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A yellow fever vaccine free from avian leucosis viruses

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The virus of yellow fever is present in large areas of tropical South America and Africa, where it circulates amongst the animal and human populations with occasional outbreaks of overt human disease, the epidemiology of which is not yet fully understood. There is, accordingly, likely to be need for an indefinite period for a protective vaccine both for travellers, as required by the International Sanitary Regulations, and, when needed, for the containment of epidemics. The well-known attenuated 17D strain of yellow fever virus is now almost universally used for vaccination and is in fact the only strain recognised by W.H.O. for use under the above Regulations. This was produced over 30 years ago by passage of the wild Asibi strain of yellow fever virus, first in mouse embryo tissue and then a large number of times in chick embryo tissue cultures, resulting in marked attenuation of the viscerotropic and, particularly, of the neurotropic properties (Theiler, 1951). A number of substrains were produced and all those in use today are of the 200th to 300th subculture in chick embryo tissue cultures or in whole chick embryos, the latter still remaining the most suitable material in which to grow the virus for vaccine production.

Because of the possible oncogenic potential in mammalian tissue of known and unknown viruses of the avian leucosis complex (Vogt, 1965), opinion is growing that these viruses should be excluded from human vaccines to be given parenterally, and this is now a requirement for measles vaccines made in chick embryo tissue (W.H.O., 1966*a*). Because they are widespread in many flocks of chickens, and no special precautions were taken to exclude them, it is probable that most substrains of 17D yellow fever virus are contaminated with avian leucosis viruses. Harris *et al.* (1966) found heavy contamination in a batch of secondary seed used for vaccine production in these laboratories, and suggested that the standards for yellow fever vaccine should conform to those for other vaccines made in chick tissues, and this opinion has also been voiced by a W.H.O. scientific group (W.H.O., 1966*b*). Piraino, Krumbiegel & Wisniewski (1967) describe the discovery of leucosis viruses in several different batches of yellow fever vaccine in the U.S.A. Like Harris *et al.* (1966) they were unable to detect neutralizing antibodies in human recipients of such vaccines, but both these teams of workers used only the Bryan standard strain of Rous sarcoma virus (see below under Methods) as the challenge virus in serum neutralization tests and, as the former comment, may have missed antibodies formed against other antigenic groups.

In any manipulations needed to clear a 17D vaccine substrain of leucosis viruses it is important that these should not involve more than a few passages of the virus

beyond the level at which it has been found to be both safe and effective as a vaccine, because of the risk of inducing undesirable variations in its properties. In the early days vaccines were commonly made by serial passage of virus from one batch of chick embryos to another; on one occasion, in Brazil, several batches of vaccine made at passage levels 22 to 25 away from a substrain of known safety were found to have increased neurotropic properties, causing encephalitis, mostly not fatal, in up to 2% of those vaccinated, all ages being affected. This was correlated with an increased neurovirulence on intracerebral inoculation into rhesus monkeys (Fox, Lennette, Manso & Aguiar, 1942; Fox & Penna, 1943). Conversely, on another occasion, some further passages beyond the 300th possibly resulted in greater attenuation with a reduction in immunizing efficiency below acceptable levels (Soper & Smith, 1938). Although in several other instances repeated passages of several substrains have not resulted in any changes (Theiler, 1951), the proven risk that these may sometimes occur led to the introduction of the well-known 'seed-lot system', now routinely applied to the production of all virus vaccines, whereby vaccine is always made at a fixed passage level. Following the introduction of this system cases of encephalitis and other undesirable reactions after vaccination with the 17D yellow fever virus have been rare and sporadic. In an account of a recent fatal case of encephalitis in a 3-year-old child (Sencer *et al.* 1966) it was estimated that about 100 million doses of the vaccine must have been given since 1942, when the 'seed-lot system' was started, but only 15 other cases of encephalitis have been reported in the world literature in this time, all in infants under a year old and none of them fatal (Stuart, 1956; Feitel, Watson & Cochran, 1960). It is unknown whether such cases are due to the occasional emergence of virus particles with increased neurotropic properties, by analogy with the happenings with attenuated poliomyelitis virus (see, for example, Permanent Section of Microbiological Standardization, 1967), or due to an unusual susceptibility of the infant nervous system to invasion by the virus, coupled, perhaps, with an anomalous antigen-antibody reaction of the type suggested by Webb & Smith (1966). The fact remains that 17D yellow fever is a potentially neurotropic virus and must be treated as such. Most countries now do not demand the vaccination of infants under a year old.

This paper describes the methods used to free a 17D substrain from contaminant leucosis viruses, with the minimum number of passages, and the production and testing of seeds and vaccine derived from it.

METHODS AND MATERIALS

The presence of leucosis viruses was tested for by the interference method (RIF test) originally devised by Rubin (1960). The yellow fever virus in the material for test was neutralized with serum from rabbits hyperimmunized with the French neurotropic strain of virus, propagated in mouse brains. The material was then inoculated on primary tissue cultures of chick embryos from a flock of chickens shown to be free from leucosis viruses and with a uniform sensitivity to Rous sarcoma viruses. The cultures were serially passaged and at the 4th subculture

both the test and negative control cultures were challenged, some with the Bryan standard strain of Rous sarcoma virus (B-RSV), of antigenic subgroup A, and others with a Rous sarcoma virus pseudotype (RSV-RAV 2) of antigenic subgroup B. Occasionally, challenges were done at the 2nd subculture. The test was considered negative if the test cultures showed a less than tenfold reduction in foci as compared with the negative control cultures when these were counted 7 days after the challenge.

The production of vaccine seeds and of final vaccine was done by methods in routine use in these laboratories. Six- to 7-day-old chick embryos were inoculated directly through the shell with 0·1 ml. of virus suspension and were incubated for 3·5–4 days at 37·5° C. The surviving embryos were then harvested aseptically, in groups of 20, and fragmented mechanically in a stainless steel blender, using 40 ml. of sterile distilled water for each group. This suspension was clarified by light centrifugation and the supernatant fluid, after taking samples for virus titration and bacterial sterility tests, was stored in glass containers at − 70° C. After completion of these tests, sufficient sterile batches were taken to make a convenient lot, they were thawed rapidly, pooled, and equal volumes filled into ampoules and freeze-dried. The chick embryos were taken from pathogen-free flocks of chickens, established by these laboratories from accredited sources, which are held in isolated quarters and repeatedly tested to confirm their freedom from leucosis viruses and other adventitious agents. Both seeds and vaccine were tested according to the W.H.O. Requirements for Yellow Fever Vaccine (1959) some of which are considered in detail later.

Titrations of yellow fever virus were done by inoculating 0·03 ml. of fourfold dilutions intracerebrally (i.c.) into groups of five to six 1-month old Swiss mice, bred in these laboratories. Some titrations were also done in monkey kidney tissue by an interference method (Draper, to be published) of almost equivalent sensitivity. Virus titres and doses are given in terms of mouse LD 50 doses (MLD 50). Neutralization (NT) tests were done by incubating, at 37° C. for 60 min., mixtures of equal amounts of test sera, previously heated at 56° C. for 30 min., and of serial tenfold dilutions of the French neurotropic strain of yellow fever virus (in its 541st passage), and these were then inoculated i.c. into groups of mice as above. The neutralization index (N.I.) of a postinoculation serum is the difference between the $-\log_{10}$ LD 50 titres of the virus in it and in the preinoculation serum. For haemagglutination-inhibition (HI) tests sera were absorbed with kaolin and then goose erythrocytes before use. Twofold dilutions starting from 1/20 were incubated at 4° C. overnight with 4 to 8 haemagglutinating units (HAU) of a yellow fever virus antigen, prepared by high-speed centrifugation (11,000 *g*) of an aqueous suspension of mouse brains infected with the French neurotropic virus, before the addition of goose erythrocytes at a pH of 6·6.

RESULTS

Production of leucosis-free seeds and vaccines

A batch of vaccine, number 1815, manufactured in 1945 by the Rockefeller Foundation Laboratories, New York, was received by these Laboratories in 1946. This material represents the 230th passage of the yellow fever 17D substrain (M. Theiler, pers. comm., 1965) and has been used as primary seed for the production of several batches of secondary seed, from which large amounts of vaccine have been made, amounting to several million doses. It was subjected to RIF tests, as described, on several occasions and found to be heavily contaminated with leucosis agents, resistance of the cultures to both of the challenge viruses being evident. Some preliminary experiments showed that it was possible to clear the material of leucosis viruses by limit dilution passages through chick embryo tissue cultures, combined with treatment with a leucosis antiserum, and this was also achieved by passage through human embryo fibroblast cultures. However, as six or more passages were needed and poor virus yields were obtained at the end, another attempt was made in whole chick embryo with a more potent antiserum, hoping thereby to reduce the number of passages and to obtain a higher yield of virus. Serial tenfold dilutions of vaccine 1815 were incubated at 37° C. for 60 min. with a chicken antiserum against the RPL12 virus strain of leucosis virus, and these mixtures were then inoculated into batches of leucosis-free chick embryos. Harvested wet material was stored at −70° C. while RIF tests and tests for yellow fever virus content and for bacterial sterility were done. On conclusion of these, one batch, made from 16 embryos which had been inoculated with a 1/1000 dilution of vaccine 1815 with antiserum, giving a dose per embryo of about 10^2 MLD50 of 17D virus, was chosen as being suitable for a new primary seed, in that it was found to be free from leucosis viruses and contained a good yield of 17D yellow fever virus. Some of this material was diluted 1/1000 and was inoculated by itself into a larger batch of chick embryos, each of which received again about 10^2 MLD50 of 17D virus. The wet pulps harvested from 117 embryos were stored in batches at −70° C. while a pooled sample from each was subjected to a RIF test and 17D virus titration, and bacterial sterility tests were done on the individual batches. All these tests proved satisfactory and the material was then further diluted with water and a large batch of ampoules freeze-dried. This was designated as a new leucosis-free secondary seed (YFS/5) to be used for the production of vaccine. The primary seed material was also freeze-dried in ampoules, for future use, and both it and YFS/5 satisfied criteria for sterility, guinea-pig toxicity, and residual moisture as defined by W.H.O. (1959), while the identity of the 17D yellow fever virus in both was confirmed by NT tests with a specific antiserum. Furthermore, quantities of YFS/5 equivalent to over 5000 egg inoculation doses were tested in primary monkey kidney tissue cultures, in chick embryo fibroblast and liver cultures, by inoculation into the allantoic cavity and the yolk sac of chick embryos, and by inoculation i.c. and intraperitoneally into mice, for the presence of other extraneous viruses, all with negative results. For these tests the 17D virus was neutralized with the rabbit antiserum described. Using a

dilution of YFS/5 such as to give an inoculum per egg of about $10^{2.5}$ MLD 50, over 30 batches of 17D vaccine have been made in pathogen-free eggs; all have been found free of leucosis viruses in the test employing two different challenge viruses. Such vaccine represents the 233rd passage level of the 17D strain.

Table 1. *Results of inoculation into Macacus rhesus monkeys of yellow fever secondary seed virus*

No. of monkey	Wt. in Kg.	Inoculum	No. occasions when viraemia detected (see text)	Highest dilution of serum in which virus detected	Days post inoculation when temp. exceeded 40 °C.	Days post inoculation when some paralysis (see text)	Maximal paralysis seen in limbs (see text)	Days post inoculation when maximal paralysis	Clinical assessment encephalitis (see text)	Neutralization index of post inoculation serum
2	7·2	$10^{4.9}$ MLD 50 i.c. of YFS/4/2 (standard seed)	Nil	Nil	5, 10–12	Nil	Nil	Nil	0	3·8
3	6·0		2	Undil.	10–13	14–18	5545	14–18	1+	2·4
4	6·2		1	1/10	14–15	14–29	5533	16–18	2+	2·1
5	5·1		Nil	Nil	Nil	12–19	5533	14	1+	3·6
6	6·0		1	Undil.	8–9, 11–13	12–18	5544	12–18	1+	3·6
9	6·9		3	1/10	8–9, 12	12–32	5533	14–22	3+	4·2
11	5·8		1	1/10	10–15	12–28	5545	15–28	1+	2·8
14	6·2		2	Undil.	8–13, 15	14–18	5554	14–18	1+	1·5
15	4·9		3	Undil.	10–12	12–28	5533	14–15	3+	1·9
16	5·1		1	Undil.	10–12	14–21	5544	14–18	1+	2·8
30	4·1	$10^{3.9}$ MLD 50 i.c. of YFS/5 (leucosis-free seed)	2	Undil.	10–11	14–24	5554	14–24	1+	3·3
33	5·0		2	Undil.	9–11	12–29	5534	14–19	3+	3·0
1011	5·0		1	Undil.	8–11	10–13	5545	10–13	1+	1·9
1012	2·8		3	1/10	5–6, 8–11	13	5554	13	±	1·8
1013	3·0		3	1/10	13	6	5544	6	±	2·2
1014	3·1		1	Undil.	8–10	11–19	5544	11–13	1+	3·0
1015	3·5		2	Undil.	8–10	12–15	5544	12	1+	2·2
1021	3·8		3	1/10	6, 10–11	11–24	5534	14	1+	3·3
1024	3·9		Nil	Nil	6, 10–12	11–20	5522	13	3+	2·4
1024	4·6		2	Undil.	9–10, 12	11–18	5534	15	1+	3·2

Tests of viscerotropism and neurotropism

The W.H.O. Requirements for Yellow Fever Vaccine (1959) state that each new batch of seed shall be subjected to these tests in not less than 10 monkeys, and Table 1 shows the results obtained in individual monkeys with two different batches of secondary seed. The first, YFS/4/2, was a new batch of standard secondary seed made directly from vaccine 1815, and the second, YFS/5, was the new leucosis-free secondary seed, the production of which has just been described. *Macacus rhesus* monkeys, which had previously been found to be free of antibodies for arboviruses of antigenic group B, were each inoculated, under anaesthesia, into a cerebral hemisphere with 0·25 ml. of a suspension of the seed for test and this was immediately afterwards titrated to determine the virus content. The requirements state that each monkey shall receive a dose of not less than 5000 MLD 50. The monkeys had rectal temperatures taken daily, and on the 2nd, 4th, and 6th days after inoculation venous blood samples were taken. The serum was rapidly separated from these and at once inoculated i.c. into groups of mice, undiluted and in tenfold dilutions up to 10^{-3}, in order to assay the degree of viraemia. The mice were observed for 3 weeks and the brains were harvested from any dying with signs of yellow fever encephalitis. The presence of the virus was confirmed in several mice from each test by its neutralization by specific antiserum. For detecting any signs of encephalitis the monkeys were removed from their cages each day over a period of a month and placed one at a time in a special exercise cage, where a full

range of movements involving running, jumping, and climbing could be seen. The same pair of observers recorded their observations throughout, and in the rare case of disagreement the verdict went in favour of recording the greater amount of impairment of movement. The scale used for grading the amount of weakness of the limbs follows that of Bodian (1948), the individual limbs being listed in this order: right arm, left arm, right leg, left leg. The figure 5 denotes normal; 4 minimal weakness; 3 marked weakness, but able to use the limb; 2 denotes inability to use the limb for climbing but able to lift it against gravity; 1 denotes inability to lift the limb against gravity; 0 denotes complete flaccid paralysis. A clinical assessment was also made of the overall picture, following that of Nathanson *et al.* (1966*a*, *b*) in which: 0 = normal; ± = doubtful (clumsy, or tremors for 1 or 2 days); 1+ = minimal (clumsy or slow, in grades 4 or 5 for 3 or more days, or tremors for 3–10 days); 2+ = moderate (grade 3 paralysis in at least one limb for 2 or more days but without residual paralysis, or tremors for 11 or more days); 3+ = severe (grade 3 or lower in at least one limb for 2 or more days with definite residual weakness after a month); 4+ = fatal. Thirty days after inoculation blood samples were again taken from the monkeys and the sera tested for neutralizing antibodies.

Table 2. *Summary of tests in monkeys of yellow fever vaccine secondary seeds*

	Standard YFS/4/2	Leucosis-free YFS/5
No. of monkeys with viraemia on all 3 days tested	2	3
No. of monkeys with viraemia on 2 days only	2	4
No. of monkeys with viraemia on 1 day only	4	2
No. of monkeys without demonstrable viraemia	2	1
Total no. of positive sera in which virus detected only when undiluted	11	16
Total no. of positive sera in which virus detected when diluted 1/10	3	3
Total no. of positive sera in which virus detected when diluted 1/100	0	0
No. of monkeys with assessment of encephalitis as 3+	2	2
No. of monkeys with assessment of encephalitis as 2+	1	0
No. of monkeys with assessment of encephalitis as 1+	6	6
No. of monkeys with assessment of encephalitis as ±	0	2
No. of monkeys with assessment of encephalitis as 0	1	0
Average number of days per monkey when temperature exceeded 40° C.	3·6	3·2
Mean N.I. of postinoculation sera	2·8	2·6

The data in Table 1 are summarized in Table 2. Regarding the degree and duration of viraemia both seeds appear to be similar though the viraemia is a little more in the case of YFS/5, perhaps related to the smaller size of the monkeys available for this test. In this test YFS/4/2 fails the criterion of the W.H.O. requirements which demands that virus shall be isolated from at least 9 out of 10 monkeys, but, as both the monkeys without demonstrable viraemia (nos. 2 and 5) subsequently became fully immune, this must have occurred at some stage. The other criteria are met that 'in no case shall 0·03 ml. of serum contain more than 500 MLD 50 and in not more than one case shall 0·03 ml. of serum contain more

than 100 MLD 50'. Regarding the important criterion of neurotropism, the W.H.O. requirements state that 'not more than 20% of the test monkeys shall develop encephalitis manifested by paralysis or inability to stand, with or without subsequent death'. If a 3+ grade of paralysis or higher as defined above, is taken as indicating such obvious clinical encephalitis both batches of seed can be deemed to fulfil the criterion. It is probable that most of the monkeys with a 1+ or even with a 2+ paralysis would not have been detected without the use of an exercise cage. There is no evidence of a change in neurotropism with the YFS/5 seed, while the febrile reaction, which has been roughly correlated with the neurotropic properties (Fox & Penna, 1943), is similar with the two seeds. All the monkeys became immune.

Table 3. *Average survival times* (A.S.T.) *of mice inoculated with varying doses of* 17D *yellow fever vaccines derived from two different secondary seeds*

$\log_{10}$ dose of virus in MLD 50	From standard seed YFS/3		From leucosis-free seed YFS/5	
	No. mice	A.S.T.	No. mice	A.S.T.
0·1–0·5	400	12·5	136	12·8
0·6–1·0	377	11·4	169	11·7
1·1–1·5	372	10·7	131	11·6
1·6–2·0	14	9·9	49	9·9

Neurotropism in mice

Average survival times (A.S.T.) have been calculated for mice inoculated intracerebrally with batches of standard vaccine derived from a secondary seed YFS/3, a sister seed of YFS 4/2, or with leucosis-free vaccine derived from YFS/5. For the calculations, only mice dying of typical yellow fever encephalitis have been included and, contrary to the occasional practice of allotting an arbitrary figure for survival time for mice remaining alive at the end of the observation period of 3 weeks, these have been omitted altogether. This follows the method of Meers (1959) in his study of the adaptation of the 17D virus to mouse brain. Table 3 shows the A.S.T.'s for 30 batches each of the two different kinds of vaccines according to the dose inoculated. As these have been calculated from routine titrations of vaccines, designed to cover the end-points, there are few observations with the larger doses. There are no differences in the neurovirulence for mice, and the figures are similar to those given by Meers (1959) for unadapted 17D virus.

Tests of antigenicity in man

As a final and necessary comparison, two comparable groups of young adults, all previously free from antibodies to group B arboviruses, were inoculated subcutaneously, one with standard vaccine and the other with leucosis-free vaccine. Several different batches of each type of vaccine were used, and in each case titration of remaining ampoules showed that from $10^{4\cdot1}$ to $10^{4\cdot4}$ MLD 50 were given. Samples of venous blood were taken at the time of inoculation and again after 1 month and these were tested for the development of neutralizing and HI

antibodies. The results are given in Table 4; there are no differences in the responses obtained. One subject in each of the two series had failed to develop detectable antibodies at the time that the second sample of blood was taken, by the conventional tests used here, and it is possible that these would have been found in a later sample. Smith, Turner & Armitage (1962) and Smith, McMahon & Turner (1963) found that in a few yellow fever vaccinees antibodies may not appear until after 28 days.

Table 4. *Neutralizing and HI antibodies in sera of human subjects inoculated with different* 17D *yellow fever vaccines*

Vaccine	No. inoculated	Mean N.I.	G.M.T. HI antibodies
Standard	38	1·6	70
Leucosis-free	59	1·7	80

DISCUSSION

The steps taken to free a substrain of 17D yellow fever virus from contaminant leucosis viruses have not caused any changes in its other properties. This, perhaps, was to be expected in view of the simplicity of the manipulations, in that it was achieved with the minimum number of passages and without recourse to other measures, such as limit dilution, which might favour the chance emergence of mutant particles. Nevertheless, because of the widespread use of this vaccine, the possibility had to be excluded.

The methods in present use for assessing in particular the neurotropic properties of a vaccine substrain of the virus are crude when compared with those used, for example, in testing attenuated strains of poliomyelitis viruses (Permanent commision of microbiological standardization, 1967). Although the 17D virus is obviously neurotropic when introduced directly into the nervous system, and although, at least in the case of some substrains, it is not in a stable state of attenuation, the very large number of vaccinations done, with remarkably few undesirable reactions, testify to the safety of its use. However, in consonance with modern trends towards improved vaccines, it might be at least reassuring if more critical tests could be adopted. A move in this direction has been made by Nathanson *et al.* (1966*a*, *b*) who have developed a feasible method for assessing the neurovirulence of arboviruses in monkeys by the examination of specific 'indicator centres' in the brain and spinal cord.

SUMMARY

1. The methods used for clearing a vaccine substrain of 17D yellow fever virus of contaminant avian leucosis viruses are described.

2. Tests in animals and human subjects showed that the characteristics of the virus remained unchanged.

The RIF tests and the preparation of the leucosis antiserum were done by Messrs A. E. Churchill and W. Baxendale, while the monkey neurovirulence tests

were done in collaboration with Dr V. Udall, all of these laboratories, whose help is gratefully acknowledged. Major-General M. H. P. Sayers and Colonel L. G. Irvine R.A.M.C. kindly arranged for the antigenicity tests in human subjects. Mr A. Jopling gave much valuable technical assistance throughout.

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