose erythrocytes to remove nonspecific goose cell agglutinins. Serum dilution neutralization (N) tests were performed in primary CEC cultures grown in disposable plastic plates with 2-cm² wells, as described previously (16).

Antigens for use in HI tests were prepared by using strain 64A87 virus in (i) CEC by the methods used in the preparation of Vero hemagglutinins (22) or (ii) sucking mouse brains (6). Both hemagglutinins gave comparable results against the same sera when diluted to contain 4 hemagglutination units/0.025 ml. For N tests, only virus from mouse brains was utilized as antigen.

An HI titer of 1:5 or greater in any plasma was considered to be due to specific antibody because no nonspecific inhibitors were detected in fractionated normal plasma of the three heron species tested at that dilution. Plasma fractions yielding a log₁₀ neutralization index of greater than 1.1 (92% plaque reduction) were considered positive. In the figures, HI titers were converted to log₁₀ to allow direct comparison with N titers, but in the text HI titers are expressed as the reciprocal of the plasma dilutions.

**Separation of 19S (IgM) and 7S (IgG) antibodies.** Since 2-mercaptoethanol partially inactivates avian immunoglobulin (Ig) G in addition to IgM (15, 19), it could not be used, as in mammals, to detect IgM. Therefore, 19S and 7S antibodies were separated by means of density gradient centrifugation (1). Plasmas found to be HI positive at titers ≥ 1:80 were diluted with an equal volume of 0.15 M NaCl, and 0.3 or 0.4 ml (depending upon titer) was layered on a 10 to 40% sucrose gradient (total tube volume of approximately 6 ml). When 0.4-ml samples were fractionated, HI units were corrected to 0.3-ml samples. After centrifugation at 35,000 rpm (100,000 x g), fractions were collected, frozen, and then tested for HI and N antibodies. Fractions tested for HI antibodies were extracted once with acetone. Fractions of plasma from GE were collected as 10 portions of approximately 0.6 ml each; fractions from SE and BCNH sera were collected as five portions of 1.2 ml each. The bottom fraction was labeled fraction 1.

**RESULTS**

Subcutaneous inoculation of young herons with VE virus generally resulted in an antibody response that was detectable by day 7 and persisted for more than 36 days. All of 15 GE and 7 SE and 18 of 20 BCNH developed HI antibodies. With the exception of two GE and two BCNH, all birds that developed antibodies had been viremic.

**GE.** The time of appearance of HI antibody in plasma was remarkably consistent in 14 of the 15 GE, including the bird inoculated at 1 year of age. In 14 birds, HI antibody was detectable by day 7 and the mean peak titer was attained by days 10 to 11. The 15th bird, although viremic on days 1 and 2 post-inoculation, developed only low levels of HI antibody (1:10) by day 28 post-inoculation and was not included in further studies. Titers decreased rapidly at first and then at a low rate through day 34 (Fig. 1A). All pre-inoculation plasmas, including those of five birds received from Florida, were negative for HI antibodies, and the Florida birds were also negative for N antibodies to VE virus. Viremia was not detected in two birds that produced only low levels of antibody, whereas all others developed titers of 1.0 to 4.5. Viremia lasted 1 to 4 days.

HI antibody activity was detected in the 19S fraction on days 7 to 9 (Fig. 1A). By days 10 and 11, maximum levels of 19S and small amounts of 7S HI antibody were present. By day 21 and thereafter, 19S antibody had disappeared and all HI antibody was 7S. HI antibody present in fraction 5 of early plasmas (day 7) was considered to be 19S, and in late plasmas (days 29 and 36) it considered to be 7S.

HI antibody results correlated well with N test results (Table 1). The N test proved to be slightly more sensitive than the HI test since it detected low levels of antibody in a few fractions that were negative by the HI test (e.g., day 7, fraction 1, and day 13, fractions 2, 3, and 5).

**SE.** In seven SE, HI antibody titers were lower than those of GE during the first 2 weeks after inoculation of virus but were similar thereafter (Fig. 1B). All SE developed viremia titers of 1.9 to 3.7.
FIG. 1. Mean (and standard error of the mean) titers of HI antibody in plasmas (curve) of 14 great egrets (A) and seven snowy egrets (B), and in sucrose gradient fractions (bars) of six great egrets (A) and four snowy egrets (B).

TABLE 1. Comparison of average VE virus HI and N antibodies in plasma fractions of two great egrets

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<th>Fraction (top of gradient)</th>
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*HI titers = log_{10} of reciprocals of plasma dilutions; N titers = log_{10} of reciprocals of plasma dilutions producing log_{10} neutralization index > 1.1.

In three of four SE tested, HI antibody at day 7 was detected only in the IgM fraction (no. 2). In one of four SE, a small amount of HI antibody, probably due to IgG, was already detectable in fractions 3 and 4 on day 7. As the amount of IgM decreased between days 7 and 11, the amount of IgG increased, although the net effect was a slight reduction in titers in
whole plasma. 7S antibody continued to increase in amount for the next 2 to 3 weeks, whereas 19S antibody persisted at least until day 27.

BCNH. Among 12 BCNH studied, two slightly different patterns of HI antibody development were observed in whole plasmas. All 12 birds responded to approximately the same extent by day 7 (Fig. 2A and B) but, as time progressed, group A produced considerably more antibody than group B. Furthermore, HI antibody titers in group A continued to rise throughout the experimental period, whereas little change occurred in the titers of group B beyond day 7.

The six birds in group A were 6 to 11 days old when inoculated with either 320 or 10⁴ PFU (two birds) of virus. Those in group B ranged from 6 to 26 days of age when inoculated with 320 PFU of virus. Differences in immune response could not be correlated with differences in age when inoculated nor with the size of the inoculum. The two birds of group A that received 1 million PFU of virus presented total immune responses similar to three others of the same age that received only 320 PFU of virus. HI antibody titers of these two birds on day 7 were 40 and 40 as compared with 20, 20, and 160 from the other three birds. On day 20 the titers were 320 and 640 as compared with 160, 320, and 640, respectively. Fractionated sera presented a similar picture in birds receiving inocula of different sizes. For example, day 14 HI titers of the 7S fraction from the two receiving the high level of inoculum and two of three receiving the lower inoculum were 5 and 20, and 10 and 40, respectively. BCNH developed viremia lasting 1 to 3 days and reaching levels of 1.0 to 4.5.

Two patterns of immunoglobulin production were discerned. On days 7 and 11, group B birds demonstrated 4 and 3.3 times as much 19S activity, respectively, as was detected in the A
IMMUNOGLOBULIN RESPONSE TO VE VIRUS

This study represents one of the few extensive surveys of the development of IgG and IgM antibodies in avian hosts responding to a replication of 19S-antibody in the brain during the first week of post-inoculation (Table 1). The detection of the wild bird could be specific to the presence of the virus in the brain. Two methods were used to detect the presence of the virus in the brain: the first was a histopathologic method and the second was a histological method. The results of both methods were similar, with the histopathologic method detecting the virus in the brain while the histological method did not detect any viral antigens in the brain.

In conclusion, this study demonstrates that the development of IgG and IgM antibodies in avian hosts responding to a replication of 19S-antibody in the brain during the first week of post-inoculation is a reliable indicator of the presence of the virus in the brain. The development of IgG and IgM antibodies in avian hosts responding to a replication of 19S-antibody in the brain during the first week of post-inoculation can be used to detect the presence of the virus in the brain.

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