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Para-influenza 2 virus infections in adult volunteers

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INTRODUCTION

Para-influenza viruses 1, 2 and 3 produce illnesses in children which range from mild upper respiratory infections to severe diseases of the lower respiratory tract (Parrott, Vargosko, Kim, Bell & Chanock, 1962). Para-influenza 2 virus is isolated from children much less frequently than para-influenza 1 or 3 viruses and does not often seem to be associated with illness other than croup (Kim, Vargosko, Chanock & Parrott, 1961). Para-influenza viruses have also been isolated from adults with mild upper respiratory infections (Evans, 1960; Holland, Tanner, Pereira & Taylor, 1960; Dick, Mogabgab & Holmes, 1961; Mogabgab, Dick & Holmes, 1961), but it has been difficult to prove that these viruses are aetiologically associated with the illnesses that have been observed. This has been due to the fact that the para-influenza viruses have been isolated along with many other different viruses and have formed only a very small portion of the total number of viruses isolated. However, para-influenza viruses 1 and 3 are able to produce upper respiratory infections of a mild cold-like nature in adult volunteers (Reichelderfer, Chanock, Craighead, Huebner, Turner, James & Ward, 1958; Tyrrell, Bynoe, Birkum Petersen, Sutton & Pereira, 1959; Kapikian, Chanock, Reichelderfer, Ward, Huebner & Bell, 1961). We present evidence that para-influenza 2 virus also produces mild upper respiratory illness in adult volunteers inoculated intranasally.

MATERIALS AND METHODS

Viruses

The para-influenza 2 virus used for volunteer inoculation was obtained from Dr M. S. Pereira (Central Public Health Laboratory, Colindale, London) as freeze-dried tissue culture fluid from a 4th monkey kidney passage. This material was reconstituted and inoculated into primary trypsin-dispersed cultures of human embryo kidney cells and these cultures were rolled and incubated at 33° C. The tissue-culture fluids were pooled on the 4th day of incubation, bovine plasma albumin was added to a final concentration of 1·0% and the pooled fluids were divided into 2·0 ml. aliquots and stored at −70° C. until used. This virus was passed once more in rhesus monkey kidney cells and was stored at −70° C. until used in neutralization tests.

The following strains propagated in tissue culture were used in haemaggluti-

nation-inhibition tests: para-influenza 2—Manganero strain; para-influenza 1—Copenhagen 222 strain; para-influenza 3—bovine strain 33; and para-influenza 3—human strain C243.

Safety tests

The virus for volunteer inoculation was neutralized by a para-influenza 2 rabbit antiserum, but not by antisera to para-influenza viruses 1 and 3. The virus ($10^{7.45}$ tissue-culture doses (TCD 50) per ml.) was inoculated intravenously (1·0 ml.) and intramuscularly (1·0 ml.) into a rabbit, intramuscularly (0·5 ml.) into guinea-pigs and intracerebrally (0·05 ml.) and intraperitoneally (0·1 ml.) into adult mice and intracerebrally (0·03 ml.) and intraperitoneally (0·03 ml.) into unweaned mice; no illnesses were observed. Tests for the presence of simian virus 40 in the para-influenza 2 virus pool were made by inoculating mixtures of virus and serum from a rabbit immunized against another strain of para-influenza 2 virus into vervet monkey kidney cells; the cultures were observed for 2 weeks and subcultures from these were also observed for 2 weeks. These tests were carried out by Dr D. McGrath (Medical Research Council Laboratories, Holly Hill, Hampstead); simian virus 40 was not detected.

Volunteers

The volunteers were between 18 and 50 years of age. They were isolated as described elsewhere (Andrewes, 1948), usually in pairs, but occasionally singly or in groups of three. After an observation period of 3½ days virus was given as nasal drops in a volume of 1·0 ml. of chilled Hanks's saline. Blood was collected 1 or 2 days before virus inoculation and again 14–16 days after inoculation when the volunteers had left the unit. Nasal washings were collected as described below. Each volunteer was examined daily by one of us (M. L. B.) who was unaware of the nature of the inoculum which each had received.

Tissue cultures

Primary trypsin-dispersed cultures of human embryo kidney cells and secondary cultures of rhesus monkey kidney cells were grown in a medium of 5% calf serum, 0·5% lactalbumin hydrolysate and Hanks's saline containing 0·03% sodium bicarbonate and antibiotics. Before use, the cultures were washed three times with Hanks's saline and then maintained in medium 199. Primary cultures of calf kidney were grown in a medium which contained 10% calf serum, 0·5% lactalbumin hydrolysate and Hanks's saline containing 0·03% sodium bicarbonate. Again, the cultures were washed three times with Hanks's saline and then maintained in 0·5% lactalbumin hydrolysate, 0·1% yeast extract and Hanks's saline containing 0·1% sodium bicarbonate. HeLa cells, obtained from Dr M. S. Pereira, were grown in medium consisting of 10% rabbit serum, 0·5% lactalbumin hydrolysate, 0·1% yeast extract and Hanks's saline containing 0·1% sodium bicarbonate and antibiotics. These cultures were also washed and maintained in medium 199.

Virus isolation

Nasal washings in Hanks's saline were usually collected 2, 3, 4 and 5 days after intranasal inoculation of virus. The washing from each volunteer was chilled in crushed ice and, within an hour of being taken, 0·2 ml. was inoculated into each of two cultures of human embryo kidney cells. The cultures were rolled at 33° C. Some washings were titrated by inoculating 10-fold dilutions made in Hanks's saline. Cultures were examined for the typical syncytial cytopathic effect of para-influenza 2 virus, but the final recording was based on the haemadsorption test (Vogel & Shelokov, 1957); 4–5 days after inoculation, 0·2 ml. of a 1·0% suspension of human group 'O' red cells in phosphate-buffered saline was added to each tube and the tubes were placed at 4° C. for 30 min. and were then read. Virus detected in this way was then identified by haemadsorption-inhibition with specific rabbit antiserum (Chanock, Parrott, Cook, Andrews, Bell, Reichelderfer, Kapikian, Mastrota & Huebner, 1958). Fresh medium was added to those cultures that did not exhibit haemadsorption and they were incubated for a further 6–7 days and retested.

Neutralization tests

Serial twofold dilutions of serum were mixed with an equal volume of a dilution of virus containing about 100 TCD 50 in 0·1 ml. After 30 min. incubation at room temperature, 0·2 ml. of each mixture was inoculated into each of two rhesus monkey kidney cultures which were then rolled at 33° C. Five days later, the cultures were tested by haemadsorption. Neutralization titres were expressed as interpolated 50% end-points. Chanock (1956) found that the neutralizing activity of sera from children was lost on heating, but partly restored on adding unheated normal serum. We did not experience this difficulty. There was no significant difference between titres obtained with sera inactivated at 56° C. for 30 min. and sera not so treated and, therefore, we used inactivated sera. Titres in all the serological tests are expressed as the reciprocal of the initial serum dilution.

Complement-fixation tests

Some para-influenza 2 complement-fixing antigen was obtained from Dr P. Bradstreet (Central Public Health Laboratory, Colindale, London) and some was prepared by ourselves in HeLa cells; the culture fluids were used as antigen. All sera were inactivated at 56° C. for 30 min. before use. The tests were done in WHO haemagglutination plates with one drop of each reagent. Two units of complement and overnight fixation at 4° C. were used.

Haemagglutination-inhibition (H.I.) *tests*

All the acute and convalescent sera were treated with cholera filtrate, and tested against para-influenza viruses 1, 2 and 3. Para-influenza 1 haemagglutinin was prepared in human embryo kidney cells; the culture fluids were used as antigen. Para-influenza 2 antigen was prepared in human embryo kidney and in HeLa cells; the cells were disrupted by blending and the fluids from both types of culture were then pooled, centrifuged at 2000 r.p.m. for 10 min. and the

supernatant used as the antigen. Haemagglutinins were prepared from the human and bovine strains of para-influenza 3 in human embryo kidney cells and in calf kidney cells, respectively; the tissue culture fluids were used as antigens. Preliminary experiments showed that the same H.I. titres were obtained when sera from adult humans were tested with either the bovine strain or the human strain. Thereafter, only the bovine strain was used because it was easier to produce haemagglutinin from it than from the human strain. H.I. tests with para-influenza viruses 1 and 3 were done in WHO plates with 0·2 ml. volumes. Four units of antigen were incubated with dilutions of serum at room temperature for 60 min.; then 1·0 % human group 'O' red cells were added and allowed to settle at 4° C. In order to save virus, tests with para-influenza 2 haemagglutinin were carried out by the micro-method of Takátsy (Takátsy, 1955) and 1·0 % chicken erythrocytes were used.

RESULTS

Clinical evidence of infection

Twenty-eight volunteers were inoculated intranasally with para-influenza 2 virus and twenty-eight were given Hanks's saline alone. From 2×10^2 to 2×10^6 TCD 50 of virus were given to each volunteer and the clinical findings are shown in Table 1. Most of the symptoms and signs were of moderate severity; and nasal stuffiness and sore throat were more evident than coryza. Eight volunteers showed enough symptoms and signs to convince the observer that they had a significant illness, but only four of these illnesses were typical experimental colds (Roden, 1958). The incubation period was 2 days for three of the colds and 4 days for the other. The other four illnesses had incubation periods of 2, 2, 3 and 3 days, respectively. Of the twenty-eight volunteers inoculated with virus, eighteen had at least one symptom or sign and there was a total of fifty-one symptoms or signs. Of twenty-eight volunteers given Hanks's saline, six had symptoms or signs of which there was a total of twelve; most of these symptoms and signs were due to one subject who had a mild cold. This evidence suggests that most of the symptoms of those subjects given the virus-containing inoculum were due to the virus it contained. Only one of six volunteers who were inoculated with 2×10^4 TCD 50 of virus became ill and no volunteer given less virus than this was ill. Three illnesses occurred in eleven volunteers inoculated with 2×10^5 TCD 50 of virus and four illnesses in six subjects inoculated with 2×10^6 TCD 50; this suggests that large doses of virus produced more illness than small ones. It is possible that the colds reported by volunteers nos. 8, 9 and 28 were due to the experimental virus infection. However, since these colds developed 7, 6 and 6 days respectively after inoculation and after the period of strict isolation they have not been included in the results.

Laboratory evidence of infection

Laboratory evidence of infection was based on the re-isolation of virus or a fourfold antibody response as determined by any of the three serological tests used.

Table 1. *The symptoms and signs, clinical assessment, virus isolations and antibody responses of twenty-eight volunteers inoculated intranasally with para-influenza 2 virus*

Volunteers (no.)	Virus dose (TCD 50) for each volunteer	Symptoms and signs: Headache	Chills	Pyrexia	Sneezing	Coryza	Max. no. of paper handkerchiefs per day	Nasal stuffiness	Sore throat	Injected fauces	Clinical assessment	Virus isolations from nasal washings. Days after inoculation of volunteer: 2	3	4	5	Reciprocal antibody titres of pre- and post-inoculation sera as shown by: Neutralization		Haemagglutination inhibition		Complement fixation	
1	2×10^2	.	.	.	.	.	.	.	.	.	.					N.T.‡		< 8	8	< 4	< 4
2		.	.	.	.	.	.	.	.	.	.					N.T.		8	12	< 8	8
3	2×10^3	.	.	.	.	.	.	.	.	+	.	N.T.			N.T.	48	64	12	12	32	32
4		.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	< 8	< 8	< 8	8	4	4
5		.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	**12**	**48**	8	12	4	8
6	2×10^4	.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	**8**	**48**	< 8	**32**	**4**	**16**
7		.	.	.	.	.	.	.	.	.	.	N.T.			N.T.	24	48	< 8	< 8	4	4
8		+	.	.	+	+	4	.	.	+	U.R.I.*					**8**	**128**	< 8	**48**	< 4	**12**
9		+	.	.	.	.	.	.	.	.	.					**24**	**96**	**8**	**32**	**16**	**64**
10		.	.	.	.	.	.	+	.	.	.					**8**	**64**	**8**	**32**	**4**	**32**
11		.	.	.	.	.	.	.	+	.	.					16	24	12	24	**16**	**64**
12		+	+	.	.	+	6	+	+	+	Cold (2)†					**16**	**96**	**8**	**32**	**4**	**32**
13		+	+	.	+	+	12	+	+	+	Cold (2)					**24**	**96**	**8**	**32**	< **8**	**32**
14		.	.	.	.	.	.	.	.	.	.					48	48	24	24	16	16
15		.	.	99·2	.	.	.	.	.	.	.					**8**	**32**	8	16	**4**	**16**
16		.	.	.	.	.	.	.	.	.	.					12	12	8	8	8	8
17	2×10^5	.	.	.	.	.	.	.	.	.	.					48	48	12	8	< 8	8
18		.	+	99·2	+	+	12	+	+	.	Cold (2)					< **8**	**64**	**8**	**48**	**4**	> **64**
19		.	.	.	.	.	.	+	.	.	.					< **8**	**48**	< **8**	**32**	**4**	> **64**
20		.	.	.	.	.	.	+	.	.	.					< **8**	**32**	< **8**	**24**		
21		.	.	.	+	.	.	+	+	.	.					< 8	< 16	8	16	16	16
22		.	.	.	.	.	.	.	.	.	.				N.T.	< **8**	**32**	**12**	**48**	< **4**	**32**
23	2×10^6	.	.	99·4	+	+	10	+	.	.	Cold (4)					64	96	24	32	**8**	**32**
24		.	.	.	.	.	.	+	.	.	.					16	24	12	24	**4**	**16**
25		+	.	.	+	.	.	.	+	.	U.R.I.					24	16	< 8	< 8	4	4
26		+	.	.	.	.	.	+	+	+	U.R.I.					12	24	8	12	8	8
27		+	+	.	+	.	.	+	+	.	U.R.I.					24	32	8	12	8	8
28		.	.	.	+	.	.	.	.	.	.					**12**	**128**	**8**	**48**	**8**	**32**

Figures in bold type in the last three columns indicate a fourfold or greater rise of antibody titre.
* U.R.I. = upper respiratory infection. † In parentheses, the incubation period. ‡ N.T. = not tested. = virus not isolated. = virus isolated.

Virus isolations

Although there was no evidence that human embryo kidney cells were more sensitive than rhesus monkey kidney cells, we used the former in order to avoid haemadsorption due to simian haemadsorbing agents. As shown in Table 1, parainfluenza 2 virus was re-isolated from all but one of the volunteers who were given 2×10^4 or more TCD 50 of virus. This correlates with the clinical picture since no volunteer given less than 2×10^4 TCD 50 of virus was considered to have clinical evidence of disease. However, fourteen volunteers were infected, as judged by virus isolation, although they did not have clinical evidence of infection. Virus was re-isolated from nine of twenty nasal washings taken from volunteers inoculated with 2×10^4 TCD 50 of virus and from twenty-four of twenty-four washings taken from volunteers inoculated with 2×10^6 TCD 50 of virus. This is a significant difference at the 1% level and suggests that virus was excreted less frequently from those given small amounts of virus.

Table 2. *The amount of virus in nasal washings of volunteers inoculated intranasally with* 2×10^6 TCD 50 *of virus*

	Virus titre (TCD 50/0·6 ml.) in washings collected at various times after original virus inoculation				
Volunteer	9 hr.	2 days	3 days	4 days	5 days
25	0	$10^{1·5}$	$10^{1·5}$	$10^{1·5}$	$10^{0·7}$
26	0	$10^{2·0}$	$10^{2·0}$	$10^{2·0}$	$10^{1·0}$
27	0	$10^{0·5}$	$10^{0·7}$	$10^{0·5}$	10^{0}
28	0	$10^{2·0}$	$10^{2·0}$	$10^{2·0}$	$10^{2·0}$

The virus that was re-isolated from volunteers was unlikely to be residual virus from the inoculum as shown in the following experiment (Table 2). Four volunteers were each inoculated with 2×10^6 TCD 50 of virus; virus could not be re-isolated from nasal washings taken 9 hr. later, but virus was isolated 2, 3, 4 and 5 days after inoculation. Further, virus was isolated on the 5th day after inoculation from sixteen volunteers (Table 1) and this virus must almost certainly have been produced in the respiratory tract and not have been residual virus from the inoculum.

It may be seen from Table 2 that volunteer no. 28, who was not ill, consistently excreted 10-fold more virus than volunteer no. 27, who had an upper respiratory illness. It appears, therefore, that the amount of virus re-isolated was unrelated to the presence of overt illness.

Antibody responses

As shown in Table 1, the four persons who had cold-like illnesses and five of the eight volunteers who were considered to have clinical evidence of infection had a fourfold antibody response in at least one of the serological tests. There was no evidence that volunteers who were given large doses of virus developed higher titres of antibody than those given the smaller doses; in fact, three of the six persons who were given 2×10^6 TCD 50 of virus had no antibody response at all.

There was a greater chance of detecting a fourfold neutralizing antibody rise in infected volunteers who had initial low levels of antibody than in those with initial high levels; ten of thirteen volunteers with neutralizing antibody titres of twelve or less showed an antibody response, whereas only three of eleven volunteers with antibody titres of sixteen or more showed a response. Antibody rises were detected in thirteen instances by neutralization tests, in eleven cases by H.I. tests and in fourteen cases by complement-fixation tests. However, although an antibody response was detected by all three tests in ten instances, it was shown by only one technique in four instances and it was necessary, therefore, to titrate antibodies by the three serological techniques in order to detect all the responses. An antibody response was a less sensitive indicator of infection than virus isolation, since virus was isolated from eight individuals who did not show an antibody response by any of the three serological techniques. In only two instances was there an antibody response without virus isolation.

As shown in Table 1, twenty volunteers had neutralizing antibody titres of eight or more before inoculation of virus so it seems likely that these subjects had been infected previously, probably in childhood, and that the illnesses observed were due to re-infection with para-influenza 2 virus in the presence of antibody. Too few volunteers were studied to establish whether a high level of neutralizing antibody could protect completely or whether a low level protected partially. It was noted, however, that low levels of antibody certainly failed to protect completely against re-infection, for persons with titres of 24 were infected by 2×10^4 TCD 50 of virus and one subject with a titre of 64 was not protected against 2×10^6 TCD 50 of virus.

Table 3. *Result of para-influenza 2 virus neutralization test carried out* in vivo

	No. of volunteers				
Intranasal inoculum	Inoculated	From whom virus isolated	With fourfold antibody rise	With symptoms and signs	With colds
2×10^5 TCD 50 of virus	5	5	4	4	1
2×10^5 TCD 50 of virus-antiserum mixture	6	0	0	1	0

When the paired sera were tested by haemagglutination-inhibition against Sendai virus and para-influenza viruses 1 and 3 no antibody rises were detected. The same paired sera were tested by Dr R. B. Heath (St Bartholomew's Hospital Medical School, London) against mumps virus by the H.I. test and again no rises were detected.

A neutralization test carried out in vivo

Because volunteers became ill even in the presence of neutralizing antibody it was possible that these illnesses were due not to para-influenza 2 virus, but to an undetected virus in the inoculum. The following experiment was, therefore, carried

out. The para-influenza 2 virus pool was diluted to contain approximately 2×10^5 TCD 50 of virus per ml. and this was mixed with an equal volume of para-influenza 2 rabbit antiserum. Virus, similarly diluted, was mixed with an equal volume of Hanks's saline. The mixtures were incubated at room temperature for 30 min. and then chilled to 4° C. The virus-antiserum mixture was inoculated into six volunteers and the virus control mixture into five volunteers. The results of this experiment are shown in Table 3. All those given virus alone were infected as shown by the re-isolation of virus or an antibody response. Four of the five subjects had symptoms or signs and one of the five had a cold. None of the six volunteers given the virus–antiserum mixture had laboratory evidence of infection, although one of them had symptoms and signs; these were insufficient to be regarded as a definite illness. This evidence suggested that the illnesses observed in all these experiments were due to para-influenza 2 virus.

DISCUSSION

It seems likely that the ability to re-isolate virus from volunteers 2 days or more after their intranasal inoculation was due to virus multiplication, since virus could not be re-isolated a few hours after inoculation. In several instances, virus was isolated from subjects with a respiratory illness and yet there was no evidence of an antibody response by any of the three serological techniques. This has been observed in natural and experimental infections with respiratory syncytial virus (Chanock, Kim, Vargosko, Deleva, Johnson, Cumming & Parrott, 1961; Kravetz, Knight, Chanock, Morris, Johnson, Rifkind & Utz, 1961; Hamre & Procknow, 1961) and in natural infections with para-influenza viruses (Chanock, Bell & Parrott, 1961). This stresses the point that a diagnosis of infection may have to be made on the evidence of virus isolation alone without an accompanying homotypic antibody response. The fact that we were not able to demonstrate heterotypic antibody responses by haemagglutination inhibition against other para-influenza viruses or mumps virus is perhaps not surprising; Heath, Tyrrell & Peto (1962) found that there was less correlation between the titres of haemagglutination-inhibiting antibody in human sera against CA virus and the other viruses of the para-influenza group than there was between the titres against para-influenza viruses 1 and 3.

It is difficult to explain the differences between our finding that satisfactory neutralization tests could be done with heated sera and the experience of Chanock (1956) who found that he could best demonstrate antibody responses if he used sera that were not inactivated by heating. The difference may have arisen because we used a different strain of virus and examined sera of adults rather than of children.

Tyrrell *et al.* (1959) found that para-influenza viruses 1 and 3 produced illnesses that fell within the range of illness produced by the rhinovirus H.G.P. in which coryza is a characteristic feature. The inoculation of volunteers with para-influenza 2 virus caused illnesses of a mild and somewhat different nature. Several volunteers had upper respiratory infections with nasal stuffiness, but without coryza, and therefore according to the criteria used at this unit these illnesses were not regarded as colds. Only four volunteers had illnesses with coryza which were

regarded as mild colds. Sore throat was quite a prominent feature of the illnesses and Mogabgab *et al.* (1961) noted this in adults from whom they isolated para-influenza 2 virus. The mild nature of the illnesses we observed might have been due to the fact that we used a virus that had had five passages in tissue culture. In one experiment, seven volunteers, all of whom possessed neutralizing antibody, were each inoculated intranasally with about 10 TCD 50 of virus which had not been passaged through tissue culture and which was contained in a throat washing. These volunteers were not infected, as judged by virus re-isolation or neutralizing antibody response, and they did not develop illness (Taylor-Robinson, unpublished). Although Tyrrell *et al.* (1959) showed that para-influenza 1 virus, which had only one passage in tissue culture, produced more infection and illness than virus that had had five passages, the severity of the illnesses was the same. It is possible that the apparently different nature of the illness produced by para-influenza 2 virus in adult volunteers is real. This would correspond with the difference noted in primary infection in children in whom para-influenza 2 virus has been shown to produce only croup, whereas para-influenza viruses 1 and 3 produce also bronchitis and pneumonia.

In our experiments, illness and infection occurred in the presence of pre-existing neutralizing antibody. This phenomenon has been observed in natural para-influenza infections of children and adults (Chanock *et al.* 1961) and in the experimental infections of adults with para-influenza viruses 1 and 3 mentioned previously. Because we found that there was no correlation between the presence of para-influenza 2 neutralizing antibody and the occurrence of illness it was important to determine whether the illnesses that we observed were due to para-influenza 2 virus or to some other agent, such as a simian virus 'picked up' by the passsage in monkey kidney cells. One of the subjects who was given para-influenza 2 virus that had been neutralized by specific antiserum prior to inoculation did present some symptoms and signs, but so did a few of the volunteers who had been given Hanks's saline. Further, there was a significantly greater number of symptoms, signs and illnesses in those who were inoculated with para-influenza 2 virus without antiserum than in subjects given the Hanks's saline and there were no illnesses in those volunteers who were not infected. We conclude from these facts that the para-influenza 2 virus caused the illnesses that we observed.

The results of these experiments seem to fulfil the third of Koch's postulates for para-influenza 2 virus as a cause of respiratory disease in adults. It must be pointed out, however, that we still know very little about how much and what sort of disease it causes under natural conditions.

SUMMARY

Twenty-eight adult volunteers were inoculated intranasally with para-influenza 2 virus and eight developed illnesses; twenty-eight volunteers were given Hanks's saline and one became ill. The illnesses occurred in volunteers given between 2×10^4 and 2×10^6 TCD 50 of virus. The most prominent symptoms were sore throat, nasal stuffiness and coryza; four of the eight volunteers had sufficient coryza to be regarded as having mild colds. Although only eight volunteers had

clinical evidence of infection, twenty-four had laboratory evidence of infection as judged by virus re-isolation or antibody response. Neutralization, haemagglutination-inhibition and complement-fixation tests on paired sera showed that sixteen individuals had a fourfold or greater antibody response by one or more tests including five of the eight volunteers who were ill. Twenty volunteers, including seven who were ill, had reciprocal neutralizing antibody titres of eight or more before inoculation of virus so it seems that the illnesses were due to re-infection in the presence of antibody. Evidence is presented which suggests that although illnesses occurred in the presence of antibody they were due to the para-influenza 2 virus and not some other agent in the inoculum. The results of these experiments seem to fulfil the third of Koch's postulates for para-influenza 2 virus as a cause of respiratory disease in adults.

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