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Interferon Administered in the Cerebrospinal Space and Its Effect on Rabies in Rabbits

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Because combined administration of intramuscular and intravenous interferon has been partially successful in the incubationary treatment of rabies, the effect of direct interferon administration into the cerebrospinal fluid space was tested. After injecting 1,800 U of interferon into the cisterna magna or the lateral ventricle, periodic samples, obtained by cisternal taps, showed that 1 to 5% remained after 24 h, as opposed to the known clearance of interferon from the bloodstream to this level within minutes. The distributions of interferon and ^{131}I-labeled albumin were similar as demonstrated by kinetics of clearance monitored over 24 h. Beginning with and after experimental infection of rabbits, daily intraventricular injections of one million units of interferon were given for as long as 3 weeks. Interferon was prepared from cell culture fluids after pressure dialysis and chromatography on Sephadex G-100. This intensive treatment did not prevent encephalitis, but prolonged the length of the incubation period by one- to two-thirds. The outcome after intraventricular administration was not as favorable as when one million units equally divided between intramuscular and intravenous injections were given at the time of challenge. Interferon administered in the subarachnoid space in this fashion is apparently inadequate to protect the rabbit against rabies. Its role as an adjunct measure, or other methods of administration in the nervous system, remains to be examined.

It is known that in rabbits, interferon given intramuscularly or intravenously, or induced by complexed polyriboinosinic polyribocytidylic acid (poly[I]·poly[C]), around the time of rabies infection, or even shortly thereafter, may protect the animal from death and paralysis (6, 7, 12, 17). Thus, 8×10^5 U of interferon divided between two intramuscular doses and one intravenous dose, injected 24 h before or up to 3 h after infection, resulted in complete protection (17). Similarly, 1 mg of poly(I)·poly(C) administered intravenously either 24 h before or 3 h after infection with 25 mean lethal dose (LD_{50}) rabies street virus protected rabbits completely (6). If more virus (as much as 3,125 LD_{50}) or less inducer (as little as 0.04 mg/kg) was used, partial protection resulted (7). The mechanism of action of poly(I)·poly(C) is presumed to be due to the high levels of serum interferon which it stimulates.

Although the above studies are promising as extrapolated to man, they are restrictive because: (i) poly(I)·poly(C) is a toxic substance, and its interferon-inducing capacity in man is limited (20); and (ii) regarding exogenous interferon, the best extrapolation from the cited reports is that a presently prohibitive amount of interferon, or at least 70 million units, is needed for one treatment in an adult male exposed to rabies. Also, there appears to be a very short time period after exposure during which treatment is effective (17). It would be highly desirable if a better agent or another method of administration could prolong this time period.

Thus we considered other methods of administering interferon. Because rabies virus is assumed to ascend via the nerve trunks from the site of infection to the central nervous system (14), methods to apply interferon directly to this system were considered. The most direct way applicable to man would be by lumbar spinal injection in the cerebrospinal fluid, bathing the subarachnoid space. Lumbar spinal tap was found to be technically impossible in rabbits. However, with the help of our neurosurgical colleagues, we were able to tap the cisterna magna and to inject substances in the cisterna magna and, also, directly into the lateral ventri-

cle. This made possible the study of the diffusion of albumin and interferon injected by these routes, representing a part of the pharmacokinetics of interferon unavailable in the literature, but essential for exploring the interferon treatment of viral diseases of the central nervous system.

MATERIALS AND METHODS

Animals. Albino rabbits of the New Zealand strain (Pel Freeze Biological Animals) weighing 1 to 2 kg were used.

Rabies virus. The rabies street virus originated from a naturally infected fox (6). It is stored as a 10% salivary gland suspension which contains 8,000 rabbit intramuscular LD_{50}/0.4 ml by previous titration (17). The virus was a gift of P. Fenje of Toronto.

^{131}Iodine-labeled albumin and radioactive sample counts. "Albumotope," radioactive ^{131}I-human serum albumin (E. R. Squibb & Sons, catalogue no. 05200), of 500 μCi/mg specific activity, was utilized. Dilutions were made so that a rabbit received about 5 μCi/0.2 ml of inoculum. Thus, only 0.01 mg of human albumin was introduced into the subarachnoid space. All cerebrospinal fluid (CSF) samples (0.2 ml) were prepared in glass tubes (16 by 125 mm) and counted in a well-type gamma counter (Picker Spectorscaler III A).

Preparation of partially purified interferon. Rabbit kidney cell cultures were grown in Falcon plastic tissue culture plates, no. 3025 (150 by 25 mm, 145-cm^2 surface area). After inoculating the CG strain of Newcastle disease virus at an input multiplicity of 5 for 1 h, 15 ml of serum-free medium (0.5% lactalbumin hydrolysate in Hanks balanced salt solution) was added to the decanted and washed cell sheet and incubated overnight. The cultured fluid was harvested, dialyzed against pH 2 buffer for 72 h, centrifuged at low and high speeds once each, and pressure dialyzed to remove dialyzable impurities and to reduce volume to 1:50 of the original. A sample of the concentrated crude preparation was saved for assay. The rest was frozen at −20 C until several batches totaling at least four million units of interferon were accumulated. The pooled concentrate was centrifuged at low and high speed once each, then was fractionated by passing through a Sephadex G-100 column. The "V_B" peak (molecular weight of 46,000) was identified and isolated as previously described (15). This semipurified preparation was stored at −70 C. Two batches of interferon were used. Their potency, based on the end point dilutions of 50% of activity against vesicular stomatitis virus (VSV) in 0.1 ml, was 8,000 and 500,000, respectively.

Interferon assay. Samples of cell culture fluid, serum, or CSF were assayed by the microassay method of Armstrong (1). Cell cultures of kidney cells from 300-g rabbits were prepared in Microtest II plates (Falcon). After overnight incubation with 0.1 ml of dilutions of samples containing interferon, the well cultures were challenged with about 400 plaque-forming units of VSV. Interferon titers obtained were standardized with the NIH rabbit reference interferon (NIH reference research reagent G-D19-901-028). Units were based on 50% end-point dilutions of activity of 0.1 ml of samples.

Implantation of intraventricular cannula. The cannula was a short-bevel disposable no. 23 hypodermic needle cut to 16 mm. The lateral ventricles were located with a stereotaxic instrument (David Kopf Instruments) according to the atlas to Fifkova and Marsala (8). A hole was drilled through the skull with a dental drill and a cylindrical diamond bit. The cannula with PE-50 tubing (Clay Adams) was lowered through the dura and brain tissue. With an infusion-withdrawal pump (Harvard Apparatus, series 900), isotonic saline was injected through the tubing and cannula at the rate of 2 to 3 μliters/min. To locate the lateral ventricle, a Statham transducer was placed between the pump and the tubing-cannula, and the change in resistance to infusion pressure as the cannula penetrated through tissue and into the ventricle was recorded by a Glass model no. 7 polygraph according to the method of Goodrich et al. (9). One screw was placed 10 mm anterior and one 10 mm posterior to the implanted cannula, and they served as fastening for the dental cement which was applied to secure the cannula. Substances, such as interferon and labeled albumin, could be easily administered repeatedly. Needle wires, which could be removed for intraventricular infusion, served as plugs and provided a tight seal. The entire procedure to cannulate one rabbit took about 3 h, but with practice was reduced to 1 to 2 h. Consequently, the number of animals which could be prepared for an experiment was limited to about twelve.

Cisternal tap. The rabbit was first anesthetized with intravenous sodium pentobarbital (Diamond Laboratories), 0.5 ml/kg. The cisterna magna was tapped with a 0.5-inch 20-gauge angiocatheter (Deseret Pharmaceutical Co., Inc.). The angiocatheter could remain taped to the rabbits throughout the experiment and was plugged between taps to prevent leakage. Thus, periodic injections could be made into the cisterna magna, and CSF samples were obtained. Routine cisternal samples, drawn at intervals for radioactivity and interferon assays, were 0.2 ml.

Serum samples. Blood was obtained by puncture of the marginal ear vein, and 0.2 ml was saved for radioactivity and interferon assays.

Scintigrams. A Picker Dyna Camera II model of the Anger camera was used to demonstrate the radioactive distribution of ^{131}I-labeled albumin in the CSF space. The cameras utilized a medium energy straight-bore columnator. The 364 KEV peak of ^{131}I was bracketed with a 20% window. Images were obtained on Polaroid film. Positive identification of the anatomical relationship of the observed CSF space to the animal's external anatomy was provided by outlining the rabbit with a flexible ^{57}Co marker.

Laboratory diagnosis of rabies in rabbits. Rabbits, found with hind-leg paralysis at around 12 days or later, depending on the dilution of rabies virus inoculated into the right quadriceps femoris muscle, were considered to have dumb rabies and were sacri-

ficed by chloroform. To confirm this diagnosis, an indirect fluorescent antibody (BBL 40604 antirabies globulin, equine, fluorescein labeled) examination of the hippocampus was performed as described by Johnson (13). When the results were borderline, the mouse inoculation test was utilized. Weanling mice received intracerebrally diluted rabbit hippocampus homogenate. When they became ill, euthanasia was performed, and their brains were examined for rabies virus antigen by using the fluorescent technique.

RESULTS

After perfecting techniques for intraventricular and intracisternal injections, a series of experiments were undertaken to determine the distribution of radioactively labeled albumin and interferon after injection in cisternal and intraventricular spaces (Table 1 and 2). When interferon was administered, it was administered together with 0.01 ml of radioactive albumin to a total volume of 0.2 ml. From each animal, 0.2-ml samples of CSF were obtained by cisternal catheter at times indicated in the tables, which sufficed for both radioactive counting and for interferon titration.

Blood samples were also obtained in some of the animals. In general, the concentrations of radioactive albumin and interferon were always lower than those in the CSF, and they were all less than 0.1% of the CSF concentrations 30 min after injection.

[^{131}I]albumin in the CSF space. [^{131}I]albumin disappeared from the CSF space with decreasing rates (Table 1). Calculation of geometric mean concentrations 30 min after injection showed that, after intracisternal injection, a dilution factor of 5.8, or 17.3% of the inoculated material, could be detected in the cisternal fluid. After intraventricular injection, 8.3% was detected. Because 0.2 ml was injected, this is consistent with a dilution space of around 2 ml, which is consistent with the size of the CSF space in a 1-kg rabbit (5). The two disparate apparent dilution spaces at 30 min may reflect the large volume of ventricular fluid relative to subarachnoid space fluid. Because of bulk flow from ventricles out into the basilar cisterns and up around the convexities, cisternal sampling reflects apparent rather than absolute dilution space.

To show better the fate of [^{131}I]albumin, the 30-min value was taken as representing 100%, the concentrations in CSF at various times were computed, and their geometric means were taken (Fig. 1). The shape of the disappearance curves of radioactive albumin after intraventricular and intracisternal injection are similar, but disappearance after intracisternal injection appears to be more rapid.

Another method was used to ascertain the distribution of [^{131}I]albumin. At the time of intraventricular injection and at 5.5 and 24 h

TABLE 2. *Distribution of interferon in cerebrospinal space*

Time (h)	Animal route and log dose[a]				
	IC (3.64)	IC (3.64)	IC (3.64)	IC (3.64)	IV (3.26)
0.5	3.00	2.38	2.82	2.55	2.25
2	1.91	1.92	2.61	2.42	1.90
5		0.95	1.74	2.34	1.08
8		None[b]		2.03	1.11
24		None		1.20	0.95

[a] Interferon was injected with [^{131}I]albumin in a total volume of 0.2 ml into the identified rabbits. Dose is expressed as $\log_{10}$ interferon units/0.1 ml. IC, intracisternally; IV, intraventricularly.

[b] None indicates no interferon detected.

TABLE 1. *Distribution of [^{131}I]albumin in the cerebrospinal space*

Time (h)	Animal route and log dose[a]									
	IC (5.40)	IC (5.40)	IC (6.06)	IC (4.82)	IC (4.82)	IC (4.90)	IC (4.90)	IV (6.46)	IV (5.40)	IV (5.40)
0.5[b]			5.26	4.16	3.85	4.13	4.28	5.38		
1	4.75	4.66	4.91							
2			4.80	3.54	3.43	3.68	3.71	4.99		
5			4.10		2.74	3.01	3.27	4.67		
8			4.00		2.51		2.89	4.25		
24	2.26	2.75	3.00		1.94		2.42	3.95	2.92	
48			2.38							3.50[c]
75										3.73[c]

[a] [^{131}I]albumin was injected intracisternally (IC) or intraventricularly (IV) in a total volume of 0.2 ml. Number of counts is expressed in $\log_{10}$ counts per min per 0.2 ml of sample.

[b] Log counts per minute of samples collected by cisternal puncture at times after initial injection.

[c] This animal received 5.40 log counts 24 and 48 h after injection. A sample at 48 h was taken before the administration.

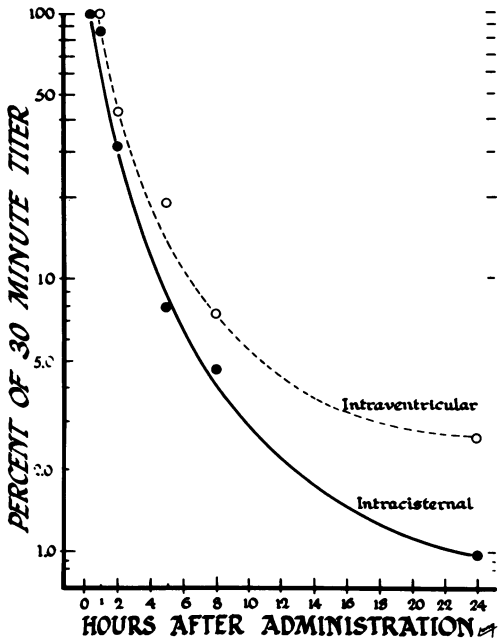

FIG. 1. [^{131}I]*albumin in the CSF space. The labeled material was injected in the cisterna magna or in the ventricle, and its disappearance was followed by analysis of CSF samples obtained through a cisternal catheter by using the mean 30-min concentration as 100%. Kinetics of disappearance of* ^{131}I *after intracisternal* (●) *and after intraventricular* (○) *injection.*

later, scintigrams were obtained as described in Materials and Methods. The 5.5-h lateral view of the animal is presented in Fig. 2B. The diffuse distribution of radioactivity in the head region and the lesser concentration in the spinal canal demonstrate the pattern of interferon distribution in the subarachnoid space. In contrast to this, a similar scan after intracisternal injection showed persistent local concentration in the area overlaying the cisterna magna for as long as 5 h (data not shown). This result suggested that the trauma following cisternal puncture may have allowed some local leakage and trapping of CSF or injected material, which may account at least partially for the more rapid disappearance curve of albumin and interferon after intracisternal injection. Accordingly, only intraventricular injections were used in the rabies experiments (see below).

Interferon in the CSF space. In Table 2, the fate of interferon after intracisternal and intraventricular injections is summarized. The geometric means of the interferon titers of CSF 30 min after injections showed that, compared with the inoculum, the dilution factors were, respectively, 8.9 (0.95 log) and 10.2 (1.01 log) after intracisternal and intraventricular injections. This is consistent with what was found with radioactive albumin.

The disappearance curve of interferon, considering the 30-min titer as being 100%, is plotted in Fig. 3. The clearance of interferon from the circulation after an intravenous injection of rabbits is included for comparison. The half-life (t 0.5) of interferon in serum after intravenous injection was about 11 min (11), and the half-life in CSF was about 1 h (Fig. 3). It should, however, be pointed out that the disappearance curve in both body spaces showed a marked tailing effect with respect to the concentrations with time.

Effect of intraventricular interferon on rabies in rabbits. From the above experiments, it appeared that interferon levels in the CSF could be maintained by daily intraventricular injections, which have the advantage of bathing the ventricular spaces as well as the cisterns and, to a lesser extent, the spinal subarachnoid space. An experiment was undertaken to test the effect of intraventricular interferon on experimental rabies in rabbits. The treated group (E in Table 3), consisting of four rabbits, each received one million units of interferon in 0.2-ml daily intraventricular doses for 11 days. The first dose was administered immediately after the animal received 0.4 ml of a 1:50 dilution of rabies virus in the right thigh. Four untreated rabbits (group B) serving as controls were similarly infected. A simultaneous virus titration on untreated rabbits was performed as follows: three rabbits were inoculated with 0.4 ml of a 1:5,000

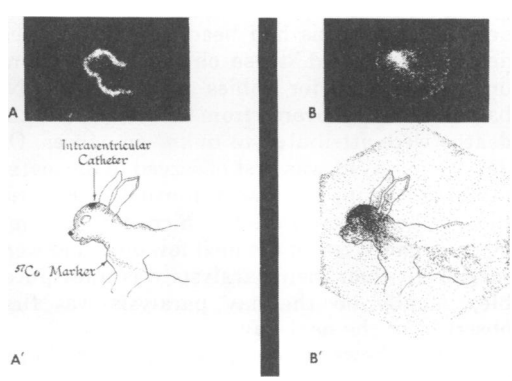

FIG. 2. *Camera scintigram of distribution of* [^{131}I]*-albumin after intraventricular injection. A, Rabbit head and neck is outlined by* ^{57}Co *marker. Position of intraventricular cannula is outlined by injected* [^{131}I]*-albumin at time of injection A#, Diagrammatic interpretation of A. B, Distribution of* [^{131}I]*albumin 5.5 h after injection. B', Diagrammatic interpretation of B.*

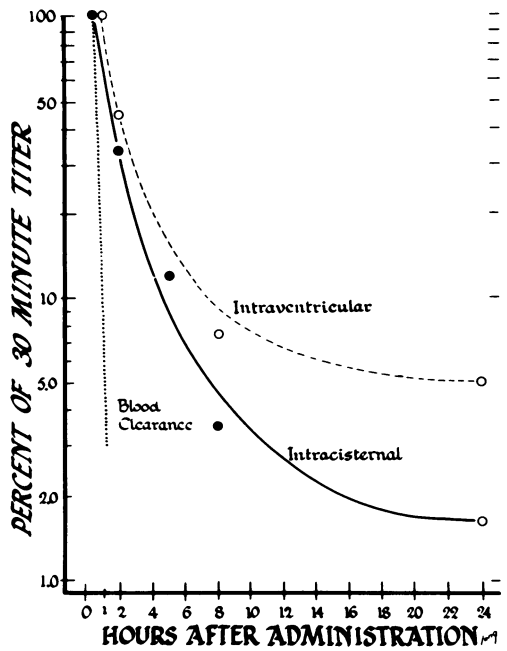

FIG. 3. *Interferon in the CSF space. Interferon was injected into the cisterna magna or into a ventricle, and its disappearance was followed by analysis of CSF samples obtained through a cisternal catheter. Kinetics of disappearance of ^{131}I after intracisternal (●) and after intraventricular (○) injection. Blood clearance after intravenous injection of interferon is inserted for comparison.*

virus suspension; similarly, another three were inoculated with a 1:500 suspension; four received a 1:50 suspension; and two received the 1:5 virus suspensions. On days 2 and 11 after infection, two interferon-treated rabbits were dead. No paralysis had been noted, and their deaths were rapid, these circumstances being uncharacteristic for rabies in this host. No bacteria were recovered from their CSF, and the deaths were attributed to unknown causes. On day 13, paralysis was first observed in untreated rabbits receiving the lowest dilution of the virus suspensions. Most rabbits which did succumb became paralyzed in the next few days and were sacrificed when their paralysis was undisputable, usually on the day paralysis was first observed or the next day.

Table 3 shows the outcome of the titration of the rabies virus inoculum and the result of interferon treatment. Comparing groups B and E, the incubation period was prolonged for 5 days in the treated group. Rabbits surviving at the end of the 6-week observation period were those inoculated with the most dilute virus suspension (1:5,000). The PD_{50} (50% paralysis dose), calculated by the Reed-Muench method from the titration data, was 1:2,830. Thus groups B and E received a challenge of 57 PD_{50}, which was somewhat more potent than planned. This was taken into account by doubling the virus dilution for challenge in the next experiment, which is described below.

In the second experiment (Table 4), the treated group again received intraventricular interferon, but controls were included which received intraventricular saline. Based on results of the previous experiment, a uniform inoculum of 0.4 ml of a 1:100 dilution (28 LD_{50}) of rabies virus suspension in the right thigh was used. Twelve rabbits were cannulated; of these, eight received intraventricularly one million units of interferon (group A), and four were infused with saline (group B) daily for 21 days. To control for the effect of interferon administered outside of the central nervous system, eight rabbits were uncannulated; of these, four were given one million units of interferon in equal intramuscular and intravenous doses concurrently with rabies virus challenge as previously described (17, group C), and four were given saline placebos (group D).

For statistical analysis of the results, the comparable groups were considered to be A and B (intraventricular interferon or saline) and C and D (parenteral interferon or saline). The parameter compared was the reciprocal of the length of incubation period. Where animals survived (group C), it was assumed that the incubation period was 2 times the observation period, or 120 days. By the Student's *t*-test, both forms of interferon treatment, that is, intraventricular or parenteral administration, significantly prolonged the incubation period at

TABLE 3. *Effect of interventricular interferon on rabies in rabbits*

Group	Virus dilution	Interferon	No. paralyzed/no. injected	Incubation period[a]	
				Mean (days)	Range (days)
A	1:5	None	2/2	14	13-15
B	1:50	None	4/4	15	13-16
C	1:500	None	3/3	27	17-39
D	1:5,000	None	1/3	23	23
E	1:50	Yes[b]	2/2[c]	20	19-21

[a] Incubation period is the mean period from the inoculation to the onset of paralysis. Observation period was 6 weeks.

[b] One million units daily for 11 days through ventricular cannula.

[c] Excludes two rabbits dying of nonspecific causes.

TABLE 4. *Effect of intraventricular interferon on rabies in rabbits*

Group	Treatment	No. paralyzed/ no. injected	Incubation period[a]		
			Mean days	Days[b]	P[c]
A	One million units IV[d] daily for 21 days	4/4[e]	25	19, 23, 25, 34	<.05
B	Saline IV daily for 21 days	3/3[f]	15	13, 15, 16	>.01
C	One million units IM[d] and IV at time of virus infection.	1/3[f]	40	40	<.01
D	Saline IM and IV at time of virus	3/3[f]	19	17, 18, 22	

[a] The mean period from the inoculation of 28 PD_{50} rabies virus until the onset of paralysis. Period of observation: 60 days.

[b] Incubation period in terms of days of specific animals.

[c] Student's *t*-test: the incubation periods of treated animals as compared with those of the appropriate control groups, viz.: groups A versus B and C versus D.

[d] IV, Intraventricularly; IM, intramuscularly.

[e] Excludes four rabbits which died from nonspecific causes.

[f] Excludes one rabbit which died from nonspecific causes.

the $P < 0.05$ level. Assumption of the length of incubation period of surviving rabbits (group C) may be arbitrary, but the efficacy of intramuscular and intravenous interferon has already been shown (17).

It was noted that all cannulated rabbits, irrespective of whether or not they received any interferon, consumed little water and food as compared with noncannulated animals. Their smaller size was grossly apparent, and periodic weighings confirmed this impression (Table 5). In addition, it is to be noted that of 12 rabbits cannulated, five died 1 to 8 days thereafter, and because no paralysis was evident prior to death, they presumably succumbed of causes unrelated to rabies. Table 4 shows that discounting nonspecific deaths, the following points may be made. (i) The insertion of an intraventricular cannula probably reduced the resistance to rabies. Thus, uncannulated control rabbits (group D) enjoyed longer incubation periods than the cannulated, saline-infused animals (group B, $P = 0.1$ by the rank sum test). (ii) Interferon injected intraventricularly (group A) lengthened the incubation period as compared with uncannulated controls (group B) by about a two-thirds increase (24 versus 15 days). This is consistent with what was observed in the first experiment, although this experiment was better controlled. (iii) As previously reported (17), interferon administered intramuscularly and intravenously protected two out of three rabbits completely (group C). The one animal with rabies had an inordinately long incubation period (40 days).

TABLE 5. *Nonspecific effect of intraventricular cannula in rabbits*

Procedure	Weight (kg)[a]		
	Day 2	Day 11	Day 15
Cannulated	1.07 (12)	0.89 (7)	0.93 (6)
Uncannulated	1.05 (8)	1.28 (7)	1.25 (7)

[a] Mean weights; number of animals is in parentheses.

DISCUSSION

Relatively little is known about the distribution and fate of interferon in various body spaces (10). Because interferon is a macromolecule which would not be expected to diffuse freely, it is particularly important to have such data in evaluating the antiviral effect of interferon in viral infections of structures related to such spaces. To induce interferon, Cathala and Baron (4) injected 1 mg of poly(I)·poly(C) intrathecally in rabbits. The interferon formed diffused poorly from the subarachnoid space into the bloodstream, but, after 24 h, interferon was still present in the CSF.

These results are consistent with what we found by directly injecting interferon in the CSF space through the lateral ventricle or cisterna magna. Interferon disappeared from CSF by a slope much shallower than the corresponding one in the bloodstream. It resembled that of albumin, although albumin is a larger molecule than the V_B fraction of rabbit inter-

feron (15) used in these experiments (molecular weight of 69,000 versus 46,000), and albumin is cleared much slower than interferon in the bloodstream (10).

In general, there are two types of viral infections which have been considered for interferon intervention. One is its use as a prophylactic-therapeutic agent in respiratory infections (16). The other is its use in serious life-threatening virus infections. Rabies is such an infection, with a definite event of exposure and a long incubation period in man which should permit adequate time for prophylactic and therapeutic intervention, including interferon (18). Several investigators showed that interferon and interferon inducers administered intramuscularly or intravenously can indeed protect rabbits, hamsters, and mice against rabies (2, 6, 12, 17, 19). The present work shows that interferon in the CSF space administered through the lateral ventricle is not as effective. The following are possible explanations. (i) The initial site of replication of rabies virus may be the exposure site (3). Although this point has not been proven by direct evidence, data showing that interferon or poly (I)·poly(C) administered into the same muscle as the virus increased the resistance is consistant with this theory (7, 12, 17). If rabies virus replicates locally, by the time it ascends into the central nervous system there may be too much virus for the interferon to deal with. These considerations suggest that a combination of intraventricular and intramuscular interferon in repeated doses should be tried. (ii) Interferon in the CSF may not get into the relevant neural tissues in which the virus replicates. Although drug levels are frequently measured in the CSF as the indication of the penetration of the "blood-brain barrier" by such drugs, strictly speaking, this is not evidence that brain cells are exposed to the drug. This is particularly true of a macromolecular substance such as interferon, in the absence of leptomeningeal inflammation. Additional studies on the diffusion of interferon or similar macromolecules from the CSF space into nervous tissues are clearly indicated. (iii) We have found that insertion of a ventricular cannula interferes with the growth of rabbits and decreases their resistance to rabies infection. Possibly the effect of interferon in the CSF space was offset by this increased susceptibility to infection. More studies are needed to determine whether this is a technical problem which may be overcome or whether it is an inevitable result of the operative procedure. (iv) Because most of the CSF in the ventricles does not circulate through the spinal subarachnoic space, administration in the ventricles or cisterna magna may result in much lower peak concentrations in the spinal subarachnoid space than could potentially be achieved by direct injection in the lumbar subarachnoid space. If ascending infection via nerve roots into the spinal cord is the main route of viral spread, injection in the lumbar subarachnoid space may offer advantages over ventricular or cisternal administration. This hypothesis should be tested further, either in man or in other animals.

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