*FROM STATENS SERUMINSTITUT, COPENHAGEN. DIRECTOR: J. ØRSKOV, M.D.*

# STUDIES ON MEASLES VIRUS IN MONKEY KIDNEY TISSUE CULTURES

## 1. *Isolation of Virus from 5 Patients with Measles.*

*By*

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In 1954 *Enders* & *Peebles* (6) reported the successful isolation of virus-like agents from blood and throat washings of patients with measles. These agents multiplied in cultures of human and simian renal cells and produced characteristic cytopathic changes. The nutrient fluid from infected cultures was found to contain antigen which fixed complement in the presence of measles convalescent sera. In addition such sera were shown to inhibit the propagation of the isolated agents in tissue cultures. These original observations by *Enders* & *Peebles* were later confirmed by *Cohen et al.* (3). These investigators isolated several virus strains and studied the intracellular localization of the virus in tissue cultures by means of the fluorescent antibody-technique.

Recently *Dekking* & *McCarthy* (4) reported that they had been able to propagate measles virus in the KB strain of human carcinoma cells. At about the same time *Black, Reissig* & *Melnick* (2) succeeded in adapting measles virus to another human cancer cell strain, HEP 2.

The present paper reports the isolation of five strains of measles virus in cultures of trypsinized monkey kidney cells. The identification of the strains by serological tests and by transmission of the disease to monkeys is also described.

### MATERIALS AND METHODS

*Tissue cultures:* Cells of trypsinized kidneys from rhesus monkeys, cynomolgous monkeys or baboons were used routinely. The technique of *Younger* (15) for the preparation of these tissue cultures was used with some modifications as previously described (10). During the outgrowth of the cells the growth medium consisted of lactalbumin hydrolysate 0.5 per cent (11) in *Hank*'s solution (9) with 2 per cent horse serum.

Before seeding with virus the medium was changed. Each tube received 1.8 ml of either synthetic medium 199 (12) or bovine amniotic fluid (5) containing phenol red as an indicator (final dilution 0.02 per cent). All media contained penicillin (100 U/ml) and streptomycin (0.1 mg/ml).

*Collection of specimens:* Isolation of virus was attempted from throat washings and blood.

*Throat washings:* Measles patients were asked to gargle either with 15 ml of a mixture of one part ox-heart infusion broth and 2 parts of buffered salt solution or, in later experiments, with 15 ml of distilled water containing 1 per cent Bacto tryptose "Difco". Penicillin, 100 U. per ml and streptomycin 0.1 mg per ml were added to the fluid. From the throats of very young children specimens were obtained by cotton swabs which were subsequently immersed into 2 ml of one of the two fluids just described. In all instances, the specimens were immediately frozen in $CO_2$-ice and then stored in an electrical deep-freeze (—60° C). Before inoculation into tissue culture the frozen materials were thawed rapidly at 37° C in running tap water.

*Blood:* In earlier experiments heparin was added to the blood (2 ml of an 0.05 per cent solution of heparin per 10 ml of blood). In later experiments the blood was allowed to coagulate. Red cells which had not become attached to the clot were resuspended in the serum, and this mixture was used as inoculum for tissue cultures. The blood samples were stored at +4° C until the time of inoculation.

*Inoculation of tissue cultures:* Tissue cultures were inoculated with 0.5 ml of throat washing material or with 0.25 ml of blood. The final amount of fluid in the tubes was about 2 ml. The cultures were kept stationary or placed in a rotating drum (1 rotation per minute).

Subcultures from the first passage were carried out between the 6th and the 16th day, usually on the 8th day after inoculation. The nutrient medium was as a rule not exchanged in the course of a passage. Subcultures from the later passages were made between the 6th and the 12th day of incubation. The passage material consisted of a suspension of cells and cell debris in culture medium. This mixture was obtained by loosening the cells still adhering to the glass through scraping with a pipette. The amount inoculated varied, but it was usually 0.2 ml, so that the final volume of fluid in the inoculated tubes was about 2 ml. Serial passages of material from control tubes were carried out in the same way.

*Complement fixation tests:* Complement fixation tests were made according to the method of *Fulton & Dumbell* (8) as modified by *Svedmyr, Enders & Holloway* (14). As antigen the undiluted suspension of cells and cell debris in the culture media from the various passages was used either immediately after harvest or after a shorter or longer period of storage at —20° C. Controls with antigen consisting of culture fluids with cells from uninoculated tissue cultures were included in each experiment. All antigens had been inactivated at 56° C. for half an hour.

The antiserum consisted of a pool of serum from about 10 measles patients bled 2–4 weeks after the development of the rash. Serum had been inactivated at 56° C for half an hour.

## RESULTS

### *Virus Isolation.*

In the spring of 1955 and of 1956 isolation experiments were carried out on 13 patients with clinical symptoms of measles (Table 1).

From 9 patients material was collected during the first 24 hours after the onset of the rash. Virus was recovered from the throat of five of these patients, in 2 instances from cotton swabs (Nos. 1 and 11) and in two cases from throat washings (Nos. 4 and 10). From one patient (No. 5) virus was recovered both from cotton swabs and from throat washings.

The throat washings were kept frozen at —60° C for varying lengths of time before inoculation into tissue cultures. As can be seen in Table 1, virus could be recovered even if 4 days had elapsed before inoculation took place (patient No. 5).

Virus was isolated from heparinized blood in only one out of three

TABLE 1

*Isolation of Measles Virus in Monkey Kidney Tissue Cultures. Virus was Recovered from 5 out of 13 Patients.*

| Patient | | | | Isolation materiel | | Virus recovered from | | |
|---|---|---|---|---|---|---|---|---|
| No. | Sex | Age years | Date of specimen | Collected hours after onset of rash | Stored (-60° C) hours before inoculation | Throat swab | Throat gargling | Blood |
| | | | 1955 | | | | | |
| 1 | M. | 6 | April | 24 | 48 | + | — | 0* |
| 2 | M. | 2 | April | 18 | 2 | 0 | — | 0* |
| 3 | F. | 1 | May | 30 | 2 | 0 | — | — |
| 4 | M. | 7 | May | 18 | 2 | — | + | +* |
| | | | 1956 | | | | | |
| 5 | M. | 21 | Jan. | 24 | 1 | + | — | 0 |
| | | | | | 24 | — | + | 0 |
| | | | | | 96 | — | + | — |
| 6 | M. | 25 | Jan. | 40 | 2 | — | 0 | 0 |
| 7 | M. | 5 | Jan | <12 | 14 | 0 | — | — |
| 8 | M. | 7 | Jan. | >24 | 14 | 0 | — | — |
| 9 | F. | 8 | Feb. | 8 | 16 | 0 | — | 0 |
| 10 | F. | 13 | Feb. | 13 | 48 | 0 | + | 0 |
| 11 | F. | 6 | Feb. | 6 | 19 | + | — | 0 |
| 12 | M. | 9 | Feb. | 6 | 19 | — | 0 | 0 |
| 13 | F. | 13 | May | >24 | 19 | — | 0 | 0 |

+ = virus isolated. — indicates: not done. 0 = virus not recovered.
* heparinized blood.

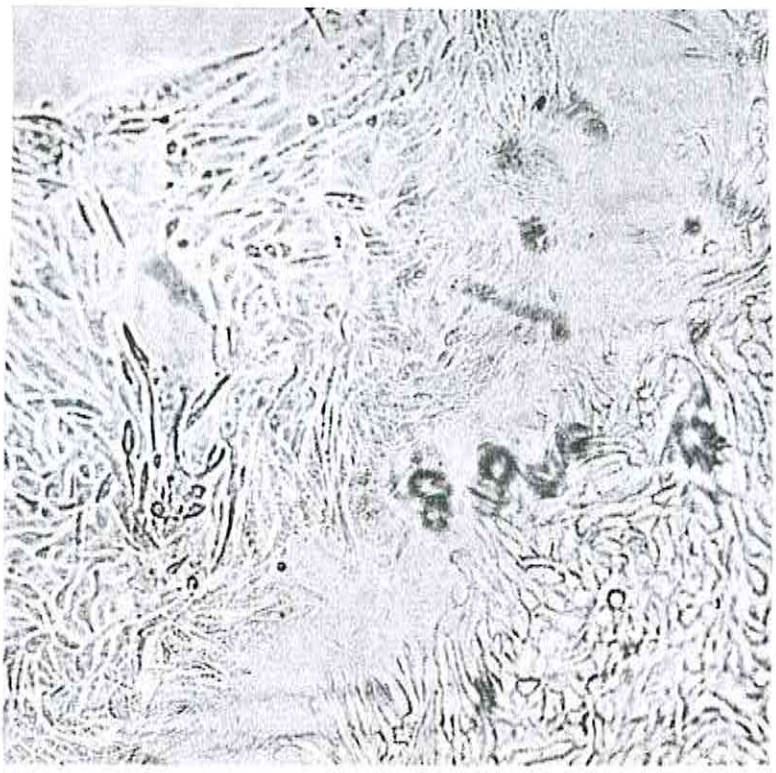

*Fig. 1.*
Outgrowth of normal epithelial cells in an uninoculated monkey kidney tissue culture.

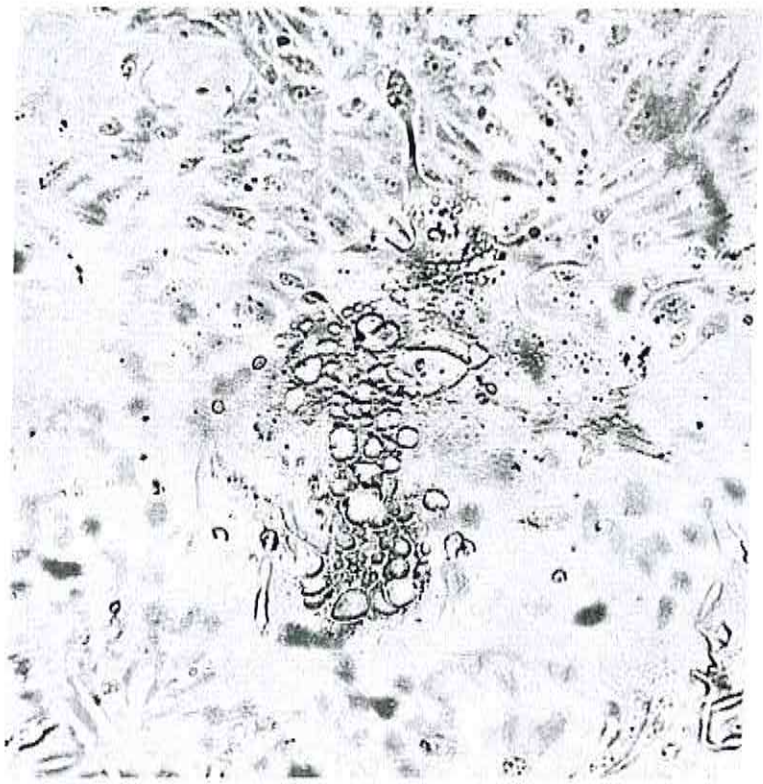

*Fig. 2.*

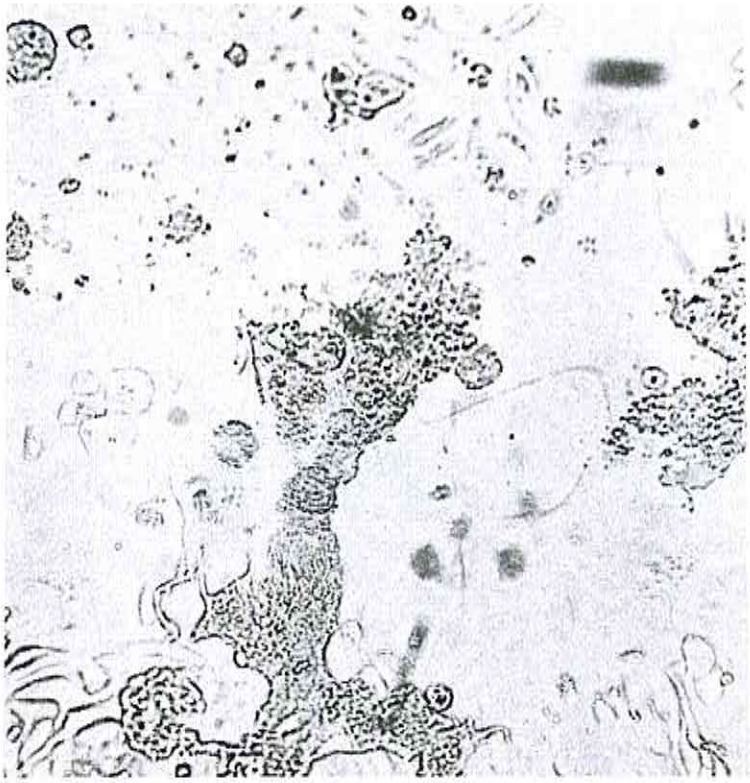

*Fig. 3.*

*Figs. 2 and 3.* Cytopathic manifestations in monkey kidney tissue cultures inoculated with measles virus. *Fig. 2.* Syncytium with vacuoles. *Fig. 3.* Later stage showing characteristic cell debris with circular formations.

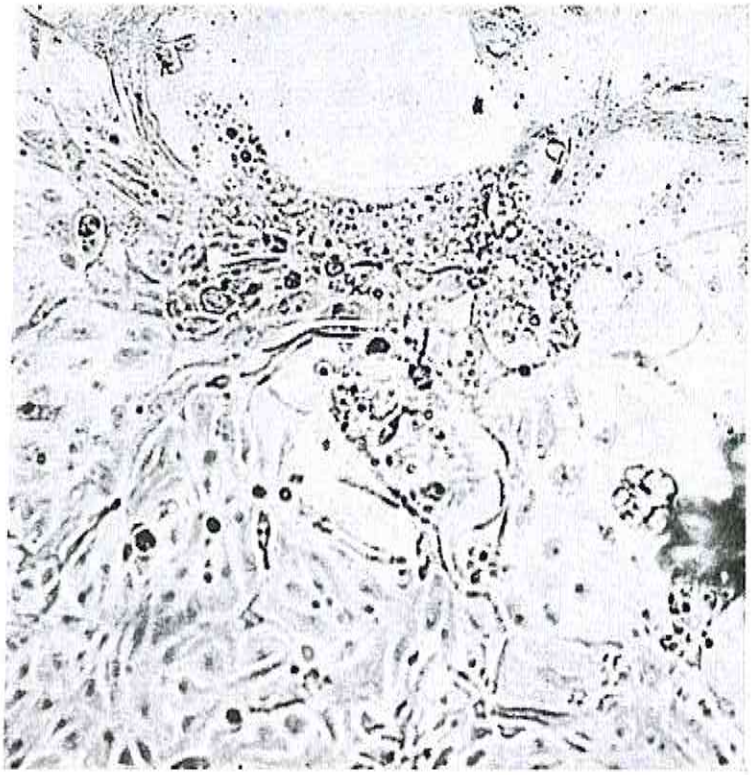

*Fig. 4.*

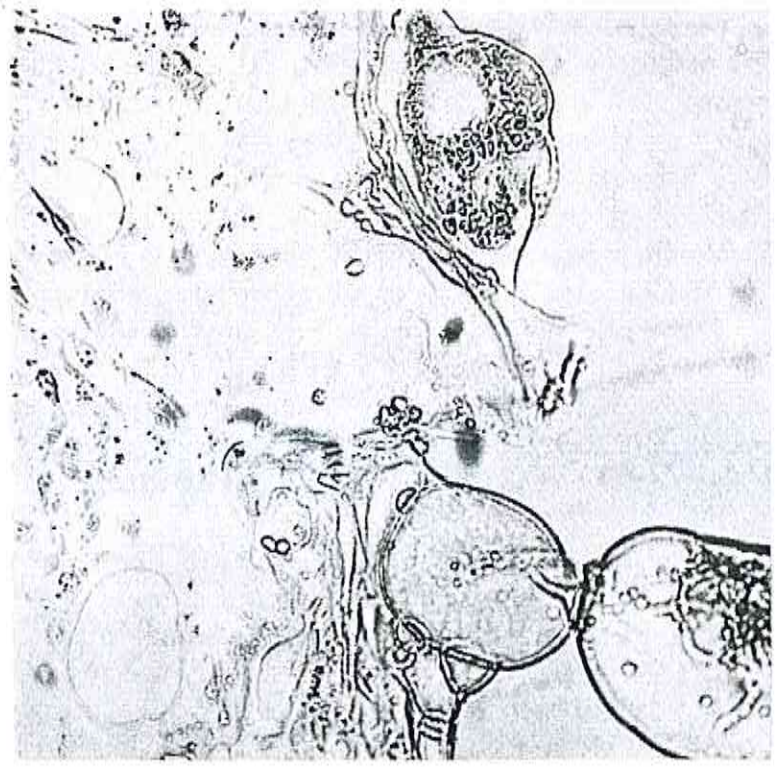

*Fig. 5.*

*Figs. 4 and 5.* Uninoculated monkey kidney tissue cultures showing vacuolated syncytial structures resembling those caused by measles virus (Fig. 4) and of somewhat different size and appearance (Fig. 5).

attempts (No. 4), and in no instance from serum (none out of 5 patients bled within 24 hours after onset of the rash).

From 4 patients virus isolation was attempted when more than 24 hours had elapsed after the appearance of the exanthem. Virus was not recovered from any of these patients.

*Cytopathic changes:* The cytopathic changes observed in the cultures on isolation of measles virus were similar to those described by *Enders & Peebles* (6): After a period of one to several days syncytium-like areas develop in the tissue. Initially, these changes are most predominant at the margin of the tissue. The syncytia seem to arise from the melting together of cells whose boundaries gradually obliterate. With continued incubation the syncytia increase in size, and new arise in other places. Very soon large numbers of small vacuoles appear centrally in the affected areas giving rise to foamy- or lace-like formations (Fig. 2).

As the degeneration continues the vacuoles disappear, and the changed areas now appear uniformly gritty and darker than the surrounding cells. The cell debris may loosen from the glass and float into the culture medium leaving an area without tissue. Usually, however, the residue of the destroyed tissue is found to be arranged like dark borders along the margin of the remaining tissue.

At this time there appear in the cultures rather large granular formations, more or less circular in outline and showing either a quite smooth or a crenated margin (Fig. 3). These formations are probably condensation products of cell debris from the specifically degenerated areas. They are seen irrespective of whether the cultures have been kept stationary or rotating. They may be fixed to the normal tissue, but frequently they float in the culture medium.

The destruction of the tissue continues as the individual syncytia increase in size and new ones appear. In monkey kidney tissue the speed of destruction and the extent of cytopathic changes may vary considerably from one passage to another. Now and then, larger or smaller areas of apparently normal tissue will remain even after 2–3 weeks. Mostly, however, the remaining tissue shows a loosening of the structure and granulation of the cells. This general over-all degeneration of the tissue may occasionally be observed already after a few days' incubation.

As described by *Enders & Peebles* (6), and later by *Rustigian et al.* (13) and by *Cohen et al.* (3) cytopathic changes similar to those caused by measles virus may be observed also in uninoculated cultures of monkey kidney tissue (Figs. 4–5). These changes are probably caused by virus-like agents, so called "foamy agents", which seem to be frequently present in kidney cells from apparently healthy monkeys. Specific measles antigen is, however, produced only in cultures infected with measles virus. In the present study the ability of tissue culture passage material to fix complement in the presence of convalescent-

phase measles serum was therefore used as a criterion for the presence of measles virus.

*Serological Studies.*

*Complement fixation tests:* Table 2 shows the results of complement fixation tests on sera from four patients with measles. Blood was taken as soon as possible after the onset of the rash, and again from 9 to 37 days later during convalescence.

TABLE 2
*Complement Fixation Titers of Sera from Cases of Measles.*

| Patient no. | Serum drawn (days after rash) | Complement fixation titer | | |
|---|---|---|---|---|
| | | Antigen † V-3 | Antigen V-14 | Antigen XI-5 |
| 5 | 1 | < 8* | — | — |
| | 18 | >32 | — | — |
| 9 | < 1 | < 8 | — | — |
| | 9 | 256 | — | — |
| 13 | > 1 | — | 32 | 32 |
| | 17 | — | 256 | 256 |
| G. | < 1 | — | <8 | <8 |
| | 5 | — | 128 | 128 |
| | 37 | — | 256 | 256 |

† The antigens employed were prepared from measles infected monkey kidney tissue cultures: V–3 and V–14 = 3rd and 14th passage, respectively, of agent from patient V, XI-5 = 5th passage of agent from patient XI.
* Titers expressed as reciprocals of serum dilution.
— indicates: not done.

*Infection Experiments in Rhesus Monkeys.*

Two Rhesus monkeys born at this Institute and about one year of age were used for the infection experiments. Before inoculation sera from both animals were examined for the presence of naturally acquired antibodies against measles. No such antibodies could be demonstrated. Both monkeys were inoculated intranasally and orally with passage material of the virus strain isolated from patient No. 11. This strain had been found to produce uniform cytopathic changes in all passages and complement-fixing measles antigen had been present in all subcultures.

During the following month the monkeys were carefully observed, and the temperature was measured daily. On the 11th day after inoculation one of the animals showed a universal rash beginning at the head and later spreading all over the body and the limbs. The skin eruption which was similar to that seen in human measles patients lasted for only 24 hours. The monkey's temperature was within normal limits. A blood sample taken the day after the disappearance of the

exanthem (day 13) showed a titer of $< 1:8$ in complement fixation tests while blood taken 8 days later had a titer of 1:256. This monkey had been inoculated with material from the 21st passage of measles virus. The antigen used in the complement fixation tests was prepared from the 5th transfer of the virus strain used for the inoculation of the monkey.

The other monkey which was inoculated with material from the 9th passage of the same measles strain showed no symptoms during a period of one month. In complement fixation tests, however, it was possible to demonstrate an increase in the serum titer from $< 1:8$ to 1:256 in blood samples taken at the time of inoculation and 17 days later, respectively. The antigen employed was the same as mentioned above. The sera from both monkeys were later retested with antigen prepared from the 14th tissue culture passage of the virus strain recovered from patient No. 5. The serum titers were identical with those obtained in the presence of the homologous antigen.

## DISCUSSION

The present work confirms the findings of *Enders & Peebles* (6) and *Cohen et al.* (3) that throat washings and blood from patients in the early stage of measles contain a virus which is able to produce characteristic cytopathic changes in cultures of human and simian kidney cells. In the inoculated cultures the same syncytical formations were observed as those described by *Enders & Peebles* (6). In addition it was found that in the tissue culture system employed in this laboratory other specific lesions developed on continued incubation. This second step of the degeneration resulted in the accumulation of cell debris with circular gritty-looking formations which had either a quite smooth or a wrinkled margin. These cytopathic changes appear to be as specific for measles virus as are the syncytia.

However, monkey kidney viruses or "foamy agents" may give rise to cellular degenerations which microscopically are indistinguishable from those caused by measles virus. For this reason the cytologic manifestations are of limited value in the study of measles and additional criteria are required to establish the identity of the cultivated agents. This can be achieved by the demonstration of intranuclear inclusion bodies in the infected cell cultures (6) or by tests for the presence of measles antigen using either the fluorescent antibody technique (3) or the complement fixation reaction (3, 6). Because of its simplicity this last mentioned method has been employed in the present study. Observations on the development of measles antigen in the various cultures will be described in detail in a forthcoming publication (1).

In the study presented in this paper measles virus was isolated from five out of nine throat washings collected within 24 hours after the outbreak of exanthem. The reason why the isolation failed in one

patient (No. 12) may be that he had a vomiting immediately before gargling. In the remaining three cases (Nos. 2, 7 and 9) no obvious explanation for the negative results can be offered. Possibly it was because these isolations were all attempted with throat swabs in young children who were very excited and the sampling thus difficult to perform.

Virus was recovered from the blood in only one out of eight attempts made within 24 hours after the onset of the rash. This isolation rate is low compared with that obtained by *Enders & Peebles* (6) who recovered virus from the blood of four out of five patients. These authors used heparinized blood from which it may be easier to recover virus than from serum containing resuspended blood cells. In our laboratory the only isolation from the blood was made from one of the three samples to which heparin had been added. Also, Enders and Peebles used a larger inoculum (0.5 ml to 2 ml) than employed in this study (0.25 ml).

The possibility was considered that the failure to recover virus from 8 of the 13 patients examined might be due to an insusceptibility to measles virus of the particular cells employed. This, however, was apparently not the case, for when tubes prepared simultaneously with those employed in the unsuccessful isolation experiments were inoculated with measles infected fluid typical cytopathic changes developed together with the appearance of complement-fixing antigen in the nutrient media.

The observations presented in this paper agree well with the assumption that the isolated agents are the cause of measles. In the first place the 5 virus strains were recovered from the throat or blood of patients in the early acute phase of measles infection. All attempts to isolate virus later than 24 hours after onset of the rash failed. Also, a definite rise in complement-fixing antibodies against the isolated agents occurred during convalescence in all patients examined. Further evidence that the isolated agents are responsible for measles was obtained by the transmission of the disease to Rhesus monkeys by administration of tissue culture virus. One of these animals developed a measles-like exanthem which appeared on the 11th day after inoculation, a time-interval which is in accordance with the development of the disease in man. It is of interest to note that this monkey had been inoculated with the 21st tissue culture passage while the other animal which showed no clinical symptoms had been given 9th passage tissue culture material of the same strain. There was thus no indication that the virus became attenuated for monkeys with prolonged passage in tissue culture. Nor could the different response of the animals be ascribed to a previously acquired immunity against measles. Both animals had been born at this institute and neither possessed antibodies to measles before inoculation. They both responded to the infection with a marked increase in complement-fixing antibodies.

The results of the transmission experiments agree well with those obtained by *Enders* (7) and by *Black, Reissig & Melnick* (2). These investigators, however, introduced the virus by intravenous or by intramuscular injections while the "natural" route of infection was used in the experiments presented in this paper.

In connection with the examination of acute- and convalescent-phase sera from measles patients it should be mentioned that patient G. (Table 2) was one of several Greenlanders who were infected during a large epidemic of measles which occurred during September–October 1955 in a big community in Greenland. Several other paired sera obtained from measles patients during this epidemic have been tested against measles antigen prepared from virus strains isolated from sporadic cases in Copenhagen. In all tests a clearcut rise in antibodies could be demonstrated (unpublished data). This observation substantiates further the etiologic relationship of the isolated agents to measles.

## SUMMARY

(1) Virus agents have been isolated in trypsinized monkey kidney tissue cultures from throat washings and blood from 5 out of 13 patients examined during the acute phase of measles. In all instances, virus was isolated from throat washings or throat swabs while only one strain was recovered from the blood. All attempts to isolate virus later than 24 hours after onset of the rash failed.

(2) The cytopathic manifestations observed in measles virus infected tissue cultures as well as in uninoculated control tubes are described. Complement fixation tests for the presence of measles antigen have been used as criterion for the presence of this virus in the infected cultures.

(3) Intranasal and oral administration of material from late passages of one of the isolated agents to two Rhesus monkeys resulted in a pronounced measles-like rash in one of the animals, and both monkeys developed antibodies against the inoculated strain.

(4) In serological studies of acute- and convalescent-phase sera from measles patients using measles virus infected cultures of monkey kidney tissue as antigen in complement fixation tests a clearcut rise in antibodies was observed in all cases.

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