Divergence of Mouse Brain Interferon Responses Following Virulent or Avirulent Newcastle Disease Virus Inoculation[1] (37243)

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Local production of interferon in mouse and rabbit brain and in rabbit cerebrospinal fluid has been demonstrated (1–3) as well as evidence that its production affects the outcome of certain viral encephalitides (4–6). However, in other experimental systems evidence suggests that the appearance of mouse brain interferon merely reflects the presence of the virus and is not important in restricting viral multiplication and disease production (7, 8). Interferon has been demonstrated in the cerebrospinal fluids of patients suffering from viral meningitis and/or encephalitis as well as from patients with bacterial meningitis (9, 10). It therefore may be one factor involved in limiting viral spread in human central nervous system infections.

Newcastle disease virus (NDV) is a potent inducer of interferon in mice (11), although multiplication of strictly egg- or chicken-adapted strains of NDV in weanling mice has not been detected (12). After intravenous inoculation NDV is cleared very rapidly via the reticuloendothelial system (13), and by most routes NDV does not produce signs of illness in mice. However, certain strains uniformly produce lethal neurologic disease if inoculated by the intracerebral route (14, 15) while other strains produce no overt signs of disease when administered by this route.

The present study compares the interferon responses elicited in mouse brain by intracerebral inoculation of a neuropathic strain (CG-179) and an avirulent strain (Hickman) and attempts to interpret the differences detected.

Materials and Methods. Viruses. CG-179 and Hickman strains of NDV were obtained from the NDV Repository, University of Wisconsin (courtesy of Dr. Joseph Spalatin). Viruses were inoculated into the chorioallantoic sac of 10-day-embryonated chicken eggs; after incubation at 39° until embryos were moribund, allantoic fluids were harvested. This procedure was repeated until virus titers in the allantoic fluid reached at least 10^8 plaque forming units (PFU/ml). Virus-infected pools of allantoic fluid were stored in small aliquots at —70° until needed. Encephalomyocarditis virus (EMC) pools were prepared by inoculating L-cell monolayers with 10^6 PFU EMC; 48 hr later supernatant fluid was harvested and stored in small aliquots at —70°.

Mice. Outbred 3-wk-old male Swiss mice were obtained locally (Rolfsmeyer Dan Co., Madison, WI).

Media and reagents. Medium 199 was used for all cell cultures. For growth medium 10% calf serum, 5% lactoalbumin hydrolysate and 0.06% sodium bicarbonate were added. For maintenance medium 3% fetal calf serum and 0.11% sodium bicarbonate were added. All media contained 200 units penicillin and 200 μg streptomycin/ml. Maintenance medium containing 0.8% tragacanth (16) was used for overlay. EAF fixative was prepared according to a formula of 600 ml 95% ethanol, 200 ml glacial acetic

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acid and 100 ml formaldehyde.

Cell cultures. Mouse embryo cell cultures (MECC) were made using 16–18 day mouse embryos which were removed from the uterus aseptically, minced, agitated at 37° for 1 hr in 0.25% trypsin, dispersed in growth medium and planted in 32 oz bottles. After 4–5 days incubation at 37° cells were removed from the glass with 0.05% trypsin and 0.05% sodium ethylenediaminetetraacetate in saline, resuspended in growth medium and planted in 1 oz bottles. Confluent monolayers formed in 2 days and were then used in interferon assays.

Chick embryo cell cultures were prepared in a similar manner, as previously described (17).

Fluorescein-labeled antiserum. Fluorescein-labeled anti-NDV chicken serum was obtained from Dr. Charles Beard, Athens, GA. At a 1:40 dilution, NDV-infected controls stained brightly by direct immunofluorescence and no nonspecific staining was seen.

Inoculation of mice and processing of tissues. Mice were lightly anesthesized with ether and 0.03 ml of NDV-infected or uninfected allantoic fluid was injected into the right frontal lobe. For cumulative mortality studies mice were observed daily for signs of neurologic disease or death. Some mice were autopsied to determine that the cause of death was not due to pulmonary consolidation. For interferon and virus assays, groups of 3 mice were sacrificed at designated intervals by cervical fracture; brains were removed, pooled, ground with sterile sand with a mortar and pestle and suspended in 10 ml maintenance medium. The extract was centrifuged at low speed and the supernatant was stored at —70° until assays were performed. Spleens were prepared for study in the same manner. Extracts for interferon assays were dialyzed against a 0.2 *M* KCl–HCl pH 2.0 solution for 24 hr and then against a 1 *M* phosphate buffer (pH 7.2) for 24 hr before freezing. Sera were obtained by bleeding from the retro-orbital plexus. For immunofluorescent studies brains were removed, bisected along the midsagital plane, frozen in egg albumin and sectioned at —20° on an International cryostat Model CT1. Sections 4 μm thick were cut parallel to the midsagital plane, placed on glass slides, fixed in acetone, and stored at —20° until stained. Staining procedure involved 30 min incubation with a 40-fold dilution of anti-NDV fluorescein-conjugated serum at room temperature in a humidified atmosphere followed by a 15 min wash with 0.85% saline solution and 5 min in deionized water. Cover glasses were mounted with buffered glycerol (9 parts glycerol:1 part phosphate buffered saline). Sections were examined for specific fluorescence under a Zeiss Photo-Microscope using a 200 W high pressure mercury vapor lamp as an ultraviolet light source with a BG 12 excitor filter and a Wratten K2 barrier filter. Whole brains were fixed in formalin, sectioned and stained with hemotoxylin and eosin for histologic examination.

Interferon assays. To assay organ extracts for interferon, serial 2-fold dilutions in maintenance medium were made and 3 ml aliquots of each dilution were pipetted onto triplicate monolayers of secondary MECC. Cells were incubated at 37° for 24 hr after which they were drained, washed with Dulbecco's saline and challenged with 30–100 PFU of EMC/bottle. Dilutions of mouse serum were handled in the same way except that only 0.5 ml of each dilution was placed on the monolayers and cells were incubated 3.5 hr before they were challenged with EMC. EMC was absorbed for 30 min; cells were covered with 4 ml of overlay and incubated at 37° for an additional 20–22 hr. The overlay was then removed; cells were washed successively with saline, EAF fixative, and crystal violet; visible plaques were counted. Interferon titers are expressed as the reciprocal of the dilution at which plaques were reduced by 50% of controls which received only diluent prior to EMC challenge. These titers were extrapolated from a graph on which the negative log of the dilution was plotted against the percentage of control plaques.

Assay for infective virus. Brain extracts were diluted 5- and 10-fold and 0.5 ml was pipetted onto primary chick embryo fibroblast monolayers which were incubated for 1 hr at 37° and then covered with 4 ml of overlay. Cells were incubated for 3 days, washed, fixed, stained as in the interferon assays, and observed for visible plaques.

Results. Comparison of mouse virulence of CG-179 and Hickman strains of NDV by the ic route. In order to verify previous reports (14) on the natural course of neurologic disease in mice following ic inoculation with these 2 strains, 40 mice were inoculated with 10^6 PFU CG-179 and 50 mice with 10^6 PFU Hickman. Figure 1 shows that by Day 9 all animals inoculated ic with CG-179 had died while the Hickman strain had not produced any deaths as late as 28 days after ic inoculation, when the experiment was terminated. No deaths were observed in this time among 13 controls inoculated with normal allantoic fluid or among 10 uninoculated mice.

When the dose of CG-179 given by the ic route was varied, it was found that CG-179 could produce lethal neurologic disease in 100% of mice when as few as $10^{3.5}$ PFU were inoculated ($LD_{50} = 10^{1.7}$ PFU) (18).

Interferon responses in mouse brain. Mice were inoculated ic with either 10^6 or 10^5 PFU of CG-179 or Hickman and were sacrificed at various intervals from 5 to 168 hr by which time animals in the CG-179 group were moribund. As shown in Figs. 2 and 3, decreasing the concentration of NDV in the inoculum by 10-fold (from 10^6 to 10^5) did not affect the interferon titers obtained. To establish that the inhibitor observed was interferon, extracts were dialyzed for 24 hr against a pH 2.0 KCl–HCl solution before interferon assays were performed. The interferon titers observed after dialysis were slightly higher than those observed before,

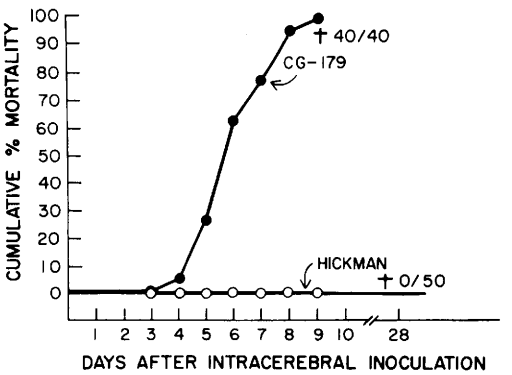

FIG. 1. Distribution of death with time after ic inoculation with 10^6 PFU CG-179 (●—) and 10^6 PFU Hickman (○).

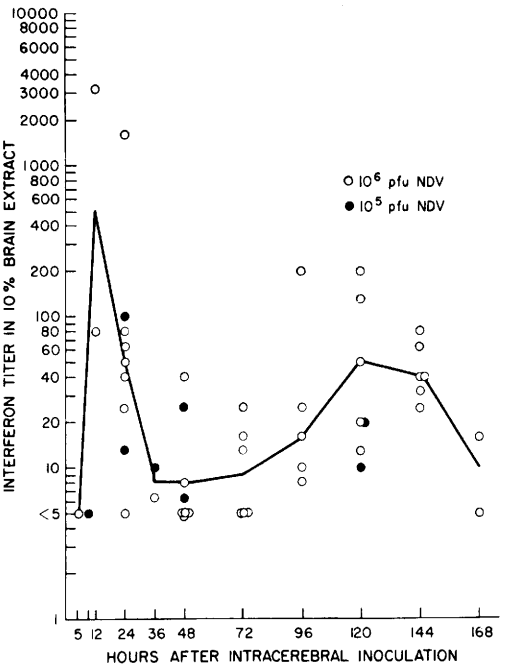

FIG. 2. Appearance of interferon in mouse brain following ic inoculation of 10^6 (○) or 10^5 (●) PFU CG-179. Interferon titers represent the reciprocal of the dilution calculated to yield 50% reduction in 30–100 PFU EMC on MECC monolayers. (—) The geometric mean of all points at a particular time.

suggesting that some antagonist to interferon activity was removed by the process.

Figure 2 shows that after ic inoculation with CG-179 interferon activity first appeared in the brain at 12 hr and peaked between 12 and 24 hr. Little or no interferon was detected between 36 and 72 hr; at 96 hr activity increased slightly; and at 120 and 144 hr a second peak was observed. By contrast, Fig. 3 shows that after ic inoculation with Hickman, some interferon activity appeared at 5 hr; activity peaked around 24 hr, decreased between 24 and 72 hr, and was absent between 72 and 168 hr. There was no difference in the magnitude of the first peak of activity obtained with the 2 strains of NDV. At comparable intervals mice inoculated with normal allantoic fluid were sacrificed; at no time was interferon activity detected in brain extracts from these mice.

Serum and spleen interferon responses. In order to determine whether the biphasic interferon response observed after CG-179

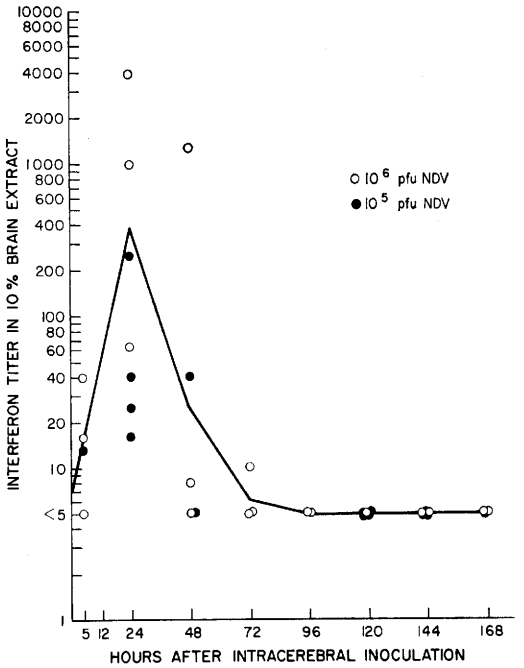

FIG. 3. Appearance of interferon in mouse brain following ic inoculation of 10^6 (○) or 10^5 (●) PFU Hickman. Interferon titers represent the reciprocal of the dilution calculated to yield 50% reduction in 30–100 PFU EMC on MECC monolayers. (—) The geometric mean of all points at a particular time.

could be detected in other organs and also to detect whether any differences existed in the ability of the two strains to induce interferon by a more standard route, mice were inoculated via the tail vein (iv) with 10^6 PFU CG-179 or Hickman. Sera were collected at 5 and 24 hr from both groups and at 120 and 144 hr from the CG-179-injected group. Spleen extracts were made from the same mice. Table I shows the results of interferon assays of these materials. There was no difference in the responses obtained at 5 and 24 hr with the two different strains of NDV. Also, no biphasic response was detected in either spleen or serum samples.

In order to determine that the biphasic response was not a peculiarity of the ic route, mice inoculated ic with CG-179 were sacrificed at 120 or 144 hr. Sera and spleen extracts obtained from these mice did not contain interferon.

Attempts to detect evidence of virus replication in brain. Brain extracts made at various intervals after ic inoculation for interferon assays were also assayed for infective virus on chick embryo fibroblast monolayers. Low titers of infective virus were detected at 5 and 24 hr in the brains of mice from both the Hickman and CG-179 groups. No infective virus was detected at 48, 72, 120, and 144 hr. Attempts to detect viral antigen by the fluorescent antibody technique in sections of brain obtained 5 to 168 hr following inoculation with either Hickman or CG-179 also yielded negative results after 24 hr; positive controls fluoresced brightly.

In mice inoculated ic with CG-179 histologic changes in formalin-fixed brain sections during the first 6 days of infection were limited to the site of inoculation where local necrosis, edema, and microgliosis was observed in infected but not in control mice. Only in one animal that had survived to 7 days was there widespread perivascular lymphocytic infiltration and areas of parenchymal microgliosis extending from the frontal lobe to the pons. No meningeal or ependymal changes were observed.

Discussion. The results of these studies show that the interferon response in mouse brain after ic inoculation of CG-179—a strain of NDV which produces a lethal encephalitis in 100% of mice inoculated by this route—is biphasic; whereas the interferon response in mouse brain after ic inoculation of the Hickman strain, which is avirulent by this route, involves only one peak of activity. Although both strains are lethal for chickens, in mice the effects of CG-179 and Hickman represent the two extremes between which there is a graded series of strains ranging from severely neuropathic by the ic route to avirulent (14). Significant concentrations of infective virus could not be detected in brains of mice at any time after ic inoculation with either CG-179 or Hickman, nor could accumulations of viral antigen be detected by immunofluorescence. The failure to demonstrate complete or incomplete virus in the brain confirms the findings of Liu and Bang (15) and supports the conclusions that NDV does not multiply in weanling mice (12).

While the mechanism by which CG-179 induces neurologic disease remains unclear, the

TABLE I. Interferon Titers of Mouse Sera and Spleens After iv Inoculation with 10^6 PFU CG-179 or Hickman NDV.[a]

	Interferon titer							
(hr):	5		24		120		144	
NDV strain	Serum	Spleen	Serum	Spleen	Serum	Spleen	Serum	Spleen
Hickman	63	136	14	20				
	200	80	50	63				
	100	400	<5	32				
	50	126						
CG-179	160	80	10	14	<5	<5	<5	<5
	40	136	25	25	<5	<5	<5	<5
	50	251	25	50				
	40	40	16	80				

[a] Each serum titer represents a pool of 4 mice. Spleen titers represent a 10% extract of a pool of 4 spleens from the same mice. Titers represent the recriprocal of that dilution calculated to yield a 50% reduction in 30–100 PFU EMC on MECC monolayers.

interferon responses described in this study may provide some clues to this puzzle. It is clear that there is not an inverse correlation between the ability of an NDV strain to cause neurologic disease and its ability to induce interferon since, at the time mice are dying with CG-179-induced disease, interferon is detectable in the brain, whereas it is not detected at this time in brains of mice inoculated with Hickman strain. Baron and Buckler (19) demonstrated that after iv inoculation of NDV, interferon activity was detectable at 1 hr and reached its peak at 4 hr. The appearance of interferon in the brain after ic inoculation is slower, reaching a peak between 12 and 24 hr. The first peaks of activity obtained with both strains are of the same order of magnitude.

The second peak of interferon activity after CG-179 is unusual. In general animals or cells experience a refractory period after interferon production; animals cannot be stimulated to produce interferon again for at least 6 days (10, 20–22). After CG-179 the second peak of activity occurs 4 or 5 days after the first. Since no biphasic response was detected in the spleen or serum after either ic or iv inoculation of CG-179, this biphasic interferon response may be peculiar to the brain. Several possibilities could explain this response: the cells initially infected could release virus or viral components which would then stimulate previously unexposed neighboring cells; previously unstimulated nonneural cells could enter the brain and subsequently release interferon; or cells initially infected could have the unusual capacity to release interferon in 2 peaks of activity following a single stimulation.

Schlesinger (23) described a partial cycle of replication after ic inoculation of nonneurotropic strains of influenza virus in mice. He demonstrated that the toxicity of the virus lies in the production of viral components which impair the integrity of the host cell, even though complete virus particles are never assembled. In the present study efforts to detect major viral antigens in the brain were not successful; however, minor protein components and/or viral RNA may have been produced. If this is the case, then the difference between the 2 strains might be that CG-179 undergoes a partial cycle while Hickman does not.

Ginsberg (24) described the development of pulmonary lesions in mice, in the absence of viral multiplication, after intranasal inoculation of NDV. Lesions continued to develop despite a continuous decrease in virus titer in the lung. One possible interpretation is that NDV triggers host immune mechanisms which damage tissue as viral titers decrease. Interferon might even enhance this process since Lindhal, Leary and Gresser (25) demonstrated that interferon preparations enhanced the specific cytotoxicity of sensitized lympho-

cytes for allogeneic target cells *in vitro*. It has been shown that sensitized lymphocytes release interferon when they contact antigen (26). In the present study, however, histologic examination of brain sections from a limited number of CG-179-treated mice showed only minimal inflammatory response localized to the site of injection, except in a single animal which survived 7 days. In this relatively long-term survivor, the changes were like those commonly associated with viral encephalitis (27–29). However, this delayed lymphocytic infiltration correlates poorly with the observed onset of neurologic disease, interferon response, and death. Therefore, it is difficult to determine whether these late changes are the cause or effect of the neurologic disease process.

In past studies of interferon production in response to viral infection, the presence of interferon has been shown either to be beneficial or insignificant. For instance, Heineberg, Gold and Robbins (30) demonstrated that adult mice produced more interferon in response to Coxsackie B1 virus and less virus than sucklings and therefore survived the infection while sucklings did not. On the other hand, Vilcek (8) demonstrated that suckling mice had higher titers of both interferon and virus after Sindbis virus infection and that adults survived while sucklings did not. He attributed the high levels of interferon to the presence of more stimulator due to greater viral multiplication. The present study is not analogous to either of the above since the second interferon peak appears in the absence of virus but in the presence of lethal neurologic disease.

It is possible that the production or release of interferon is in some way directly related to the neurologic disorder. There is increasing evidence that interferon has effects on cells other than those related to its antiviral activity. Specifically, O'Shaughessy, Lee and Rosee (31) have shown that interferon in moderate amounts can have both lethal and growth depressing effects on cells *in vitro*. The second peak of interferon activity produced after ic inoculation of CG-179 may actually contribute to the presumed cytotoxicity resulting in death.

The exact mechanism responsible for CG-179 encephalitis is still unclear as are the events which lead to the second peak of interferon activity. These two may be related and any explanation of one must take the other into account.

Summary. Mice were inoculated ic with CG-179, a strain of NDV which by this route causes 100% lethal encephalitis within 9 days, and with Hickman, a strain which is avirulent by this route. Measurement of interferon activity in the brain at daily intervals following inoculation revealed that following CG-179 two peaks of activity occurred—one at 24 hr and the other at 120 to 144 hr. This unusual biphasic response did not occur in the brains of mice inoculated with Hickman strain. In these mice interferon activity in the brain peaked at 24 hr and then disappeared rapidly. Neither infective virus nor viral antigen could be detected in the brains after inoculation with either strain. There appeared to be no difference between viral strains in the order of magnitude of the first peak of interferon activity. No biphasic activity was detected in spleens or sera of mice after either ic or iv inoculation of CG-179. Possible differences between the 2 strains of NDV are discussed as well as possible explanations for the occurrence of CG-179-induced neurologic disease and the biphasic interferon response.

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