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Propagation of Measles Virus in Cultures of Chick Embryo Cells.* (23637)

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Cultivation of measles virus in cultures of chick embryonic tissues and in developing hens' eggs has been reported by a number of workers. Others in similar attempts failed to obtain convincing evidence that the agent could be thus propagated(1,2,3,4,5). These discrepancies depended at least in part upon the fact that in nearly all these studies the only criterion of viral multiplication formerly available in the laboratory consisted in the development of signs of measles in monkeys following inoculation of materials suspected to contain the virus. Since a varying proportion of "normal" monkeys does not develop overt disease even when inoculated with materials known to contain the agent(1,6), it is understandable that the findings of different investigators were not always in agreement.§ In 1954, a more dependable index of viral multiplication was recognized by Enders and Peebles in the characteristic changes which the virus produces in primate epithelial cells (7). Accordingly, a reinvestigation of its capacity to multiply in chick embryonic tissues was undertaken.

At first attempts were made to cultivate in embryonated eggs several strains of measles virus that had been subjected to only a few passages in human kidney cells. No evidence was obtained that any of them multiplied in this host. One of these strains (Edmonston) was added to cultures of chick embryo tissues prepared according to the procedure that Plotz described(8), and with which Rake and his co-workers also reported successful results (3). But again no indication of increase or even persistence of the virus was noted(7). Ultimately adaptation to chick embryos of this strain was attained by Milovanovic, Enders and Mitus(9) after many passages in human renal and amnion cells. In preliminary experiments this chick embryo-adapted virus was found to multiply readily in cultures of chick cells(9), where its behavior will now be described in detail.

Materials and methods. Virus. The Edmonston strain was isolated from the blood of a child in the early acute phase of measles (7). When this investigation was initiated it had been passed serially 24 times in human kidney and 28 times in human amnion cell cultures, and 6 times in embryonated eggs.

Storage of virus. Virus suspensions in the form of infected tissue culture fluids were

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§ At least one of the factors responsible for variation in response of monkeys has lately been defined. Thus Peebles and his co-workers(6) have demonstrated in a high proportion of "normal" rhesus and cynomolgus monkeys maintained under laboratory conditions neutralizing and complement fixing antibodies that react specifically with measles virus. These antibodies presumably arise during the course of unobserved spontaneous infections with this virus or a closely related agent.

stored in the CO_2 cabinet at approximately −58°C. *Titration of infectivity.* Tenfold dilutions of infected tissue culture fluids were made in bovine amniotic fluid medium (BAF see below). Aliquots of 0.1 ml of each dilution were added to 3 tissue cultures. The cultures were examined at frequent intervals for cytopathic changes. Final readings were made as routine on the 14th day after addition of the inoculum. Titers are expressed as log TCD_{50}/0.1 ml as calculated by the method of Reed and Muench. *Neutralization tests.* Acute and convalescent phase sera from a patient with measles and sera taken from a cynomolgus monkey before and 25 days after inoculation with the Edmonston strain of the 23rd passage in human kidney cell cultures (6), were employed. The sera were inactivated at 56°C for ½ hour and a series of dilutions increasing by a factor of 2 were prepared in isotonic phosphate buffer solution. To each dilution an equal volume of virus suspension containing a calculated 200 TCD_{50}/0.1 ml was added. The mixtures were allowed to stand for 1 hour at about 6°C. 0.1 ml of each was then added to each of 3 cultures. Serum neutralization titers were calculated by the method of Reed and Muench from readings of cytopathic changes taken on the 14th day. *Tissue cultures.* Cultures of trypsinized human amnion and human kidney cells were prepared and maintained according to procedures previously described(9). Roller tube cultures of fragments of whole 9-day chick embryos were initially used and later cultures of trypsinized chick embryo cells. The latter were prepared according to the technic of Dulbecco(10) as modified by Dr. Donald N. Medearis, Jr. in this laboratory. Embryos from eggs incubated at 100°F for 7 to 9 days were washed in phosphate buffer solution after removal of the eyes and transferred to an Erlenmeyer flask containing about 50 ml of 0.25% trypsin solution at pH 7.8. The flask, after standing for 30 minutes at 37°C or for 60 minutes at room temperature, was shaken for 1 minute. The suspension of cells was separated from the remaining coarse debris and centrifuged at 1000 rpm for 10 minutes. The fluid was discarded and replaced with an equal volume of BAF medium. After deposition in the centrifuge the cells were resuspended in this medium to yield a concentration of 200,000 to 400,000 per ml. Sufficient cells were usually obtained from a single embryo to provide about 100 cultures. Aliquots of 1 ml of the cell suspension were added to 150 x 16 mm test tubes, which were set at an angle of about 5° and incubated for 48 to 72 hours at 37°C when suitable monolayers were formed. The medium was changed before addition of virus and at intervals of 7 to 10 days thereafter. *Tissue culture medium.* Bovine amniotic fluid medium containing 5% normal horse serum was employed as routine(9) for growth and maintenance of cultures of chick cells. During the phase of outgrowth human amnion cells were nourished with a horse serum-glutamine salt mixture(9) and thereafter with BAF medium.

Experimental. Multiplication in cultures of chick embryonic cells. A series of passages in chick cell cultures was initiated with a 10% suspension of amniotic membranes from the 6th chick embryo passage of the Edmonston strain(9). Infectivity titer of this suspension in human amnion cells was 520 TCD_{50}/0.1 ml. Three roller tube cultures of chick tissues were each inoculated with 0.1 ml of the suspension. A pool of undiluted fluids removed on the 19th day was used as inoculum for the 2nd passage in roller tube cultures. The 3rd passage was initiated in trypsinized chick cell cultures with a pool of fluids collected on the 14th day from cultures of the 2nd passage. Thereafter trypsinized cell cultures were inoculated with pooled fluids from the previous passage taken 7 to 14 days after virus was added. Infectivity of fluids removed successively during each passage, with the exception of the second, was assayed in human amnion cells. In Fig. 1 the infectivity titers of fluids from the 5th and the 8th passages are presented graphically to show how the virus increased and declined. The curves differ significantly in respect to the time at which the maxima were attained. Maximal titers determined during each of 19 serial passages are listed in Table I with the times at which the fluid was harvested. In 11 of the first 12 passages the titers represent the true

maxima within the limits of the experimental conditions. From the later passages only fluids taken during the first 11 to 14 days were tested. In these instances the titers may not represent the true maxima, although this seems probable, since they are within the range of the highest observed in this or in any other system we have employed(7,9).

The data included in Fig. 1 and Table I show that the egg-adapted measles virus multiplied readily in this system. That the biological properties of the agent were in certain respects modified to meet the conditions presented by this new environment is suggested by inspection of the various times at which it was present in highest concentration. Thus during the first 6 passages for which information is available the viral content of the fluid did not reach its peak until about the 30th day following inoculation. (Average: 27 days.) In contrast the maxima for the next 6 passages, with one exception, were reached within 14 days or less (Average: 12 days). Additional evidence of an altered biological characteristic emerging during these passages is to be found in the assumption of cytopathogenicity for chick cells.

Cytopathogenic effects in chick cells. Throughout the first 4 serial passages no changes definitely attributable to the virus were noted. Its presence and increase were determined solely by effects produced in human amnion cells exposed to fluids taken from

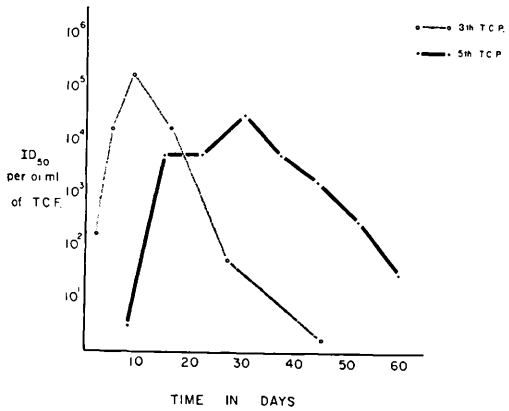

FIG. 1. Infectivity titers as determined in cultures of human amnion cells of fluids removed at various intervals from the 5th and 8th passages of measles virus in chick embryo cell tissue cultures.

TABLE I. Maximal Titers Found in Fluids of Serial Passages of Measles Virus in Chick Embryo Tissue Cultures.

Passage	Titer* (TCD_{50}/0.1 ml)	Days after inoculation
1	2.5	27
2	VP†	
3	3.5	33
4	3.8	28
5	4.5	30
6	4.5	29
7	4.8	13
8	5.3	9
9	3.8	7
10	3.8	11
11	3.5	19
12	4.8	14
13	4.8	6
14	4.5	11
15	4.3	9
16	4.8	11
17	3.8	8
18	4.3	10
19	4.8	7
20	4.3	7

* As determined in cultures of human amnion cells.

† Presence of virus confirmed but concentration not determined.

chick cell cultures. On the 8th day of the 5th passage distinctive changes became evident, consisting of increased refractility of cells in circumscribed areas. Many affected cells exhibited a fusiform or stellate configuration owing to the formation of filamentous cytoplasmic processes, which varied in length, and often appeared to be finely beaded. In Fig. 2 A is shown such changes in cells of the 22nd passage 10 days after addition of virus. The appearance of cells in a control culture is illustrated in Fig. 2 B. In fixed and stained preparations eosinophilic intranuclear and cytoplasmic inclusion bodies were observed that closely resembled those found in infected primate cells(7,9,11) as shown in Fig. 3. As the process advanced the affected cells became rounded, pyknotic and eventually disintegrated. In succeeding passages these changes appeared sooner and proceeded more rapidly to involve a large proportion of the cell population. Complete destruction of all cells, however, even after many weeks has not been observed and virus production has continued at low levels. It is noteworthy that shortly after the cytopathogenic effect became evi-

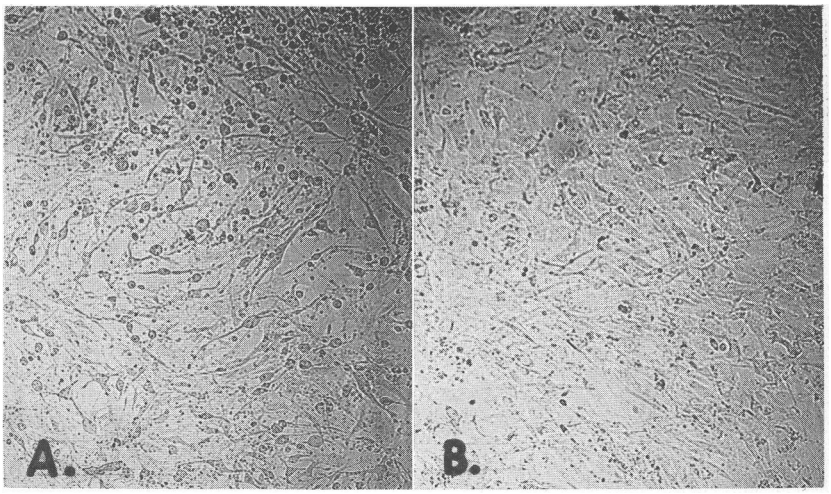

FIG. 2A. Cytopathic changes in cells 10 days after addition of measles virus. Edmonston strain 22nd passage in chick cell tissue cultures. Unstained. Mag 147 ×.

FIG. 2B. Cells in control culture maintained under same conditions but without addition of virus. Unstained. Mag 147 ×.

dent the time required by the virus to attain its maximal concentration in the fluid was reduced.

Identification of the virus. The agent propagated under these conditions was identified as measles virus by its behavior in cultures of human amnion cells and in neutralization tests with acute and convalescent phase measles sera. Addition of infected fluids from chick cell cultures of all passages to primary cultures of human amnion cells was followed by the appearance of cytopathic changes characteristic of measles virus(9). Infectivity titers of the fluids from later passages as determined simultaneously in amnion and in chick cells, were of the same magnitude as shown in Table II. These findings indicate that the same agent was responsible for the cytopathogenic effects observed in both kinds of cells.

TABLE II. Infectivity Titers of Fluids from Various Passages of Virus in Chick Cells as Determined in Cultures of Chick and Human Amnion Cells.

Passage	Day fluid harvested*	Titer† Chick	Titer† Amnion
6	14	3.8	3.8
7	13	4.5	4.8
8	9	5.5	5.3
10	11	4.5	3.8
13	6	4.3	4.8
17	8	4.3	3.8

* Day after inoculation fluid harvested from serial passages of virus in chick cell cultures.
† TCD_{50}/0.1 ml.

TABLE III. Results of Neutralization Tests with Human and Monkey Measles Sera and Virus of the 7th Passage in Chick Cells.

Cells used in test	Serum	Serum titers* Acute or preinfection	Serum titers* Convalescent
Human amnion	Human	7	710
Chick	"	<8	840
Human amnion	Monkey	<4	575

* Reciprocal of final dilution of serum inhibiting cytopathogenic effect of the virus in one-half of the cultures as calculated by the method of Reed and Muench.

The results of neutralization tests are summarized in Table III. The acute phase serum from a measles patient possessed little capacity to inhibit the cytopathogenic effects of the agent in either human amnion or chick embryo cells; the convalescent phase serum prevented these effects in high dilution. Similar results were obtained in amnion cell cultures with sera of a monkey taken before and after inoculation of the Edmonston strain before adaptation to the chick embryo.

Antibodies in rabbits with virus propagated in chick cells. It was determined that antibodies reacting specifically with the virus were induced in rabbits by injection of fluid from infected cell cultures. Two animals of 6 and 7 pounds respectively were inoculated with

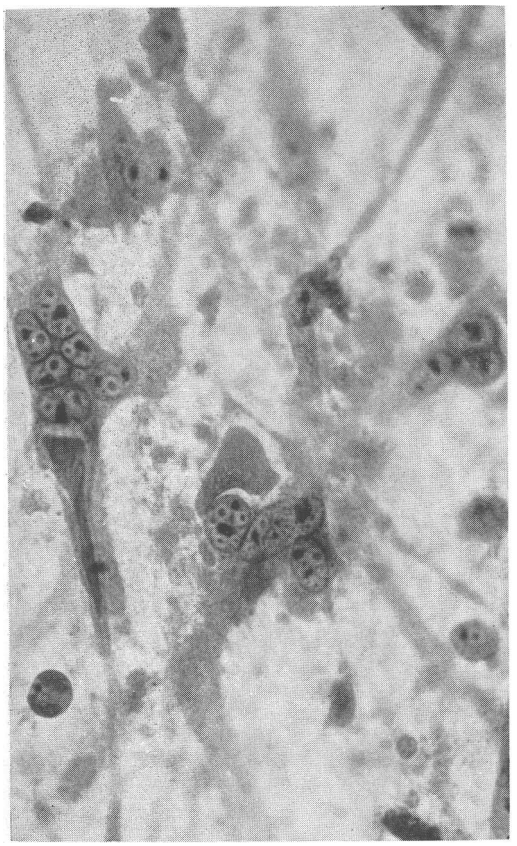

FIG. 3. "Multinuclear giant cells" in a culture of chick embryo cells 19 days after addition of measles virus. Edmonston strain, 7th passage in chick cell tissue cultures. Bouin's fixative; collodion imbedding; H & E stain. Mag 880 ×. Note eosinophilic intranuclear inclusions and large irregular cytoplasmic masses.

undiluted fluid removed on the 8th day from a 15th chick-cell passage. To minimize the development of antibodies against other constituents, the following medium was employed instead of the usual BAF mixture:

Hanks' balanced salt solution (with phenol red)	50%
Chick amniotic fluid (from 8 day eggs)	50%
Penicillin	50 units/ml
Streptomycin	50 μg/ml
Mycostatin	50 "

The procedure recommended by Ramos-Alvarez and Sabin for production of antisera to ECHO viruses(12) was followed. Neutralization tests were carried out in cultures of human amnion cells with the rabbits' sera drawn just before immunization and 24 days thereafter. The Edmonston virus used in these tests had been passed 26 times in human renal cells and then 29 times in human amnion cells but had not been cultivated in chick cells. Neutralizing antibodies were not detected in the initial specimen of serum of either rabbit in a final dilution of 1:4. The titers found for the 24th day specimens were respectively 1:128 and 1:180. These results indicate that anti-measles antibodies can be produced in rabbits by inoculation of the chick cell-adapted virus. They also afford additional evidence for the identity of the chick-cell-adapted virus and the agent originally isolated from a patient with measles.

Cultivation with media of known composition. Chick cells originally grown in BAF medium were washed 3 times with Hanks' solution at pH 7.6 and divided into 4 sets each of 6 cultures. Each set was maintained with one of the following solutions:

(a) Hanks' balanced salt sol
(b) Hanks' sol, 3 parts; ox-serum ultrafiltrate,‖ 1 part
(c) Medium 199 (Morgan and Parker)
(d) BAF medium

To each of 3 cultures in each set was added 0.1 ml fluid from the 20th chick-cell-passage of the virus. (Titer = $10^{4.3}$ TCD_{50}/0.1 ml in human amnion cells.) Cytopathic changes were apparent on the 4th day in all tubes to which virus was added. The fluids from infected cultures in each set were collected and pooled on the 7th day. At this time the cells in the control cultures remained in fair to good condition. The titer of each pool in human amnion cell cultures was determined as follows:

Medium	TCD_{50}/0.1 ml
(a)	$10^{2.5}$
(b)	$10^{2.8}$
(c)	$10^{3.3}$
(d)	$10^{4.3}$

While the quantity of virus in cultures with medium 199 is smaller, it nevertheless approaches that often encountered in those of primate or chick cells maintained with BAF medium. It is unlikely that in this experi-

‖ Microbiological Associates, Washington, D.C.

ment the virus found in the fluids represents that introduced as inoculum. In unpublished experiments we have observed in agreement with data reported by Ruckle(13) that decay of measles virus at 37°C is very rapid. Therefore if the agent had failed to multiply one would not expect to encounter it in appreciable quantities at the end of 7 days.

Complement fixing antigen in cultures of chick cells. Antigen fixing complement with antibody developing during convalescence from measles regularly appears in the fluid of infected primate cell cultures(7,9). Repeated attempts have so far failed to demonstrate this antigen in the fluid of infected chick cell cultures. Recently, however, in a preliminary experiment antigen reacting specifically with convalescent measles serum has been prepared by repeated (3 times) freezing and thawing of whole cultures infected 40 days previously with the virus. It is probable, therefore, that complement fixing antigen present in the cells is released by this procedure.

Discussion. It is not surprising that measles virus previously adapted to the chick embryo proliferates in the cells of this host when they are maintained in culture. For most if not all viruses capable of multiplying in the developing egg can also be propagated *in vitro* in cells derived from its tissues. Accordingly, the principal significance of these results lies elsewhere. First, they confirm, at least in principle, the early findings of Plotz and Shaffer and Rake. The only indicator then available in the laboratory of the presence of this agent was the development of overt disease in the monkey. The data here presented were obtained through the application of criteria for its recognition which appear to be more reliable and specific. There is, therefore, little reason longer to doubt the conclusion of these workers that the measles virus can be cultivated in chick embryo cells either *in vivo* or *in vitro*.

We have not, however, succeeded in reproducing their results under conditions approximating those which they employed. Thus their original inocula consisted of virus present in materials derived from patients or monkeys with measles and it would seem *a priori* that in this state the agent would tend to behave more like our strains which after isolation were subjected to only a few passages in primate cells. As previously emphasized, we have repeatedly failed to maintain such strains in either chick embryos or chick cell cultures. Perhaps this remaining discrepancy will be resolved when the effect of inoculating chick cells with blood or throat washings of measles patients is determined by means of the newer criteria for viral activity. For possibly a few passages of the virus in primate cells may interfere with its adaptation to chick embryo systems. If so, any effect of this sort must be transitory, since we have succeeded in cultivating the virus in the chick embryo only after it has undergone many passages in human epithelial cells.

The acquisition of cytopathogenicity for chick cells in culture is also of interest. Its sudden emergence during the 5th serial passage at first suggests a mutation. It may, however, reflect the capacity of the agent to increase more rapidly within the cells of this unnatural host which was gained at about the same time. Thus the phenomenon, if it can be elicited with regularity, might prove useful in the future analysis of the factors that in general underlie viral cytopathogenicity.

From the practical standpoint the development of cytopathogenicity is of value since it makes possible the application of a technic to the assay of infectivity and the titration of antibodies which is more convenient, rapid and economical than the culture of primate cells. A greater utilitarian significance, perhaps, may be the demonstration that quantities of virus are produced in chick cells even when maintained with a medium of known composition that is comparable to the concentrations found in cultures of primate cells. Fluids from such cultures might provide appropriate material for the preparation of vaccine. Such vaccine would contain no foreign proteins or other macromolecular substances save the small amounts derived from the chick cells. That chick embryo proteins in low concentration may be injected with very little risk is indicated by extensive experience in

man with yellow fever or influenza virus vaccine prepared in eggs.

Summary. An egg-adapted strain of measles virus has been propagated in cultures of chick embryo cells throughout 24 serial passages. Beginning with the 6th passage an abrupt decrease occurred in the time required to attain maximal concentration of virus in the fluid. The concentration of virus was comparable to that found in cultures of infected primate cells. In the 5th chick cell passage the virus began to produce cytopathic changes closely resembling those occurring in human epithelial cells infected with measles virus. The identity of the agent was confirmed in neutralization tests with measles antisera. In chick cells maintained with a medium of known composition virus production approached that in cultures nourished with serum and tissue extracts. Inoculation of rabbits with the virus propagated in chick cells was followed by development of neutralizing antibodies specific for the agent of measles.

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Increase of Urinary Citrovorum Factor Activity in Patients Receiving Methotrexate (Amethopterin) (23638)

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Regression of metastatic trophoblastic tumors has been observed in women treated with methotrexate, a folic acid antagonist(1). The mechanism of action of the folic acid antagonist has been shown to include an inhibition of the synthesis of citrovorum factor (CF) in liver and prevention of its utilization by tissues in laboratory animals(2,3). It, therefore, was of interest to attempt to correlate the daily excretion of urinary CF activity with tumor response in patients with trophoblastic tumors receiving methotrexate therapy.

Methods. All patients received a constant diet composed of a liquid mixture of milk, cream, eggs and dextrose during the period of study. The mixture contained approximately 1,000 calories, 5 μg of folic acid and 6 μg of CF/liter. The dietary intake of these patients ranged from 1,800 to 2,500 calories/day. Daily 24 hour urines were carefully collected and refrigerated. 25-35 mg of methotrexate was given daily for 5 days either by mouth or intramuscularly. Toxicity, manifested by anorexia, nausea, oral ulcerations and occasional emesis was present but did not interfere with the constant dietary intake. The CF activity was assayed by the microbiological technic of Sauberlich and Baumann (7) using Difco assay medium. Turbidimetric readings were made after 16 to 18 hours incubation at 37°C. Since *Leuconostoc citrovorum* #8081 (*Pediococcus cerevisiae* ATCC 8081) was susceptible to methotrexate inhibition, a methotrexate resistant strain *L. citro-*