GROWTH OF MEASLES VIRUS IN NERVOUS TISSUES

IV. NEUROVIRULENCE OF WILD MEASLES AND SSPE VIRUSES IN MONKEYS

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SUMMARY: Neurovirulence of in vivo-passed wild measles virus and that of the cell-associated SSPE virus were compared by intracerebral inoculation into monkeys. The wild measles virus was found to lack neurovirulence without producing neurological signs or significant histological changes in the brains, whereas the virus was confirmed to preserve the properties characteristic of the wild virus. In contrast, inoculation of the SSPE virus induced rapid onset of neurological signs with mild but definite histological changes including degeneration of nerve cells. The fact that SSPE virus exhibited neurovirulence in monkeys indicated importance of the current assay system for neurovirulence of measles vaccine by intracerebral inoculation into monkeys.

INTRODUCTION

Measles virus has been found to be responsible for subacute sclerosing panencephalitis (SSPE) (Payne, Baublis and Itabashi, 1969) and also for acute measles encephalitis (Adams, Baird and Filloy, 1966). Since different neurovirulences were noted among several strains of measles virus in experimental infections of rodents (Wear and Rapp, 1970; Albrecht and Schumacher, 1971; Janda, Norrby and Marusyk, 1971; Shishido et al., 1973), estimation of neurotropism of live measles virus vaccine in humans has become an important problem for the safety of the vaccine. The current neurovirulence test of measles vaccine by intracerebral inoculation into monkeys is based mainly on the findings of Buynak et al. (1962) who noticed severer histological lesions in the central nervous system (CNS) of monkeys inoculated with virulent measles virus at low passage level in tissue culture than those with attenuated virus.

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However, Albrecht *et al.* (1972) reported that both wild and attenuated measles viruses failed to produce significant histological changes in monkeys and that the hamster-brain-adapted measles virus produced encephalitis with the histological lesions resembling partly to those of SSPE.

It has been pointed out that the throat swabs obtained from measles patients or the lymphoid tissue extracts from monkeys infected with the measles patient's materials produce typical lesions consisting of giant cell formation in the lymphoid tissues of monkeys by subcutaneous inoculation, and that most of the laboratory strains of measles virus including those at low passage levels in *vitro* tissue culture were less virulent in monkeys (Yamanouchi *et al.*, 1970, 1973). Therefore, the *in vivo*-passed measles virus was considered to preserve the properties of wild virus to a higher degree than the *in vitro*-passed laboratory strains.

On the other hand, viruses isolated from the patients with SSPE are suspected to have acquired high neurotropism during the long latency in the CNS. The Niigata-1 strain of SSPE virus was shown to be highly neurovirulent in rodents producing encephalitis not only in newborn animals but also in adult ones (Doi *et al.*, 1972; Katow *et al.*, 1973).

In the present study, neurovirulence of *in vivo*-passed wild measles virus and that of the SSPE virus were compared by intracerebral inoculation into monkeys to provide basic information for the neurovirulence test of measles virus vaccines in monkeys.

MATERIALS AND METHODS

Monkey: Cynomolgus monkeys (*Macaca fuscicularis*) at estimated ages of 1–3 years were imported from Southeast Asia. After they had been confirmed to be free from measles virus infection by the hemagglutination-inhibition test, they were inoculated with virus into the thalamus or into both the thalamus and cisterna magna.

Viruses: Details of preparation of wild measles virus were described previously (Yamanouchi *et al.*, 1970). In brief, throat swabs were obtained from measles patients within few days after onset of rash, and inoculated subcutaneously into monkeys. Seven days later, the spleen and lymph nodes were collected and made into 20% homogenates, which were used as wild virus stock. Since this virus does not adapt to grow in tissue cultures, titration for infectivity was not applicable. Instead, the stock virus had been confirmed to induce typical lesions consisting of giant cells in the lymphoid tissues about one week after subcutaneous inoculation into monkeys (Yamanouchi *et al.*, 1973).

As for SSPE virus, Niigata-1 strain (Doi *et al.*, 1972) was employed. Since no cell-free virus was released, trypsinized cell suspensions from the virus-

infected Vero cell cultures were used as inocula for monkeys. The infectivity titer was assayed by co-culture with Vero cells and expressed as a number of infectious centers per milliliter. To minimize possible side effects caused by the growth of injected Vero cells constituting the major population in the inoculum, the cell suspensions were treated in some experiments by incubation with mitomycin C at 10 $\mu\text{g/ml}$ for 3 hr at 37 C as described previously (Yoshikawa et al., 1975).

Hemagglutination-inhibition (HI) test: The procedure was described previously (Shishido et al., 1967). Reciprocals of the highest dilutions of sera inhibiting hemagglutination by measles virus were taken as HI antibody titers.

Immunofluorescent staining: The tissues were quickly frozen in hexane chilled in a dry-ice-acetone bath. Thin sections were made by cryostat, air-dried, and fixed in acetone at -20°C for 20 min. The fixed sections were stained by the direct method using measles convalescent monkey serum conjugated with fluorescein isothiocyanate as described previously (Yamanouchi et al., 1973).

Histopathological examination: The tissues were fixed in 10% formalin, dehydrated and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (HE) and luxol fast blue (LFB).

Virus isolation: The brain tissues were trypsinized and mixed with a Vero cell suspension for co-culture. The cultures were observed for cytopathic changes for three passages. When cytopathic changes were observed, the cultures were frozen, thawed and inoculated into another culture of Vero cells to examine for the recovery of cell-free or cell-associated virus.

TABLE I

*Neurovirulence of wild measles virus in monkeys**

Monkey No.	Autopsy (weeks)	HI anti-body at autopsy	Histological changes in						
			Submandibular nodes	Axillary nodes	Inguinal nodes	Mesenteric nodes	Spleen	Lung	CNS
9672	1	<8	+	+	+	+	+	—	—
9673		<8	—	—	—	—	—	—	—
9674	2	256	—	—	—	—	—	—	—
9675		32	—	—	—	—	—	—	—
9676		8	—	—	—	—	—	—	—
9677	3	2048	—	—	—	—	—	—	—
9678		1024	—	—	—	—	—	—	—
9679		64	—	—	—	—	—	—	—
9681	4	1024	—	—	—	—	—	—	—
9682		1024	—	—	—	—	—	—	—

* None of the animals developed neurological signs.

** Giant cell formation.

TABLE II

Neurovirulence of Niigata-1 strain of SSPE virus in monkeys

Exp.*	Monkey No.	Inoculum	Day of onset of neurological signs	Autopsy (days)	HI antibody at autopsy	Virus recovery from		
						CBR**	CBL	MO
1	10826	SSPE-Vero cells	7	7	<8	—	—	—
	10828		7	7	<8	—	—	+
	10829		6	9	<8	—	—	—
	10830		8	8	<8	—	—	—
	10831		9	9	<8	—	—	—
	10832		7	8	<8	—	—	+
	10833		8	8	<8	—	—	—
	10834		12	12	<8	—	—	—
	10835		9	10	<8	—	—	—
	10836		8	9	<8	—	—	—
	10837		10	10	<8	—	—	—
	10838		12	12	<8	—	—	—
	10839		6	7	<8	—	—	—
	10840		5	8	<8	—	—	—
	10682	Vero cells	—	14	<8			
	10683		—	14	<8			
2	12663	Mitomycin-treated	7	9	<8	—	—	—
	12666	SSPE-Vero cells	7	9	NT***	NT	NT	NT
	12667		9	10	<8	—	—	+
	12670		8	9	<8	—	—	—
	12672		8	10	<8	—	—	—
	12844	Mitomycin-treated	—	20	<8			
	12856	Vero cells	—	20	<8			
	12857		—	20	<8			
	12859		—	20	<8			
	12860		—	20	<8			

* Exp. 1.: Monkeys were inoculated into the bilateral thalami either with SSPE-Vero cells or with 5.6×10^5 normal cells.

Exp. 2.: Monkeys were inoculated into the unilateral thalamus either with mitomycin-cells and 1.7×10^5 normal cells or with 5.5×10^5 normal cells.

** CBR: cerebrum, CBL: cerebellum, MO: medulla oblongata.

*** NT: not tested.

RESULTS

Neurovirulence of Wild Measles Virus

Ten monkeys were inoculated with wild measles virus, and two to three monkeys were sacrificed in 1, 2, 3, and 4 weeks after inoculation. None of them showed any neurological signs. As summarized in Table I, development of HI antibody was demonstrated in the monkeys autopsied later than 2 weeks with similar patterns to that of natural infection (Yamanouchi *et al.*, 1970). Histo-

Virus antigen in		
CBR	CBL	MO
—	—	—
+	+	+
+	—	—
—	—	+
—	—	—
—	+	+
—	—	—
+	—	—
—	—	—
—	+	—
—	—	+
+	—	—
—	—	+
—	+	—

—	—	—
NT	NT	NT
—	—	—
—	—	—
—	—	—

containing 72 infected cells and 9.2×10^5 normal

treated SSPE-Vero cells containing 600 infected

pathological examinations revealed typical giant cells characteristic of infection with wild measles virus (Yamanouchi et al., 1973) in the lymphoid tissues of one monkey (#9672) autopsied in one week. However, histological changes were not found in the CNS in any of the monkeys except the presence of a few glial cells around the needle tracks of inoculation in the cerebrum of the monkeys autopsied in 3 and 4 weeks. These results indicated that the wild measles virus lacked neurovirulence in monkeys.

Neurovirulence of Niigata-1 Strain of SSPE Virus

Two experiments were conducted as summarized in Table II. In Exp. 1, 14 monkeys were inoculated into the bilateral thalami with a virus-infected Vero cell suspension containing 72 infected cells and 9.2×10^5 normal Vero cells, and two monkeys with 5.6×10^5 normal Vero cells. In Exp. 2, five monkeys were inoculated into the unilateral thalamus with a mitomycin C-treated virus-infected Vero cell suspension containing 600 infected cells and 1.7×10^5 normal cells, and other five monkeys with 5.5×10^5 normal cells treated with mitomycin C. In both experiments, all the monkeys inoculated with the virus-infected cells developed neurological signs such as apathy and paralysis in 5–12 days and became moribund or died within 1–3 days. The cell-associated virus was isolated from the medulla oblongata of two monkeys (#10828 and 10832) in Exp. 1 and of one monkey (#12667) in Exp. 2 by co-culture with Vero cells. Immunofluorescent staining revealed the presence of the measles virus antigen in the cerebrum, cerebellum, and medulla oblongata of 10 out of 14 monkeys in Exp. 1 but none of four monkeys examined in Exp. 2. Development of measles HI antibody in the sera was not observed at autopsy in any of 18 monkeys inoculated with the virus. None of the control monkeys inoculated with the normal cells showed any clinical signs during the observation periods of 14 days in Exp. 1 and of 20 days in Exp. 2.

Fig. 1. Mild spongy degeneration associated with decreased nerve cells in the hippocampus, #10833. H.E. $\times 40$

Fig. 2. Degeneration of nerve cells and nodular accumulation of mononuclear cells indicating neuronophagia in the midbrain, #12670. H.E. $\times 100$

Fig. 3. Mild perivascular cuffings in the paraventricular areas in the pons. The upper part shows the cerebellum, #10838. H.E. $\times 40$

Histological examinations were made on the brains obtained from the virus-inoculated monkeys sacrificed at the moribund stage or at the time of death 5–12 days after inoculation, and from the control monkeys autopsied in the thalamus or the nucleus caudatus or both. Around the needle tracks, focal hemorrhages and softenings associated with spongy degeneration, vascularization, infiltration of macrophages, glial proliferation, and mild perivascular cuffings were observed in both the virus-inoculated and control monkeys.

Except the lesions around the needle tracks and occasional demonstration of mild cuffings, no significant changes were found in the control monkeys. In the virus-inoculated monkeys, histological lesions were relatively mild and localized in spite of acute severe clinical signs. Local degeneration and decreased nerve cells with spongy degeneration and mild glial proliferation were occasionally demonstrated in the hippocampus, pons and medulla oblongata (Fig. 1). The nodular accumulation of glial cells with or without showing the characteristics of neuronophagia was demonstrated in some cases (Fig. 2). Mild perivascular cuffings were observed in the subarachnoid space and brain parenchyma, especially in the paraventricular areas and the areas distant from the needle traumas (Fig. 3).

DISCUSSION

Intracerebral inoculation of monkeys with *in vivo*-passed wild measles virus failed to produce significant histological changes in the CNS, whereas such virus was shown to retain the properties of wild virus as indicated by induction of typical lesions of giant cells in the lymphoid tissues and by the pattern of antibody response. Invasion of measles virus into the CNS during the uncomplicated course of measles infection has been suspected from the abnormal pattern of electroencephalograms in natural measles (Pampiglione, 1964). However, measles encephalitis was roughly estimated to occur in one out of 1,000 measles patients (Adams *et al.*, 1966). Whether such a relatively low rate of onset of measles encephalitis reflects low neurotropism of wild measles virus or rare access of the virus through the blood-brain-barrier is unknown. The present results may be taken as a partial evidence for the former possibility.

The cell-associated SSPE virus was found to be highly neurovirulent as indicated by the rapid onset of neurological signs in all the monkeys inoculated. In spite of severe clinical signs, the histological lesions consisted mainly of localized degeneration of nerve cells with mild inflammatory lesions. The inconsistent isolation of virus from the brains as well as the demonstration of the virus antigens in limited areas indicated localized virus growth in the CNS. In the control monkeys inoculated with the normal cells, neither clinical signs nor histological lesions were observed. Therefore, the neurological signs in the virus-

inoculated monkeys must have resulted from virus-induced destruction of nerve cells, although the virus-affected areas seemed to be rather limited. Similar observations were made on adult hamsters inoculated with the same virus (Katow et al., 1973).

Schumacher et al. (1971) failed to detect any significant histological changes in monkeys inoculated with cell-free Ro-SSPE-1 strain of SSPE virus. In a previous study, we noted lack of neurovirulence in cell-free Mantooh strain of SSPE virus in ferrets, in contrast to the high neurovirulence of Niigata-1 strain in the same species of animals (Yoshikawa et al., 1975). Similarly, several strains of cell-associated SSPE virus were shown to produce severer neurological signs than cell-free SSPE virus in various animals including rodents, sheep, cattle and dogs. Lack of antibody development in monkeys inoculated with the cell-associated SSPE virus in the present study may indicate that failure in immune mechanism to eliminate virus from the CNS is partly responsible for the high neurovirulence in addition to neurotropism of this virus.

The wild measles virus was found to lack detectable neurovirulence in monkeys. Previously it was shown that the same stock of wild virus lacked neurovirulence in newborn hamsters and mice, whereas some other strains of measles virus adapted to grow *in vitro* were found to exhibit high neurovirulence (Shishido et al., 1973). The level of neurovirulence in the rodents did not necessarily correlate with that of attenuation, and it was suggested that some vaccine strains might exhibit higher neurovirulence than wild virus in humans as well. Thus far, none of the vaccine strains that had been examined in this laboratory showed neurovirulence in monkeys (unpublished data). However, Albrecht et al. (1972) demonstrated high neurovirulence of the hamster-brain-adapted measles virus in monkeys. In the present study, the cell-associated SSPE virus was found to be highly neurovirulent in monkeys. These findings indicate the possibility of appearance of neurovirulent variants of measles virus during *in vitro* or *in vivo* passages. Subsequently, the current assay system for neurovirulence of measles vaccine by intracerebral inoculation is considered to be useful in detecting such neurovirulent measles virus, although the assay system was originally developed on the assumption that wild measles virus holds higher neurovirulence than vaccine strains.

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