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Preliminary Communications

Some Attempts to Produce an Experimental Vaccine with Rhinoviruses

By the use of primary human kidney cells maintained in roller tubes at 33° C. and with a *p*H of about 7, viruses which produce a cytopathic effect can be isolated from some human subjects with colds (Tyrrell and Parsons, 1960). They resemble the enteroviruses in their size and density, but differ from them in some other physical properties (Dimmock and Tyrrell, 1962). They have been named rhinoviruses (Andrewes *et al.*, 1961) and have been grouped together with the enteroviruses in the picornavirus family by the International Nomenclature Committee (paper in press).

Rhinoviruses can be isolated from 20–30% of common colds (Tyrrell and Bynoe, 1961 ; Hamre and Procknow, 1961 ; Kendall *et al.*, 1962 ; Hilleman *et al.*, 1962 ; Johnson *et al.*, 1962). Some will grow only in human-embryo-kidney (H.E.K.) cultures or human-embryo-lung (H.E.L.) fibroblasts (H strains), while others will grow in monkey-kidney cells as well as in human-embryo cells (M strains). There are several different serological types of both M strains and H strains (Taylor-Robinson and Tyrrell, 1962a ; Hilleman *et al.*, 1962), and specific antibody to these serotypes is produced in humans in response to infection (Tyrrell and Bynoe, 1961 ; Hamparian *et al.*, 1961). It has been found that volunteers with high levels of serum antibody against H.G.P. virus, an M strain, can rarely be infected with H.G.P. virus, while volunteers with low levels of serum antibody are usually susceptible to infection (Bynoe *et al.*, 1960).

We have attempted to produce high levels of serum antibody without producing symptoms of the common cold, using P.K. (Sal/1/56M), a strain serologically identical with H.G.P. (Tyrrell and Bynoe, 1961), and administering it by various routes.

Materials and Methods

Virus.—The virus used was the P.K. strain isolated in 1956, and later passed seven or eight times in H.E.K. One batch of virus was given a seventh passage in H.E.L. fibroblasts. The virus showed adaptation to tissue cultures by giving a more rapid cytopathic effect in H.E.K. and monkey-kidney cultures than did the unpassed virus. A formolized E.C.H.O. 28 vaccine was used for comparative purposes. This was supplied by Dr. J. Holper, of Abbott Laboratories Ltd. E.C.H.O. 28 is similar to the M type rhinoviruses. The vaccine was given as two intramuscular injections of 1 ml. administered one week apart as recommended by the makers.

Tissue Cultures.—H.E.K. cell cultures were prepared as described earlier (Tyrrell *et al.*, 1960). They were washed three times before inoculation and maintained in medium 199, the *p*H of which was adjusted to about 7.2 by adding CO_2 in the gas phase. A human diploid fibroblast cell strain was prepared from embryo-lung tissue as described by Hayflick and Moorhead (1961), but using medium 199 instead of Eagle's medium. The cultures were maintained as described above.

Virus Pools.—Bottle or tube cultures were inoculated with virus and rolled at 33° C. until the cell sheet had completely degenerated. The medium was stored at −70° C. and tested for the presence of contaminating bacteria. The purity of the virus stock was shown by the fact that the virus was completely neutralized by antiserum against H.G.P. virus and no contaminating virus grew out in H.E.K. cells.

Virus Isolation and Neutralization Tests.—These were carried out in monkey-kidney-cell cultures as described earlier (Tyrrell and Parsons, 1960 ; Taylor-Robinson and Tyrrell, 1962b).

Volunteers.—Volunteers between the ages of 18 and 45 years were housed and observed as described by Andrewes (1948). Serum was collected on arrival at the unit, and a further specimen was collected by the volunteers' general practitioner two weeks after inoculation. In most cases nasal washings were taken daily from the second day after the virus was given until the fifth day. Faeces were collected from some volunteers. Control groups given normal tissue-culture fluid were included in all trials.

Results

Inoculation of Volunteers with Live P.K. Virus by Different Routes.—P.K. virus grown in H.E.K. cultures was inoculated into groups of volunteers by one of three routes: each volunteer received 1 ml. of culture fluid as intranasal drops, or orally in 25 ml. of milk, or by intramuscular injection. The volunteers were observed for symptoms of the common cold, and attempts were made to isolate virus from the noses of volunteers inoculated intranasally and intramuscularly, and from the faeces of volunteers inoculated orally. The results

are shown in Table I. It can be seen that virus given intranasally produced colds, and a few rises in neutralizing antibody, indicating that it was not attenuated. One of the volunteers who was given virus

TABLE I.—*Number of Colds and Antibody Rises in Volunteers Inoculated by Different Routes*

Route of Inoculation	Dose TCD_{50}	Total Volunteers	No. of colds	Virus Isolations from		Antibody Rises
				Nasal Washings	Faeces	
Nasal	$10^{4.4}$	5	2	2	—	2/5
Oral	$10^{4.0}$	14	1	0	0	0/11
Intramuscular ..	$10^{5.2}$	8	0	1	—	7/7

by the oral route developed a cold, but no virus was isolated from a nasal washing from this subject or from faeces from any of these volunteers and there were no antibody rises. There was therefore no evidence that the virus infected by this route. When the virus was given intramuscularly no colds occurred, but virus was isolated from the nose of one volunteer on the third and fourth days after inoculation. The antibody titre of all the volunteers who were given virus intramuscularly rose after inoculation regardless of the antibody level at the time of inoculation.

Effect of Giving Diluted Virus Intramuscularly.—A pool of virus which induced rises in the antibody level of all volunteers to whom it was given intramuscularly was diluted 1/4 and 1/40, and the dilutions were given by the same route to further groups of volunteers. The results are shown in Table II. The undiluted virus (batch A) produced antibody rises in all seven of the volunteers to whom it was given, and the mean rise in

TABLE II.—*Effect of Intramuscular Inoculation of P.K. Virus Propagated in H.E.K. or H.E.L. Cells*

Batch:	A			B	C	D
Tissue Culture:	H.E.K.	H.E.K.	H.E.K.	H.E.K.	H.E.K.	H.E.L.
Dose (TCD_{50}/ml.)	$10^{5.2}$	$10^{4.6}$	$10^{3.6}$	$10^{3.2}$	$10^{3.7}$	$10^{2.7}$
Dilution ..	1/1	1/4	1/40	1/4	1/1	1/1
Antibody rises ..	7/7	4/5	1/3	4/11	2/4	4/6
Mean-fold rise ..	64·9	18·8	2·36	5·3	33·9	14·3

Batch B was freeze-dried and reconstituted before inoculation.

titre was 65-fold. When diluted 1/4 the virus produced antibody responses in four out of five volunteers; the geometric mean rise of titre in the five serum pairs was 23-fold. Virus diluted 1/40 gave fewer antibody rises (one out of three volunteers) and these were smaller (a mean rise of 7.0-fold). There was therefore a marked dilution effect in both the number and the size of the antibody rises.

Effect of Administering Intramuscularly Virus Produced in Different Batches of Cells.—P.K. virus pools were made in three different batches of H.E.K. cells, and in H.E.L. fibroblasts. The result of giving these virus pools intramuscularly is also shown in Table II. It can be concluded that $10^4 TCD_{50}$, or more, virus produced an antibody response in virtually all volunteers, and that virus produced in H.E.L. fibroblast cells is also a potent antigen.

Effect on Antibody Responses of Inactivating the Virus.—The infectious virus given to the volunteers might have acted as a true live vaccine by multiplying in the body or it might have been behaving as a non-multiplying antigen. To distinguish between these possibilities P.K. virus produced in H.E.K. cultures was inactivated by one of three methods, so that all, or most of, the infectivity was lost and its effectiveness as an antigen was then observed. Virus was inactivated by heating at 56° C. for 30 minutes or at 37° C. for three days. The remainder was inactivated by incubating with a final dilution of 1/4000 formalin at 36° C. for six hours. The formalin was neutralized with a sodium metabisulphite solution. No infectivity could be detected in either of the heated preparations when inoculated into H.E.K. After formalin treatment 97% of the infectivity was lost. The results of giving these virus preparations intramuscularly to volunteers are shown in Table III. The virus heated for 30 minutes at 56° C. was no longer antigenic. The virus inactivated by formalin or by more gentle heating at 36° C. for three

TABLE III.—*Effect of Heat or Formalin Treatment on Antibody Responses*

		No. of Volunteers Inoculated	No. of Antibody Rises Observed	Mean-fold Rise
Batch C	No treatment	4	2	33·9
	Formalin-treated ..	5	5	22·4
	Heated (37° C. for 72 hours)	5	4	16·6
Batch A	No treatment	7	7	64·9
	Heated (56° C. for 30 minutes)	4	0	0

days produced antibody responses of the same order of magnitude in four out of five and in five out of five volunteers respectively. It was concluded that the live virus acted as a non-multiplying antigen.

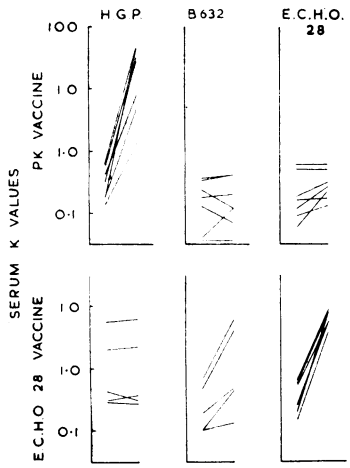
Antibody Levels After Intramuscular Vaccination Compared With Those Found After Intranasal Infection.—Any procedure used for experimental vaccination should induce antibody levels at least as high as those found in convalescence after a natural infection. We therefore compared the antibody levels produced after intramuscular injection of P.K. with those found after an infection of the respiratory tract with a pool of the same virus which had not been adapted to tissue culture. Volunteers were inoculated intranasally with a washing containing P.K. virus which had not been passed in tissue culture. Seven developed colds and showed evidence of infection. The antibody levels of these subjects and of the two volunteers (Table I) who were successfully infected with tissue-culture passed virus are summarized in Table IV. As was expected (Bynoe *et al.*, 1960) these volunteers all had relatively low antibody levels (K<2) before infection; the antibody levels after infection were about the same as those of 19 subjects who had similar levels and were then vaccinated. (The vaccinated subjects included those receiving batch A diluted 1/1 and 1/4; batch C untreated, heated, and formalin-treated; and batch D.) Volunteers who already possessed substantial amounts of antibody (K from 2.2 to 24.5) produced even more,

TABLE IV.—*Comparison of Serum K Values After Intranasal Infection or Intramuscular Vaccination*

Final Antibody Level (K Value)	Volunteers with Initial K Value of			
	Less than 2		Greater than 2	
	Infected	Vaccinated	Infected	Vaccinated
0·1–1	1	0	0	0
1–10	5	11	1	6
10–100	2	8	0	2
>100	0	0	0	5
0·1–>100	8	19	1	13
Geometric mean titre	4·52	9·73	—	26·4

and some developed titres of over 100—much greater than those we have found in sera collected at random or following experimental colds.

Heterologous Antibody Responses.—As there are numerous serotypes of rhinoviruses it was of interest to know whether heterotypic antibody responses occurred after the inoculation of one serotype. Sera from volunteers who had developed antibody against H.G.P. after intramuscular inoculation of P.K. were tested with two viruses—namely, B632 and E.C.H.O. 28—which are antigenically related to each other but unrelated to H.G.P. (Taylor-Robinson and Tyrrell, 1962a). For comparison some volunteers were given two injections of a formolized E.C.H.O. 28 vaccine, and their sera were titrated for antibodies against E.C.H.O. 28, B632, and H.G.P. The results are presented in the Chart. The volunteers showing rising titres against H.G.P. virus had no significant rises to either E.C.H.O. 28 or B632. Volunteers inoculated with E.C.H.O. 28 vaccine who showed antibody responses to E.C.H.O. 28 virus also showed rising titres against B632, although the rises were smaller; rising titres against H.G.P. virus were not observed. It was therefore concluded that the antigenic cross-reactions already demonstrated using sera from immunized rabbits were sufficient to lead to similar heterotypic antibody responses in man.



The antibody levels before and after intramuscular inoculation of P.K. virus and of formalin-inactivated E.C.H.O. 28 virus vaccine are shown. P.K. virus induced an antibody response against H.G.P., with which it is antigenetically identical. E.C.H.O. 28 vaccine induced antibody responses against itself and the related B632 virus, but not against H.G.P.

DISCUSSION

These serological experiments show that inactivated or untreated fluids from tissue cultures infected with rhinoviruses are antigenic for man, although the infectivity titres before inactivation may be low. The experiments designed to produce a symptomless infection with rhinoviruses were not successful, but further work on this line would be worth while. It is also clear that, although H.E.K. cells will not yield enough virus for large-scale experiments, satisfactory material might be obtained from human diploid strains.

Earlier experiments by Price (1957) suggested that a formalin-inactivated vaccine protected against illness due to E.C.H.O. 28 virus observed in a naturally occurring epidemic of E.C.H.O. 28 infection. Colds occurred in 3 out of 50 children who had been given the vaccine and in 23 out of 50 children who had received a placebo.

Preliminary experiments at this unit suggest that vaccination with batch B of virus (Table II) did not protect all volunteers against an experimental inoculation with the homologous viruses. We therefore need to find means of increasing the frequency and size of antibody responses to the intramuscular injection of viruses, perhaps by the use of adjuvants.

These preliminary experiments, using only three viruses, suggest that the antibody response to P.K. is specific. Nevertheless, because of the heterotypic response observed after inoculating E.C.H.O. 28 virus it is still possible that by means of a polyvalent vaccine one might be able to induce antibody against substantially more serotypes than were included in the vaccine. At the moment we know too little about the frequency of antigenic crossings between rhinovirus types to predict whether this will be possible, but it would be interesting to look for such cross-reactions and to try to exploit them.

SUMMARY

When tissue-culture fluid containing live-tissue culture-passed P.K. virus was administered to five volunteers intranasally, some developed symptoms of the common cold and had rises in serum neutralizing antibody; it was administered orally to 14 volunteers who remained symptom-free and had no antibody rises. P.K. virus, either live or inactivated by formalin or by heating at 37° C. for three days, when given as an intramuscular injection produced no symptoms and induced homologous antibody responses in volunteers. Heterologous antibody responses to B632 virus were induced by an E.C.H.O. 28 formolized vaccine; P.K. vaccine induced no antibody responses to either of these viruses.

We conclude that it is now necessary to try to improve the antigenic potency of tissue-culture material and to determine whether it can induce immunity to experimental colds. If it does it may still be very difficult to find virus strains which will give a useful degree of protection against natural colds.

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JENNIFER E. DOGGETT, B.Sc.
M. L. BYNOE, M.B., D.T.M.&H., D.Obst.R.C.O.G.
D. A. J. TYRRELL, M.D., M.R.C.P.

From the M.R.C. Common Cold Research Unit, Salisbury, Wilts.

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