BIKEN JOURNAL Vol. 18, 123-133, 1975

COMPARATIVE STUDIES ON SUBACUTE SCLEROSING PANENCEPHALITIS VIRUS AND A USUAL MEASLES VIRUS WITH SPECIAL REFERENCE TO THEIR CAPACITIES TO PRODUCE VIRAL HEMAGGLUTININ IN VERO CELLS[1]

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(Received January 7, 1975)

SUMMARY Various comparative studies were made on the capacities of a cell-associated subacute sclerosing panencephalitis agent (SSPE agent, Niigata/1 strain) and a usual measles virus (Toyoshima strain) to produce measles virus hemagglutinin (MV-HAnin) in Vero cells. The results obtained by various methods, such as the hemagglutination test (HA), hemadsorption test (HAd), antibody blocking test, antibody inducing test and fluorescent antibody technique (FAT) using monospecific antibody to MV-HAnin, consistently suggested that the SSPE agent was an incomplete measles-like virus having little if any MV-HAnin, but sharing immunologically closely related viral components other than MV-HAnin with those of the usual measles virus. The significance of these findings is discussed with respects to the latency of the SSPE agent.

INTRODUCTION

The possible involvement of measles-like virus in the pathogenesis of SSPE has been suggested from extensive studies on patients with this disease (Dawson, 1933; Connolly et al., 1967; Freeman et al., 1967; Dayan et al., 1967; Baublis and Payne, 1968; Jabbour et al., 1969; Katz et al., 1969). SSPE agents have been isolated from viable affected cells from the brains of patients either by cocultivation (Baublis and Payne, 1968; Horta-Barbosa et al., 1969; Chen et al., 1969; Payne et al., 1969; Sato et al., 1972; Ueda et al., 1975) or by further artificial fusion (Katz et al., 1970; Barbanti-Brodano et al., 1970) with susceptible indicator cells, and the characteristics of SSPE agents have been intensively studied by comparison with usual measles viruses in vivo and in vitro. Although these agents resemble measles virus in many respects, they also differ in some important respects (Jen Yeh, 1973; ter Meulen et al., 1972; Oyanagi et al., 1971; Hamilton et al., 1973; Kato et al., 1972). The main difference is that the degree of association of these agents with host cells is general-

1 This work was reported at the Annual Meeting of the Society of Japanese Virologists, in Tokyo, on Nov. 4, 1973.

ly closer than that of usual measles viruses (Katz and Koprowski, 1973; Doi et al., 1973). Recently Sato et al. (1972) succeeded in establishing a cell line carrying an extremely cell-associated SSPE agent by cocultivation of SSPE brain cells with Vero cells. Characterization of this SSPE agent might provide information for understanding the latency of the SSPE agent.

This paper reports results of comparative studies on the capacities of the SSPE agent (Niigata/1 strain) and usual measles virus (Toyoshima strain) to produce MV–HAnin in Vero cells.

MATERIALS AND METHODS

1. *Virus and cell culture*

The Toyoshima strain of a usual measles virus (Toyoshima et al., 1959) was used as a representative of productive infection. A cytopathic SSPE agent, Niigata/1 strain, isolated from a brain biopsy specimen of a patient with SSPE by cocultivation with Vero cells (Sato et al., 1972) was kindly supplied by Dr. Y. Doi of The Japan Poliomyelitis Research Institute. This agent has never been demonstrated as a cell-free infectious virus during extensive subculture after establishment of the cell line carrying the SSPE agent (SSPE cells) and it was used as a representative of latent infection at the 41–45th cell passage level after 10 subsequent passages in our laboratory. The SSPE cells were passaged by cocultivation with fresh Vero cells at intervals of 2–5 days and incubated at 37 C. Vero cells, a continuous line derived from African green monkey cells, were also supplied by Dr. Y. Doi. Vero cell lines were grown in a mixture of equal volumes of Eagle's MEM and YLE (Earle's BSS containing 0.5% lactoalbumin hydrolysate, 0.1% yeast extract and antibiotics) supplemented with 5% calf serum. A human ammnion cell line (FL) was grown in the same medium containing 10% calf serum and used as host cell for preparation of purified MV–HAnin.

2. *Preparation of infected cells and their lysates*

Confluent monolayer cells were infected with the usual measles virus at a m.o.i. of 0.01. The SSPE cells were concultivated with fresh Vero cells at a appropriate ratio to produce confluent CPE. Three to 4 days after infection or subculture, when almost all the monolayer showed the desired degree of CPE, the cells were harvested by scraping them off with a rubber policeman. Then they were washed 3 times and suspended in PBS (2×10^7 cells/1.5 ml). To prepare cell lysates cells were subjected to 5 cycles of freezing and thawing and then centrifuged at low speed. Uninfected materials were prepared by the same processes.

3. *Preparation of antiserum*

The antiserum used for determination of complement fixing (CF) antigen and for the indirect FAT was obtained from a patient with typical measles and had a reciprocal CF antibody titer of 400. Antiserum against human gamma-globulin was prepared by repeated inoculation of rabbit with the mixture of antigen and equal volume of incomplete Freund's adjuvant.

Antiserum against purified MV–HAnin was prepared as follows. The usual measles virus grown in FL cells was concentrated by centrifugation at 25,000 rpm for 1 hr and then disrupted by treatment with Tween 80 and ether (Norrby, 1962). The degraded MV-HAnin recovered from the aqueous phase was concentrated on Sephadex, dialysed against PBS, and then purified by sucrose gradient centrifugation and adsorption onto African green monkey (AGM) erythrocytes (Iinuma et al., 1972). Then 0.5 ml of the treated HAnin was layered onto 4.5 ml of a linear gradient of 5–20% sucrose and centrifuged at 25,000 rpm for 2.5 hr in a Spinco L SW39 rotor at 4 C. Eight fractions were collected from the top of the tube. The upper 3 fractions were pooled and dialysed against PBS. One volume of the HAnin was mixed with one-fifth volume of 50% AGM erythrocyte suspension for 30 min at 37 C. After two cycles of adsorption, the AGM erythrocytes were collected, washed and suspended in PBS. This erythrocyte preparation was injected into the heart of an AGM free of measles antibody, whose erythrocytes were used to absorb the HAnin,

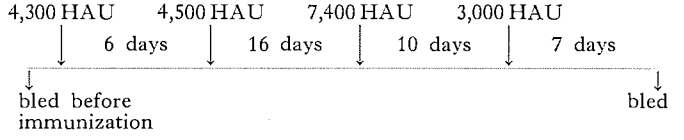


FIGURE 1. *Immunization schedule of monkeys*

as indicated in the schedule of immunization (Fig. 1).

4. *Preparation of antigens for serological tests*

To test the specificity of the antiserum to purified MV–HAnin, purified MV–HAnin as immunogen and measles virus CF antigen (1: 8) lacking HA activity were used. The latter was prepared by absorbing the usual measles virus-infected cell lysate with AGM erythrocytes. In other serological studies the usual measles virus-infected cell lysate was used as antigen.

5. *Hemagglutination (HA) test and hemagglutination-inhibition (HI) test*

The HA and HI tests were performed in tubes (9×90 mm).

1) HA test

PBS containing 0.1% bovine serum albumin and 0.01% gelatin was employed as diluent. Volumes of 0.2 ml of two-fold serially diluted virus suspension were mixed with an equal volume of 0.5% AGM erythrocyte suspension and incubated at 37 C.

2) HI test

Nonspecific materials in the serum interfering with the HI test were removed by adsorption onto kaolin and AGM erythrocytes following method of Stewart et al. (1967). Volumes of 0.1 ml of two-fold serially diluted serum were mixed with 4 units of the antigen in 0.1 ml and the mixtures were incubated at 37 C for 1 hr. Then 0.2 ml of 0.5% AGM erythrocyte suspension was added and tubes were stood at 37 C.

6. *Complement-fixation (CF) test*

The microtiter method was employed using a plastic tray and a syringe (Hirasawa MFG Co.). In this method, 0.1 ml of reagent in Kolmer's method corresponds to one drop (0.02 ml) from the springe, and 10 units of hemolysin were used (Kunita et al., 1963).

7. *Hemadsorption (HAd) test*

On the 3rd day after inoculation of the usual measles virus or subculture of the SSPE cells, the cell monolayer was washed twice with PBS. Then 1 ml of 0.5% AGM erythrocyte suspension was added to the infected cells in a Leighton tube. The tube was incubated for 1 hr at 37 C, rinsed 3 times with PBS and examined for HAd under a light microscope.

8. *Infectivity titration and neutralization test (NT)*

Viral infectivity and neutralizing antibody titers were determined using microtiter plastic plates (Falcon Plastics) and Vero cells. Volumes of 0.2 ml of serial ten-fold dilutions of the virus or the SSPE cell suspension in growth medium were each inoculated into 5 holes. For the neutralization test, serial two-fold dilutions of serum were mixed with an equal volume of challenge virus (100 $TCID_{50}$/0.1 ml). After incubation for 1 hr at 37 C, 0.2 ml volumes of the mixture were each inoculated into two holes. The final reading was taken after incubation for 2 weeks and the 50% effective point was determined.

9. *Antibody blocking test*

Undiluted lysates of infected cells were mixed with equal volumes of serial two-fold dilutions of the patient serum containing 32 to 1 HI units and the mixtures were stood for 1 hr at 37 C. After centrifugation at 40,000 rpm for 2 hr, residual HI antibody in the supernatant was determined. The titer of HI antibody blocking activity of the lysate was expressed as the highest concentration of HI antibody showing hemagglutination.

10. *Antibody inducing test*

Guinea-pigs free of antibody to measles virus were divided into 6 groups and treated as indicated in Fig. 2. They were inoculated three times intracerebrally (i-c) or intraperitoneally (ip) at intervals of 7 or 8 days. Their sera were collected 11 days after the last injection and tested for the presence of antibody to measles virus.

11. *Fluorescent antibody test (FAT)*

1) Preparation of fluorescein isothiocyanate (FITC)-conjugated antibody

The serum gamma-globulin fraction from antiserum against purified MV–HAnin or human gamma-globulin was conjugated with FITC by the method of Marshall et al. (1958). The conjugate was purified by the method of McDevitt et al. (1963) and absorbed with an acetone power of monkey liver as described by Coons et al. (1955).

2) Staining procedures

Monolayers of Vero cells on cover slips in Leighton tubes 2–3 days after infection of the usual measles virus or subcultures of SSPE cells were washed with PBS and treated with cold acetone for 15 min. The cover slips were covered with the

Group No.	No. of animal	Immunogen	Route	Volume (ml)	Inoculum		
					1st	2nd	3rd
1	6	MV cells[a]	i-c[b]	0.1	6.4 HAU	3.2 HAU	3.2 HAU
2	6	SSPE cells	i-c	0.1	360 PFU	361 PFU	364 PFU
3	4	MV cells	ip[c]	1.0	64 HAU	32 HAU	32 HAU
4	4	SSPE cells	ip	1.0	3,600 PFU	3,610 PFU	3,640 PFU
5	4	MV lysate	ip	1.0	64 HAU	32 HAU	32 HAU
6	4	SSPE lysate	ip	1.0	0 PFU	0 PFU	0 PFU

[a] *Measles virus-infected cells.*
[b] *Intracerebral inoculation.*
[c] *Intraperitoneal inoculation.*

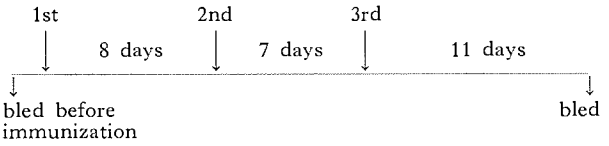


FIGURE 2. *Immunization schedule in the antibody inducing test in guinea-pigs*

conjugate diluted to a staining titer of 4 units. After incubation in a humidified chamber at 4 C overnight, excess conjugate was removed by washing the cells 3 times with PBS. In the indirect FAT method unlabeled serum from a patient with measles was used for the first reaction. The cover slip was mounted in buffered glycerol and examined under a fluorescence microscope (Olympus Optical Co.). Photographs were taken on Kodak high speed ektachrome film.

RESULTS

1. *Biological activities of infected cells*

Various biological activities of SSPE cells were compared with those of cells infected with the usual measles virus. As shown in Table 1, in SSPE cells HAnin was undetectable, while measles virus CF antigen was definitely detectable although its titer was low. In cells infected with the usual measles virus, the ratio of the titer of HAnin to CF antigen had a constant value of 8, although the yield of HAnin varied with different batches of cells. Infec-

TABLE 1. *Biological activities of infected cell lysates*

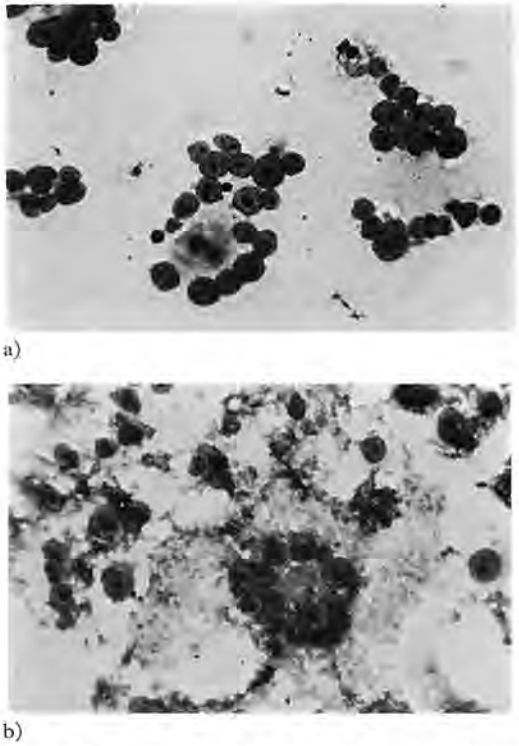
Virus harvest	Biological activities of				Ratio of HA to CF
	Lysate			Cells	
	HA	CF	$TCID_{50}$[a]	PFU[b]	
MV 3.10[c]	32[d]	4	5.0	ND[e]	8
MV 5.15	128	16	6.2	ND	8
SSPE 41[f]	<1	4	<0	3.8	<0.25
SSPE 45	<1	2	<0	3.6	<0.50

[a] 50% tissue culture infective dose, $\log_{10}$ $TCID_{50}$/ml.
[b] Plaque forming unit, $\log_{10}$ PFU/ml.
[c] Date when cells infected with the usual measles virus were harvested.
[d] Reciprocal of titer.
[e] Not done.
[f] Passage level at which the SSPE cells were used.

tivity of the SSPE agent could only be demonstrated as infective centers associated with affected viable cells, not as cell-free infective agent.

2. *Hemadsorption of infected cells*

To demonstrate the presence of HAnin associated with the surface of infected cells, the HAd test was carried out on monolayers of SSPE cells or cells infected with the usual measles virus. Occasionally weak hemadsorption was observed at the earlier cell passage level (≧9th) of SSPE cells (Doi et al., 1972), but from the 60th passage level of the same SSPE cells results were consistently negative in agreement with findings in electron microscopic and immunoperoxidase studies (Dubois-Dalcq et al., 1974). Monolayers of SSPE cells did not adsorb erythrocytes at all, even in areas showing severe CPE consisting of multinucleated giant cells, like those seen on infection with the usual measles virus (Fig. 3a). However, in monolayers infected with the usual measles virus, adsorbed erythrocytes completely covered the surface of degenerated cells (Fig. 3b). No adsorption to uninfected monolayers was observed and, in the presence of antiserum to measles virus adsorption to monolayers infected with the usual measles virus was completely inhibited.



a)

b)

FIGURE 3. *Hemadsorption of infected cells (Giemsa staining). (a) SSPE cells. (b) Cells infected with the usual measles virus.*

3. *HI antibody blocking activities of infected cell lysates*

HAnin could not detected in SSPE cells in HA and HAd tests. However it seemed possible that SSPE cells might contain a structural component of HAnin which had no HA activity itself but could react with HI antibody causing blocking of the HI test. The HI antibody blocking activities of various lysates are shown in Table 2. Lysates of SSPE cells and uninfected cells caused no blocking at the lowest titer of HI antibody tested, but lysates of cells infected with the usual measles virus blocked up to 32 HI units.

4. *Antibody inducing activities of infected cells and their lysates in guinea-pigs*

The presence of HAnin in various materials was examined by testing whether they induced serum HI antibody to measles virus when inoculated into guinea-pigs. Table 3 shows the titers of HI, CF and NT antibody to measles

TABLE 2. *HI antibody blocking activities of infected cell lysates*

Source of lysate	Absorbing antigen dose		HI antibody blocking activity
	HA	CF	
MV cells	128[a]	16	32 HIU
SSPE cells	<1	4	<2 HIU
Vero cells	ND[b]	ND	<2 HIU

[a] Reciprocal of titer.
[b] Not done.

virus induced in the serum of individual guinea-pigs. Groups of guinea-pigs were inoculated with SSPE cells (Groups 2 and 4) or their lysate (Group 6). Of the guinea-pigs inoculated with SSPE cells, one (No. 11) died the day after, and another (No. 12) three days after the last intracerebral inoculation. The SSPE agent was recovered from the brain of the latter case by cocultivatioh with Vero cells, but no characteristic pathological features were ob-

TABLE 3. *Antibody inducing activities of infected cells and their lysates in guinea-pigs*

Group No.	Immunogen	Route	Volume (ml)	Individual No.	Antibody titer to measles virus		
					HI	CF[a]	NT[b]
1	MV cells	i-c[c]	0.1	1	256[d]	32	90
				2	512	32	180
				3	256	64	128
				4	128	32	64
				5	256	32	128
				6	512	32	180
2	SSPE cells	i-c	0.1	7	<4	8	2
				8	<4	2	1
				9	<4	4	1
				10	<4	8	1.4
				11[e]	ND[g]	ND	ND
				12[f]	<4	ND	ND
3	MV cells	ip[h]	1.0	13	512	64	360
				14	1,024	32	256
				15	512	32	180
				16	512	32	180
4	SSPE cells	ip	1.0	17	<4	8	8
				18	<4	2	<1
				19	<4	2	<1
				20	<4	4	4
5	MV lysate	ip	1.0	21	128	8	45
				22	1,024	64	360
				23	1,024	64	512
				24	1,024	64	360
6	SSPE lysate	ip	1.0	25	<4	2	<1
				26	<4	2	<1
				27	<4	2	<1
				28	<4	2	<1

[a] Sere were absorbed with Vero cells before the CF test.
[b] Neutralizing antibody titers of sera from guinea-pigs immunized with the SSPE materials as averages of results in triplicate tests.
[c] Intracerebral inoculation.
[d] Reciprocal of antibody titer.
[e] Died the day after the last inoculation.
[f] Died 3 days after the last inoculation.
[g] Not done.
[h] Intraperitoneal inoculation.

served in this brain specimen. Moreover neither the latter animal nor any of survivors showed detectable HI antibody even at the lowest dilution (1: 4) of their sera, although they demonstrated definite CF antibody. Low neutralizing activity was detected in some sera. The specificity of this activity to the structural components of measles virus, especially HAnin, has not yet been elucidated. In the groups inoculated with the usual measles virus (Groups 1, 3 and 5), specific antibody to measles virus was strongly induced in the sera of all individuals.

5. *Immunofluorescent staining of infected cells*

An attempt was made to prepare monospecific antiserum against purified MV–HAnin, since use of FITC-conjugated antibody prepared from this antiserum was considered the best method for detection of the corresponding antigen in SSPE cells.

1) Preparation of antiserum and its specificity

Usual measles virus grown in FL cells (HA titer of 80) was used as starting materials for preparation of immunogen. Successive concentration by centrifugation, Sephadex and treatment with Tween 80 and ether resulted in 10, 2 and 4-fold increase in HA titer, respectively. The distribution of HAnin on sucrose gradient centrifugation is shown in Fig. 4. After two cycles of adsorption, 96% of the HAnin in the combined 3 upper fractions was adsorbed onto AGM erythrocytes. Antiserum to purified MV–HAnin reacted specifically with purified MV–HAnin, but not with viral components other than HAnin or contaminants derived from AGM erythrocytes and host cells. The antiserum showed antibody titers with purified MV–HAnin of 256 in the HI test and 2 in the CF test (Table 4). The conjugate had a molar ratio of FITC to gamma-globulin of 1.16 and a staining titer of 8.

2) Immunofluorescent staining

Fig. 5a shows the immunofluorescent staining of SSPE cells. No specific immunofluorescence was observed in the amorphous area, consisting almost entirely by multinucleated giant cells. The bright cells in the upper left corner

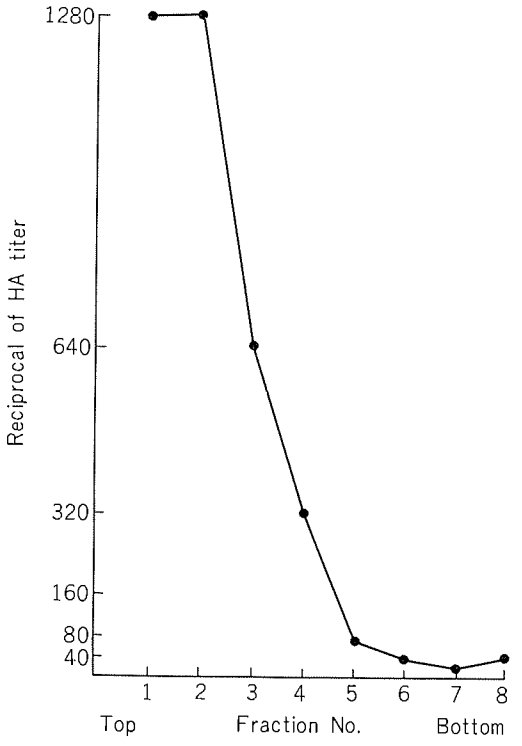


FIGURE 4. *Distribution of degraded HAnin in a sucrose gradient. A 0.5 ml of degraded HAnin was layered on 4.5 ml of a linear gradient of 5–20% sucrose (w/v) and centrifuged at 25,000 rpm for 2.5 hr in a Spinco L SW39 rotor at 4 C. Fractions were collected from the top of the tube and tested for HA activity.*

TABLE 4. *Specificity of antiserum to purified MV-HAnin*

Antigen	Antibody titer	
	HI	CF
Purified MV-HAnin	256[a]	2
Measles virus-infected[b] Vero cell lysate other than the HAnin	—	<1

[a] Reciprocal of antibody titer.

[b] A lysate of cells infected with the usual measles virus was absorbed 5 times with AGM erythrocytes. After absorptions the lysate had antigen titers of CF (8) and HA (<1).

of the photograph can be clearly differentiated from cells showing a positive reaction with specific immunofluorescence because they show blue autofluorescence and appear intact rather than syncytial. On infection with the usual measles virus, both multinucleated giant cells and infected, nonsyncytial cells showed intense homogeneous immunofluorescence, especially in the cytoplasm (Fig. 5b). When SSPE cells were subjected to the indirect FAT using serum from a patient with measles, extensive granular immunofluorescence was seen to be confined to the plaques of the SSPE agent and was mainly observed in the cytoplasm of the giant cells of these plaques (Fig. 5c).

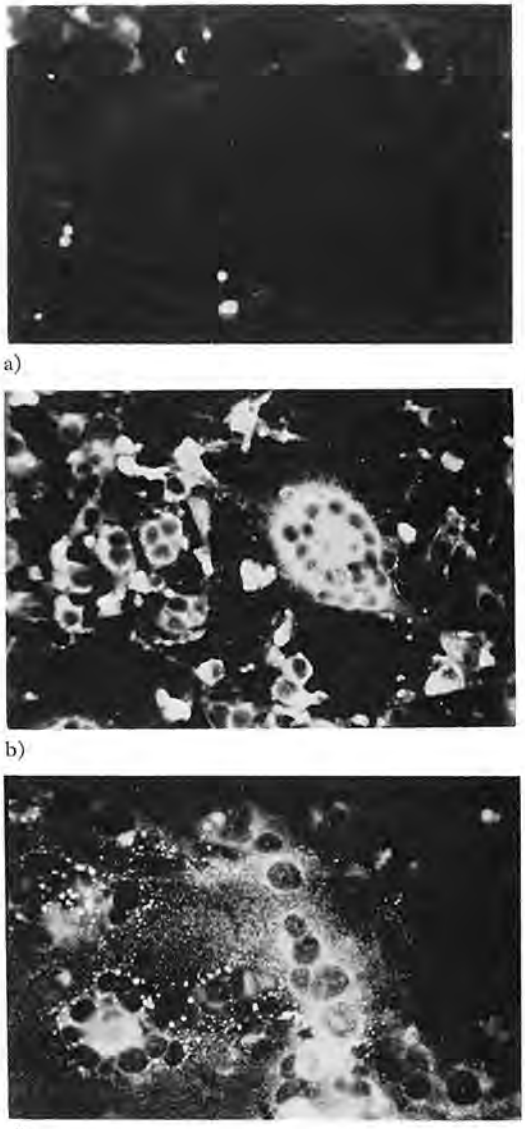


FIGURE 5. *Immunofluorescent staining of infected cells. (a) SSPE cells stained by the direct method with monospecific antiserum to purified MV–HAnin. (b) Cells infected with the usual measles virus stained by the direct method with monospecific antiserum to purified MV–HAnin. (c) SSPE cells stained by the indirect method with serum from a patient with measles.*

DISCUSSION

Several hypotheses have been postulated to explain the pathogenesis of SSPE (Burnet, 1968; Johnson, 1970; Kolar, 1968; Koprowski et al., 1970). In one of these, it is postulated that a strain variant of measles virus causes slow infection (Johnson and Johnson, 1968; Johnson, 1970). Agents recovered from patients with SSPE generally show some extent of variation (Jen Yeh, 1973; Hamilton et al., 1973) and the general properties of these variants seem to be closely associated with the host cells (Katz and Koprowski, 1973; Doi et al., 1973). Thinking that an SSPE agent (Niigata/1 strain) which is extremely closely associated with the host cell would provide a good system for investigating these properties, we compared the capacities of the SSPE agent and the usual measles virus to produce the HAnin essential for viral infectivity in Vero cells.

First a cell lysate was tested by the HA test, antibody blocking test and antibody inducing test. Results consistently showed that SSPE cells contained an undetectable amount of the HAnin and supposed monovalent HAnin. The neutralizing activities were very low in sera of guinea-pigs inoculated with SSPE materials, which suggests that the dose of antigen was very low, or that viral structural components with fusion activity are not directly related to induction of neutralizing activity.

The recovery of HAnin in cell lysates after

disruption of the infected cells by freezing and thawing may reflect the degree of its association with cell components rather than the amount that is actually present, and its association with cell components may differ in SSPE cells and those infected with the usual measles virus. Therefore, in this work HAnin on the surface of infected cells was examined by the HAd test. However, no even occasional weak hemadsorption, like that observed at an earlier passage level ($\geqq$9th) of SSPE cells (Doi et al., 1972), was observed with the SSPE cells used in the present study. Dubois-Dalcq et al. (1974) also recently reported negative results on the same SSPE cells at more than the 60th passage level.

In the present study the direct FAT using monospecific antibody to purified MV–HAnin was used to detect intracellular HAnin. No specific immunofluorescence was observed, even in the amorphous cytoplasm of multinucleated giant cells induced by the SSPE agent. In a parallel test on the same SSPE cell preparation by the indirect FAT using serum from a patient with measles specific granular immunofluorescence was demonstrated, located predominantly in the cytoplasm of affected cells (ter Meulen et al., 1972). This result shows that immunofluorescence in the SSPE cells is due to viral components other than the HAnin.

These results suggest that this SSPE agent is an incomplete measles-like virus which has little or no HAnin, but shares immunologically closely related structural components other than HAnin with those of the usual measles virus. The fact that no cell-free infectious SSPE agent (Niigata/1) was released may be attributed partly to the lack of ability or impaired ability of this agent to produce HAnin as a structural component.

If only the defective variant described in this paper persisted in a patient for a prolonged period after primary measles, the HI antibody in the patient's serum should inevitably decrease. However, the serum of the patient with SSPE we examined, showed very high HI antibody levels. These findings suggest the following possibilities. (1) The SSPE patient may have a heterogeneous population of complete and defective viruses. The latter is predominant in the brain and so was found on brain biopsy, while a small amount of complete virus persists in other part of the brain and other tissues (Horta-Barbosa et al., 1971) and continues to cause antigenic stimulation. (2) Reinfection or activation of latent complete virus may occur. (3) In vitro passages of the agent may cause the complete virus to become defective.

A carrier state of cultured cells associated with an incomplete measles virus has been obtained by passage of complete virus in the presence of the antibody (Rustigian, 1962, 1966). A similar phenomenon in the presence of a host control mechanism, such as a very high level of antibody, may result in vivo in a defective variant devoid of the HAnin and the budding process in virus maturation (Sato et al., 1972) but still having potent fusion activity. This defective variant may be transmitted slowly to adjacent cells through an entirely intracellular route which is thus not affected by the presence of the antibody, and infected cells which do not have the main viral HA antigen on their surface may reduce the sensitivity of the target cells to specific immunocytes (Saunders et al., 1969; Mizutani et al., 1970). Thus the defective variant may persist for a prolonged period and consequently predominate in a heterogeneous population.

ACKNOWLEDGMENTS

We are grateful to Dr. Y. Odagiri of Osaka Prefectural University for performing the pathological examination and to Dr. T. Kurimura of our laboratory for helpful advice.

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