A CYTOPATHOGENIC AGENT ISOLATED FROM A CASE OF AN UPPER RESPIRATORY ILLNESS RESEMBLING AN ADENOVIRUS INFECTION

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Several cytopathogenic agents have already been reported to be isolated from upper respiratory illnesses (Price, 1956; Chanock, 1956; Pelon, Mogabgab, Phillips and Pierce, 1957) besides adenovirus group. During the studies of adenovirus infections and other respiratory illnesses, a cytopathogenic agent has been isolated by the present authors from an infant suffering from a febrile illness accompanied with pharyngitis. The present paper is to describe a report of the patient and certain characteristics of the isolated agent.

MATERIALS AND METHODS

Virus isolation was carried out by HeLa cell inoculation. Detailed techniques for handling and inoculating HeLa cell cultures were already given previously (Fukumi, Nishikawa, Nakamura, Watanabe, Kitayama and Fujita, 1957).

Infectivity (TCID50) was estimated by inoculating 0.1 cc of 10 fold dilutions of the agent into groups of 3 to 5 HeLa cell tube cultures. Cytopathogenic effects were checked by every other day through microscopy of low magnification for 10 days.

Quantitative neutralization was done by inoculating mixtures of virus suspension and 10 fold dilutions of serums to be tested into groups of 2 HeLa cell tube cultures. Virus dilution, which caused cytopathic changes in HeLa cells just 2 days after inoculation, was employed for the test. Neutralization titers of serums were determined on the basis of inhibition of cytopathogenic effects in the way that cytopathic changes in the test cultures were compared with those in the control cultures, which were inoculated with virus suspension (2 times diluted in Hanks' ballanced salt solution) alone.

The procedure employed for testing the presence of neutralizing antibodies against the agent in question is as follows: Two HeLa cell tube cultures were inoculated

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with mixtures of serums to be tested (2 times diluted in Hanks' ballanced salt solution) and virus dilution (the same dilution as that employed for the quantitative neutralization). If HeLa cell cultures thus inoculated showed the same degree of cytopathic changes as those control HeLa cell cultures, that were inoculated with virus suspension alone, the serum tested was determined to contain no neutralizing antibody. Those serums, by which cytopathogenic effects were delayed or completely inhibited in HeLa cell cultures, were determined to contain more or less neutralizing antibodies.

Estimations of hemagglutination inhibition titer against influenza viruses were made after destroying non-specific inhibitors by receptor destroying enzyme (RDE) prepared by us from cholera filtrate.

Examination of cytopathic changes in stained HeLa cells was made by hematoxylin and eosin staining after fixation with Zenker's solution of infected HeLa cells cultivated on cover glasses.

REPORT OF THE CASE

3 years old, male—His parents said that he had been attacked by the influenza virus of A/Asia/1957 type on July 2nd or 3rd, 1957. In Tokyo, the first epidemic of the influenza of this type began at the beginning of May, 1957 and temporarily subsided in the end of July, 1957. They also announced that his fever had subsided on the next day. After that, however, his body temperature rose again up to 38° to 39°C every 2 or 3 days, and on July 16 again he had a fever of 39°C at night. On the next day he visited hospital though his fever subsided.

On the day of his visiting hospital, his throat and tonsils were remarkably injected and his both conjunctivas also considerably injected. Throat swab was taken for suspicion of adenovirus infection.

His acute and convalescent serums were taken on July 17, and September 17, respectively. He had no intervening illness during this period. He recovered from his illness just after his visiting hospital.

LABORATORY EXAMINATIONS

**Virus Isolation**

The throat swab from the patient was inoculated into 3 HeLa cell tube cultures. About 8 days after inoculation, a questionable change was recognized in a part of the HeLa cell sheats and it was somewhat intensified and extended until 11 days after inoculation. The supernate of the cultures was passaged to 5 HeLa cell tube cultures. Cytopathic changes were this time unquestionably recognized 2 days after inoculation. Four days after inoculation the supernate medium of these cultures were collected and pooled together and its infectivity titration was made. It showed a value of 10^5.8/cc in TCD_{50}. Serial passage of the isolate was carried out further successfully in HeLa cell cultures.

**Behavior of the Isolated Virus in Tissue Cultures**

Though on primary isolation in HeLa cell cultures questionable cytopathic changes were first recognizable 8 days after inoculation, unambiguous changes were already observed 24 hours after inoculation and yet quite distinctive after second passage if virus was inoculated in sufficient dose. But with limiting
infective doses production of observable changes required about 5 to 7 days or sometimes more. When large does of virus was inoculated, cytopathic changes were completed in a few days after the onset of the changes and the degenerated cells fell off from the glass wall.

Cytopathic changes caused by this agent were somewhat similar to those due to polioviruses or Coxsackie B viruses. The first changes seen in infected HeLa cells was the rounding of cells in scattered areas (Fig. 1). Some of the rounded cells fell off from the glass surface separately and others got together to make small cell clumps and then gradually fell off (Fig. 2 and Fig. 3).

Further details of the cytopathic changes due to the virus in question were obtained through microscopy of stained preparations. At first, slight deformation of the nucleus was recognized and almost at the same time, the cytoplasm retracted from the normally spread state (a in Figs. 4, 5, and 6). The cytoplasmic retraction was observed to progress further and as the result, the cytoplasm became stained thick. With parallel to these cytoplasmic changes, the nuclear deformation also proceeds, and the nucleus was finally observed to be picnotic and stained thick with hematoxylin (b in Figs. 4, 5, and 6). Sometimes, in the cytoplasm rather near the nucleus, a somewhat eosinophilic area was observed (c in Figs. 5 and 6), just as those described in the case of poliomyelitis viruses (Reissig, Howes, and Melnick, 1956). Those cells whose cytopathic changes went fairly far were very apt to fall off from the glass surface and sometimes they attached to it in a single spot so as to shift their position onto nearby standing cells during the staining processes (d in Fig. 6).

The virus is now tentatively called by us as 57-67 virus, because it was isolated from the patient No. 67 of our virus-material list in 1957. The infectivity in the supernate of the HeLa cell cultures infected with 57-67 virus and showing remarkable cytopathic changes was quite considerable. It was of the order of $10^7$/cc in average.

The 57-67 virus showed no pathogenicity to adult mice through intracerebral inoculation. It also caused no abnormality in suckling mice through intracerebral or intraperitoneal inoculation. The 57-67 virus produced no pock on chorioallantoic membrane of embryonated hens eggs. The 57-67 virus produced no hemagglutinin to chicken red blood cells through intraallantoic or intraamniotic inoculation into embryonated hens eggs. These eggs showed no abnormality or no death.

**Differentiation and Classification of the Virus 57-67**

Experiments were carried out to determine whether the 57-67 virus was neutralized by the antisera of the following virus strains.

a) **Myxoviruses.**

Influenza A/Shinjuku/1/1956, B/Yamanashi/5/1956, A/Adachi/2/1957 (A/Asia/1957 type), Influenza C 1233, mumps virus (Enders strain), Newcastle Disease virus (Tokyo strain), HVJ (Fukumi, Nishikawa and
b) Coxsackie group of viruses.
Coxsackie viruses A 1 through A 10, A 12 and A 14.
Coxsackie viruses B 1 through B 4.

c) Polioviruses.
Polioviruses types I, II and III.

The antisera against myxoviruses were all prepared from roosters. The antisera against Coxsackie group of viruses were prepared from mice or rabbits. The antiserums against polioviruses were obtained from the regional poliocenter in Japan by the courtesy of Dr. M. Kitaoka. These antisera were all employed at a dilution of 1:10. An adequate dilution of the 57–67 virus was mixed with 1:10 diluted antisera and then inoculated into HeLa cell tube cultures. Virus dilution causing cytopathic changes in HeLa cells just 2 days after inoculation was employed.

It was found by these experiments that the 57–67 virus was not neutralized by the antisera against the above mentioned viruses.

The 57–67 virus is considered to be different from CA virus (Chanock, 1956) and adenoviruses from the morphological point of view because the cytopathic changes in HeLa cells due to the 57–67 virus are quite different from those of CA or adenoviruses infected cultures.

The 57–67 virus is considered to be different from herpes simplex virus because the former is not pathogenic for chorioallantoic membrane of embryonated hens eggs or mice, and further because that cytopathogenic effect is morphologically quite different from the latter.

The 57–67 virus is considered to be different from the virus isolated by Price (1956) and from the virus isolated by Pelon, Mogabgb, Phillips and Pierce (1957), because these viruses are reported not to be cytopathogenic to HeLa cells according to the authors.

ECHO group of viruses are said to be got adapted to HeLa cells (Archetti, Weston and Wenner, 1957), but primary isolation of this group of viruses through HeLa cells is reported to be quite rare. We did not try to differentiate the 57–67 virus immunologically from the ECHO group of viruses, because the antiserums against these viruses were not available.

Berge. England, Mauris, Shuey and Lennette (1955) isolated an “unidentified non-APC agent” from upper respiratory ill cases producing cytopathic changes in HeLa cells in a military recruit. It is reported to be different immunologically from HE–1 through HE–5 strains of the ECHO groups of viruses (Ramos-Alvarez and Sabin, 1954). It is not quite certain whether the 57–67 virus differs from this virus or not, because the descriptions of the characteristics of the virus by Berge and his coworkers are far from completeness and there is even no mention of the characteristics of cytopathic changes in HeLa cells caused by the virus. However, the facts that they isolated it from throat
and that it was isolated by HeLa cell inoculation, suggest strongly the importance of the comparison between the 57-67 virus and the virus isolated by Berge and his coworkers.

**Examination of the Patient's Serums**

It has already been mentioned that serums were taken from the patient on July 17 and September 17. These serums were examined for their titers against various serodiagnostic antigens. The results are summarized in Table 1. Streptococcus MG agglutinin titers do not seem to be significant for the patient's illness. His both acute and convalescent serums do not contain more than 1:2 of complement fixation antibody titer against adenoviruses. The complement fixation antigen for adenoviruses employed here was prepared from adenovirus type 5 (Mayeda strain) infected HeLa cell cultures. This indicates that his illness was not due to adenovirus infection.

He does not seem to have been attacked by the old A type or the current B type of influenza viruses. The influenza virus A/Kojiya/1/1952 was one of the strains isolated from the A type epidemic of influenza in 1953 in Tokyo and of the so-called Scandinavian type (Issacs and Andrewes, 1951), while the influenza B/Meguro/9/1956 was also isolated from influenza B epidemic in Tokyo in 1956. His both serums had no appreciable amount of hemagglutination inhibition antibodies against these influenza viruses.

As already mentioned above, the influenza virus A/Adachi/2/1957 was of the type A/Asia/1957, isolated in Tokyo. The acute serum of the patient taken on July 17, already showed a hemagglutination inhibition titer of 1:512. The influenza of A/Asia/1957 type started in Japan in the beginning of May, 1957. Therefore, it is certain that he had experienced an infection of this type virus during the period of the beginning of May to July 17, 1957. But, he had never had an upper respiratory infection except for the illness which began in the beginning of July. Thus, it is suggested that the illness of the patient on 2nd of July must have been due to A/Asia/1957 type virus (according to our ex-

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The serum of the patient taken on July 17, had a neutralizing antibody titer of 1:16 against the virus 57–67, while his serum taken on September 17, had a titer of 1:256. The antibody rise in the convalescent stage is very remarkable. This is reasonably considered to indicate that his illness in question must be due to the infection of the 57–67 virus. It is already suggested that his illness on July 2, is due to A/Asia/1957 type virus. Then, the question, when the 57–67 virus infection started in this patient, is raised. Already on July 17, he had neutralizing antibodies against this virus though of a small amount, and this may suggest that his illness due to the 57–67 virus began 2 or 3 days after the subsidence of his influenzal infection, namely probably on July 4 or 5, when his intermittent fever started according to his parents' announcement.

At any rate, it is very probable that his illness following influenza infection was due to the 57–67 virus infection.

Serological Surveys of the Antibody Distribution of the 57–67 Virus in Normal Populations

So far we have had only one case of the 57–67 virus infection, just described above. The next problem seems to us to know how this virus is distributed in the people, and so, at first, we tried to know it by examining the distribution of serum antibodies against this virus in a few groups of people. Examinations were made only to know presence or absence of the neutralizing antibodies qualitatively, and therefore no quantitative titrations were carried out.

a) Examinations of students in the Nurse School of the Tokyo First National Hospital: Twenty six students of the first school year were selected at random and their bloods were taken. They were in average 18 years of age. Among them 10 had no recognizable antibody against the 57–67 virus, therefore the rate of the antibody positive students is calculated to be approximately 61%.

b) Examinations of infants in Ninomiya Branch Nursery of the Tokyo First National Hospital: Thirty two infants (about 2 to 3 years of age in average) were examined for the 57–67 virus antibodies in their bloods. As 13 infants among them had no antibodies, the rate of the antibody positive infants is calculated to be about 59%.

In short, it may be presumed that in a considerable part of the population at least in Tokyo and its suburban areas people have neutralizing antibodies against the virus 57–67.

DISCUSSION

Further studies are requested to decide whether the 57–67 virus is a new virus or not, but it is quite possible that this virus was responsible to a patient who had a fever and a kind of pharyngitis.
It is suggested by our serological surveys that the 57–67 virus is distributed fairly widely. Accordingly, it is considered to be important to know a clinical feature which this virus causes commonly. Though it is clear that the virus infects upper respiratory tracts in human beings, whether or not such a simple clinical feature is a commonest occurrence for this virus remains to be studied.

The cytopathic changes caused by this virus in HeLa cells are rather similar to those due to polioviruses or Coxsackie B group of viruses, but quite distinctive from those due to adenoviruses or herpes simplex virus. To establish a position of the 57–67 virus in the virus classification, further studies must be made, especially about its size and shape of virus particle.

**Summary**

The 57–67 virus, probably a new virus, has been isolated from a febrile upper respiratory illness by cytopathogenic effects in HeLa cells.

Serological surveys of healthy people reveal that the virus is fairly widely spread at least in Tokyo and its suburban areas.

**References**


Figs. 1 to 3.
HeLa cells infected with the 57-67 virus. Unstained.
Figs. 4 to 6.
HeLa cells infected with the 57-67 virus. Stained with hematoxylin and eosin after fixation with Zenker's solution.