

PATHOGENICITY OF CRYOPHILIC AND THERMOPHILIC STRAINS OF INFLUENZA TYPE A2 VIRUS FOR DEVELOPING CHICK EMBRYOS AND ALBINO MICE

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Summary. — Strains of influenza A2 virus acquire different virulence for developing chick embryos after continuous cultivation at high (40–41° C) or low (26–28° C) temperatures. Influenza viruses passaged at 40–41° C became more virulent for chick embryos, while passages at 26–28° C resulted in a decrease of the virulence. A precise estimation of different virulence of cryophilic and thermophilic variants can be made on the basis of the intensity of their reproduction in the developing embryos at the temperature of 41° C. The phenomenon of an increased virulence of cryophilic variants for chick embryos was not observed in experiments on albino mice.

Introduction

Long-term cultivation of viruses at supra- (>37° C) or infraoptimal (<37° C) temperatures affects their reproduction activity and appears to be one of the most effective means of changing biological properties of the agent and, in particular, their virulence for susceptible animals. Multiple passages of viruses in developing chick embryos or tissue cultures at the temperature of 23–28° C have resulted in a decrease of their pathogenicity for man and susceptible animals, thus providing a possibility to prepare useful vaccine strains of influenza, poliomyelitis, measles or other viruses. This method of attenuation was used in this laboratory for preparation of additionally attenuated strains of influenza virus useful for an active immunization of children (Aleksandrova and Kugel, 1961; Aleksandrova and Smorodintsev, 1965). In contrast, passages of laboratory strains of poliomyelitis or Japanese encephalitis viruses at high temperatures increased their virulence (Lwoff, 1959; Inoue and Kato, 1963).

The aim of this investigation was to compare the pathogenicity of cryophilic and thermophilic variants of influenza A2 viruses for susceptible animals.

Materials and Methods

Viruses. The following viruses were tested: two inhibitor resistant strains of influenza A2 virus (A2/65/151 and A2/65/147); their variants obtained after 45 serial passages in developing chick embryos incubated at low (26–28° C) or high (40–41° C) temperatures; and control

Table 1. Pathogenicity and infectivity of two strains of influenza A2 virus variants for chick embryos

Variant	Strain A2/65/151						Strain A2/65/147					
	Day 3		Day 5		Day 7		Day 3		Day 5		Day 7	
	I	P	I	P	I	P	I	P	I	P	I	P
Original (3 passages at 36° C)	8.5	3.8	5.0	5.2	1.5	5.8	8.5	3.5	4.5	5.5	1.0	6.0
Control (45 passages at 36° C)	9.0	6.0	5.5	7.0	2.0	8.0	8.5	6.0	5.0	7.8	1.5	8.0
Thermophilic (45 passages at 40–41° C)	9.5	9.5	5.5	9.5	2.0	9.5	9.5	9.2	6.0	9.2	1.8	9.2
Cryophilic (45 passages at 26–28° C)	9.0	2.2	5.5	3.7	1.5	4.0	9.0	2.7	5.0	4.0	1.5	4.0

I = Infectivity in log EID₅₀ values.

P = Pathogenicity in log ELD₅₀ values.

lines passaged at 36° C. The viruses were cloned by three serial passages in developing chick embryos using the endpoint dilution technique.

Chick embryos. Ten- to 12-day-old developing chick embryos of Leghorn hens from the same stock were used. The embryos were thoroughly examined under the ovoscope before inoculation, and those with the signs of haemorrhages, injured or pale vessels were discarded. The inoculated embryos were incubated at 36.5–37° C.

Titration of virus infectivity in chick embryos. Groups of 16–18 chick embryos were simultaneously inoculated with 10-fold dilutions of each of the variants tested. An equivalent group of embryos was injected with saline. Virus infectivity was determined 3, 5 or 7 days later. At the days indicated, three embryos from each group were taken, chilled at 4° C for 18 hours and the occurrence of haemagglutinins in the allantoic fluid was recorded. To obtain more precise infectious titres, one additional passage of negative samples of the allantoic fluid from chick embryos inoculated with the endpoint dilutions of the viruses was attempted. The embryonic infectious dose (EID₅₀) was calculated by the method of Reed and Muench.

Determination of pathogenicity for chick embryos of the influenza virus strains tested. Different dilutions of the influenza virus strains tested were inoculated into allantoic sacs or amniotic cavities of developing embryos examined daily to determine the dates of the amnion deaths. Concurrent observations were carried out on the time of death of uninfected control embryos. Pathogenicity was calculated by the method of Yoshino *et al.* (1959) taking into account the mortality of uninfected embryos of not more than 5% for the embryos injected with saline and of 10% for those inoculated into the amniotic cavity. The number of deaths among embryos inoculated with different dilutions of viruses was calculated according to the formula $X = A - KB$, where X = actual number of deaths among embryos; B = number of embryos inoculated; and K = correction coefficient (0.05 for embryos inoculated into allantoic sac and 0.1 for those inoculated into amniotic cavity). The median lethal dose of the virus for inoculated embryos (ELD₅₀) was calculated by the method of Reed and Muench.

Determination of virus reproduction in developing chick embryos. Groups of 25 chick embryos were simultaneously infected with different virus variants (10²–10³ EID₅₀). An equivalent control group was injected with 0.2 ml amounts of saline. At the days 1, 3, 5 or 7, three embryos were taken from each group, chilled at 4° C for 18 hours and the respective allantoic fluids pooled. The virus concentration in the samples was determined by titrations in 10- to 12-day-old chick embryos. Lethality of embryos infected with similar doses of the virus was studied at the indicated time intervals. Deaths of embryos in both infected and control groups were recorded, indices of lethality calculated and expressed in per cent values.

Examination of virus pathogenicity for albino mice. Newborn or adult albino mice were infected intranasally under mild anaesthesia with virus-containing allantoic fluid, undiluted or diluted in 10-fold steps. The animals were observed for 12 days after infection.

Determination of virus toxicity for albino mice. Mice were infected by injections of 1.0 ml amounts of sterile virus-containing allantoic fluid into the tail vein. Difference between lethality of animals with no signs of pulmonary lesions 3 days after injection of normal infectious or allantoic fluid was considered to be due to the effect of influenza toxin.

Results

Virulence of cryophilic and thermophilic strains of influenza A2 virus for developing chick embryos

Table 1 shows the correlation between infectivity and pathogenicity of A2/65/151 or A2/65/147 strains of influenza virus and their cryophilic or thermophilic variants for developing chick embryos inoculated intraallantoically. All lines of the strains A2/65/151 or A2/65/147 tested intensively reproduced in the tissues of chick embryos and were similar in respect to the rate of their development and to concentration of the viral particles in the allantoic fluid. Peak values of this concentration were observed on day 3 (9.0 log EID₅₀) and then gradually decreased. One median lethal dose of the original viruses (A2/65/151 and A2/65/147) was equivalent to 2.5 to

3.5 log EID₅₀ of the virus, thus providing the basis for an explanation of the marked difference between their infectious and lethal activities. After 45 passages at 40–41° C, virulence of the viruses sharply increased and approached the values of infectivity (9.3–9.5 log EID₅₀/0.2 ml). In contrast, cryophilic variants adapted to reproduction at 26–28° C were characterized by the lowest pathogenicity for embryos: one median lethal dose for embryos (1 ELD₅₀) corresponded to 10⁵ EID₅₀.

Pathogenicity of the viruses was unaffected by the route of inoculation of embryos (into the allantoic sac or amniotic cavity), showing similar quantitative characteristics.

Differences in the level of virulence of these viruses were reflected by the degree of divergence between infectious and lethal activity. Injection of minimal doses (approximately 1 EID₅₀) of thermophilic strains into amniotic or allantoic sacs of embryos caused their deaths. Cryophilic strains of low virulence caused deaths of embryos when injected but in a massive dose (10⁵ EID₅₀), which was 100,000 times as large as the minimal lethal dose of thermophilic virus.

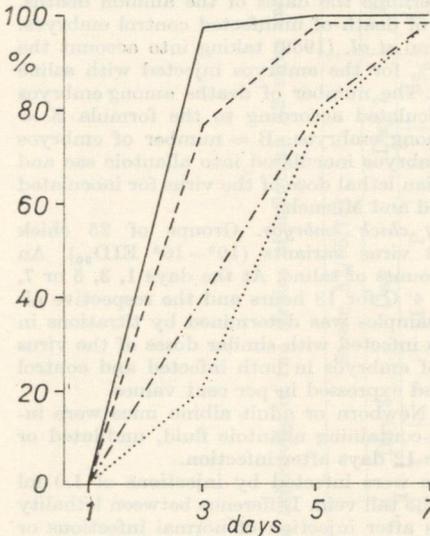


Fig. 1.
Mortality of chick embryos inoculated with 10³
EID₅₀ of influenza A2/65/151 virus variants
Variants: ——— thermophilic, - - - control,
- · - · - original, ····· cryophilic
Ordinate: mortality per cent

Differences in pathogenicity of influenza virus variants adapted to reproduction at low or high temperature was also manifested by the time-dependent development of a lethal infection in embryos.

Fig. 1 summarizes the results obtained in experiments carried out to estimate the time of death of chick embryos infected with a constant dose (10³ EID₅₀) of different variants of influenza A2 virus strains. Thermophilic variants caused lethality of 88–98% of embryos 3 days after injection of virus. Time of death of embryos infected with original and especially cryophilic viruses was delayed with the peak of lethality occurring not earlier than 7 days after infection.

Distinct differences in the rate of lethality of the embryos inoculated with the variants described were most marked 3 days after injection of the virus into allantoic or amniotic cavities. At this time the majority of the embryos infected with thermophilic variants were dead while not more than 22 to

32% of deaths were observed among those injected with cryophilic strains. The control variant adapted to reproduction at 36° C caused deaths of 78 to 80% of embryos.

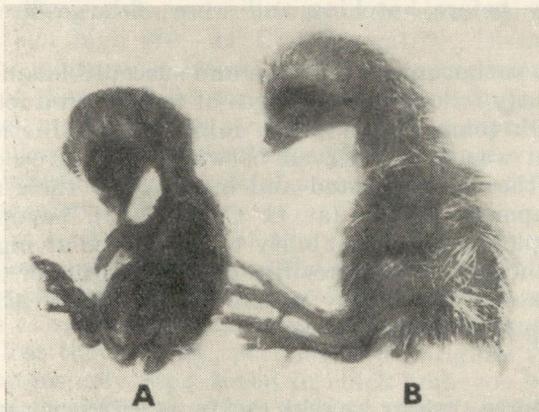


Fig. 2.

Chick embryos incubated for 3 days at 36.5° C

A — embryo inoculated with thermophilic influenza A2/65/151 virus variant; B — embryo injected with saline

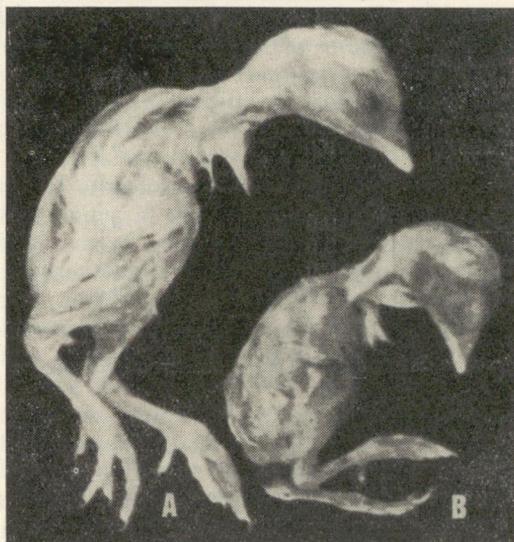


Fig. 3.

Chick embryos incubated for 7 days at 36.5° C

A — intact control embryo; B — embryo inoculated into the amniotic cavity with cryophilic influenza A2/65/151 virus strain

Deaths of the embryos infected with thermophilic strains occurred at early times and the embryos exhibited pronounced toxicosis, hyperaemia of the cerebral vessels and liver; they were of small size and had jelly-like general appearance (Fig. 2). The embryos inoculated with cryophilic strains were killed shortly before hatching and were of large size and feathered (Fig. 3).

Changes of virus pathogenicity for man and susceptible animals are known to be not infrequently followed by changes of their reproduction at low and, more often, at high temperature which inhibits reproduction of avirulent strains. Correlation was also observed between the degree of virulence for chick embryos of the viruses tested and intensity of their reproduction at supra-optimal temperature zone (at 41° C, Table 2). Cryophilic strains of low virulence completely lost the ability to reproduce at high temperature, while the most pathogenic strains readily reproduced under these conditions reaching peak values of infectivity which was similar to that observed in titration tests at optimal temperature.

Table 2. Correlation between virulence for chick embryos of temperature variants of influenza A2 viruses and intensity of their reproduction at 41° C

Virus strain	Variant	log EID ₅₀ /0.2 ml of allantoic fluid at the indicated temperatures of reproduction		log ELD ₅₀ /0.2 ml of allantoic fluid*
		36° C	41° C	
A2/65/151	Original	9.0	5.0	5.8
	Control	9.5	5.0	8.0
	Cryophilic	9.0	1.0	4.0
	Thermophilic	9.5	9.0	9.5
A2/65/147	Original	9.0	5.5	6.0
	Control	9.0	6.0	8.0
	Cryophilic	9.0	1.5	4.0
	Thermophilic	9.5	9.0	9.2

* Pathogenicity of viruses was determined in chick embryos incubated of optimal temperature (36° C).

All variants of influenza type A2 virus tested actively reproduced, like original viruses, in the lungs of albino mice but did not kill adult or newborn animals. No variation was found in the intensity of the virus development in the lung tissue of albino mice simultaneously infected with the test virus strains injected in 3—4 log EID₅₀ amounts. Tests on toxicity and virulence of the viruses for albino mice were also negative and showed no difference between cryophilic and thermophilic strains of influenza type A2 viruses.

Discussion

The experiments were carried out with the aim to compare virulence of influenza type A2 virus variants for susceptible animals. These variants were obtained after prolonged cultivation in developing chick embryos at low (26–28° C) or high (40–41° C) temperature. Distinct differences were observed in the pattern of infection of developing chick embryos induced by cryophilic or thermophilic variants and, hence, in pathogenicity of these viruses for chick embryos. The strains adapted to reproduction at 40–41° C were more virulent for chick embryos than original viruses and especially the variants obtained after 45 serial passages at 26–28° C, which possessed minimal pathogenicity.

An increase in pathogenicity of the viruses in the process of passages at high temperature and a decrease in the level of their initial virulence at low temperature appeared to be well correlated with the virus reproduction in chick embryos at supra- or infraoptimal temperatures. Cryophilic strains of low virulence lost their ability, peculiar to all original viruses, to reproduce at 41° C but intensively reproduced in chick embryos incubated at optimal or low temperature. Thermophilic variants were remarkable for high virulence for chick embryos and sharply increased reproduction activity at the temperature of 41° C, this temperature being most suited for their reproduction.

Changes of biological properties of influenza type A2 virus variants were due to two different factors: an influence of high or low temperature, on the one hand, and adaptation to sensitive cells of a chick embryo, on the other. The temperature factor seems to be the most important in decreasing virulence during passages at low temperature, which is substantiated by the lower virulence of cryophilic strains as compared to control strains passaged at the temperature routinely used for influenza viruses. Adaptation of viruses to chick embryos was no less important than temperature conditions in the mechanism of enhanced pathogenicity of viruses cultured for a long time at the temperature of 40–41° C. This is borne out by the fact that the level of virulence of control viruses after 45 serial passages at 36° C appeared to be higher than that of the original strains.

The experiments carried out showed that passages of viruses at high or low temperature resulted in changes of the level of their pathogenicity for chick embryos but did not affect their pathogenicity for albino mice. Lack of detectable changes in pathogenicity of the viruses for albino mice after continuous passages in chick embryos at high temperature provides an extra evidence that species specificity of a susceptible organism, selected as an experimental model to study variability of biological properties of viruses, plays an important role in the results of temperature adaptation.

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