Establishment of a method to isolate influenza virus by using a cell culture and application of the method for epidemiological analysis

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Epidemics of influenza in Japan usually occur suddenly in winter season and spread explosively and this pattern repeats every year.

However much is left unknown on the detail of the epidemics.

In order to know exactly details of the epidemics and its correlation to antigenic mutation of the virus, it is neccessary to accumulate the epidemiologic analysis consisting careful detection of the epidemic virus. Clarification of the year-to-year mutation of the virus and ascertainment of the direction of the virus evolution.

When we try to isolate influenza viruses for this purpose, the present conventional method is so tedious and time consuming that it is difficult to manage many clinical samples effectively and rapidly collected during long period.

For this reason, I studied a method of stirring suspension culture of MDCK and ESK cell lines which will provide enough amount of cells which can be used immediately, and investigated the practical application of the method for isolation of influenza virus.

In addition, I investigated a method with which the cell preserved in frozen state could be directly used for the isolation of virus, which could be supplementally employed in place of the fresh suspension cell culture.

According to the results of this experiment, the new method seemed to be sufficiently applicable for the epidemiologic study, as many specimens could be managed effectively and rapidy with the method.

Especially it was found that ESK cell were more sensitive than MDCK

cell for isolating virus from clinical specimens.

To detect the haemagglutinating activity of influenza virus is an indicator of the virus growth in fertile hen's egg or cell cultures, and chicken red cells are conventionally used for the work. In this study, I also investigated the efficiency of guinea pig red cells to detect the haemagglutination by influenza virus, in order to develop a more efficient method. Application of the neuraminidase activity, which in a biological character of influenza virus and an indicator of the virus growth, for the field work of isolating virus was also investigated.

By the methods described above, influenza-like diseases in Nagano Prefecture were continuously surveyed and its epidemiologic analysis was undertaken.

In order to know the immune state before epidemics, blood specimens were collected from haelthy people in Nagano Prefecture in October every year, and the antibody titer of the blood sera was measured.

Influenza virus isolates were analyzed antigenically by using reference antisera in order to clarify the antigenic variation of the viruses. Influenza epidemics usually occur in Japan in winter season.

However viruses isolated after or at the end of epidemics in spring are antigenically different from the main epidemic viruses in the preceeding winter, and it will become the prevailing epidemic virus in the next epidemic season, which is called the hypothesis of the influenza herald wave. Phenomenon of the herald wave was observed during my follow-up studies of influenza,i.e., antigenic similarity between herald and epidemic virus strains were confirmed by using the haemagglutination inhibition [HI] test. In order to clarify the genetic relatedness between them, base sequences of viral genomes of the herald and epidemic viruses were analyzed. This experiment was performed in both of H1N1 and H3N2 type A viruses.

Following is the outline of this report.

1. Investigations on the method of isolating influenza virus.

The basal medium for cultivating cells was MEM Joklik suspension culture-fluid supplemented with 10% of newborn calf serum and 3% of a methyl cellulose solution(30 mg/ml). A 10% of tryptose phosphate broth (29.5 mg/ml) was added to the basal medium for the culture of MDCK cells, and 5% of polypeptone solution (100 mg/ml) was added to the basal medium for the culture of ESK cells. The cells grew well in spinner flasks stirred at 200 rpm in a 37℃ incubator, and enough amount of dispersed cells in a nice conndition could be obtained.

Reference strains of influenza viruses were inoculated into various concentrations of the cell suspensions and the highest HA (haemagglutinin) production was observed at the concentrations of 5.5×10^6 and 6.0×10^6 cells/ml of MDCK and ESK cell, respectively.

Isolation of virus from clinical specimens obtained during influenza epidemics was undertaken by using the optimal concentration of the each cell suspension, and any results of the isolating virus were not worse than that by using the fertile hen's eggs or the monolayer cell culture.

More than 95% of MDCK cells were alived at -80℃ for 6 months, if 10 % of dimethyl-sulfoxide was added as a preservative. When the thawed suspensions of the freez-preserved cells were inoculated with reference influenza viruses and HA titers of the inoculated cell cultures were measured, sensitivity of the cells to the inoculated viruses was estimated equivalent to that of the monolayer cell culture or of the freshly cultured cell suspension.

2. Investigations on markers of the virus growth during the work of virus isolation.

Usefulness of guinea pig red blood cells for detection of the viral haemagglutinin and efficiency of the NA (neuraminidase) activity as makers of the virus growth during the virus isolation was studied.

All of the isolated viruses agglutinated the guinea pig red cells but a considerable number of the virus isolates did not agglutinate the chicken red cells. This was obvious in type A(H1N1) viruses and up to 48.8% of H1N1 isolates agglutinated the guinea pig cells but not the chiken cells. On the contrary, in case of the type A(H3N2) and type B viruses, almost no difference was observed between the guinea pig and chicken cells as far as the efficiency of the virus isolation concerns. But many viral strains of H3N2 and type B showed lower haemagglutinating activity to the chiken red cells than to the guinea pig ones, and provided the chiken cells were used, the haemagglutinating titers of the primary cultures of the many viral isolates were too low to be employed for the HI (haemagglutination inhibition) test.

Consequently the guinea pig red cells had a higher sensitivity to the viral haemagglutinin than the chiken red cells had, and the former was better than the latter cells to detect the virus growth during the work of isolating the virus.

Efficiency of the haemagglutination and the neuraminidase tests as a marker of the virus growth in the primary culture of the virus isolation were compared, and it was found that all of the cultures with the positive haemagglutinin titers(1:4 or higher) gave the corresponding positive neuraminidase titer but some of the samples with negative haemagglutinin titer (less than 1:4) showed the positive neuraminidase activity. Moreover, inoculated cultures, the absorbance of which was 0.05 or more in the nuraminidase test, had to be passaged one more time, before their sufficient haemagglutinin titers were obtained.

Therfore, rate of the virus isolation could be improved,if a primary culture, which showed 0.05 or more absorbance value in the neuramni-

dase test, were passaged one more time without discarding.

3. Epidemiological study of influenza epidemics

Virologocal examination was performed on patients with influenza -like diseases at two fixed medical points in Nagano Prefecture during the period from January, 1986 to July,1989. In addition, patients with influenza-like diseases in Chiang Mai, Thailand was also examined in Augast, which is the interepidemic period in Japan, from 1986 to 1989.

The epidemics of influenza and other viruses were surveyed based on the isolated viruses, and the epidemic periods of influenza were estimated with the results of the virus isolation. Moreover, in each epidemic of influenza defined from results of the virus isolation, serum antibody level of the patients and their history of natural infection and of vaccination were analysed from the epidemiological viewpoint.

Blood samples were collected from healthy peaple in Nagano Prefecture in October every year and the antibody level was measured, in order to study the immunity level before the epidemics.

Result of the virus isolation : An epidemic of each virus and its duration in each geographical area was exactly determined with isolation of virus in fixed medical points throughout the year. Almost all of the prevailing influenza viruses were recognized in the herald waves before the epidemics.

Isolation of pathogenic viruses other than influenza was attempted at the same time, and it was clarified that there were very small number of cases of the non-influenza cold at the peak period of the influenza epidemics and that there were many cases with the cold infected with viruses other than the influenza before and after the period of influenza epidemics. Epidemics of the enteroviruses prevailing viruses of which varied every year, were demonstrated, also.

With isolation of the viruses in Chiang Mai, Thailand, moving of the influenza virus during the interepidemic period of Japan was clarified and many foreseeing suggestions were obtained on the influenza epidemics in Japan in the next winter seasons. In case of viruses other than the influenza, isolated viruses were of similar kinds to those isolated in Japanese fixed medical points, and good correlations of epidemic viruses were found between Chiang Mai and Japan in case of the enterovirus suggesting a possible global current of prevailing viruses like in case of the influenza virus.

Correlation of serum antibody titers of the patients during the epidemic period to the influenza infection and the vaccination : Epidemiological analysis of data obtained at fixed medical points, where clinical specimens were collected during the epidemic periods, gave following results. Time of beginning and ending of influenza epidemics varied from year to year by about two months, and it differed by one or two weeks between Matsumoto and Suzaka.

Rate of the isolation of influenza virus from patients was generally high in the young age group, but sometimes the virus was isolated at a high rate even from the old age group. This finding may suggest that not only school children and pupils, which is an amplifying group of the influenza epidemic and subjected to the specific public health programme, but also adults and aged people should be necessarily included to the sensitive group and subjected to the prevention programme of influenza.

The serum antibody titer of patients infected with influenza virus were very low at the acute phase irrespective of their vaccination history, and few of them had the antibody titer over 1:128 to the prevailing virus. The antibody titers of patients not infected with influenza were obviously higher at the acute phase of disease than those of the people infected with influenza and most of the people,

who had been injected the vaccine and protected from influenza, were considered to have acquired the high immunity which could be sufficient to prevent the infection. Some patients were found infected by influenza even though they had received the vaccine, but most of them had low titers of the antibody,i.e., lower than 1:128.

Basic antibody titer of young age groups were high, and after the infection they acquire higher antibody titers owing to a sensitive immune responsibility. But adults, especially aged people had a low basic antibody and many of them did not show a significant increase of their antibody titer after the infection.

Thus, it was found that aged people were infected with influenza at a rate not lower than young people, that their basic antibody titers were low, and that they had not been actively immunized with the vaccine. Therefore,considering a measure for adults and especially for aged people, it seems necessary to raise their basic antibody titers with an intensitive vaccination, because they easily suffer from a fetal sick after the infection of influenza.

Relationship between influenza epidemics and the antibody level of people before the epidemics : It was difficult to obtain any information to forecast the prevailing virus from the antibody level before the epidemic season, becouse the antigenic mutations of any prevailing viruses during 1986 throuh 1989 were of continuous and actually no new antigenic type was detected.

However,the epidemic cycle of influenza viruses,theory of the herald wave and results of the virus isolation in Thailand gave suggestion on a virus which might prevail in Japan in the coming next winter, so that, to a certain extent, scale of the coming Japanese influenza epidemics could be estimated in advance. Against all the studied viruses, the low age group and especially young people of 5 to 19 years of age had relatively high antibody level both in percentage of

individuals with the positive antybody and in their antibody titers.

But adults had a very low antibody level in terms of the percentage of the antibody-peritive and of the antibody titer. Therefore, it will be noticed that the adult group, especially aged group, should be subjected to a some measure to raise the antibody titers.

4. Antigenic mutation and evolution of influenza viruses

Antigenic analysis with the cross HI (haemagglutination inhibition) test : Influenza viruses isolated in Japanese fixed medical points and at Chiang Mai were antigenically analyzed by using antisera raised to the viral strains contained in Japanese vaccines or other reference strains.

Viruses isolated at the fixed medical points during the epidemic period of 1986/87 and viruses isolated at the herald wave in 1986 were antigenically similar to the A/Yamagata/120/86 conteined in the vaccine for the 1986/87 season.

A virus strain, isolated as an expected herald wave virus in May, 1987, was antigenically similar to A/Osaka/156/87 which is somewhat different from A/Fukuoka/C29/85 contained in the vaccine for the 1987/88 season. Viruses of type A(H3N2) isolated in the winter of 1987/88 consisted two variants, one of which was antigenically similar to A/Fukuoka/C29/85 contained in the vaccine of 1987/88 and the other was similar to A/Osaka/156/87 which was somewhat different from A/Fukuoka/C29/85, but the main prevalent was A/Osaka/156/87-like viruses. Type B viruses isolated during the epidemic season of 1987/88 consisted B/Nagasaki/3/87-like and B/Yamagata/16/88-like ones, but the main prevailing virus was B/Nagasaki/ 3/87-like one.

Most of the type A (H1N1) viruses isolated in the epidemic season of 1988/89 and in the herald wave in 1988 were antigenically similar to A/Yamagata/120/86, but slightly different variants circulated together

with A/Yamagata 120/86-like virus. A virus of type A (H3N2) isolated as an expected herald wave virus in March, 1989 was antigenically similar to A/Hokkaido/20/89 which was somewhat different from the virus contained in the vaccine. A herald wave virus of type B isolated in April,1989 was B/Aichi/5/88-like one.

A virus of type A (H3N2) isolated from a clinical specimen collected in Chiang Mai, Thailand in Augast,1986 was A/Yamagata/120/86-like one, which is an evidence that an antigenically identical viruses prevailed in Chiang Mai before it prevailed in Japan.

From clinical specimens of Chiang Mai in Augast, 1987, type A (H1N1) and type B viruses were isolated. It was found that, on the type A (H1 N1) viruses, the A/Yamagata/120/86-like viruses which were isolated in Chiang Mai in Augast, 1987 and prevailed in Japan in the winter of 198 7/88 had a same antigenic pattern as viruses isolated in Japan in the epidemic season of 1988/89.

Type B viruses in Chiang Mai were antigenically similar to B/Nagasa ki/3/87. The herald wave virus of B/Nagasaki/3/87-like virus was not found medical fixed point in Nagano Prefecture, but B/Nagasaki/3/87-like one existed in Chiang Mai five months before it prevailed in Japan.

Type A(H3N2) viruses isolated from clinical specimens in Chiang Mai in Augast, 1988 were antigenically close to or somewhat dufferent from A/Fukuoka/C29/85. The virus antigenically close to A/Fukuoka/C29/85 was considered antigenically nearly same as those isolated in Japan in the season of 1987/88, and the virus different from the A/Fukuoka/C29/85 was supposed to be close to those isolated in Japan the season of 1989/90.

From clinical specimens in Chiang Mai in 1989,three kinds of viruses were isolated.i.e., type A (H1N1), A (H3N2), and B.

The type A (H1N1) viruses consisted A/Yamagata/120/86-like viruses

and viruses somewhat different from A/Yamagata/120/86.

The type A (H3N2) viruses consisted A/Sitchuan/2/87-like virus,A/Akita/4/88-like virus which prevailed in Japan in 1987/88 season, and Japanese prevailing virus in 1989/90 which were antigenically slightly different from A/Sitchuan/2/87 or A/Akita/4/88. All of type B viruses were B/Yamagata/16/88-like ones. All of the virus isolated in Japan in the epidemic season of 1989/90 were antigenically similar to B/Yamagata/16/88-like viruses isolated in Chiang Mai in Augast, 1990.

Briefly, some mutant viruses had prevailed in Thailand in the preceeding Augast before they prevailed in Japan in the epidemic season of the following winter. In addition, viruses which prevailed in Japan one or two years before also cocirculated in Thailand.

Study of evolution of influenza viruses at the molecular level by analysing the base sequence of HA genes : When the herald wave viruses were confirmed with HI test, the genetic relationship between the herald wave virus and the dominant epidemic virus in the following winter was clarified by constructing the evolutionary tree based upon the analysis of nucleotide sequence of the HA gene.

In one case in H1N1 viruses in 1986, the spring isolates (herald wave viruses) were genetically close to some of the winter isolates and both isolates were considered to have originated from a same parental virus. After the epidemic in 1986/87 ceased, A/Nagano/1396/88 was the first type A (H1N1) isolate in Japan in 1988, A/Nagano/1396/88 was observed to have achieved two mainstreem change at positions 303 and 553 from A/Yamagata/120/86 which was isolated in the 1986/87 influenza season. But,epidemic viruses in the 1988/89 influenza season were located on another branch than that of A/Nagano/1396/88.

Therfore, 1988/89 epidemic viruses were unlikely to be any direct descenclents of A/Nagano/1396/88 which circulated in the preceding spring. A/Nagano/1046/87, the only H3N2 isolate in Japan in the spring

of 1987 after the 1986/87 influenza season, epidemic viruses in the following winter of 1987/88, and A/Chiang Mai/156/88 were located closely to each other on three branches and these three were considered genetically close and derived from a same origin. Similarly, viruses isolated in Tokyo and Chiba in the season of 1987/88 and those in Kanagawa in Augast, 1988 were determind to have derived from other origins than that of a group of viruses represented by A/Nagano/1046/87, and considered close to A/Sitchuan/2/87, while A/Nagano/184/88, and A/Kobe/768/88, both of which were isolated during the epidemic period of 1987/88, were located on the same branch derived for the stem on a mainstream change away from A/Sitchuan/2/87,i.e., they had another origin then the two former groups of H3N2 viruses.

Thus, A/Nagano/1046/87,a herald wave virus in the spring of 1987 was a parental virus of a part of epidemic viruses, but other viruses derived from other origins also cocirculated in the influenza season of 1987/88.

It will be necessary to continue this kind of the epidemiological survey of influenza centered in the study of the pathogenic viruses, to ascertain the antigenic mutations of influenza viruses and direction of the viral evolution and to prepare for appearance of a new type of the virus. In the genetic study of some harald wave viruses, their relationships to the succeedingly prevailed viruses were clarified by analysing the base sequenses of viral genes and a possible assumption was made on the survival of influenza virus during the interepidemic period, but on the viruses obtained in some herald waves further clarification of the ecological meaning of their appearance will be urgently needed. In addition, detail of the influenza infection of adult, especially of aged people, should be clarified and establishment of the measure to cope with the disease of that age

group will be necessary.