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Chick embryo lethal orphan (CELO) virus as a possible contaminant of egg-grown virus vaccines

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INTRODUCTION

Viruses which are able to establish persistent subclinical infections in their natural hosts may present serious problems in the preparation of vaccines. These viruses may be present, undetected by conventional virus isolation techniques, in tissue cultures prepared from laboratory animals. Of particular importance in this respect are viruses with oncogenic potential and it is now well established that formaldehyde-inactivated poliovirus vaccines made in monkey renal-cell cultures may contain an oncogenic virus of simian origin, SV40 virus (Goffe, Hale & Gardner, 1961; Gerber, 1967). In addition, viruses of the avian leucosis group are common contaminants of fertile hens' eggs and such viruses have been detected in live attenuated yellow fever vaccines prepared in eggs (Harris *et al.* 1966).

Since fertile eggs are employed in the production of a number of different attenuated or killed virus vaccines, latent viruses of chickens and their eggs merit detailed investigation. Such a virus is Chick Embryo Lethal Orphan (CELO) virus, which was first isolated by serial passage of allantoic fluid from apparently normal fertile eggs (Yates & Fry, 1957). Sero-epidemiological studies have shown that CELO infection is widespread in flocks of fowls in the U.S.A. and Japan (Yates & Fry, 1957; Kawamura *et al.* 1963). More recently CELO virus has been isolated in England from eggs laid by an apparently normal flock with exceptionally high egg production (Cook, 1968). Sarma, Huebner & Lane (1965) have described the induction of fibrosarcomas in hamsters inoculated with CELO virus when newborn and the oncogenic properties of the virus have been confirmed in this laboratory with two different strains of CELO virus (Schild, Oxford & Potter, to be published). CELO virus is a potential contaminant of egg-prepared vaccines and because of its stability (Petek, Felluga & Zoletto, 1963) may survive vaccine virus inactivation procedures.

In the present study the inactivation kinetics of CELO virus in the presence of 1/4000 formaldehyde have been investigated. This concentration of formaldehyde has been used in the preparation of certain killed virus vaccines (Gerber, Hottle & Grubbs, 1961). Further, we have tested a number of sera collected from flocks of chickens for the presence of CELO virus neutralizing antibody in order to investi-

gate whether infection with the virus is widespread in England. Finally, we have searched for CELO virus antibody in the sera of persons who have received killed influenza vaccines.

METHODS

Virus

The 'Phelps' strain of CELO virus was supplied by Dr J. S. Garside, Houghton Poultry Research Station, Huntingdon. The virus was diluted 1/100 in phosphate buffered saline and 0·2 ml. inoculated allantoically in 7-day-old fertile hens' eggs. Allantoic fluids were harvested after 5 days incubation at 35° C. and stored at −70° C. in ampoules. The virus pool had a titre of $10^{9\cdot0}$ ELD 50/ml. in eggs and $10^{8\cdot5}$ TCID 50/ml. in tissue cultures of chick kidney.

Serum samples

Chicken sera were kindly supplied by Dr J. E. Wilson, Ministry of Agriculture, Fisheries and Food, Veterinary Laboratory, Lasswade, and Dr D. P. McHugh, Pfizer Co., Sandwich, Kent. Other sera were from our own flock of hens.

Rabbit sera were from animals immunized with three weekly intravenous injections of the stock CELO virus egg allantoic fluid pool. The rabbits were bled 2 weeks after the final injection.

The human sera were collected during trials of various influenza vaccines.

R.A.F. Halton Trial. Sera were taken 18 months after the subcutaneous injection of 0·25 ml. of a bivalent influenza vaccine containing 2000 haemagglutinating units of influenza A 2/Singapore/1/57 and B/England/939/59 viruses. The vaccines contained either vegetable-oil adjuvant (A 65) or Drakeol no. 6 mineral-oil adjuvant.

Reed Paper Group trial, Maidstone. The sera were collected 12 months after subcutaneous injection of bivalent vaccines containing influenza A 2/Jap/170/62 and B/Maryland/1/59 viruses. Volunteers were given either an aqueous (saline) vaccine containing 500 haemagglutinating units of each virus, or an adjuvant vaccine (adjuvant A 65, lot 140) containing 250 haemagglutinating units per 0·5 ml. dose.

Sera were also tested which had been collected in 1961–3 during trials of attenuated poliomyelitis vaccines. For the purpose of the present study these were designated 'normal' sera, there being no known history of administration of virus egg vaccines to these individuals.

Tissue cultures

Chick kidneys were removed aseptically from fowls aged 2–4 weeks, trypsinized and seeded into tubes at a concentration of 0·1 ml. centrifuged cell pack per 100 ml. of medium. Chick embryo fibroblast cells were prepared by mincing and trypsinizing decapitated embryos from 10-day-old fertile hens' eggs and seeded into tubes at a concentration of 0·1 ml. of centrifuged cell pack per 100 ml. of medium. The cell growth medium was Eagle's minimal essential medium (M.E.M.) containing 10 % inactivated calf serum and 0·44 g./l. sodium bicarbonate. The maintenance medium contained 5 % inactivated calf serum and 0·88 g./l. sodium bicarbonate in M.E.M.

Neutralization tests

Equal volumes of serum dilution and CELO virus containing 100 TCID 50 were incubated for 1 hr. at room temperature and 0·2 ml. inoculated in each of four chick kidney tube tissue cultures. Neutralization end-points were taken as the highest serum dilution completely inhibiting virus cytopathic effect after 7 days incubation at 35–36° C.

Formaldehyde inactivation of CELO virus

Formaldehyde was diluted to 1/4000 in phosphate buffered saline in stoppered conical flasks and CELO virus added to give a final concentration of $10^{7.5}$ TCID 50/ml. The flasks were shaken periodically to wash any virus from the vessel walls. Samples were withdrawn at various time intervals, the formaldehyde neutralized with sodium bisulphite and the fluids titrated for CELO virus in chick kidney tissue cultures. A control flask containing virus but no formaldehyde was tested in parallel and the experiment carried out at 4° C. and 36° C.

Table 1. *Comparison of chick kidney cells and chick embryo fibroblast cells for quantitation of CELO virus*

tre of virus ($\log_{10}$ TCID 50/ml.) and C.P.E. after infection of cells with different virus multiplicities.)

me after ıfection cultures (days)	1·0 TCID 50 per cell*		0·01 TCID 50 per cell	
	Chick kidney	Chick embryo fibroblast	Chick kidney	Chick embryo fibroblast
0	4·5†(0)	4·5 (0)	1·5 (0)	1·5 (0)
½	6·5 (±)	5·3 (0)	1·8 (0)	1·5 (0)
1	7·8 (+++)	5·5 (0)	3·5 (0)	1·8 (0)
2	7·8 (++++)	5·5 (±)	5·7 (+)	2·8 (0)
4	8·5 (++++)	5·8 (+++)	7·5 (++++)	4·5 (0)
7	8·5 (++++)	5·8 (++++)	7·5 (++++)	5·3 (+)
10	8·5 (++++)	5·8 (++++)	7·5 (++++)	5·3 (+++)

- ++, +++, ++, +, Cytopathic effect (C.P.E.) in 100 %, 75 %, 50 % and 25 % of cells respectively.
virus adsorbed for 2 hr. at room temperature, cells washed 3 times to remove unadsorbed virus and ıbated at 36° C.
Titre of virus expressed as $\log_{10}$ TCID 50/ml. from three pooled tissue culture tubes.

RESULTS

In preliminary experiments we compared the relative sensitivity of chick kidney cells and chick embryo fibroblast cells for the detection of CELO virus cytopathic effects and for growth of the virus. At two multiplicities of virus infection (0·01 and 1·0 TCID 50 virus per cell) cytopathic changes were noted earlier, and virus titres in the supernatant fluids reached higher levels, in chick kidney cell cultures as compared to chick embryo fibroblasts (Table 1). In addition, a single pool of CELO virus was titrated in parallel in tenfold dilution steps using these two types of cell. The titre of the virus read at 10 days by cytopathic end-point was $10^{8.3}$ TCID 50/ml. in chick kidney cells and $10^{6.5}$ TCID 50/ml. in chick embryo fibroblast cells. Chick kidney cells were therefore used for all subsequent studies with CELO virus.

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Formaldehyde inactivation of CELO virus

The inactivation of CELO virus in the presence and absence of 1/4000 formaldehyde is shown in Fig. 1. In control fluids, containing no formaldehyde, the infectivity titre of CELO virus did not decline significantly after 14 days incubation at 4° or at 36° C. In contrast, virus inactivation at 36° C. in the presence of 1/4000 formaldehyde was relatively rapid, and no CELO virus was detected after 24 hr. incubation. Inactivation of CELO virus by formaldehyde at 4° C. was less efficient; $10^{2.5}$TCID 50/ml. of virus was still present in the fluids after 14 days incubation.

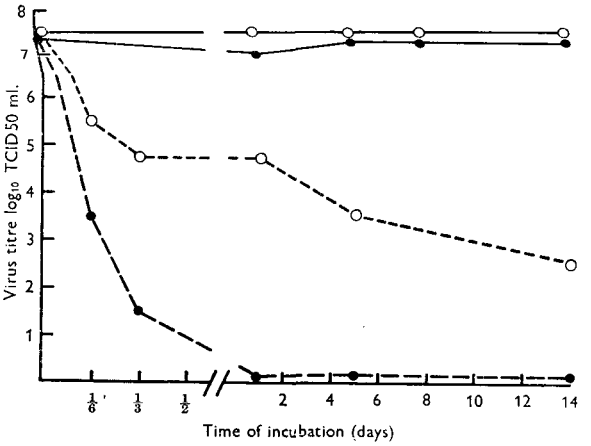

Fig. 1. Formaldehyde inactivation of CELO virus. ○——○, PBS at 4° C., ●——●, PBS at 35–36° C., ○- - -○, formaldehyde 1/4000 at 4° C., ●- - -●, formaldehyde 1/4000 at 35–36° C.

Neutralizing antibodies for CELO virus in sera

Chicken sera from a number of flocks were tested at a dilution of 1/8 for the presence of neutralizing antibody to CELO virus and the results are shown in Table 2. Neutralizing antibody was detected in a proportion of each group of chicken sera tested ranging from 20 % to 88 %. In a further experiment twenty-nine chicken sera from two groups were titrated in twofold dilution steps to find the range of neutralizing antibody titres (Table 3). CELO virus neutralizing antibody titres ranged from 1/10 to 1/640 and the modal titre of neutralizing antibody was between 1/40 and 1/160.

No CELO virus neutralizing antibody was detected in the 142 normal human sera examined at a dilution of 1/8 from a random section of the population. Similarly, no CELO virus neutralizing antibody was detected in the sera tested from 229 individuals who had been immunized with inactivated influenza virus vaccines prepared by two different manufacturers. Rabbits which were immunized with three successive doses of CELO virus developed high titres of neutralizing antibody ranging from 1/512 to 1/2048. This suggested that CELO virus was a potent antibody inducer in species additional to its natural host.

Table 2. *CELO virus neutralizing antibody in human and animal sera*

Source of sera	No. tested	No. of sera with neutralizing antibody at serum dilution 1/8
A. Human sera		
After influenza vaccine, Maidstone trial	141	0
After influenza vaccine, R.A.F. Halton trial	88	0
Normal individuals: 10 months to 5 years of age	30	0
Normal individuals: 18 years of age and over	112	0
B. Chicken sera		
Pfizer, Kent	10	2
Lodge Moor, Yorks.	9	8
D945 Lasswade, Scotland	25	15
D801 Lasswade, Scotland	12	6
D.3, Lasswade Scotland	20	15
C. Rabbit sera		
Immunized with CELO virus	4	4

Table 3. *CELO virus neutralizing antibody in chicken sera*

No. of sera tested*	No. of sera with neutralizing antibody at indicated serum dilution							
	<1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
29	6	3	1	6	4	6	2	1

* Chicken sera from Lodge Moor and Lasswade D.3.

DISCUSSION

Previous studies with CELO virus have described the cytopathic effects of the virus growing on chick kidney cells (Chomiak, Luginbuhl & Helmboldt, 1961). However, chick embryo fibroblast cells are a more readily obtainable source of chick cells and were, therefore, compared with chick kidney cells for their relative sensitivity for the growth of CELO virus. The latter were found to be superior both for the growth and titration of CELO virus; higher titres of virus were produced and cytopathic changes occurred more quickly in chick kidney cells. Chick kidney cells therefore appear to be more sensitive for the detection of small quantities of CELO virus in vaccines, for example, than would be chick embryo fibroblast cells.

The inactivation kinetics experiments with allantoic fluids containing high initial infectivity titres of CELO virus indicated that no detectable virus survived the inactivation period of 24 hr. with 1/4000 formaldehyde at 36° C. However, the inactivation process at 4° C. was less complete and infective CELO virus was detected even after 14 days. The present formaldehyde inactivation studies with

CELO virus did not show a linear reaction. Thus, the major portion of the CELO virus population was inactivated in the first 8 hr. at 4° C. in the presence of formaldehyde. After this initial inactivation the titre of infective virus continued to decrease more slowly over the subsequent test period of 14 days. CELO virus appears to be more efficiently inactivated than another stable potential contaminant of certain vaccines, SV 40 virus; Gerber *et al.* (1961) demonstrated that some SV 40 virus infectivity was retained even after 14 days inactivation at 33° C. with 1/4000 formaldehyde.

The present survey of human sera for CELO virus neutralizing antibody failed to detect any serological evidence that persons who had received egg-prepared inactivated influenza virus vaccines were also injected concurrently with CELO virus. These negative results, however, do not demonstrate unequivocally that the original vaccines were free of CELO virus. Harris and his colleagues (1966) were able to demonstrate the contamination of yellow fever vaccine with an avian leukosis virus but were unable to detect any antibody to the latter virus in immunized volunteers. In contrast, antibody for an avian leukosis virus was detected after repeated immunization of chickens with these vaccines.

CELO virus-neutralizing antibody was detected in the serum of a proportion of hens in each of the five flocks tested. This suggests dissemination of the virus in England and Scotland. In addition, studies in another laboratory have also detected CELO virus neutralizing antibody in chicken sera in Great Britain. (D. A. McMartin, Veterinary Laboratory, Lasswade, Midlothian—personal communication.) Recently CELO virus has been isolated from eggs laid by an apparently normal flock in England (Cook, 1968). Similar studies in the U.S.A. and Japan have also noted a proportion of hens from different flocks with CELO virus neutralizing antibody (Yates & Fry, 1957; Kawamura *et al.* 1963). More studies are thus indicated to attempt CELO virus isolation from eggs and chickens, particularly in flocks used for vaccine production. It may then be possible to circumvent the problem of potential contamination of vaccines with this virus by only using eggs from virus free, seronegative flocks.

SUMMARY

The inactivation kinetics of CELO virus were studied in the presence of 1/4000 formaldehyde. Inactivation of the virus by formaldehyde at 4° C. was not complete after 14 days incubation. Formaldehyde inactivation at 36° C., however, was rapid and no virus was detected after 24 hr. incubation.

Neutralizing antibody to CELO virus was detected in 20–88 % of sera tested from five flocks of hens. This suggested dissemination of the virus in England and Scotland. However, no CELO virus neutralizing antibody at a serum dilution of 1/8 was detected in 142 normal human sera or in 229 sera from persons who had been immunized with egg grown, inactivated influenza virus vaccine.

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