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Laboratory studies on a strain of Asian influenza virus used as a living vaccine. (A report to the Medical Research Council Committee on Influenza and other Respiratory Virus Vaccines*)

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Several clinical trials of living influenza virus vaccine prepared from the Russian A 2 strain Iksha have been conducted in this country since 1960 under the auspices of the M.R.C. Committee on Influenza and other Respiratory Virus Vaccines. In the initial trials (McDonald, Zuckermann, Beare & Tyrrell, 1962) the vaccine pool represented the 6th allantoic passage (pass L_6) of the virus. Most subjects given intranasal drops of this material developed symptomless infection with slight or moderate rises in haemagglutination-inhibiting (HI) antibody titres, and were resistant to subsequent experimental reinfection. However, in a further trial of Iksha vaccine prepared in exactly the same way (Andrews, Beare, McDonald & Zuckermann, 1966) virus was recovered from only a few inoculated volunteers and only one-third of the group (mainly those with pre-existing HI antibody) showed a significant serological response. In subsequent trials (Beare *et al.* 1967) the vaccine pools were prepared somewhat differently; Iksha virus which had been recovered in the throat washings of a volunteer inoculated with pass L_6 material was given one further allantoic pass in eggs from a flock of hens known to be free from infection with avian leucosis agents. Adult volunteers given this vaccine pool (pass $L_6Hu_1L_1$) showed no evidence of established experimental infection, and few developed significant rises in HI antibody. A vaccine pool of the virus after one further allantoic passage (pass $L_6Hu_1L_2$) was administered to children, and this also seemed ineffective.

No obvious differences in the composition of the volunteer groups or in the method of vaccine administration emerged to account for these discrepant results, and it seemed probable that the virus strain itself might have undergone some change in character during the production of the different vaccine pools. There is thus an obvious need to define laboratory criteria which would allow direct recognition of influenza virus pools which are suitable for use as living vaccine from those which are not. Accordingly, at the request of the MRC Committee on Influenza and other Respiratory Virus Vaccines, it was decided to compare the

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response to some laboratory tests of Iksha pass L_6 used in the earlier trials with that of the pass $L_6Hu_1L_2$ virus used in the less successful later trial in children.

MATERIALS AND METHODS

Seed pools of the Iksha strain of Influenza A 2 virus, at the passage levels L_6 and $L_6Hu_1L_2$ described above, were given a single further egg passage; 0·2 ml. of a 10^{-4} dilution of the seed pool was inoculated into the allantoic cavity of 11-day-old chick embryos; infected allantoic fluids were harvested after 42 hr. incubation at 35° C. and stored in 2 ml. vol. at − 70° C. after clarification by low-speed centrifugation. All subsequent tests were on these first laboratory pools, unless otherwise indicated in the text.

Haemagglutination (HA) and haemagglutination-inhibition (HI) tests were performed by the WHO plastic plate method (WHO Expert Committee on Influenza, 1953); a 1 % suspension of human group O cells was used except where otherwise stated. Rabbit antisera to various influenza viruses were as previously described (Hobson, 1966).

The kinetics of adsorption to and elution from red cells was studied by methods based on those of Lief & Henle (1958).

Infectivity titrations by conventional methods in the allantoic cavity of 11-day-old developing fertile eggs, or in isolated pieces of chorioallantoic membrane (Fazekas de St Groth & White, 1958) were carried out at various temperatures, using specially controlled incubators (Bedson & Dumbell, 1961).

Infectivity titrations in Swiss white mice were performed by methods previously described (Lindenmann, Lane & Hobson, 1962).

RESULTS

Growth in eggs at various temperatures

It is known that the highest temperature at which viruses will grow in tissue culture or eggs may vary characteristically from strain to strain and, with the poxviruses, these ceiling temperatures offer a useful means of characterization (Bedson & Dumbell, 1964). Differences in the ability of polioviruses to grow at high temperatures has been employed as a means of differentiating virulent from attenuated strains, and cryophilic variants of poliovirus have been shown to be of decreased virulence (Dubes & Wenner, 1957); similar findings with influenza viruses have been shown by Alexandrova & Smorodintsev (1965). Accordingly, the growth characteristics of the two pools of Iksha virus were examined at various temperatures of incubation.

After incubation of inoculated eggs at 35° C for 48 hr. both the pools L_6 and $L_6Hu_1L_2$ showed similar infectivity titres (approx. $10^{9.5}$ EID 50/ml.); neither strain caused death or obvious lesions in the chick embryos within this time, and HA/EID 50 ratios were similar for both pools.

A single dilution (10^{-4}) of each pool was inoculated into eggs, and after incubation at various temperatures from 25 to 42° C. for 16 hr., allantoic fluids were

harvested and titrated for infectivity in chorioallantoic membrane (CAM) cultures maintained at 35° C. for 48 hr. At temperatures between 35 and 39° C. both strains grew equally well, to final titres of $10^{8.5}$–$10^{9.0}$ EID 50/ml. At lower temperatures, a progressive reduction of growth occurred; although titres were generally 0·3–1·0 $\log_{10}$ lower with $L_6Hu_1L_2$ than with L_6 virus over this range the behaviour of the two types of virus could not be clearly differentiated, and titres of $10^{1.4}$ and $10^{1.7}$ EID 50/ml. respectively were found at the lowest temperature, 25° C. At higher temperatures of incubation, the growth of both strains was equally affected over the range 39–40° C., but the degree of inhibition was comparatively small (titres = $10^{6.7}$ EID 50/ml.). Between 40·5° and 42° C. it was difficult to obtain closely comparable results in different experiments, owing probably to the poor viability of chick embryos at these high temperatures; however, in all tests Iksha L_6 remained capable of growth to titres of approx. 10^4 EID 50/ml. at 42° C., whereas $L_6Hu_1L_2$ virus grew erratically, and titres of individual eggs were usually lower than $10^{1.5}$ EID 50/ml. It seemed possible that growth of Iksha virus pools at 25–27° C. or at 40–42° C. might have led to the selection of variant fractions of the original uncloned virus populations. However, the virus progeny of the two strains grown at these extreme temperatures for up to 48 hr. showed no difference from the parent strains in their growth-temperature range, nor in any of the properties to be described below, and would thus appear to be randomly-selected survivors of the original virus pools.

Nutritional factors

Certain differences in nutritional requirements for the growth of different strains of influenza virus in chorioallantoic membrane cultures have been shown by Eaton and his colleagues (Daniels, Eaton & Perry, 1952: Eaton, Adler & Perry, 1953) and by Veeraraghavan, Kirtikar & Sreevalsan (1961). However, in the present experiments, the growth of Iksha viruses in CAM cultures was not affected by the substitution of medium 199 for the simple glucose-saline solution of Fazekas de St Groth & White (1958), the replacement of glucose by sodium pyruvate (Levine, Bond & Rouse, 1956) or the addition of folic acid, glycine and sodium molybdate (Veeraraghavan *et al.* 1961) and attempts to find nutritional differences between the two virus pools have not been pursued further at this stage.

Mouse infectivity

Neither of the virus pools caused any deaths or signs of infection after intracerebral inoculation. After intranasal instillation both L_6 and $L_6Hu_1L_2$ pools gave similar results. Deaths with gross pulmonary consolidation occurred within 3–6 days after inoculation of either undiluted or a 10^{-1} dilution of infected allantoic fluids; no deaths occurred in mice inoculated with dilutions of 10^{-2} or 10^{-3} but when the survivors were killed, 8 days later, scattered pulmonary lesions were found in most animals; higher dilutions gave no evidence of infection.

Emulsions of lungs obtained from infected mice were directly titrated for their virus content in further batches of mice and in eggs. The mouse ID 50/EID 50 ratio of the emulsions was closely similar to that of the original virus pools. Thus,

there was no evidence that variants of significantly enhanced virulence had been selected by single passage through mice.

Iksha viruses which had been passaged in eggs incubated at 27° C. showed mouse ID 50/EID 50 ratios similar to those of the parent strain.

Haemagglutination characteristics

The range of activity of influenza virus haemagglutinins against various species of red blood cell, their thermostability and sensitivity to mucoid inhibitors, and the reversibility of haemagglutination, probably as an effect of viral neuraminidase, may differ from strain to strain. These differences have been shown by Burnet (1951) to behave as heritable properties of certain virus strains in genetic recombination experiments. The L_6 and $L_6Hu_1L_2$ pools of Iksha virus were, therefore, examined to determine whether significant differences in the behaviour of their haemagglutinins could be found.

Both virus pools gave similar HA titres when tested with human red blood cells (RBC) of group O, A, B and AB or horse, sheep and fowl RBC at 4° C., room temperature or 37° C.; after standing for a further 2 hr. at these incubation temperatures, some disaggregation was found, but only in HA shields produced by the higher concentrations of virus, and only at 37° C. Mouse RBC were agglutinated equally well by either pool but the HA shields were unstable and almost complete reversion of agglutination occurred rapidly at room temperature and at 37° C.

A more quantitative study of the elution of virus from RBC revealed some difference between the two Iksha strains. Washed human group O RBC were added in a final concentration of 10 % to 50 ml. volumes of fresh allantoic passages of L_6 and $L_6Hu_1L_2$ viruses, each diluted in saline to contain 320 HA units per 0·25 ml. After incubation for 1 hr. at 4° C., with constant stirring, the RBC were recovered by centrifugation in the cold at 1000 rev./min. for 10 min. and twice washed with cold saline. No detectable haemagglutinin remained in the original supernatant fluid or the saline washings. Each RBC mass was resuspended in 5 ml. M/50 phosphate buffered saline and incubated with stirring at 37° C.; samples taken at serial intervals were centrifuged briefly in warmed containers. The supernatant fluids of each sample were tested by HA titration for eluted virus. The results (Fig. 1) show that $L_6Hu_1L_2$ virus eluted faster and more completely than the L_6 passage.

The thermal resistance of the haemagglutinin of the two Iksha pools was compared by incubating undiluted infected allantoic fluids for various times at temperatures from 37° to 56° C., and subsequently titrating at room temperature for residual HA activity. The two virus pools differed in behaviour as shown in Fig. 2; HA of both pools was rapidly destroyed at 53–56° C. but at temperatures of 49–51° C. the HA of the L_6 pool was inactivated more quickly and completely than that of $L_6Hu_1L_2$ virus.

To exclude the possibility that these were merely chance variations between the two egg pools, it was decided to investigate several further pools of L_6 and $L_6Hu_1L_2$ virus obtained by passage at or near their terminal infective dilutions. Groups of 11-day-old chick embryos were inoculated allantoically with 0·2 ml. of a 10^{-8} or

10^{-9} saline dilution of L_6 or $L_6Hu_1L_2$ pool. Allantoic fluids were harvested separately after 42 hr. incubation at 35° C. and tested for haemagglutinin. Samples of each positive fluid were retitrated after being incubated at 49·5° C. for 30 min. and 50° C. for 60 min. The results (Table 1) confirmed the sharp differences in thermal stability of HA of the two virus strains. In many cases, heating at 49·5° C. for

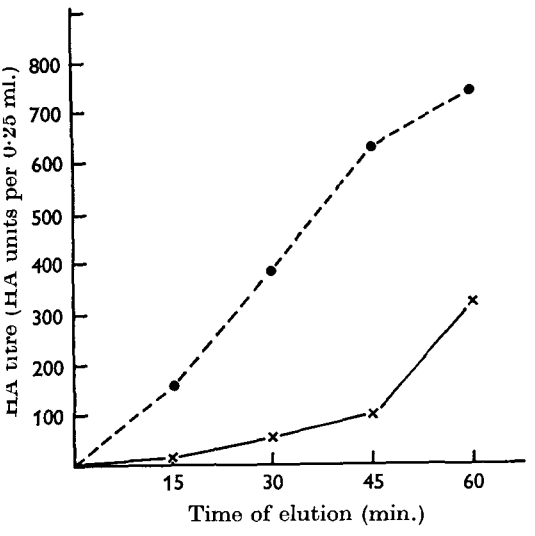

Fig. 1

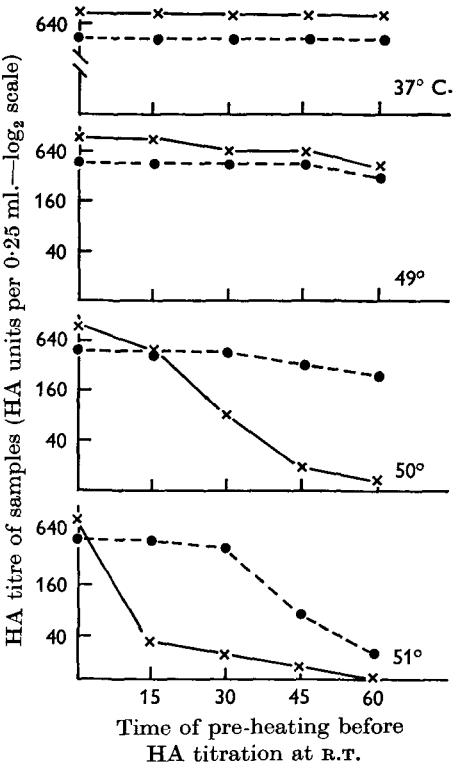

Fig. 2

Fig. 1. The rate of elution of two pools of Iksha influenza virus from haemagglutinated human RBC ×—×, Early pass, L_6; ●---●, later pass, $L_6Hu_1L_2$.

Fig. 2. The effect of heat on the HA titre of two pools of Iksha influenza virus. ×—×, Early pass, L_6; ●---●, later pass, $L_6Hu_1L_2$.

30 min. gave a rise in HA titre, possibly as a result of virus disaggregation or removal of non-specific inhibitors in the fluid; on continued incubation of the HA tests at room temperature, shields of agglutination produced by heated L_6 virus rapidly crumbled whereas HA shield-patterns produced by heated $L_6Hu_1L_2$ were stable; no dissociation occurred in titrations of fresh virus pools of either strain, and it was presumed that heating had affected the strength of bonding of virus to RBC. At 50° C. for 60 min. 0/7 pools of L_6 showed more than a trace of residual HA, whereas 6/7 pools of $L_6Hu_1L_2$ were virtually unaffected by this temperature.

The stock pools of L_6 and $L_6Hu_1L_2$ virus were tested for HI at a constant dosage of 8 HA units against various animal sera. There was no inhibition of haemag-

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glutination by normal horse, mouse or rabbit serum or by specific rabbit antisera against A (Swine), A O (WS and PR 8) or A 1 (FM 1) strains of influenza virus. With standard antiserum against A 2/Jap/305/57/EFME, HI titres were 1/4096 against the homologous virus, 1/1024 against L_6 virus and 1/512 against the $L_6Hu_1L_2$ strain. A rabbit antiserum prepared against L_6 virus showed an HI titre of 1/320 against the same strain, but 1/192 against $L_6Hu_1L_2$ virus. It would thus appear that the avidity of the two virus strains for antibody differs, or that there may be slight differences in antigenic constitution. HI tests with each of the terminal dilution pools of each virus showed that all those derived from L_6 were more sensitive to inhibition by the antiserum prepared against L_6 virus than were those derived from the $L_6Hu_1L_2$ strain.

Table 1. *The thermostability of HA of various limiting-dilution clones of two pools of Iksha influenza* A_2 *virus*

Parental virus	Clone no.	HA titres of allantoic fluid suspensions of virus clones: Unheated	After heating: 49·5°/30 min.	After heating: 50°/60 min.	Stability of HA of heated virus
Early pass, L_6	− 8, a	480	320	20	—
	− 8, b	1920	1280	20	—
	− 8, c	480	320	20	—
	− 8, d	960	1280	20	—
	− 9, e	960	960	< 20	—
	− 9, f	100	< 20	< 20	
	− 9, g	1920	< 20	< 20	
Later pass, $L_6Hu_1L_2$	− 8, a	240	240	480	+
	− 8, b	960	1280	640	+
	− 8, c	240	240	160	+
	− 8, d	480	960	1280	+
	− 8, e	320	480	240	+
	− 9, f	320	640	640	+
	− 9, g	960	1280	60	+

− = HA titration showing disaggregation of RBC shields after a further 1–2 hr. at R.T.

DISCUSSION

The aim of the present experiments was to assess certain laboratory characteristics of two different pools of the Russian A2 virus Iksha, one of which ($L_6Hu_1L_2$) appeared to be more attenuated than the other (L_6), in the sense that fewer infections and a lesser serological response could be demonstrated after inoculation into volunteers. As would be expected, the two close passage levels of this single virus strain did not differ in most respects. However, the more 'virulent' pool, i.e. the L_6 strain used fairly successfully in clinical trials, could be differentiated to some extent by laboratory tests. The HA of L_6 virus was less thermostable than that of the later passage $L_6Hu_1L_2$, and terminal dilution clones derived from each strain behaved similarly to the uncloned parent pools. The rate of elution of virus from human RBC was slower for L_6 than for $L_6Hu_1L_2$, and this may

indicate differences in the neuraminidase activity of the two strains; however, both were fully resistant to non-specific serum inhibitors of haemagglutination. In HI tests with specific Asian antisera L_6 was slightly more sensitive than the later passage, but serological cross-adsorption studies would be required before this could be assumed to be a true antigenic difference. The L_6 pool showed a slightly greater capacity to multiply in the chorioallantoic membrane at temperatures above 40° C. than the apparently more attenuated $L_6Hu_1L_2$ pool. Recently, Alexandrova & Smorodintsev (1965) have shown that cryophilic variants of certain A2 and B strains of influenza virus, which are incapable of growth at 38°–40° C., are of lower human virulence than the original parent strains.

The precise definition of genetic markers which may be associated with the infectivity of influenza viruses for man has, however, been limited by the lack of suitable experimental animal infections which are a realistic model of the human disease, and by the lack of tissue culture plaque techniques with a sufficiently high plating efficiency for exact quantitative studies. However, Burnet (1951) showed that, with respect to neurovirulence for mice, the avirulent AO strain WSM could be differentiated from the virulent variant NWS by several laboratory markers, particularly by the reduced thermostability of HA of the neurotropic strain. More recently, Soloviev, Orlova, Porubel & Vasilieva (1961) compared the behaviour of egg-grown strains of A2 influenza virus of known difference in human virulence. In general, moderately attenuated strains which were acceptable as living vaccines showed greater HA-thermostability than strains of greater virulence, which had produced untoward reactions on inoculation into volunteers. The attenuated strains also eluted more readily from red blood cells, were less sensitive to non-specific HA-inhibitors and gave higher HA titres with mouse and horse RBC than the virulent strains. It cannot be determined whether these were merely chance differences between unrelated virus strains or whether they were correlated in any way with the observed differences in virulence.

In the present experiments, the two virus pools L_6 and $L_6Hu_1L_2$ were derived from a single parent strain with only a few intervening steps. Here it is perhaps more reasonable to assume that their differences in laboratory behaviour may in fact be due to modifications of genetic constitution, and that these changes may be linked in some way with the observed change in clinical performance. The range of virulence of L_6 and $L_6Hu_1L_2$ (i.e. from satisfactory attenuation to over-attenuation) is below the range investigated by Soloviev *et al.* (1961), but the trend of increasing thermostability and increasing ease of elution with decreasing infectivity for man is similar to that shown by the Russian workers.

However, influenza viruses are notoriously labile on laboratory manipulation, and it has yet to be shown that the different properties of L_6 and $L_6Hu_1L_2$ will breed true over controlled serial passage. Before any significant relationship could be assumed between these markers and virulence, it would be necessary to show that clones of virus with the laboratory markers of $L_6Hu_1L_2$ could be segregated from the L_6 pool and subsequently shown to be avirulent, or conversely that L_6-like virus could be segregated from the $L_6Hu_1L_2$ pool and shown to have regained virulence. It would also be of interest to study the changes in genetic markers of

further influenza viruses over the whole course of their development from 'wild-type' strains to living vaccine strains of clinically satisfactory attenuation.

SUMMARY

Two pools of living influenza vaccine derived from the Russian A2 strain Iksha had given discrepant results in clinical trials. The less effective pool showed an increased thermostability of haemagglutinin, an increased elution rate from red blood cells and a reduced ability to grow in chorioallantoic membranes at temperatures above 40° C. The relationship of these findings to the virulence of influenza viruses is discussed.

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