Contaminant viruses in two live virus vaccines produced in chick cells

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Attention has been focused on the need for particular care in the preparation of virus vaccines since the discovery that a virus (SV 40) present as a contaminant in Salk-type poliomyelitis vaccines could produce tumours following inoculation into rats and hamsters (Eddy, Borman, Berkeley & Young, 1961; Girardi, Sweet, Slotnick & Hilleman, 1962) and could, moreover, escape the inactivation procedures used in the preparation of such vaccines.

Our attention has been directed towards live virus vaccines containing virus grown in eggs or in chick embryo cell cultures. Fowls are known to harbour leukosis viruses which are transmitted from hen to egg so that the embryos are carriers of the virus. The use of the eggs for the growth of viruses such as 17D strain yellow-fever, or attenuated measles, might result in a considerable contamination of the vaccine with leukosis-group viruses. Very few flocks of chicken are known to be completely free from leukosis and, at the time these tests were made, the yellow-fever and measles viruses were *not* being propagated in tissue from such flocks.

Two experimental approaches were adopted. The first was to neutralize the yellow-fever virus with a large excess of monkey antiserum (from a monkey inoculated with a *non-avian* strain of yellow-fever virus) and the measles virus with rabbit antiserum and to assay the residual material for leukosis virus using the interference test devised by Rubin (1960). The second was to immunize adult chickens of a flock maintained in isolation with the neutralized viruses and test the sera for capacity to neutralize Rous sarcoma virus of the Bryan strain (RSV(B)) by the general procedure of Simons & Dougherty (1963).

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MATERIALS

Preparation of the vaccines

The two vaccines studied were prepared by different methods. Both are live-virus vaccines and use the seed-lot systems (Fox, Kossobudzki & da Cunha, 1943). In the case of yellow-fever vaccine (YFS 2) the virus was grown by inoculating seed virus into 7-day-old chick embryos with continued incubation in the intact egg for a further 4 days; the infected embryos were then harvested, homogenized in saline and the clarified embryonic pulp freeze-dried in batches of convenient size. The sample tested was taken before freeze-drying from the seed lot used for routine production between 1958 and 1964 and was composed of a pool of approximately 2000 embryos derived from seven commercial flocks in southern England. It had been stored in sealed glass containers at $-70°$ C. until immediately before test. The YF virus content was $10^{5·8}$ mouse LD 50 per ml. and was only one chick embryo passage from original Rockefeller Institute virus prepared by Dr Max Theiler in 1944.

The experimental vaccine (MV 16) was prepared from the fluid medium of chick embryo cell cultures infected with an attenuated measles virus (Goffe & Laurence, 1961). The virus strain was derived from Enders' Edmonston 'B' strain and was in its third tissue culture passage after a series of thirty serial chick-embryo passages by the intra-amniotic route. The tissue cultures were prepared by trypsinizing decapitated 8-day chick embryos and growing the cells in a monolayer. This batch was grown from a pool of thirty-five embryos from a single flock. Virus was added on the third day, and incubation continued in a serum-free medium for a further 8 days. The medium containing the virus and some degenerated cells was harvested and clarified by centrifugation; the supernatant fluid together with a stabilizer was freeze-dried to form the final vaccine. The sample tested was in the final freeze-dried form exactly as used for the inoculation of children (Benson et al. 1964; Watson, 1965).

METHODS AND RESULTS

Interference test for leukosis virus in yellow fever vaccine YFS 2

A sample of yellow fever vaccine YFS 2 was neutralized with monkey antiserum by incubation of equal volumes of virus and antiserum (diluted 1/10) at 4° C. for 24 hr. Secondary chick embryo fibroblast cultures were set up from leukosis-free embryos and infected in suspension with 0·1 ml. of the undiluted vaccine-serum mixture or a 1/10 dilution according to Rubin (1960). These cultures and uninfected controls were then passaged and challenged with RSV (B) 5 days later. The results (Table 1) show that the relative sensitivity to RSV (B) of the infected cultures was much reduced compared with the controls. This indicates that the neutralized yellow fever vaccine contains very significant amounts of a virus which, in this test, acted like a member of the fowl leukosis group.

Two similar tests for interference with RSV (B) were made with measles vaccine, using Measles Vaccine 16 Unspun, 11,15.3.62 (25 ml.) neutralized with Measles

Rabbit Immune Serum Pool no. R. 10 (3 ml.) The results are shown in Table 2. In these tests four tissue culture passages of the infected cells were required before their relative sensitivity to RSV(B) approximated to that produced by only one passage of the cells infected by the yellow fever vaccine-serum mixture (Table 1). Since the purpose of the passages is to increase the titre of the interfering virus, this means that the measles vaccine contained less interfering virus than the yellow-fever vaccine.

Table 1. *Interference test on yellow-fever vaccine*

(A relative sensitivity of 0·001 means that the cultures previously infected with the interfering virus required 10^5 focus-forming units (F.F.U.) of RSV(B) to produce the same number of foci as 10^2 F.F.U. in the controls (or $10^3 : 1$).)

Infected with	Tissue culture passage no. 1
YFS 2 vaccine-serum mixture	0·001*
YFS 2 vaccine-serum mixture diluted 1:10	0·002

* Relative sensitivity to challenge with (RSV(B).

Table 2. *Interference test on measles vaccine*

	Tissue culture passage no.			
Infected with	1	2	3	4
MV 16 virus-serum mixture	0·5*	0·02	0·02	—
MV 16 virus-serum mixture	—	0·1	—	0·008

* Relative sensitivity to challenge with RSV(B).

Both yellow-fever and measles virus are cytopathic for chick embryo cells in culture. If the viruses are inadequately neutralized lytic plaques appear. For this reason antiserum was kept in all the cultures at each passage. Under these conditions, which were based on model experiments, no 'break-through' of the neutralization could be detected. It would appear, therefore, that the observed interference cannot be attributed to residual yellow fever or measles virus. Moreover, interference of this type, increasing in extent with the number of subcultures of the cells, has been shown by Hanafusa, Hanafusa & Rubin (1964) to be dependent upon antigenic similarity between the interfering virus and the challenge RSV(B) stock. A virus with these properties is most likely to be a virus of the avian leukosis group.

Immunity tests

Equal numbers of adult male and female Edinburgh Brown Leghorns were randomly allotted to four groups. Each group, consisting of fourteen or fifteen birds, was housed in isolated arks kept in the open on a concrete apron. Food and water were provided *ad libitum.*

Sera from all birds were collected 24–31 days before the primary inoculation, which consisted of the following preparations:

Group 1: Freund's adjuvant plus undiluted neutralized yellow fever vaccine.

Group 2: Freund's adjuvant plus undiluted neutralized measles vaccine.

Group 3: Freund's adjuvant plus dilute lymphoid leukosis virus.

Group 4: Freund's adjuvant plus concentrated lymphoid leukosis virus.

Each bird received 2·0 ml. of the relevant preparation, 0·5 ml. being given intramuscularly at each of four sites in the pectoral muscles. Twenty-six days later sera were collected. On the same day the birds were given the following treatment as a second inoculum:

Group 1: One ml. of neutralized yellow fever vaccine comprising 0·5 ml. subcutaneously into each wing web.

Group 2: 0·5 ml. of neutralized measles vaccine subcutaneously into the right wing web.

Group 3: One ml. of dilute lymphoid leukosis virus, 0·5 ml. subcutaneously into each wing web.

Group 4: One ml. of concentrated lymphoid leukosis virus, 0·5 ml. subcutaneously into each wing web.

Seven days later sera were again collected.

Birds of groups 1, 2 and 3 were killed 6 months later and the birds of group 1 examined for the presence of tumours. No tumours were found.

The lymphoid leukosis virus used was HPRS F42, which has been shown to interfere with the multiplication of RSV(B) virus *in vitro* and to reproduce lymphoid leukosis following inoculation of day-old chicks (Biggs & Payne, 1965).

For the neutralization tests on these sera the virus used was a Moloney-type preparation—a single batch of RSV(B) stored in small volumes at $-70°$ C. in sealed ampoules. The infectivity of this preparation for the chorioallantoic membrane of eggs of the Brown Leghorn strain was such that an inoculum of 0·1 ml. of a dilution of $1·5 \times 10^{-4}$ gave about 100 pocks. Before admixture with the virus the sera were diluted 1/5 with medium DI (phosphate-buffered saline containing 2 % inactivated calf serum and 100 units/ml. of penicillin and streptomycin) and inactivated at $56°$ C. for 30 min. Equal quantities of virus and serum were mixed, incubated overnight at $4°$ C. and 0·1 ml. volumes inoculated into groups of six eggs by the method of Simons & Dougherty (1963). The infected eggs were incubated at $38°$ C. for a further 7 days, chilled at $0°$ C. overnight, the chorioallantoic membranes removed, and the pocks counted.

The results may conveniently be expressed as percentage reduction of pock count as between untreated virus and virus incubated with pre-immunization or primary or secondary challenge sera. Evidence that the serum has significant virus neutralizing capacity will be accepted if the pock count in the challenge group is 15 % or less of that of the control figures. Table 3 sets out these data for birds of group 1.

Since the birds in groups 2, 3 and 4 showed less indication than this of having been immunized, the results for these groups are only summarized in Table 4.

All groups of chickens were treated with preparations which had been shown by the RSV(B)-interference test to contain an avian leukosis virus. However, chickens treated with measles vaccine (group 2) and dilute and concentrated preparations

of HPRS F 42 (groups 3 and 4) did not produce antibody to RSV (B) during the period of this experiment.

Failure to elicit antibodies with concentrated leukosis virus (HPRS F 42) under these conditions may be a result of antigenic differences between F 42 and RSV (B), or of failure to achieve adequate immunization of the fowls. Accordingly, nine birds of group 4 were re-inoculated 180 days later with HPRS F 42 and bled 21 days afterwards. Eight of the nine sera had significant neutralizing capacity for RSV (B).

Table 3. *Neutralizing capacity for RSV (B) of sera from chickens in group 1*

| | \ % Control pock count | | | |
	Pre-bleed	Primary	Secondary	Inhibitory
Female 1	70	31	40	
2	38	40	38	
3	38	24	25	
4	69	27	15	3/7
5	73	60	47	
6	100	37	15	
7	77	27	15	
Male 1	32	37	—	
2	84	3	1	
3	32	45	41	
4	68	10	14	6/7
5	87	6	0	
6	90	41	15	
7	41	8	1	
8	87	65	3	

Table 4. *Neutralizing capacity for RSV (B) of sera from chickens in groups 2–4*

	Sex	Inhibitory
Group 2	Female	0/7
	Male	0/8
Group 3	Female	0/6
	Male	0/8
Group 4	Female	1/6
	Male	0/6

Another group of ten birds was subjected to a different immunization procedure with the same virus, as follows: day 1, inoculated with 2×10^4 focus-forming units (FFU) of HPRS F 42 intravenously; day 7, and at 7-day intervals for five weeks, given 5×10^4 FFU of virus intramuscularly. On the 14th day one bird showed antibodies to RSV (B). On day 21 four birds, and on day 56 all 10 birds had antibodies to RSV (B).

Two different leukosis virus strains (HPRS F 45 and HPRS B 15) administered to the same schedule gave respectively 4 out of 9 and 5 out of 8 neutralizing sera on the 51st and 48th days respectively.

With the collaboration of a number of R.A.F. men who consented to being bled before and after standard vaccination against yellow fever, it was possible to test

for antibodies to avian leukosis virus in man using the same time relationships as for group 1 birds but without re-immunization. None of the twelve volunteers developed any antibodies to RSV (B) in his serum as a result of his vaccination.

DISCUSSION

We conclude from this investigation that these samples of yellow fever and measles vaccines each contained a virus, presumably acquired from infected chick embryo tissues, with the biological properties of an avian leukosis virus. Adult male volunteers vaccinated with the yellow-fever vaccine did not develop detectable antibodies to this contaminant virus. Some strains of Rous sarcoma virus, which is a member of this chicken tumour virus group, will infect human and simian tissues *in vitro* (Jensen, Girardi, Gilden & Koprowski 1964), produce chromosome abnormalities in human leukocytes (Nichols *et al.* 1964) and even tumours in monkeys (Munro & Windle, 1963).

The regulations for the manufacture of live measles vaccine both in Britain and the U.S.A. now require that the fertile eggs for chick embryo cell cultures shall be derived from leukosis-free flocks and that the final vaccine shall be tested for the presence of leukosis virus. Our results show that the interference test is a much more sensitive indicator of the presence of leukosis virus than the immunity test, but both require embryos or birds from leukosis-free flocks.

There is no evidence that the contaminant virus in yellow-fever vaccine is dangerous—it will not be present in measles vaccine—but, nevertheless, it would now be prudent to adopt similar precautions for its manufacture to those in force for measles vaccine. At the same time it would be worth while looking for any positive (or negative) association between human malignancy, especially leukaemia, and prior yellow-fever vaccination, especially as some 20 years have now elapsed since the introduction of this vaccine.

SUMMARY

Samples of yellow-fever vaccine prepared from homogenized chick embryos, and of an experimental measles vaccine prepared from chick embryo cells, have each been shown to contain a contaminant virus similar in properties to an avian leukosis virus. Young adult males injected with the yellow-fever vaccine did not develop neutralizing antibodies for Rous sarcoma virus.

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