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ATTENUATION OF TURKEY MENINGO-ENCEPHALITIS VIRUS IN BHK21 CELLS

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ABSTRACT

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Turkey meningo-encephalitis virus was adapted to BHK21 cell culture. Cytopathic effects were characterized by rounding and detachment of cells within 48 hours. Attenuation was achieved by 41 successive passages in BHK21 cell cultures. Turkeys and Japanese quail (*Coturnix coturnix japonica*), kept under laboratory conditions and inoculated with the attenuated virus, did not develop symptoms of turkey meningo-encephalitis but reacted by the production of haemagglutination inhibition antibody. They resisted intracerebral challenge with pathogenic strains of turkey meningo-encephalitis virus.

Résumé

L'ATTÉNUATION DU VIRUS DE LA MÉNINGO-ENCEPHALITE DE LA DINDE DANS LES CELLULES BHK21

Le virus de la meningo-encephalite de la dinde a été adapté à la culture de cellules BHK21. Les effets cytopathogéniques furent caractérisés par l'arrondissement et le détachement des cellules dans les 48 heures. L'atténuation fut réalisée par 41 passages successifs dans des cultures de cellules BHK21. Les dindes et la caille japonaise (Coturnix coturnix japonica) *placées sous des conditions de laboratoire et inoculées avec le virus atténué ne développèrent pas de symptômes de la méningo-encéphalite de la dinde mais elles réagirent par la production d'un anticorps inhibiteur d'haemaglutination. Elles résistèrent à une épreuve intracérébrale avec des souches pathogénes du virus de la méningo-encéphalite de la dinde.*

INTRODUCTION

Turkey meningo-encephalitis (TME), a disease of turkeys caused by a flavivirus, was recently diagnosed in South Africa (Barnard, Buys, Du Preez, Greyling & Venter, 1980). Komarov & Kalmer (1960) developed the first vaccine against TME by attenuating the virus through 34 passages in embryonated hens' eggs. The attenuated virus was used for vaccine production in embryonated hens' eggs and was used in Israel for a number of years with good results. Later on, because of low titres of virus in commercial vaccines, even flocks repeatedly vaccinated were not protected (Ianconescu, Hornstein, Samberg, Aharonovici & Merdinger, 1975). These authors attempted to improve the vaccine by including greater amounts of virus, but this resulted in severe paralytic and lethal post-vaccinal reactions. They then adapted TME virus to Japanese quail (*Coturnix coturnix japonica*) and attenuated the virus by 11 passages in quail kidney cell cultures. This attenuated virus was subsequently used for vaccine production in embryonated hens' eggs.

The behaviour of TME virus in various cell systems was investigated by Nir (1972). In most cell systems no virus multiplication could be detected either by direct observation of the cells for cytopathic effect (CPE) or by inoculation of the culture medium into suckling mice. Of the systems tested only chicken embryo fibroblasts allowed virus replication as well as CPE. Replication occurred in BHK21 cell cultures, but no CPE was observed. When Barnard *et al.* (1980) adapted TME virus to BHK21 cultures, high titres of virus were obtained and CPE was discernible within 24 h after infection of cell cultures. In this paper the attenuation and some characteristics of the attenuated TME virus are described.

MATERIAL AND METHODS

Animals

Eggs were obtained from a commercial turkey flock. The turkeys hatched from these eggs and locally bred quail were reared in isolation until 6–8 weeks old, when they were used in various experiments. Before being used, each bird was tested for the absence of haemagglutination-inhibition (HI) antibody against TME virus.

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One-day-old mice were used for virus titrations and virus multiplication.

Virus

A field strain of TME virus, isolated from a diseased turkey during 1978 (Barnard *et al.*, 1980), was passaged 3 times in 1-day-old mice and stored as a freeze-dried suspension of 10% infected mouse brain in phosphate buffer containing 10% peptone and 5% lactose (BLP). The freeze-dried virus was stored at 4 °C and used both as challenge virus and for the production of haemagglutination-inhibition antigen. It was further attenuated by the inoculation of BHK21 cell monolayers in roller tubes, incubation at 37 °C and subculturing after 4 days, when the cells were in various stages of degeneration. The culture medium of every 10th passage was freeze-dried for use later.

The H3 strain of TME virus*, passaged 4 times in 1-day-old mice and stored as a 10% suspension of infected mouse brain in BLP, was used as challenge virus in some experiments.

Virus titrations

Virus titrations were carried out by inoculating serial tenfold virus dilutions intracerebrally into 1-day-old mice or onto BHK21 cell cultures in roller tubes. In the various experiments the amount of virus inoculated into each bird is expressed as $\log_{10}$ TCID50. The titres were calculated according to Reed & Muench (1938).

Serological tests

The haemagglutination-inhibition technique (HI) (Clarke & Casals, 1958) was used to determine antibody titres. A sucrose-acetone extract of infected mouse brain was used as antigen and goose red cells were used in the test, which was performed at pH 6,4. Titres of 1:20 or higher were considered positive.

Determination of pathogenicity and immunogenicity

The 3rd mouse brain passage of the field strain and the 21st, 31st and 41st passage in BHK21 cell monolayers were inoculated into the breast muscle of various groups of 6-week-old poults and/or quail.

* Kindly supplied by Dr M. Ianconescu, The Kimro Veterinary Institute, Beit Dagan, Israel

Each bird was inoculated with 0,05 mℓ of a virus suspension containing various amounts of virus. In the immunogenicity experiments, the 41st passage only was used and uninoculated birds of the same age were kept in contact with inoculated birds as controls. The birds were observed for 14 days and the number of sick and dead birds was recorded daily. The sick ones were only recorded on the day they were first seen to be sick and the dead ones on the day of death. Before inoculation, 14 days after inoculation and, in some instances, also 21 days after inoculation, blood samples were collected and the sera tested for HI antibodies against TME virus. The immunity was challenged on 14, 21 or 28 days after inoculation by intracerebral inoculation of at least $10^{5,5}$ mouse ID50 of either the 3rd mouse brain passage of the field strain or the mouse brain passage of the H3 strain of TME virus.

Thermostability

Suspensions of harvested medium of the 41st passage of TME virus in BHK21 cell cultures were either kept at 4 °C or freeze-dried. Either 1% m/v bovine albumin fraction V or 25% BLP was used as stabilizer for freeze-drying. Freeze-dried virus was either kept at 4 °C or exposed to 37 °C for 7 days. The suspensions and the freeze-dried virus were titrated at various intervals in both 1-day-old mice and on BHK21 cell monolayers in roller tubes.

Results

Virus titrations

The results shown in Table 1 indicate that adaptation of TME virus to BHK21 cell cultures causes a loss of infectivity for mice. While in the early passages, virus titres of $10^{8,0}$ MID50/mℓ were obtained; in the later passages they dropped to $10^{4,5}$ MID50/mℓ.

TABLE 1 Virus titres in 1-day-old mice and on BHK21 cell cultures of TME virus at various passage levels on BHK21 cell culture

Passage level	Virus titres in 1-day-old mice(1)	Virus titres in BHK21 cell cultures(2)
Mouse brain 3	$10^{8,0}$	No CPE(3)
BHK21 6....	$10^{5,5}$	$10^{5,6}$
BHK21 10...	$10^{6,3}$	$10^{7,5}$
BHK21 21...	$10^{5,8}$	$10^{9,0}$
BHK21 31...	$10^{5,0}$	$10^{9,0}$
BHK21 41...	$10^{4,5}$	$10^{8,5}$

(1) Expressed as $\log_{10}$ MID50/mℓ
(2) Expressed as $\log_{10}$ TCID50/mℓ
(3) No specific CPE effect noticed and cultures not titrated

Pathogenicity

The results summarized in Table 2 show that 6 out of 10 poults inoculated intramuscularly with the 3rd passage in mouse brain developed symptoms of TME and that 2 of them died, while none of the 40 poults inoculated with the 31st and 41st passages in BHK21 cell cultures died or developed symptoms. Out of the 20 birds which had been inoculated with the 21st passage, 2 only developed symptoms. Quail reacted in a similar way. Out of 10 birds inoculated with the 3rd passage in mouse brain, 2 died and 5 developed symptoms, while none reacted clinically to the inoculation of the 41st passage in BHK21 cell cultures.

TABLE 2 Pathogenicity of BHK21 cell culture-adapted TME virus in turkeys and quails

Passage level	Species inoculated	Post vaccinal reaction	
		Sick(1)	Deaths
3 (Mouse brain)...	Turkey	6/10	2/10
3 (Mouse brain)...	Quail	5/10	2/10
21 (BHK21).....	Turkey	2/20	0/20
31 (BHK21).....	Turkey	0/20	0/20
41 (BHK21).....	Turkey	0/20	0/20
41 (BHK21).....	Quail	0/10	0/10

(1) Number sick or dead/number inoculated

Immunogenicity

When tested 14 days after inoculation with at least 5×10^2 TCID50 of the 41st passage in BHK21 cell cultures, 104 out of 104 poults were positive for the presence of HI antibodies against TME virus. Only 4 out of 10 birds inoculated with 5×10^1 TCID were positive 14 days after inoculation, but when they were retested 7 days later, all were positive. This holds good for 11 out of 12 control birds tested. Control birds tested after 14 days were consistently negative. The results are reflected in Table 3.

Three out of 34 poults challenged 14 days after inoculation developed symptoms only. They showed a slight incoordination but recovered within 3 days. Nineteen out of 28 birds in the control groups developed symptoms, and 6 died. None of the 80 inoculated birds challenged 21 or 28 days after inoculation showed any signs of TME. Control birds which developed HI antibodies also resisted challenge.

Thermostability

The results of the thermostability tests are presented in Table 4. The virus titres of suspensions of the 41st passage in BHK21 cell cultures held at 4 °C remained constant for at least 29 days and then dropped from $10^{8,0}$ to $10^{7,5}$ TCID50/mℓ during the next 34 days. When BLP was used as stabilizer during freeze-drying, the virus titres dropped from $10^{8,0}$ to $10^{7,5}$ and remained constant during exposure to 37 °C for 7 days. When bovine albumin fraction V was used as stabilizer, the virus titre dropped from $10^{8,0}$ to $10^{7,0}$ TCID50/mℓ during freeze-drying and to $10^{5,5}$ TCID50/mℓ during exposure to 37 °C for 7 days.

Discussion

Vaccine production in embryonated hens' eggs is an expensive and cumbersome procedure. Although the multiplication of TME virus in chick embryo fibroblasts (Nir, 1972) and in Japanese quail kidney cell cultures (Ianconescu, Aharanovici & Samberg, 1974) have been demonstrated, TME vaccine is still commonly produced in embryonated hens' eggs. This is probably due to the lack of a cell culture system suitable for the production of TME vaccine.

In the present investigation the adaptation and the attenuation of TME virus were achieved by successive passage in BHK21 cell cultures. The high titres of virus obtained in this way and the decreased infectivity for mice by the adapted virus (Table 1) endorse this procedure.

TABLE 3 Immunogenicity of the 41st passage of TME virus on BHK21 cell culture

Experiment	Virus inoculated per turkey	Challenge strain	Immunological reactions					
			HI antibody post-inoculation at day		Reactions post-challenge on			
					Day 14		Day 21 or 28	
			14	21	Sick	Dead	Sick	Dead
1.....	$10^{4,5}$(1) None(5)	H3(2) H3	17/17(3) 0/15	ND(4) ND	2/17 11/15	0/17 2/15	ND ND	ND ND
2.....	$10^{4,5}$ None	Field(6) Field	17/17 0/13	ND ND	1/7 8/13	0/17 4/13	ND ND	ND ND
3.....	$10^{3,0}$ None	Field Field	40/40 0/10	40/40 6/10	ND ND	ND ND	0/40 2/10	0/40 2/10
4.....	5×10^{4} None 5×10^{3} None 5×10^{2} None 5×10^{1} None	Field Field Field Field Field Field Field Field	10/10 0/3 10/10 0/3 10/10 0/3 4/10 0/3	ND 3/3 ND 3/3 ND 3/3 10/10 3/3	ND ND ND ND ND ND ND ND	ND ND ND ND ND ND ND ND	0/10 0/3 0/10 0/3 0/10 0/3 0/10 0/3	0/10 0/3 0/10 0/3 0/10 0/3 0/10 0/3

(1) Expressed as $\log_{10}$ $TCID_{50}$
(2) H3 strain of TME virus. Fourth passage in mouse brain
(3) Number positive, sick or dead/number of birds tested
(4) ND = Not done
(5) None = Uninoculated control birds
(6) Field strain of TME virus. Third passage in mouse brain

TABLE 4 Thermostability of the 41st passage of TME virus in BHK21 cell culture

Time in days	Titres of virus suspensions at 4°C(1)	Titre of virus freeze-dried with:			
		Bovine albumin at		BLP at	
		4 °C	37 °C	4 °C	37 °C
1	$10^{8,0}$	$10^{7,0}$	ND	$10^{7,5}$	ND
7	$10^{8,0}$	$10^{7,0}$	$10^{5,5}$	$10^{7,5}$	$10^{7,0}$
29	$10^{8,0}$	ND	ND	ND	ND
63	$10^{7,5}$	ND	ND	ND	ND

(1) Expressed as Log_{10} TCID50/mℓ
ND = Not done

The decreased pathogenicity for both turkey and quail during successive passages is indicated in Table 2, where it is shown that no deaths or symptoms resulted from the inoculation of TME virus after the 31st or the 41st passage in BHK21 cells.

No detectable loss of immunogenicity was observed in various tests using the 41st passage of TME virus in BHK21 cell cultures. All the birds inoculated with 5×10^{2} TCID50/mℓ or more of virus reacted by the production of HI antibodies within 14 days after inoculation. The late production, i.e. after 14 days of antibodies, in 6 out of 10 birds inoculated with 5×10^{1} TCID50, (Experiment 4, Table 3) may be the result of viral spread from other birds. The mechanism of the spreading of the virus to these birds and to the in contact controls is unknown at this stage, as it was shown (Ianconescu, Aharonovici, Samberg, Hornstein & Merdinger, 1973) that TME virus does not spread by direct contact except in older birds whose sexual activities result in a high incidence of superficial wounds which serve as port of viral entry.

The protective value of the 41st passage in BHK21 cell cultures was demonstrated by the good results obtained after the laboratory infections (Table 3). After severe challenge, i.e. intracerebral inoculation of virulent virus, no deaths or symptoms of TME were noticed in the inoculated birds in contrast to high morbidity and mortality in the control birds.

The thermostability of a vaccine is important. The results presented in Table 4 show that TME virus suspensions are fairly stable at 4 °C with some loss during freeze-drying but with insignificant loss during subsequent exposure to 37 °C for 7 days, when BLP was used as stabilizer.

Because of the small numbers of birds used in the various experiments and the lack of field trials, the evidence for the attenuation of TME virus by 41 passages through BHK21 cell cultures cannot yet be regarded as sufficient to ensure the safety of a vaccine in all circumstances. Nevertheless, the adaptation and attenuation achieved, the stability of the freeze-dried virus and its immunogenicity would give good grounds for the future use of BHK21 cells in the production of TME vaccine.

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