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Persistent Infection of Measles Virus in Mouse Brain Cell Cultures Infected *in vivo*

By

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With 5 Figures

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Summary

Cell lines have been established from measles infected mouse brains. They grow more readily than do cultures from similar uninfected animals and cytoplasmic inclusions, typical of measles, are present in H and E stained preparations. Minimal amounts of measles virus can be isolated from the culture fluids and measles infection in a proportion of the cells can be demonstrated by immunofluorescence. No detectable interferon is produced and the cells are not damaged by measles antiserum and complement.

1. Introduction

Recent studies suggest strongly that measles virus is in some way associated with subacute sclerosing panencephalitis (SSPE) (FREEMAN *et al.*, 1967; LEGG, 1967). High titres of measles antibodies have been reported in the serum and cerebral spinal fluid of cases with SSPE, while measles antigens have been demonstrated in the brains (CONNOLLY *et al.*, 1967, 1971; DAYAN *et al.*, 1967) and infectious measles virus has been isolated from brain biopsies (CHEN *et al.*, 1969; HORTA-BARBOSA *et al.*, 1969).

An experimental model of a persistent infection with measles virus in brain tissue should, therefore, prove useful in the study of this and other "slow" infections of the nervous system. RUSTIGIAN (1966 a, b) produced a persistent infection in HeLa cells with measles virus which could be demonstrated by immunofluorescence. Infectious virus was released from these cells, but when cultured in the presence of measles antiserum, a clonal line was obtained, characterized by the synthesis of incomplete measles virus even when the cells were afterwards cultured in antibody-free medium.

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The present work describes persistent infection with measles virus in cell lines established from the brains of mice infected *in vivo* with a mouse adapted strain of measles virus.

2. Materials and Methods

2.1. Virus Strains

Mouse adapted measles virus was received from Professor F. Rapp (Pennsylvania State University) and passaged three times in one- to three-day-old Webster Swiss mice by intracerebral inoculation of a 10% brain suspension. The Edmonston strain of measles virus was kindly supplied by Dr. S. D. Gardner (Public Health Laboratory Service) while a locally isolated (RVI) strain was obtained from Dr. P. S. Gardner. Both strains were passaged in secondary rhesus monkey kidney cells (RMK) (received from the National Institute for Medical Research, Holly Hill, London) and titred in LLC-MK_2 cells.

2.2. Antiserum

Two young rabbits were bled from the ear and the pre-immunization serum stored at −70°C. Each rabbit was then inoculated with 1 ml of RVI measles virus in the lateral ear vein twice weekly for four weeks. Ten days after the last inoculation, the rabbits were again bled from the ear and the immune serum stored at −70°C.

The neutralisation titre of the antiserum against 100 TCD_{50} of measles virus (Edmonston strain) in LLC-MK_2 cells was 1:1280. An aliquot was adsorbed with homogenized human foetal brain overnight at 4°C, prior to use in immunofluorescence testing.

2.3. Growth of Cell Cultures

Eight one- to three-day-old Webster Swiss mice were inoculated intracerebrally with 0.03 ml of a 10% mouse brain suspension of mouse adapted measles virus. Four control mice were inoculated with the same amount of a 10% normal mouse brain suspension. After four to six days, when the measles-inoculated mice were beginning to show signs of illness, infected and control animals were killed and the brains removed.

Explants (eight to ten pieces, approximately 1 mm cubes) of the brains were placed in 6 cm Petri plates (Escoplastic) containing medium 199 with 20% foetal bovine serum and incubated at 36°C in a humidified 5% CO_2 atmosphere. Medium was changed once weekly and stored at −70°C for virus assay. The explants grew out and when the monolayers became confluent, the cells were trypsinized (0.125% trypsin Difco 1:250 in 0.02% versene) and redispersed in plastic Petri plates. Once established as continuously growing cultures, they were passaged when the monolayers became confluent.

Monolayers of cells grown on glass coverslips were fixed in cold acetone for immunofluorescence, or Formal Ringer for histological staining.

2.4. Immunofluorescence

Following fixation in cold acetone, the frozen coverslips bearing cell monolayers were immersed in a buffered saline solution to warm at room temperature. Excess saline was drained and the coverslips placed in a moist chamber where measles antiserum (adsorbed against normal mouse brain tissue) was layered on to one of each pair and left for one hour at 20°C. The other was covered with adsorbed pre-immunization (normal) serum. The coverslips were then washed in buffered saline for 15 minutes, fixed in cold absolute alcohol for 10 minutes followed by buffered saline for 5 minutes. After draining excess fluid the coverslips were replaced in the moist chamber, and an anti-rabbit gamma globulin-fluorescein isothiocyanate (FITC) (Hoechst Pharmaceuticals) was applied to each for half an hour at 20°C. The coverslips were then washed in 2 changes of buffered saline for 5 minutes each and a third change for one hour. They were mounted in buffered glycerol on glass slides for microscopic examination.

2.5. Attempts to Isolate Measles Virus

Culture fluids stored at −70°C were tested for the presence of measles virus. Cells were also frozen and thawed in an attempt to recover the virus. Each sample of fluid was inoculated in 0.2 ml amounts into tube cultures of human embryo brain (HEB), human embryo kidney (HEK), rhesus monkey kidney (RMK) or LLC-MK_2 cell cultures. The tube cultures were kept for periods of up to 35 days and were examined regularly for the presence of measles cytopathic effect (CPE) or syncytia formation.

2.6. Neutralization

Fluids from the cultures which had produced CPE in RMK and LLC-MK_2 cells were tested for neutralization of CPE by measles antiserum. Infected fluids from the first passage of these cultures in LLC-MK_2 monolayers were also neutralized and tested for the presence of measles virus.

Undiluted samples of the supernatant fluids were mixed with equal amounts of either pre-immunization rabbit serum or measles antiserum (diluted 1:64) for one hour at room temperature. Each mixture was then inoculated in 0.4 ml amounts into LLC-MK_2 rolled monolayer cultures and examined daily for two weeks for measles CPE.

2.7. Interferon Assay

Fluid from two of the cultures was removed 15 days following trypsinization and six days after a change of medium. The supernatant fluids were taken from two other cultures 24 hours after trypsinization. All four samples were tested for the presence of interferon.

The samples were diluted in two-fold dilutions, 1:2 to 1:8 in medium 199. The interferon assay was performed on four-day-old secondary mouse embryo fibroblasts (MEF). Each dilution was inoculated in 2.5 ml amounts onto 6 cm plastic Petri plates containing confluent monolayers of MEF cultures and incubated overnight at 37°C. The MEF cultures were then washed with phosphate buffered saline, challenged with 60—80 PFU of Semliki Forest virus and overlaid with an agar overlay medium. After 48 hours the cell cultures were stained with neutral red (0.01%) and the plaques counted.

2.8. Effect of Antibody and Complement

The effect of antibody and complement on the cell cultures was determined by adding adsorbed antiserum (diluted 1:64 in growth medium) and 20 units of guinea pig complement. Control cultures received antiserum alone, complement alone or growth medium alone. The cultures were incubated at 35°C and examined microscopically over a period of seven days.

3. Results

3.1. Growth of Cultures

Table 1 summarizes the growth history of the control and measles infected mouse brain cultures. Of the four controls, one did not grow. Two grew for periods of six to nine weeks but then began to degenerate and were discarded between 15 and 19 weeks. The remaining culture is still growing well after ten months and is now at its fifth passage level.

Of the eight cultures grown from explants of mouse brains infected *in vivo* with measles virus, three of the cultures are growing well after 10 months. Two of the cultures began to degenerate after 13 and 15 weeks and were discarded at 16 and 22 weeks, respectively. The remaining three cultures became contaminated after 9, 33 and 34 weeks of growth. One of these contaminated cultures (N 61) had been passaged ten times when it was discarded. These results show that explant cultures of measles-infected brains grew significantly better ($P =$

Table 1. *History of Mouse Brain Explant Cultures*

Culture		Number of Passages	Duration of cell growth	Time to degeneration
Control Cultures	N 53	1	6 weeks	15 weeks
	N 54	1	9 weeks	19 weeks
	N 55	0	no growth	
	N 56	5	10 months	growing+
Measles-Infected Cultures	N 57	1	13 weeks	16 weeks
	N 58	0	9 weeks	oo
	N 59	1	33 weeks	oo
	N 60	1	15 weeks	22 weeks
	N 61	10	34 weeks	oo
	N 62	6	10 months	growing+
	N 63	11	10 months	growing+
	N 64	9	10 months	growing+

oo Cultures were discarded because of contamination.
+ Cultures are still growing.

0.036) than did similar cultures from uninfected mice when comparison of their survival times was made by the Mann-Whitney non-parametric test for small samples (SIEGEL, 1956).

No CPE was detected at any time in any of the cell cultures although occasionally large multinucleate cells were seen in the measles-infected cultures. There was a tendency, too, for these measles infected cells to overlie one another rather than form true monolayer cultures, though no clear plaques of "transformed" cells were visible. The appearance of the cultures following haematoxylin and eosin staining is shown in Figures 1 a and 1 b. One or more cytoplasmic

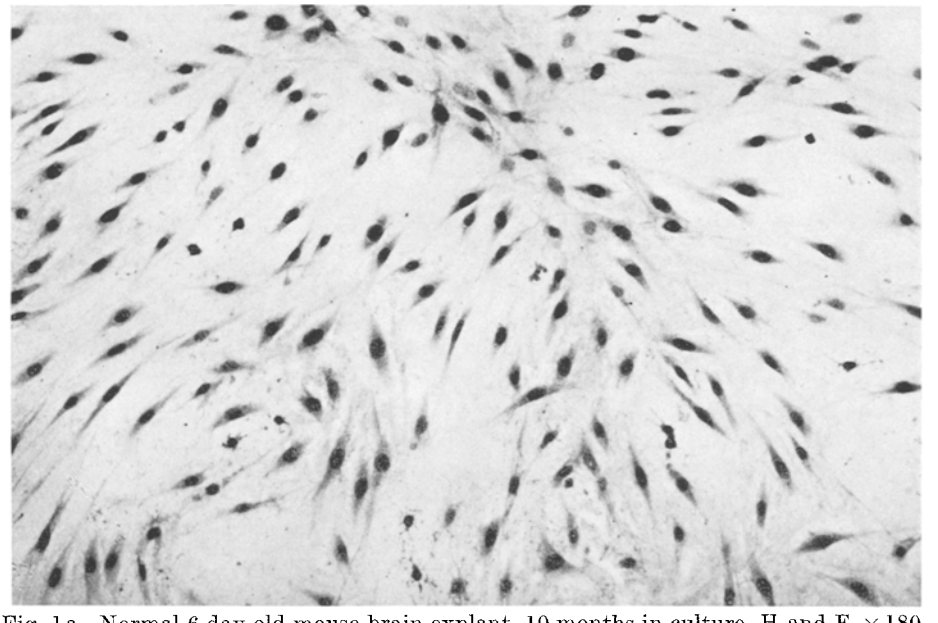

Fig. 1 a. Normal 6 day old mouse brain explant, 10 months in culture. H and E ×180

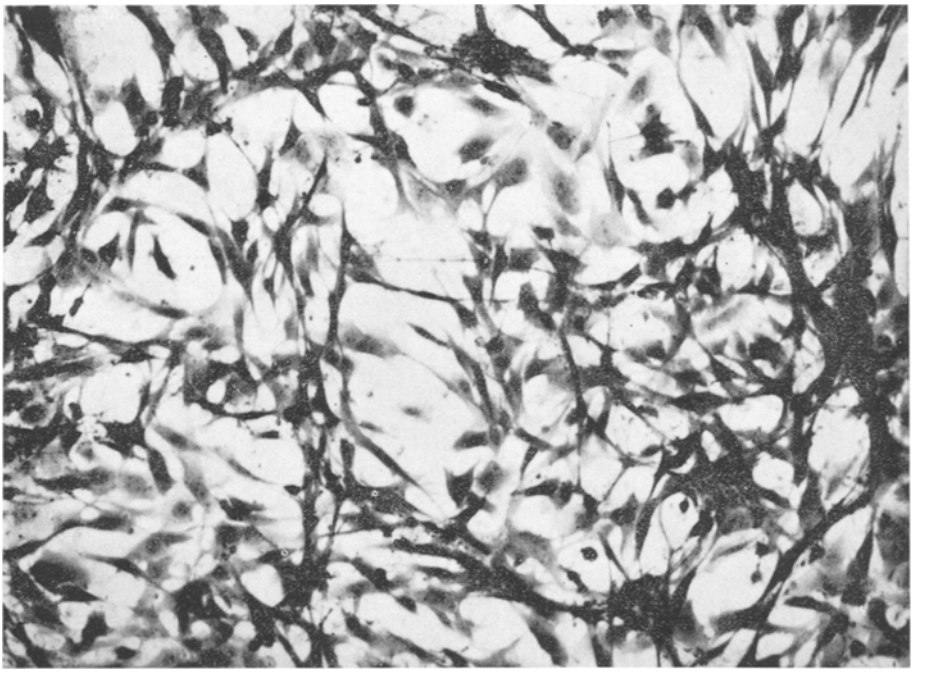

Fig. 1b. Brain explant from 6 day old mouse infected with mouse adapted measles virus. 10 months in culture. Note the high cellularity and the tendency of cells to overlie one another. H and E $\times 180$

inclusions, typical of measles virus, were present in approximately one quarter of the cells in the infected culture (Fig. 2).

3.2. Fluorescent Antibody Studies

The control cell culture, N 56, was examined by immunofluorescence for measles antigen after the second and third passages. No fluorescence was detected on either occasion.

The measles-infected cultures, N 61, N 62, N 63, and N 64 all showed intracytoplasmic fluorescence when treated with measles antiserum (Fig. 3). Cells which appeared morphologically similar sometimes showed markedly different virus content (Fig. 4). No fluorescence was detected when the cells were treated with the pre-immunization antiserum (Fig. 5). When examined under phase contrast microscopy the cells which showed immunofluorescent staining tended to be more granular than those which did not stain.

Only a small number of the cells in each culture was stained and the fluorescence tended to be perinuclear. No fluorescence was observed in the passage four level of the N 64 cells. However, at passage seven, occasional cells showed intracytoplasmic fluorescence. The large multinucleate cells were invariably stained. No intranuclear fluorescence was observed in any of these cultures.

3.3. Attempts to Isolate Measles Virus

Several attempts have been made to isolate measles virus from the cells of explants up to 10 months old. Virus was detected only occasinally and then at

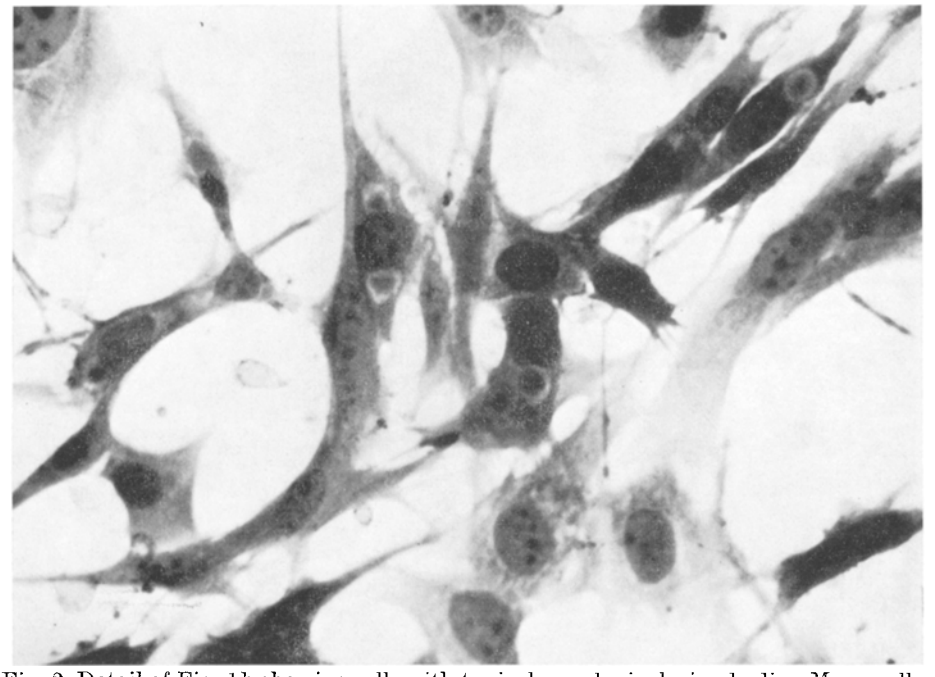

Fig. 2. Detail of Fig. 1b showing cells with typical measles inclusion bodies. Many cells contain multiple inclusions, eosinophilic or occasionally rather basophilic. H and E $\times 720$

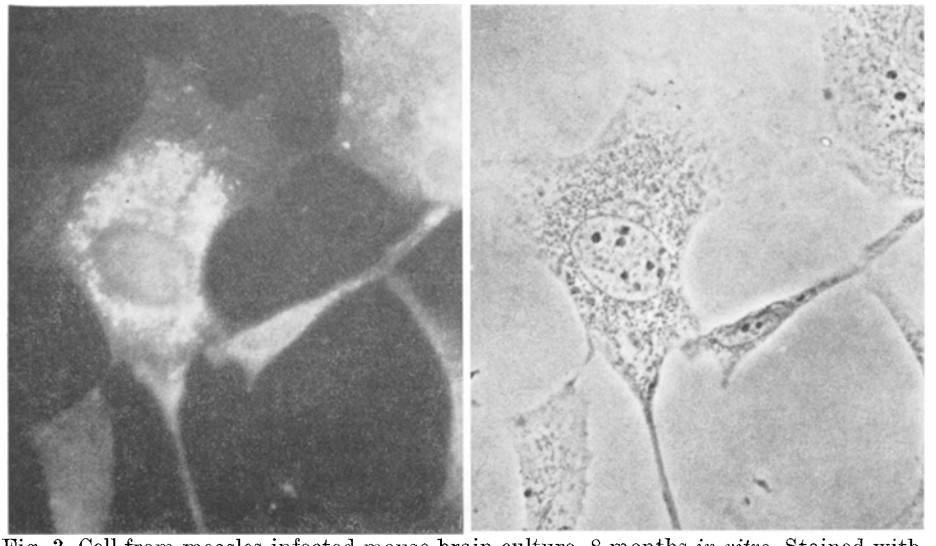

Fig. 3. Cell from measles-infected mouse brain culture, 8 months *in vitro*. Stained with anti-measles hyperimmune rabbit serum followed by anti-rabbit gamma globulin (fluorescein iso-thiocyanate conjugated)

(a) viewed in U.V. microscope

(b) in phase contrast

Note perinuclear measles antigen material co-extensive with the more granular part of the cytoplasm $\times 1600$

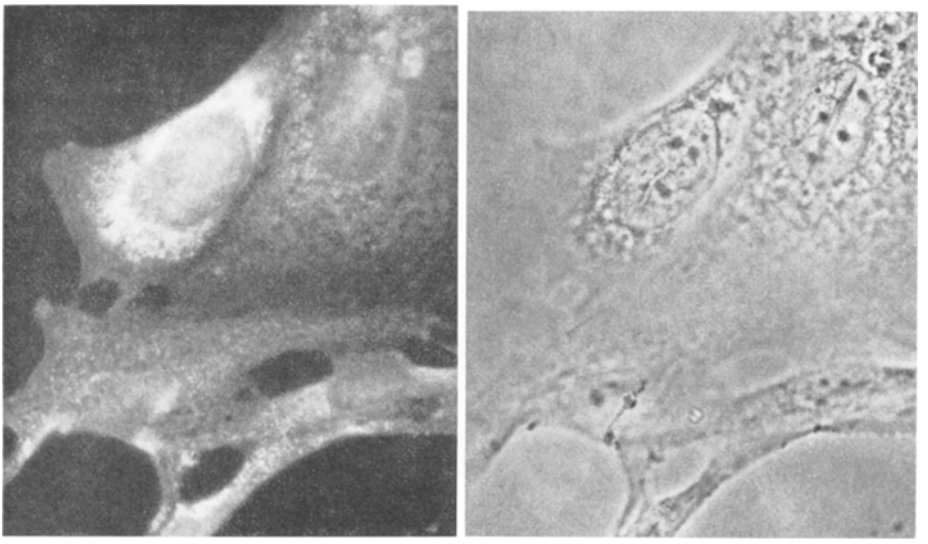

Fig. 4. Culture similar to Fig. 3. Note cells with identical morphology but markedly different virus antigen content
(a) viewed in U.V. microscope
(b) in phase contrast $\times 1600$

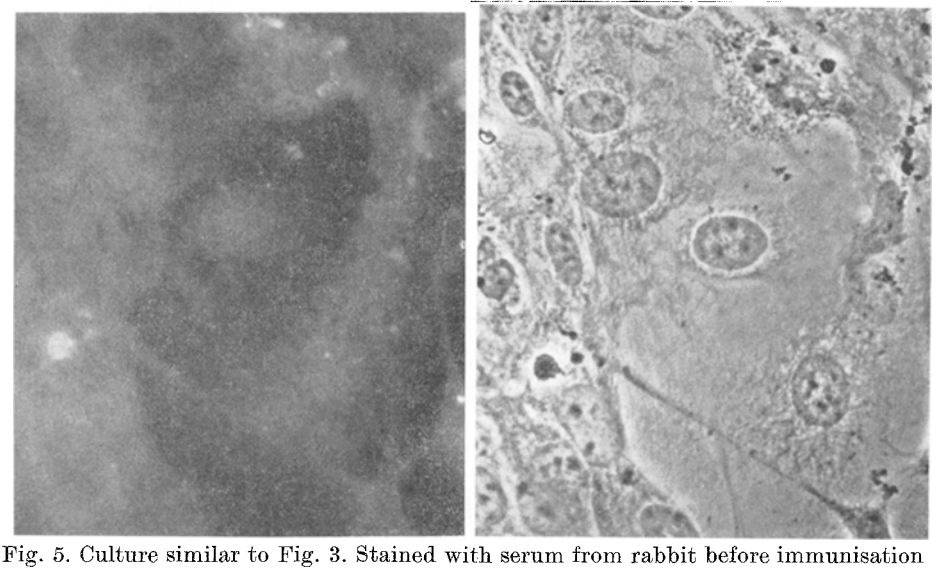

Fig. 5. Culture similar to Fig. 3. Stained with serum from rabbit before immunisation with measles virus. Note absence of fluorescence. $\times 1600$
(a) viewed in U.V. microscope
(b) in phase contrast

the limit of assay sensitivity (0.5—2 TCD_{50}/0.1 ml TC fluid). This particular strain grows very poorly in tissue culture and it appears that measles virus is released from these cell lines in minute amounts. Table 2 illustrates the isolation attempts.

Table 2. *Attempted Isolation of Measles Virus from Cultures*

Time after Explanting	Culture							
	N 57	N 58	N 59	N 60	N 61	N 62	N 63	N 64
2 weeks	—	—	—	—	N	N	N	N
5 weeks	N	+	+	+	+	+	N	N
3 months	—	C	+	+	+	—	—	—
4 months	D	C	N	N	—	N	N	N
6 months	D	C	N	D	+	N	N	N
8 months	D	C	C	D	C	N	+	+

\+ Measles Virus isolated.
— No Virus Isolated.
C Culture lost by contamination.
D Culture degenerated.
N Not Tested.

In all cases tested measles antiserum neutralized the cytopathic effect of the isolated virus.

3.4. Attempts to Detect Interferon

The cell cultures do not appear to produce detectable amounts of interferon.

3.5. Antiserum and Complement

There appeared to be no effects of antiserum and complement on the cell cultures. After one week of exposure no visual cell damage could be observed in the light microscope.

4. Discussion

This report describes the establishment of cell lines derived from the brains of mice inoculated intracerebrally with a mouse-adapted strain of measles virus. The cultures are characterized by the discontinuous production of very low yields of measles virus, cytoplasmic inclusions typical of measles, and the presence of measles antigen. Immunofluorescent studies revealed that only a small proportion of cells possessed measles antigen. Those which contained measles antigen were more granular than the others and granulation and fluorescence both tended to be perinuclear. The large multinucleated cells which were occasionally seen also contained measles antigen.

HORTA-BARBOSA *et al.* (1969) were unable to obtain complete measles virus from human brain cells cultivated from the biopsy of a case of SSPE although measles virus antigen was demonstrated in the cells by immunofluorescence. However, when these cells were mixed with HeLa cells and cultured together, complete infectious virus was released. In the present work, it was possible to detect minimal virus production without cell-cell association procedures.

The adaptation of measles virus to grow in mouse brain renders it much less cytopathic than the Edmonston or wild strains. It appears that the natural adaptation in man which occurs with the strains present in SSPE is very similar.

The presence of measles neutralizing antibody and complement had no visible effect on the cells. This may be because too few of the cells are infected although a 10% CPE should have been obvious. Alternatively, there might be incomplete

cell infection, with insufficient measles antigen incorporated into the cell membrane for damage to occur.

In SSPE the measles antibody levels (both IgG and IgM) are high at the onset of the illness and in some cases the titres continue to rise as the disease progresses (ADELS *et al.*, 1968; CONNOLLY *et al.*, 1971). If there is a slow release of minimal quantities of complete infectious virus in SSPE, as there is in the measles infected mouse brain tissue cultures, then the rise in antibody levels to a hyper-immune state with persisting IgM is readily explained. Similarly, as the artificially produced rabbit hyperimmune serum has no effect on the *in vitro* measles-infected brain cells, it is reasonable to suppose that there will be no *in vivo* effect.

Unpublished work (BELL, COWSHALL, and FIELD) suggests that different strains of measles virus may produce different degrees of morphological change in human embryo brain cell cultures and that varying amounts of infectious measles virus may be produced. It seems possible that subtle strain differences may contribute to varying persistence in the nervous system, those subjects in whom the virus adapts well and becomes a "slow" agent going on to develop SSPE or perhaps even multiple sclerosis (reviewed by ADAMS and BROWN, 1969).

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