Newcastle Disease as a Model for Paramyxovirus-Induced Neurological Syndromes: Pathogenesis of the Respiratory Disease and Preliminary Characterization of the En ensuing Encephalitis

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Various clinical, virological, immunological, and morphological aspects of velogenic Newcastle disease were defined in chickens inoculated by natural routes with the Missouri-(H) Len 1950 strain. The disease initially appeared as a severe pneumonitis from which most birds recovered. Several days later, many of these birds developed severe encephalitic signs, largely referable to-inflammatory changes in the cerebellum. During the pneumatic stage, virus was easily isolated in relatively high titers from the brains of all chickens, and viral products were easily detected in Purkinje neurons. However, when the encephalitis developed, virus was isolated irregularly and in low titers from brains, and morphological evidence for the presence of viral products could no longer be obtained. The encephalitic disease is discussed in relation to encephalitic syndromes induced by other neurotrophic viruses.

An etiologic role for paramyxoviruses in encephalitic syndromes of diverse types is now well appreciated. In man, the most common agents involved are mumps virus, which usually induces a self-limiting meningitis (only rarely damaging the brain parenchyma [10, 11]), and measles virus, which is responsible for more serious syndromes including postinfectious encephalitis (11, 13) and subacute sclerosing panencephalitis (cf. review by Weiner et al. [15]). In addition, over many years, evidence has accumulated which strongly suggests that there is a causal relationship between measles virus and multiple sclerosis (3). From these considerations, it is clear that a spectrum of neurological diseases ranging from benign meningitis to severe and fatal illnesses are induced by these agents. Classically, such illnesses follow by some days or longer a more common form of disease induced by the agent in one or more other organ systems.

The pathogenesis of these diseases is obviously complex and their importance justifies the extensive study of diverse experimental systems. To date, most of these systems have involved the human agents given by unnatural routes in unnatural hosts, usually intracrere-

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brally to young hamsters (4, 9, 14). Velogenic Newcastle disease in the chicken is a natural model that has not been studied in this context. Therefore, we chose to define the pathogenesis of this disease in some detail and to concentrate ultimately on several aspects of the central nervous system (CNS) disease. In this communication, we describe the pathogenesis of the acute pneumonitis and more generalized infection and present an initial characterization of the encephalitis that follows some days after resolution of the primary illness.

MATERIALS AND METHODS

Embryonated hen’s eggs and chickens. Hatching eggs (type 2 SPF) were obtained from Heisdorf and Nelson Farms, Redmond, Wash., and hatched in our laboratories. To obviate accidental infection, the birds were reared in a building separated from the laboratory by personnel who were not allowed to contact infected birds.

Virus. The virus employed was Missouri-(H) Len 1950, a velogenic strain kindly supplied by the Newcastle Disease Virus (NDV) Repository, Department of Veterinary Science, University of Wisconsin, Madison. Virus stocks were prepared by standard procedures in the chorionallantoic sac of SPF eggs. These stocks possessed titers of about 5 × 10⁶ plaque-forming units (PFU)/ml and were stored at −70 C. Viral assays were performed on secondary chick embryo fibroblast monolayer cultures maintained in
minimal essential Eagle medium (MEM) and 10% newborn calf serum in plastic flasks (Falcon Plastics, 25-cm growth area). After adsorption of 0.2 ml of a virus dilution for 1 h, the cells were overlayed with minimal essential Eagle medium plus 10% tryptose phosphate broth, 10% newborn calf serum, and 0.6% Noble agar (Difco). After 2 days of incubation at 39 C, the overlay was poured off, the monolayer was stained with crystal violet, and plaques were enumerated.

Inoculation of chickens. Chicks, 2 to 4 weeks old, were used throughout the experiments. The virus stock was diluted to 10,000 PFU/ml, and 1 drop (from a 26-gauge needle) was placed on each eye and each external nasal opening.

Processing of tissues. (i) Viral titrations. At appropriate intervals, similar tissues were removed from each of four birds, pooled and ground in Ten Broeck homogenizers as 10% (wt/wt) suspensions. The choroid plexi were prepared as 0.1% suspensions, and whole blood was allowed to clot and ground in a mortar with sand also as a 10% (wt/wt) suspension. For assays of blood components, the cellular components from heparinized blood were separated and assayed after homogenization (10% suspensions), and plasma was assayed directly. All suspensions were centrifuged for 5 min at 3,000 x g in a Sorvall RC-2B refrigerated centrifuge, and the supernatant fluid was used for viral assays on secondary chick embryo fibroblasts as described above.

(ii) Standard histopathology. Appropriate tissues were fixed in neutral buffered Formalin, sectioned, and stained with hematoxylin and eosin.

(iii) Immunofluorescent techniques. For maximum sensitivity, the indirect immunofluorescent method was used to detect NDV antigens in frozen sections of appropriate tissues. Sections of tissue were cut in a cryostat at 6 μm and fixed in acetone before staining. A chicken anti-NDV serum was used as the primary reagent, and the secondary reaction employed rabbit-derived fluorescein-conjugated anti-chicken immunoglobulins. The chicken serum was prepared from birds that had recovered from experimentally induced Newcastle disease and were subsequently hyperimmunized with virus in Freund adjuvant. At a 1:100,000 dilution, the serum collected from these birds neutralized about 25% of the added PFU of virus after 1 h at 37 C. Appropriate control tissues indicated that the technique was specific for viral antigens in all but lymphoid tissues and cells (which normally possess levels of chicken immunoglobulins that are stained by these methods).

Electron microscopy. Since it was necessary to perform additional tests on the same tissues to be examined ultrastructurally, infected birds were not perfused with fixative. Only tissues that were found to contain significant amounts of viral-specific antigens (as shown by immunofluorescent methods) were chosen for study by electron microscopy. Birds were sacrificed by decapitation and a portion of the appropriate tissue was placed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer and immediately chopped into small pieces. These were stored in fresh fixative until the extent of viral involvement of the tissue was evaluated. At that time, the blocks were prepared for electron microscopy as was previously described (6).

Detection of serum-neutralizing antibodies. Chickens were bled from the heart before they were sacrificed for collection of other tissues, and the serum obtained was frozen at -70 C. When all samples had been collected, each sample was combined with the sera from other birds sacrificed on the same day and the pool was assayed by standard methods for viral-neutralizing antibody.

RESULTS

Clinical course of infection. The accumulated results of several experiments (a total of more than 300 birds inoculated) serve as a basis for description of the clinical illness. By day 4 after infection, all chicks inoculated on the nasal and ocular mucosa (as described in the previous section) began to develop severe respiratory signs with difficult breathing, mouth breathing, and gasping. In addition, all showed obvious generalized signs such as drooping wings, ruffled feathers, and anorexia. Over the next 3 days, one-third to one-half became somnolent, a few developed head tremors or clonic convulsions, and most of these birds died. Thus, in individual experiments, all birds developed respiratory signs and one-fourth to one-half died by day 7. At about 7 days, the respiratory and generalized disease began to stabilize in the remainder of the birds and a resolution of signs began. However, beginning at about 11 or 12 days after infection, about one-third of those remaining developed severe signs in the CNS. Most of these birds presented with torticollis and ataxia, and some had unilateral or bilateral paralysis of wings or legs. Although some of these birds died rapidly, or with a more prolonged downward course (3 to 4 weeks), the conditions of most stabilized, and a few eventually recovered.

Viral assays of various tissues. To establish the general features of viral pathogenesis in these birds, the birds were killed and pertinent tissues were homogenized and assayed for virus at intervals after infection. In these experiments, only living birds were assayed, and they were randomized as to degree of illness. In assays from day 12 on, most groups had one to two birds with severe CNS signs included. The results of such an initial experiment are presented in Fig. 1. Here, after replication in the tissues at the site of entry, virus appeared by 3 days in the trachea, lungs, and blood, and by day 5, the brain was infected. This experiment also suggests that peak titers of virus were reached in most tissues by day 5. By day 7, virus became undetectable in all tissues except...
Most of the virus was leukocyte associated at the time of peak titers (day 4) and disappeared from all tissues, again by day 7.

(ii) Lymphoid organs. Viral assays of the bursa of Fabricius, thymus, and spleen are presented in Fig. 3. In general, the findings mirror those described for leukocytes from peripheral blood, although it appears that less virus is associated with the thymus, and disappears from this organ earlier than from the other two tissues studied.

(iii) CNS. Selected portions of the CNS were separated and assayed. As shown in Fig. 4, cerebellum and choroid plexi contained the most virus, and by day 4 the agent could be detected in all parts of the brain. As with other tissues, the virus could not be detected in the brain by day 9.

Viral assays of brains from individual birds. The brains from birds sacrificed during the period of acute pneumonia (5 days after infection), or on the day that CNS disease developed (11 to

Fig. 1. Amount of NDV present in various tissues assayed at intervals after intranasal and conjunctival inoculation of chickens. Similar tissues from four chickens were pooled and assayed at each interval noted. Details concerning methods are given in the text. The level of sensitivity for these assays is 50 PFU/μg of tissue.

lung, where it disappeared by day 9. Although the tissues for assay were not taken at daily intervals, this experiment puts the virological aspects of this disease into a general time frame. Of particular significance to subsequent considerations is the fact that virus was easily detectable in all tissues examined at 5 days after infection but could not be recovered from any tissue by day 9.

Detailed virological studies of selected tissues. Since we were more interested in those aspects of the disease associated with the central nervous and hematological systems, a more detailed virological study of these tissues was completed. In all cases, chickens were infected and tissues were processed as described earlier.

(i) Peripheral blood. When the three main components of peripheral blood were separated and assayed for virus (Fig. 2), it was found that a considerable amount of virus was associated with leukocytes and erythrocytes by day 3, but none was detectable in plasma until day 4.

Fig. 2. Amount of NDV present in hematological components assayed at intervals after intranasal and conjunctival inoculation of chickens. Similar tissues and fluids from four chickens were pooled and assayed at each interval noted. Further details concerning methods are given in the text. The level of sensitivity for these assays is 50 PFU/μg for blood cells and 5 PFU/μg for plasma.
17 days after infection), were assayed individually for virus. In these more sensitive and precise assays, virus could be detected in small quantities (50 to $10^2$ PFU/g of tissue; average, $2 \times 10^2$ PFU/g) in only one-half of the brains (6 of 12) of birds sacrificed on the day that CNS signs developed. On the other hand, all birds studied (12 of 12) that were killed on day 5 of infection had virus in the brain and in significant amounts ($2 \times 10^2$ to $4.8 \times 10^4$ PFU/g of tissue; average, $1.6 \times 10^4$). This latter group was selected so that more than one-half would have survived the acute disease.

Appearance of neutralizing antibody. Neutralizing antibody became detectable by days 4 to 5 after infection (neutralization constant $K$ at $37^\circ C$ $[1] = 0.2$ ml/min at 4 days and 0.4 ml/min at 5 days) and had apparently not reached a plateau by day 15 when the experiment was terminated ($K = 7.3$ ml/min).

Pathological effects. (i) Light and fluo-
folia and nuclei, were noted in all birds (Fig. 5). The meninges of the cerebellum demonstrated an increased number of mononuclear cells, and all areas of the brain were edematous and congested. By day 9, there was disappearance of Purkinje cells and focal areas of glial and mononuclear inflammatory cell accumulation, particularly in the molecular layer (Fig. 6) and at the junction between the molecular and granular layers in most birds. Such focal lesions were also seen in optic lobes, cerebrums, brainstems, and spinal cords of some birds. Perivascular mononuclear cell infiltration in the white matter continued to be a prominent lesion in the cerebellum at this time also. Such infiltrations were also seen in optic lobes, cerebral cortices, brainstems, and spinal cords. The cerebellar lesions persisted through day 15, and were present in all birds with CNS signs. Finally, Weil strains performed on brain sections taken from birds with CNS signs failed to demonstrate noticeable demyelinating lesions.

In immunofluorescent studies, the lesions seen most consistently were again present in the cerebellum, where they were most prominent in Purkinje cells. Here, between 4 and 6 days after infection, nearly all birds demonstrated viral antigens in at least a few Purkinje cells, and in some, viral antigens were present in almost all Purkinje cells in certain folia (Fig. 7). By day 7, antigens could be detected in neither Purkinje nor any other cells in the CNS. By ultrastructural methods, viral-specific effects and viral products were found only in the cerebella, and then at 5 days postinfection. Sampling errors undoubtedly account for our inability to locate viral-infected cells elsewhere. As was expected from the immunofluorescent studies, the Purkinje neuron was the cell most often seen to be infected, although infected astrocytes near these neurons were also observed. In addition, an occasional extracellular virion could be seen in the neuropil. Virions were frequently seen to be “budding” from the plasma membrane of Purkinje cell somas; less frequently this process was ob-

Fig. 5. Photomicrograph of cerebellar white matter from a chicken processed 6 days after the bird was inoculated with NDV. Two vessels demonstrating perivascular inflammatory changes are prominent (×280).

Fig. 6. Photomicrograph of cerebellar cortex from a chicken processed 11 days after the bird was inoculated with NDV. A large glial nodule is present in the molecular layer, and the Purkinje cells beneath this nodule have disappeared (×280).
served at dendritic processes. Figure 8 represents a survey micrograph of an infected Purkinje neuronal soma. Here, budding Newcastle virions are present at the plasma membrane and a dense aggregate of nucleocapsid-like structures is present in the nucleus. The outlined portion of Fig. 8 is presented in greater magnification in Fig. 9, where nucleocapsids are seen within and beneath a budding virion. A similar area in a Purkinje dendrite is shown in Fig. 10. In general, the cytoplasm of infected neurons was more condensed than that in uninfected cells and contained swollen organelles and viral nucleocapsids.

The dimensions of nucleocapsids within virions (Fig. 11) were compared with morphologically similar components observed elsewhere in the cell. Large and small masses of loosely arranged nucleocapsids were seen scattered in the cytoplasm of many neurons (Fig. 12). In addition, nuclei of these cells contained dense accumulation of structures that differed distinctly from condensed chromation or nucleoli (Fig. 13 and 14). These forms have the structure and dimensions (16 to 18 nm in diameter) of the nucleocapsids seen in Fig. 11 and 12. The nuclear forms differed from cytoplasmic nucleocapsids only in their more orderly and compact arrangement and frequent proximity to the nuclear membrane. Although such ultrastructural evidence of NDV components in nuclei has not been reported by others, the presence of antigens observable by immunofluorescent methods has been claimed (7).

**DISCUSSION**

This communication establishes the general virological and histopathological aspects of acute velogenic Newcastle disease that precede the disease of particular interest—the encephalitis. An initial characterization of this latter syndrome was also completed. To summarize, after conjunctival and intranasal inoculation, virus rapidly spread to the lungs and induced a severe pneumonitis in all birds, and many succumbed. At this time, significant amounts of virus could be isolated from blood, lymphoid organs and all parts of the brain, and viral-specific products were readily detected in Purkinje neurons. In the brain, the most consistently seen and extensive lesions were present in the cerebellum, where perivascular accumulations of round cells in the white matter, focal accumulations of glial cells in the molecular layer, and disappearance of Purkinje neurons were prominent. This predilection for Purkinje neurons has also been noted by others (5). A detailed immunofluorescent and ultrastructural study of these infected neurons during the acute pneumatic phase of the disease indicated that they were productively infected. In general, no correlation could be made between severity of the generalized illness and extent of the viral-induced changes in the CNS.

By 9 days after infection, virus could no longer be detected in any of the pooled tissues studied in these birds, and the acute disease in the survivors had begun to resolve. However, 2 to 8 days later, a significant number of these birds abruptly developed the severe CNS signs described in the previous section. At the time that CNS disease became evident, virus could be isolated from the brain of only about one-half of the birds, and then in comparatively small amounts. In addition, when immunofluorescent methods were employed at this time, no evidence of virus infection in any cell type in the brain parenchyma was discernible.
Fig. 8. Survey electron micrograph of a Purkinje neuron from a chicken infected with NDV. Budding virions can be seen at the plasma membrane (arrows and outlined area), and a dense aggregate of nucleocapsid-like structures is present in the nucleus (×20,000).
FIG. 9. The outlined portion of Fig. 8 at higher magnification. An accumulation of nucleocapsids is present beneath the budding virion on the right (×66,000).

FIG. 10. Electron micrograph of a Purkinje cell dendrite from a chicken infected with NDV. Nucleocapsids and a budding virion are present (×57,000).
FIG. 11. Electron micrograph of an NDV-infected Purkinje neuron. Nucleocapsids present in virus forming at the plasma membrane can be compared with similar structures present elsewhere in the cell (Fig. 12–14) (×92,000).

FIG. 12. Electron micrograph of Newcastle disease nucleocapsids present in the cytoplasm of a Purkinje neuron (×92,000).

FIG. 13 and 14. Electron micrographs of NDV nucleocapsid-like structures present in the nuclei of Purkinje neurons (×92,000).
Since the antiviral antibody present in these birds undoubtedly neutralized some virus in the brain both in situ and during preparation of material for assay, these results are quite compatible with the hypothesis that a continuing and smoldering virus infection resulted ultimately in the appearance of clinical disease. The temporal sequence of events also leads to a suspicion that processes in addition to a direct cytolytic effect of virus on various cells in the CNS might be contributing to the clinical disease noted. In this regard, we have been unable to find any pertinent literature relative to paramyxoviruses, but it appears that when most neurotrophic viruses are given by peripheral routes, the development of clinically apparent encephalitis correlates temporally with the concentration of virus in the brain (2, 8, 12, 16).

Since the present system yielded different results, it is possible that inflammatory and immune responses were playing important detrimental roles contributing to the development of Newcastle encephalitis.

Taken together, the results indicate that Newcastle encephalitis is different from mumps meningocencephalitis and has some similarities to measles-virus-induced postinfectious encephalitis. However, perivenular demyelination, which is generally considered to be a hallmark of postinfectious encephalitis (13), was not observed to be a prominent feature in the birds examined at the time CNS disease developed. Studies concerning additional virological, immunological, and pathological aspects of this encephalitis and its more long-term consequences are now in progress.

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LITERATURE CITED