INFECTION AND IMMUNITY, Oct. 1971, p. 419-424

Vol. 4, No. 4
Printed in U.S.A.

Intracerebral Inoculation of Rhesus Monkeys with a Strain of Measles Virus Isolated from a Case of Subacute Sclerosing Panencephalitis

HEINZ P. SCHUMACHER,[1] PAUL ALBRECHT, RONALD G. CLARK, RUTH L. KIRSCHSTEIN, AND NICOLA M. TAURASO

Laboratory of Virology and Rickettsiology and Laboratory of Pathology, Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 1 April 1971

Measles virus isolated from the brain of a patient with subacute sclerosing panencephalitis was injected intracerebrally (ic) into 34 rhesus monkeys. Groups of these animals were injected with measles antigen in Freund's complete adjuvant or treated by schedules used for suppression of the general or cell-mediated immune responsiveness. In another group of animals, experimental allergic encephalitis was induced parallel with measles infection. Measles virus was isolated from the brains of monkeys up to 13 days after ic inoculation. No virus was detected in the central nervous system after 3 to 4 weeks, the longest postinoculation period examined. It was concluded that the subacute sclerosing panencephalitis-derived virus either lost its neurotropic properties at the passage level at which it was used or that it submerged into a silent stage and escaped detection. Neither immunosuppression nor concomitant autoimmune encephalitis had an effect on the survival of measles virus in the central nervous system. The histology of the nervous tissue was basically normal except for characteristic lesions of experimental allergic encephalitis in animals receiving the respective treatment.

Encephalitides in the postinfectious period of several common exanthematous diseases of childhood or after vaccination against rabies and smallpox are thought to depend largely on immune reactions directed against brain antigens, viral antigens, or both, in the brain tissue proper (12, 21). The frequent failure to isolate an infectious agent from the central nervous system (CNS) in fatal cases of encephalitis has supported the above view (21).

With improved virus isolation techniques, several isolations of measles virus were made from brains of patients with subacute sclerosing panencephalitis (SSPE) and these viruses were shown to possess altered physical and cultural properties (5, 6, 9-11, 18). When one of the strains was injected intracerebrally into monkeys, the animals developed serum antibody but failed to show neurological symptoms over a 3-month observation period (10). The possibility was considered that, as in the acute forms of parainfectious encephalitides, chronic measles infection of the CNS may be associated with an altered humoral or cell-mediated immune response of the host (7).

[1] Present address: Universitäts-Kinderklinik, 78 Freiburg/Br., Germany.

We wanted to investigate whether measles virus isolated from human brain tissue with lesions of SSPE would infect the CNS and cause clinical or morphological encephalitis in normal or in immunologically enhanced or suppressed rhesus monkeys.

MATERIALS AND METHODS

Virus. The Ro-SSPE-1 strain of measles virus, isolated from the brain of a patient with SSPE (18), was obtained from F. E. Payne in its 18th passage in BS-C-1 cells. Stock virus used in these studies was passaged twice in Vero cells and had a titer of 1.4×10^6 plaque-forming units per 0.1 ml.

Animals. Monkeys (Macaca mulatta) were obtained commercially from New Delhi, India, and quarantined upon arrival in one large room with one or two animals in a cage. A total of 34 juvenile monkeys (1.8 to 3.5 kg) shown to be free of measles virus and measles antibody was selected, and four to five animals were assigned to each of eight experimental groups as indicated in Fig. 1. Each animal was observed twice daily for respiratory symptoms, conjunctivitis, nasal discharge, cough, rash and malaise, and for symptoms of experimental allergic encephalomyelitis (EAE), such as eye symptoms, weakness, tremor, ataxia, and paralysis.

Cell culture. The Vero (23) African green monkey

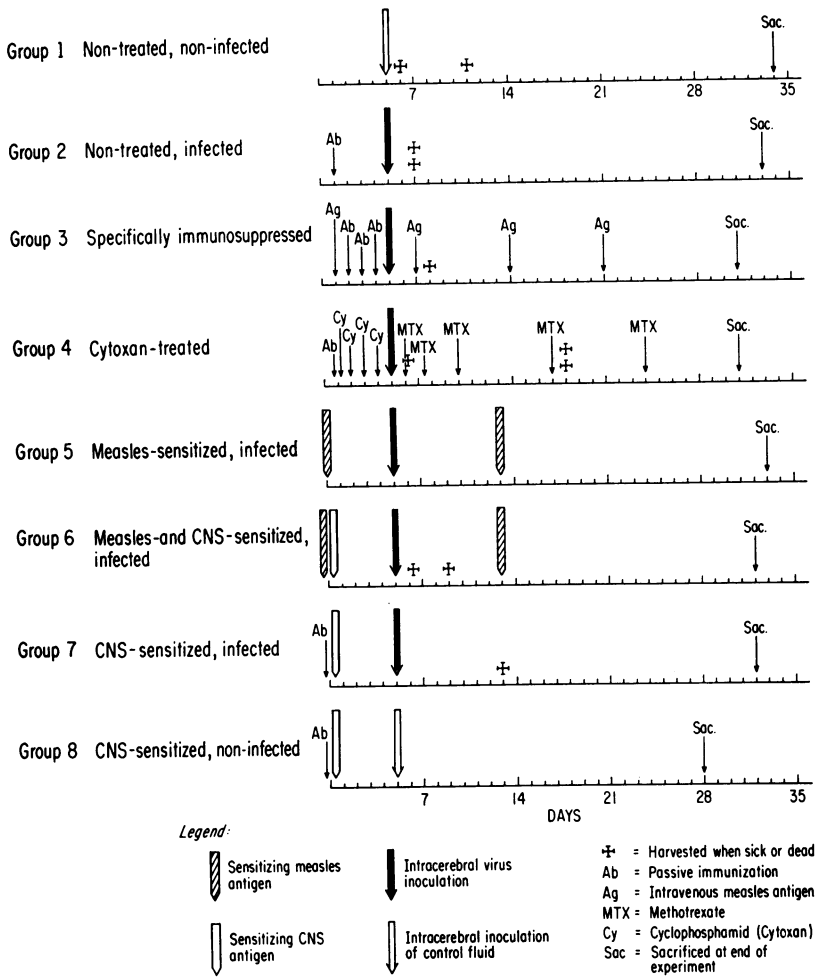

FIG. 1. *General design of experiments related to intracerebral inoculation of rhesus monkeys with the Ro-SSPE-1 strain of measles virus.*

kidney cell line was obtained at passage 160 and propagated in medium 199 containing 10% fetal bovine serum (FBS), penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml).

Virus inoculation. Two kinds of virus inocula were employed. (i) Stock virus (BS-C-1/18, Vero/2) suspension was diluted 1:10, and 0.5 ml was inoculated into the left thalamic area; (ii) Vero cells infected with the stock virus pool were harvested by trypsin dispersion 3 to 5 days later and taken up in cell culture medium as a 10% cell suspension. An inoculum of 0.5 ml of this suspension (BS-C-1/18, Vero/3) was introduced into the right thalamic area. In all of these inoculations, the standard technique of the intracerebral (ic) inoculation of monkeys as used in safety testing of viral vaccines was employed (15).

Virus isolation technique. Brain tissue removed at autopsy was minced, washed three times with Dulbecco's saline, and dissociated by trypsinization as described previously (20). A 10% brain cell suspension was prepared in Eagle's minimum essential medium (MEME) containing Earle's balanced salt solution base, 10% FBS, penicillin G (100 units/ml), and streptomycin sulfate (100 μg/ml); 0.2 ml of this suspension was seeded into each of three petri dishes (35 mm diameter, Falcon Plastics) containing 3 ml of MEME. In addition, a mixture of 0.6 ml of the 10% brain cell suspension and 9 ml of a Vero cell suspension (100,000 cells/ml) was seeded in three 35-mm petri dishes (co-cultivation). The cultures and co-cultures were observed periodically for at least 3 weeks. When cytopathic changes were noticed, the cultures were examined by the fluorescent-antibody technique for the presence of measles virus. When no cytopathic changes were noticed, the cultures were typsinized, passed into new petri dishes, and examined in the same way as the first passage.

Sensitization. Some monkeys were hyperimmunized by intramuscular injection into each leg of 0.25 ml of a mixture of equal volumes of killed

measles virus reference vaccine (DBS lot 2) and Difco Freund's complete adjuvant (FCA). The potency of the antigen was tested in rats (Lewis strain) and in guinea pigs (Hartley strain). Animals injected with 0.1 ml of the sensitizing antigen and skin-tested 14 days later developed indurations measuring 6 to 15 mm in diameter (*see below*).

Other animals were inoculated intradermally into five sites on the shaved back with a total of 0.5 ml of a CNS antigen consisting of a mixture of equal volumes of 40% guinea pig spinal cord homogenate and FCA.

Immunosuppression. The schedule of Santos (19) for immunosuppression of graft rejection was employed to achieve general immunosuppression of the monkeys. Essentially, each animal received a total of 180 mg of cyclophosphamide per kg (Cytoxan; Mead Johnson Laboratories, Evansville, Ind.) divided into four daily intravenous injections. This was supplemented with six injections of amethopterin (Methotrexate; Lederle Laboratories, Pearl River, N.Y.) given 2, 4, 6, 13, 20, and 27 days after the last injection of Cytoxan (Fig. 1).

To suppress delayed hypersensitivity without affecting the antibody response, a protocol based on studies of Asherson and Stone (3) and Axelrad (4) was used. This included various series of intravenous injections of antigen and antibodies as indicated in Fig. 1. The antigen was a Tween 80-ether-treated (16) preparation used also in the hemagglutination-inhibition antibody test described below. The antibody preparation was hyperimmune monkey serum with an antibody titer of 1:20,000. A single intravenous injection consisted of 3 ml of antigen and 1 ml of the antibody preparation.

Hemagglutination-inhibition test. Heat-inactivated (56 C/30 min) sera were treated with kaolin and then adsorbed overnight with 50% rhesus monkey red blood cells. The test was performed with 4 hemagglutination units of Tween 80-ether-treated measles hemagglutinin antigen (titer 1:800, reference 16). A reference hyperimmune monkey serum was included in each test.

Skin test. Tests for hypersensitivity to measles and CNS antigens were performed by intradermal injection of 0.1 ml of test antigen. Shaved pigment-free areas were used and the test results were recorded 48 hr later. Measles antigen consisted of formalin-killed, alum-adsorbed measles vaccine. The CNS antigen was a 20% monkey brain homogenate in phosphate-buffered saline, clarified by centrifugation at 1,000 × *g* for 10 min.

Preparation of CNS tissue for histopathology. At autopsy the brains were removed and sectioned transversely. One section from each brain was used for virus isolation, and the remaining tissue was immersed into 10% formalin-saline containing 10% acetic acid. The tissue was then embedded into paraffin, sectioned, and stained with either Luxol fast blue-MBS, Weil, gallocyanin, cresylecht violet, or hematoxylin-eosin. A minimum of 15 to 20 transverse sections from each hemisphere and 10 sections from the lower brain stem were examined.

RESULTS

General experimental design. The general design of the study is illustrated in Fig. 1. The animals in group 2 were injected ic with measles virus, and the clinical course of disease, laboratory data, and histopathology were compared with the results obtained in group 1 animals inoculated with tissue culture fluid only. Groups 3 and 4 consisted of specifically suppressed animals or those in which suppression was induced by Cytoxan, respectively. The effect of hypersensitivity against measles antigen, encephalitogenic antigen, or both, on measles infection was studied in the last four groups. The ic injection of measles virus in all groups was scheduled so that maximal ic virus multiplication would coincide with maximal expected hypersensitivity or immunosuppression.

From recent observations of newly arrived monkeys and based on previous studies, it was expected that on arrival at the laboratory the animals would be in an early stage of measles incubation. It was anticipated that starting treatment immediately on arrival would produce the above immunological effects. To prevent possible interference of viral replication after ic inoculation of Ro-SSPE-1 virus strain by circulating natural wild-type virus, monkeys in groups 2, 4, 7, and 8 received an initial intravenous dose of 1 ml of a high-titered measles hyperimmune monkey serum.

Clinical course of disease. Rash was observed in 12 of 32 animals between day 2 and 6 of the experiment (Table 1). It was most pronounced on the face and resulted in a small degree of scaling a few days later.

In the untreated, uninfected group 1, two animals died on days 1 and 6 after ic inoculation, respectively (Fig. 1). Virus isolation attempted only from the second animal was unsuccessful (Table 2). In the untreated, measles-infected group 2, two animals died 2 days after ic inoculation, probably as a result of trauma. Measles virus was isolated from the brain of both animals. One of the remaining two animals developed a slight hemiparesis several days before sacrifice.

In the specifically suppressed group 3, one animal died 3 days after inoculation and one animal developed weakness of the left arm 4 days before sacrifice. In the Cytoxan-treated group 4, one animal died 1 day after inoculation. Two animals died 13 days after ic inoculation in a state of generalized debilitation, and virus was isolated from the brains of both animals. The last animal in the group was sacrificed 23 days after ic injection. The facial rash present in this group of monkeys at the start of the treatment with Cytoxan changed into a diffuse erythema persisting throughout the observation period.

TABLE 1. *Cumulative data obtained in eight groups of monkeys injected intracerebrally with the Ro-SSPE-1 strain of measles virus*[a]

Group	Prophylactic passive immunization	Inoculation (ic) of Ro-SSPE-1	Rash	HI-antibody titer on day					Skin test		CNS histology
				0	7	13	21	29–35	Measles antigen	Brain antigen	
Adolescent animals											
Untreated	No	No	0/4	0	0	0	40[b]	512	0	0	2/3[c]
Untreated	Yes	Yes	0/4	0	0	0	48	320	1.5[d]	0	1/3[c]
Specifically immunosuppressed	Yes	Yes	3/4	0	0	27	75	43	ND	ND	1/5[c]
Cytoxan-treated	Yes	Yes	4/4	0	0	0	ND	8	ND	ND	0/4[c]
Young adult animals											
Measles-sensitized	No	Yes	1/4	0	24	35	132	214	0.5	0	1/4[c]
Measles- and CNS-sensitized	No	Yes	3/4	0	43	128	181	192	1.5	4.0[e]	3/5[f]
CNS-sensitized	Yes	Yes	0/4	0	0	20	48	192	0.5	6.5	4/4[f]
CNS-sensitized	Yes	No	1/4	0	3	50	96	192	ND	ND	4/4[f]

[a] IC = Intracerebral; HI = hemagglutination inhibition; CNS = central nervous system; ND = not done.
[b] Reciprocal of the average HI titers for the group at a given time.
[c] Number of animals with mild, nonspecific lesions/number of animals examined.
[d] Difference in skin reactions between the area injected with measles and control antigen (mm). Average of group response.
[e] Difference in skin reaction between area injected with brain antigen and control antigen (mm). Average of group response.
[f] Number of animals with experimental allergic encephalitis lesions/number of animals examined.

TABLE 2. *Isolation of measles virus from the brains of rhesus monkeys sacrificed during the first 14 days after ic infection*[a]

Group	Treatment	Time after ic infection (day)	Time to first visible CPE (day)		Measles-specific fluorescent-antibody test	CNS histology
			Primary brain cultures	Brain-Vero cell co-cultures		
6	Measles and CNS-sensitized	1	17	8	Positive	Neg
2	Untreated	2	Neg[b]	7	Positive	Heavy cuffs near inoculation site
2	Untreated	2	Neg	7	Positive	Neg
6	Measles- and CNS-sensitized	4	23	5	Positive	Neg
1	Untreated	—[c]	Neg	Neg	Neg	Several small focal lesions
7	CNS-sensitized	8	Neg	10	Positive	Neg
4	Cytoxan-treated	13	22	5	Positive	Neg
4	Cytoxan-treated	13	22	6	Positive	Neg

[a] ic, Intracerebral; CPE, cytopathic effect; CNS, central nervous system.
[b] Neg, no virus isolated by that technique.
[c] Monkey in group 1 was inoculated with control tissue culture fluid (Eagle's minimum essential medium).

In the measles-sensitized, infected group 5, one animal developed transient weakness and jerkiness of the left arm 6 days after ic inoculation. Of the total of 12 animals sensitized with guinea pig cord (groups 6, 7, and 8), 3 died, 4 and 8 days after ic infection, respectively, and measles virus was isolated from the brains of all three. Of the remaining nine animals, seven developed pronounced symptoms of EAE (e.g., visual disturbances, impaired pupillary reflexes, nystagmus, generalized weakness, tremor, cerebellar ataxia) which persisted until their sacrifice. The four ani-

mals in group 6, which were sensitized with measles antigen in addition to receiving brain tissue, developed symptoms of EAE comparable to those of animals sensitized with brain antigen only.

Results of virological and immunological investigations. In Table 1, the results of serological studies and the skin tests for delayed hypersensitivity are shown. The untreated, uninfected animals in group 1 developed measles antibodies during the third week of the experiment. Seroconversion was considered the result of the spontaneous infection which the animals apparently acquired shortly before they were used in the experiment. A similar antibody response developed in the untreated, ic measles-inoculated animals in group 2 and in animals (group 3) receiving the treatment for specific suppression of delayed hypersensitivity. An almost complete suppression of the humoral antibody response was achieved in the Cytoxan-treated animals (group 4). Seroconversion in the young adult animals (groups 5 to 8) generally started earlier, indicating that the spontaneous infection in these animals was probably more advanced than in the adolescent animals (groups 1 to 4).

Sensitization of groups 5 and 6 with measles antigen in FCA did not produce the desired effect. Although the antibody response appeared accelerated, the 24-hr skin test failed to show evidence of a significant reaction for delayed hypersensitivity (Table 1).

Eleven animals became sick and died during the course of the experiment. Histological study of the brain tissue and virus isolation attempts were performed in eight of these animals. Virus was isolated from the CNS of all seven animals inoculated ic with *measles* virus (Table 2). Virus was not isolated in the animal inoculated ic with control fluid. The ability to isolate virus was greater when brain cells were co-cultivated with Vero cells, and cytopathic effect appeared earlier in these cultures than in primary brain cell cultures. Measles virus was not isolated from any of the remaining animals sacrificed at the termination of the experiment, i.e., 3 to 4 weeks after ic infection. However, adventitious, syncytia-forming viruses were recovered from brain cells cocultivated with Vero cells. Identification of these viral isolates is presently being performed in this laboratory.

Histopathology. No significant lesions were observed in the CNS of monkeys in groups 1 to 5, whether sacrificed intercurrently or at the end of the experiment. Occasionally, nonspecific lesions were noted in these animals (Table 1). Such lesions consisted of mild perivascular infiltrates of only a few vessels in the periventricular tissue. They were not different in extent or character from lesions found routinely in control inoculated or uninoculated rhesus monkeys. A careful search failed to reveal characteristic viral inclusions (1, 8).

Lesions of EAE in monkeys were observed in groups 6, 7, and 8 (Table 1). Typically, these lesions consisted of extensive, diffuse perivascular infiltrates throughout the brain stem and cerebrum (14, 22). Two of the animals in group 8 had more acute coalescent, demyelinating lesions. Significantly, no inclusion bodies were found in any of the infiltrates. The lesions in all three groups were similar and apparently were not modified by measles infection or sensitization.

DISCUSSION

There is accumulating evidence that acute and chronic measles encephalitis in man is the consequence of a direct invasion and multiplication of measles virus in the CNS (1, 10, 18). In acute encephalitis, hypersensitivity to measles antigen coupled with an autoimmune reaction to damaged brain tissue was thought to be an essential component of the disease (12, 17). In contrast, the chronic form of measles encephalitis (SSPE) was thought to develop as a consequence of an acquired defect of cell-mediated immunity in the presence of a normal antibody response (7).

Circumstances which determine the persistence and pathogenicity of measles virus in the nervous tissue can be only indirectly implied from observations in laboratory animals and in cell cultures.

To investigate the effect of altered immunological responsiveness on measles infection, we attempted to induce a state of hypersensitivity as well as of general or partial unresponsiveness in monkeys. The failure of the animals injected with measles antigen in Freund's complete adjuvant to develop delayed hypersensitivity was surprising, since the same antigen produced good responses in guinea pigs and rats. In recent (*unpublished data*) experiments, rhesus monkeys with acute measles were injected with sensitizing measles antigen at different stages of infection. Although delayed hypersensitivity could readily be elicited before infection started, it was suppressed or abolished in animals which received the sensitizing antigen shortly before or after the eruptive stage. Apparently, generalized measles infection in the present experiment was too advanced for the sensitizing antigen to be effective.

The advanced stage of infection might also explain the failure of passive immunization to prevent rash in animals so treated. In fact, it was found that immune serum given to patients in an advanced stage of measles infection precipitated a typical skin rash (13).

Strain Ro-SSPE-1 was isolated from the brain of ic-infected monkeys up to 13 days after inoculation (Table 2). The unsuccessful attempts to isolate the virus at later intervals may reflect a failure of the virus to become adapted to grow in nervous tissue and, thus, to escape elimination by the developing antibody response. A similar mechanism for suppression of the potential neurotropism of distemper virus in experimentally infected puppies was postulated by Appel (2). None of the altered immunological situations induced in monkeys had a perceptible effect on the result of ic inoculation of the Ro-SSPE-1 strain.

The absence of specific morphological lesions in the CNS of the monkeys and the failure to isolate the virus 3 to 4 weeks after ic inoculation indicates that at this stage the virus was either eliminated or entered into a latent state. However, one cannot exclude the possibility that a small focus of persisting viral activity may have escaped morphological or virological detection.

ACKNOWLEDGMENTS

We thank F. E. Payne for the Ro-SSPE-1 strain of measles virus.

H. P. S. was a recipient of Public Health Service International Postdoctoral Research Fellowship I FO 5 TWO 1369-01 from the Fogarty International Center, National Institutes of Health.

LITERATURE CITED

1. Adams, J. M., C. Baird, and L. Filloy. 1966. Inclusion bodies in measles encephalitis. J. Amer. Med. Ass. 195:290–298.
2. Appel, M. J. G. 1969. Pathogenesis of canine distemper. Amer. J. Vet. Res. 30:1167–1182
3. Asherson, J. L., and S. H. Stone. 1965. Selective and specific inhibition of 24-hour skin reactions in the guinea pig. I. Immune deviation: description of the phenomenon and the effect of splenectomy. Immunology 9:205–217.
4. Axelrad, M. A. 1968. Suppression of delayed hypersensitivity by antigen and antibody. Immunology 15:159–171.
5. Barbanti-Brodano, G., S. Oyanagi, M. Katz, and H. Koprowski. 1970. Presence of two different viral agents in brain cells of patients with subacute sclerosing panencephalitis. Proc. Soc. Exp. Biol. Med. 134:230–236, 1970.
6. Baublis, J. V., and F. E. Payne. 1968. Measles antigen and syncytium formation in brain cell cultures from subacute sclerosing panencephalitis (SSPE). Proc. Soc. Exp. Biol. Med. 129:593–597.
7. Burnet, F. M. 1968. Measles as an index of immunological function. Lancet 2:610–613.
8. Dawson, J. R., Jr. 1942. Cellular inclusions in cerebral lesions of epidemic encephalitis. Arch. Neurol. Psychiat. 47:353–363.
9. Horta-Barbosa, L., D. A. Fuccillo, R. Hamilton, R. Traub, A. Ley, and J. L. Sever. 1970. Some characteristics of SSPE measles virus. Proc. Soc. Exp. Biol. Med. 134:17–21.
10. Horta-Barbosa, L., D. A. Fuccillo, W. T. London, J. T. Jabbour, W. Zeman, and J. L. Sever. 1969. Isolation of measles virus from brain cell cultures of two patients with subacute sclerosing panencephalitis. Proc. Soc. Exp. Biol. Med. 132: 272–277.
11. Katz, M., L. B. Rorke, W. S. Masland, G. Barbanti-Brodano, and H. Koprowski. 1970. Subacute sclerosing panencephalitis: isolation of a virus encephalitogenic for ferrets. J. Infec. Dis. 121:188–195.
12. Koprowski, H. 1962. The role of hyperergy in measles encephalitis. Amer. J. Dis. Child. 103:273–278.
13. Mitus, A., J. F. Enders, T. M. Craig, and A. Halloway. 1959. Persistence of measles virus and depression of antibody formation in patients with giant-cell pneumonia after measles. N. Engl. J. Med. 261:882–889.
14. Morgan, I. M. 1946. Allergic encephalomyelitis in monkeys in response to injection of normal monkey cord. J. Bacteriol. 51:614–615.
15. Technical Committee. 1956. The monkey safety test for poliomyelitis vaccine. Amer. J. Hyg. 64:104–137.
16. Norrby, E. 1962. Hemagglutination by measles virus. IV. A simple procedure for production of high potency antigen for haemagglutination-inhibition (HI) tests. Proc. Soc. Exptl. Biol. Med. 111:814–818.
17. Paterson, P. Y. 1969. Immune processes and infectious factors in central nervous system disease. Annu. Rev. Med. 20:75–100.
18. Payne, F. E., J. V. Baublis, and H. H. Itabashi. 1969. Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosing panencephalitis. N. Engl. J. Med. 281:585–589.
19. Schumacher, H. P., and P. Albrecht. 1970. Optimal conditions for isolation of neurotropic measles virus from brain tissue. Proc. Soc. Exp. Biol. Med. 134:393–402.
21. Scott, T. F. McN. 1967. Postinfectious and vaccinia encephalitis. Med. Clin. N. Amer. 51:701–717.
22. Wolf, A., and E. A. Kabat. 1947. The pathology of acute disseminated encephalomyelitis produced experimentally in the rhesus monkey and its resemblance to human disease. J. Neuropathol. Exp. Neurol. 4:333–357.
23. Yasumura, Y., and Y. Kawatika. 1963. Studies on SV 40 virus in tissue culture cells. Nippon Rinsho 21:1201–1215.