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An outbreak of streptococcal sore throat and rheumatic fever in a Royal Air Force Training camp; significance of serum antibody to M-associated protein

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SUMMARY

A large outbreak of streptococcal sore throat in a Royal Air Force Training Camp resulted in five cases of rheumatic fever among the 16- to 18-year-old apprentices, and one case in a 33-year-old airman. The most prevalent type of group A streptococcus isolated from throat swabs was M-type 5 and there was serological evidence that at least four of the rheumatic fever (R.F.) cases were due to this type.

Among the patients with uncomplicated throat infection the anti-streptolysin O (ASO) and anti-deoxyribonuclease B (anti-DNAase B) responses were in general rather low, even where there was evidence of protective antibody against type 5. However, a combination of the results of the ASO and anti-DNAase B tests gave an estimate of the extent of streptococcal infection 15–25% higher than did either test alone.

The titres of antibody to M-associated protein (MAP) were $\geqslant 60$ in all the R.F. patients, and in about 50% of the other patients with ASO titres $\geqslant 200$. This figure is unusually high compared with data from several other outbreaks of streptococcal infection due to different serotypes and also greatly exceeds comparable figures for cases of sporadic sore throat and acute glomerulonephritis.

INTRODUCTION

In the spring of 1970 a large outbreak of streptococcal sore throat occurred among the apprentices in the Royal Air Force Training Camp at Halton, Bucks. Over 400 of the 1750 youths aged 16–18 years suffered from streptococcal sore throat and five of them had attacks of rheumatic fever. In the latter part of the outbreak, and subsequently, we took advantage of this situation to study the antibody response in rheumatic fever patients and to compare it with the response in other apprentices who escaped this complication.

MATERIALS AND METHODS

Diagnosis and treatment of streptococcal infection

Before 16 April 1970 the practice had been to examine clinically all apprentices reporting to the Station Sick Quarters with sore throat; if there was objective evidence of acute tonsillitis, the patient was admitted, a throat swab was collected, and penicillin treatment was begun; if not, he was given an analgesic and an antiseptic gargle, but was admitted only if the clinical condition warranted this. Routine penicillin treatment comprised phenoxymethyl penicillin 125 mg, 4 times a day for 7 days. From 16 April to 21 May all patients complaining of sore throat were treated with penicillin and a throat swab examined from each.

Throat swabs were cultured on horse-blood agar and representative β-haemolytic colonies were examined. Before 16 April these were screened for bacitracin sensitivity (Maxted, 1953) but after this date they were grouped serologically (Maxted, 1948) and a selection of the group A streptococci were sent to Colindale for typing.

Streptococcal typing

Streptococci were typed by T-agglutination (Griffith, 1934) and the M-precipitin method (Swift, Wilson & Lancefield, 1943). Rabbit antisera were prepared by the Streptococcus Reference Laboratory, Colindale.

Human sera

Serial samples of serum from patients with rheumatic fever and single samples from 45 cases of sore throat and 91 symptomless apprentices were tested for anti-streptolysin O, anti-DNAase B, anti-MAP, and type-specific antibodies against M-types 5, 18 and 58.

Anti-streptolysin O. ASO titres were determined by a spectrophotometric method based on that of Gooder & Williams (1961) and Gooder (1961).

Anti-deoxyribonuclease B. Anti-DNAase B titres were determined by the micro-method of Nelson, Ayoub & Wannamaker (1968).

Antibody to M-associated protein. Anti-MAP titres were determined by a complement fixation test (CFT) with the purified M-protein of a type 30 strain (Widdowson, Maxted & Pinney, 1971). The use of a type 30 M-protein virtually rules out the possibility of fixation of complement by a patient's serum due to the presence of type-specific antibodies, since type-30 infections are extremely rare in Britain. The CFT titre of a serum was therefore taken as a measure of antibody to the non-specific part of the M-protein complex.

The bactericidal test. This test for M-antibody was done as described by Maxted, Widdowson & Fraser (1973). All sera were tested for the presence of type-specific M-antibody to the three types of streptococci prevalent in the camp during the outbreak. These were M-types 5, 18 and 58. The sera were first treated with penicillinase (Burroughs Wellcome & Co. Ltd.) to destroy any penicillin present. Antibody to the type under test was considered to be present if + + + + growth in the control was reduced to −, +, or + + growth in the test. Sera which

showed only slight depression of growth (+ + +) were retested using 0·04 ml of serum. If the depression of growth was then increased, the serum was considered to contain antibody. None of the sera tested showed inhibition of the growth of all three types, which ruled out the possibility of killing by a non-specific mechanism, e.g. presence of residual antibiotics in the serum.

RESULTS

History of the outbreak

The frequency of sore throat began to increase late in March, and by the second week in April had risen from the usual level of about 3 cases a day to 15 cases a day. Up to 16 April about 150 of the apprentices had reported sick with sore throat. Three cases of rheumatic fever were diagnosed between 8 and 10 April and at about the same time a fourth case, a 33-year airman from a neighbouring unit whose only previous contact with Halton had been attendance at the Ear, Nose and Throat Out-patient Clinic, was admitted to the Sick Quarters with the disease.

Between 16 April and 21 May a further 270 cases of sore throat were reported and group A streptococci were isolated from over half of them. Two more cases of rheumatic fever occurred on 21 and 24 April respectively. A random selection of 34 group A streptococci isolated from patients with tonsillitis during this period were typed. Blood samples were collected from 92 of the apprentices on or about 25 April, about 4 weeks after the beginning of the outbreak.

On 21 May the apprentices went on Whitsun leave and when they reassembled at the beginning of June the outbreak had subsided. At this time, random throat swabs were collected from a sample of the boys and 60 group A streptococcal strains were typed; samples of serum were collected from 44 boys who had not reported sick with sore throat during the outbreak, and who had negative throat swabs.

Throat swabs had not been examined for four of the rheumatic fever patients during the initial respiratory infections; bacitracin-sensitive β-haemolytic streptococci were isolated from the remaining two cases, but were not typed.

Types of streptococci isolated

The outbreak of sore throat lasted about 10 weeks, but information about the types of group A streptococci prevalent during the first four weeks is lacking. M-type 5 predominated in the sample of strains isolated in weeks 5 and 6 of the outbreak, accounting for over 50% of the total, but smaller numbers of M-types 18 and 58 were also present (Table 1). In early June when the outbreak had subsided, type 5 was still the most prevalent, but the other two types had disappeared.

Table 1. *Types of streptococci isolated from throat swabs during the period 22 April to 8 June 1970*

	Type		Throat swabs	
Date	T	M	No. positive/total positive for group A	% positive
22 April to 7 May 1970	5/27/44	5	19/34	56
	—	18	6/34	18
	25/Imp 19	58	6/34	18
		Others	3/34	9
8 June 1970	5/27/44	5	38/60	63
	9	ND	5/60	8
		Others	17/60	28

Rheumatic fever cases; clinical histories

Patient B.M.P. aged 17 years reported sick on 4 April with a sore throat which lasted 3 days. He received no penicillin treatment and on 21 April rheumatic fever with cardiac involvement was diagnosed.

Patient T.A.G., aged 16 years, first reported sick on 13 March with swollen ankles. This persisted for about 1 week, and recurred on 27 March with a sore throat and backache. These symptoms subsided, but on 8 April he had pain in both elbows and was admitted to hospital with a diagnosis of mild rheumatic fever (no cardiac involvement) on 10 April. He had received intermittent penicillin treatment for 4 weeks before admission.

Patient A.P., aged 17 years, had tonsillitis on 13 March and received oral penicillin treatment for only 2 days. On 20 April he had a mild sore throat and was admitted to hospital on 27 April with a diagnosis of typical rheumatic fever and active carditis.

Patient P.G., aged 17 years, had a mild sore throat on 20 March which was untreated, and lasted only 48 hr. On 8–10 April he developed swollen painful joints and was admitted to hospital with a diagnosis of typical rheumatic fever, without cardiac involvement, on 13 April.

Patient C.C., aged 17 years, developed a sore throat and cough on 7 March. He received 7 days' penicillin treatment and the symptoms abated. Sore throat, fever and headache recurred on 7 April and penicillin was given for a few days. Between 10 and 20 April he developed pain and swelling in both ankles and knees and stiffness in his hands and shoulder. He was admitted to hospital late in the course of his disease, which was diagnosed as classical rheumatic fever with no evidence of cardiac damage.

Patient P.M.E., aged 33 years, developed otitis media in February and a β-haemolytic streptococcus was isolated but not typed. He had no further symptoms until 7–10 April when he developed a painful swollen left knee and a sore throat. He was admitted to hospital on 12 April with typical rheumatic fever but no evidence of carditis.

Thus, there was a history of antecedent sore throat in five of the six cases, but

Table 2. *Streptococcal antibody titres of rheumatic fever patients*

Patient number	Age (years)	Rheumatic fever	Date of bleeding	Weeks* after onset	Titre of anti-streptolysin O	Titre of anti-DNAase B	Titre of anti-MAP	Presence of type 5 M-antibody in serum taken on 13. v. 70
1	17	Typical: aortic valve damage	24. iv. 70	½	570	400	128	
			13. v. 70	3	720	1600	256	
			21. v. 70	4	520	400	256	+
			10. vi. 70	7	280	—	256	
			4. ix. 70	21	160	400	128	
2	16	Mild: no heart damage	16. iv. 70	1†	590	800	64	
			13. v. 70	4	470	800	64	
			21. v. 70	7	340	1600–3200	64	−
			10. vi. 70	10	340	1600–3200	64	
3	17	Typical: active carditis, aortic systolic murmur	29. iv. 70	1	280	800	32	
			13. v. 70	3	640	1600–3200	64	
			21. v. 70	4	590	1600	64	−
			10. vi. 70	7	300	400	64	
4	17	Typical: no heart damage	16. iv. 70	1	580	—	64	
			13. v. 70	5	560	6400	64	
			21. v. 70	6	600	—	128	+
			10. vi. 70	9	520	400	64	
5	17	Typical: no heart damage	1. v. 70	3	550	800	256	
			13. v. 70	5	630	6400	256	
			21. v. 70	6	530	3200	256	+
			10. vi. 70	9	540	400	256	
6	33	Clinically 'fairly typical' no heart damage	16. iv. 70	1	305	3200–6400	128	
			5. v. 70	4	320	—	256	
			13. v. 70	5	300	6400	128	+
			3. vi. 70	8	330	800	128	
			4. ix. 70	20	155	400	128	
		Upper limit of normal			200	250	20	

* Weeks after approximate onset of rheumatic fever symptoms.
† History indefinite; rheumatic fever may have begun 3 weeks earlier.

Table 3. *Percentages of sera with raised streptococcal antibody titres from various groups of cadets in R.A.F. Halton*

	Symptomless with				Sore throat with				Type 5M isolated from throat†		Rheumatic fever cases
	negative TS*		positive TS†		negative TS†		positive TS†				
Antibody	No.	%	No.	%	No.	%	No.	%	No.	%	
ASO titre > 200	11/44	25	17/47	34	7/22	31	6/23	26	7/16	43	6/6
Anti-DNAase B titre > 250	19/39	49	14/44	32	6/20	30	6/23	26	6/16	37	6/6
Anti-MAP titre > 20	18/44	41	26/47	55	11/22	50	10/23	43	12/16	75	6/6
Anti-M antibody to types 5, 18 or 58 present	14/44	32	20/47	40	5/22	23	8/23	31	12/16	75	4/6
Antibody to type 5 present	9/44	21	15/47	30	2/22	9	6/23	26	11/16	68	4/6
Antibody to type 18 present	5/44	12	8/47	17	2/22	9	3/23	13	1/16	6	0/6
Antibody to type 58 present	1/44	2	0/47	0	2/22	9	1/23	4	1/16	6	0/6

Negative TS = throat swab negative for β-haemolytic streptococci.
Positive TS = throat swab positive for β-haemolytic streptococci.
* Sera collected on 7 June.
† Sera collected on 25 April.

this was mild and indefinite in four cases; in two there was more than one episode of sore throat, making it impossible to determine the length of the latent period. In only one case was penicillin given for 7 days.

Antibody titres of rheumatic fever patients

The findings are summarized in Table 2. All six patients showed a moderate rise in ASO titre and a somewhat more dramatic rise in anti-DNAase B titre, which in most cases began to decline during the period of investigation. The anti-MAP titre was raised in the first serum of each series and in general showed less tendency to decline. Sera taken on 13 May from all six patients were tested in the bactericidal test and four out of six had type-specific antibody to type 5. None of the sera had M-antibodies to type 18 or 58.

Antibody titres in sera from cadets with uncomplicated respiratory infection and from symptomless controls

The data derived from ASO, anti-MAP, anti-DNAase B and type-specific antibody tests were analysed by dividing the sera into five groups. Table 3 shows the percentage of sera in each of these groups that had antibody titres above the upper limit of normal in the ASO, anti-DNAase B and anti-MAP test. The upper limit of normal for the ASO was taken as 200 (Gooder & Williams, 1961). A value of 250 was taken as the upper limit of normal for the anti-DNAase B titres. In a survey by Ayoub & Wannamaker (1962), this value was exceeded

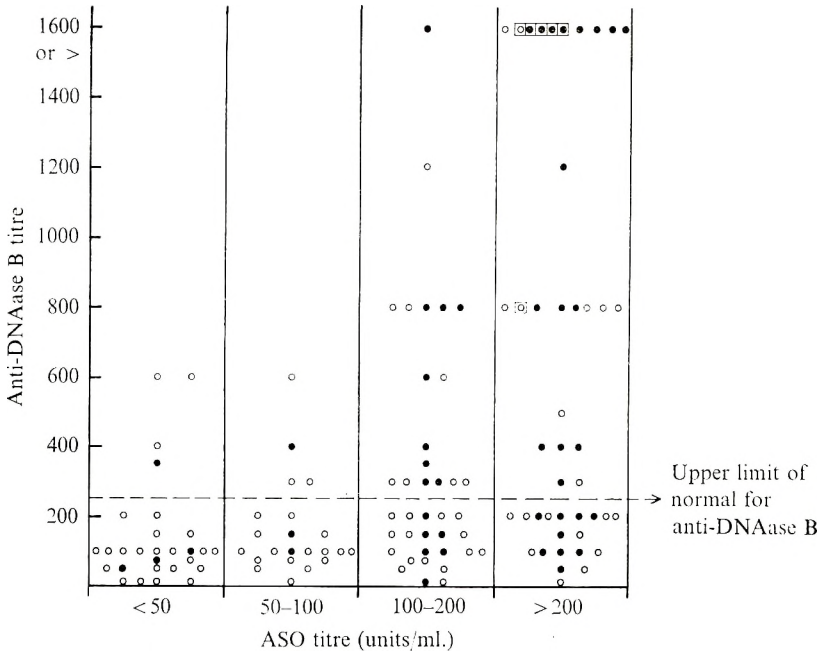

Fig. 1. The relationship of anti-DNAase B titre with ASO titre and the presence of type-specific antibodies. ●, Denotes presence of antibody to M types 5, 18 or 58. ○, denotes absence of type-specific antibody to these types. □, rheumatic fever cases.

by 15% of normal subjects tested. In similar tests in our laboratory a titre of 250 was exceeded by about 14% of persons without a history of recent streptococcal infection. In the anti-MAP test all sera with titres greater than, but not including 20, were considered to be above the upper limit of normal. About 20% of presumed normal sera examined have titres exceeding 20. Most normal individuals have titres of < 10 (Widdowson *et al.* 1971, and unpublished).

Table 3 shows the percentage of sera with evidence of protective antibody to types 5, 18 and 58. The results for the sera of the rheumatic fever patients are included for comparison, and these were derived from the antibody titres of sera taken on 13 May (see Table 2) between 3 and 8 weeks after the onset of rheumatic fever. The results for the ASO, anti-DNAase B and anti-MAP titres in Table 3 indicate very little difference between cadets with positive or negative throat swabs, or between cadets classified as symptomless and those who reported sick with sore throats, despite the difference of 6 weeks in the date of collection of the serum samples. The percentage of sera with type-specific antibody against type 5 appeared to be lower (9%) in cadets with sore throats but negative throat swabs, than in any other group, but the difference was not statistically significant at the 5% probability level ($\chi^2 = < 3{\cdot}84$).

The percentage of patients with elevated ASO and anti-DNAase B titres in the group which had type 5 M isolated from the throat did not differ significantly from the other groups in Table 3 ($\chi^2 < 3{\cdot}84$). However, the greatest difference between this group and the rest was in the number of sera with type-specific

Table 4. *Relationship of ASO and anti-DNAase B titre with the presence of type-specific antibodies*

Anti-DNAase B titre	Sera with antibody to type 5 out of total sera with ASO titre of:			Sera with antibody to types 5, 18 or 58 out of total sera with ASO titre of:		
	< 100	100–200	> 200	< 100	100–200	> 200
> 400	0/3 (0)	4/9 (44)	11/20 (55)	0/3 (0)	5/9 (55)	12/20 (60)
250–400	1/5 (20)	3/8 (38)	3/5 (60)	2/5 (40)	4/8 (50)	4/5 (80)
< 250	2/42 (5)	3/21 (14)	7/19 (37)	5/42 (12)	6/21 (28)	9/19 (47)

Figures in parentheses represent the percentage of sera in each group with type-specific antibody.

antibody to type 5; 68% compared with an average of 21·5% for the other four groups ($\chi^2 > 6{\cdot}6$ – the difference is significant at the 1% probability level). The percentage of sera with raised anti-MAP titres (75% compared with an average of 47% for the other groups) was also significantly high ($\chi^2 > 6{\cdot}6$).

Correlation of ASO titres with anti-DNAase B titres

The anti-DNAase B titres of all the sera were plotted as a scattergram in four categories of ASO titre (Fig. 1). Although there was a general tendency for the anti-DNAase B titre to increase with the ASO titre, there were 25 of 134 sera with anti-DNAase B titres > 250 whose ASO titres were < 200, and 19 of 134 sera with ASO titres ⩾ 200 whose anti-DNAase B titres were below the upper limit of normal. Among the sera in which a raised ASO was not confirmed by a raised anti-DNAase B titre, or vice versa, 20 of 44 had type specific antibody to types 5, 18, or 58 which suggested possible recent infection with these types in this outbreak.

Table 4 shows the relationship of ASO titre and anti-DNAase B titre with the presence of type-specific antibody to types 5, 18 or 58 and to type 5 considered alone. The presence of type-specific antibodies of any of the three types in 5 of 42 (12%) sera, without evidence of recent streptococcal infection (ASO < 100, anti-DNAase B < 250) probably indicates the 'background level' of type-specific antibodies to these three types in the community. Only 2 of 42 (5%) of these sera had type 5 antibodies. Bactericidal antibodies are known to persist for many years in man (Lancefield, 1959), but a small survey among workers in our laboratory revealed that only 1 out of 10 had antibody to types 5 or 18 and none had antibody to type 58. Table 4 shows that the percentage of sera with type-specific antibody was appreciably higher than this background level in groups with raised ASO titres, but low anti-DNAase B titres. Among sera with raised anti-DNAase B titres, but low ASO titres only those with ASO titres between 100 and 200 showed a high percentage of type-specific antibodies. Among the sera with ASO titres < 100 and anti-DNAase B titres > 250, only 1 of 8 had type-specific antibody to type 5, indicating that a raised anti-DNAase B titre may not be significant if the ASO is < 100.

Table 5 shows the effect of combining the results of ASO and anti-DNAase B

Table 5. *Percentage of sera with raised ASO and/or anti-DNAase B titres*

Antibody titre	All sera (except samples from rheumatic fever patients)	Sera from patients with TS positive for type 5 M	Sera with type 5 antibody
ASO > 200	33·3	43·0	55·5
Anti-DNAase B > 250	37·9	37·5	66·9
ASO and/or anti-DNAase B raised	52·3	62·5	83·3
Anti-MAP > 20	47·0	75·0	75·0

TS = throat swab.

Table 6. *Relationship of ASO and anti-MAP titre with the presence of type-specific antibodies*

Anti-MAP titre	Sera with antibody to type 5 out of total sera with ASO titre of:			Sera with antibody to types 5, 18 or 58 out of total sera with ASO titre of:		
	< 100	100–200	> 200	< 100	100–200	> 200
> 80	0/0 (0)	0/0 (0)	11/14 (79)	0/0 (0)	0/0 (0)	13/14 (93)
20–80	1/12 (8)	6/21 (28)	9/22 (41)	2/12 (16)	8/21 (38)	12/22 (55)
< 20	2/40 (5)	6/21 (28)	1/12 (8)	6/40 (15)	8/21 (38)	2/12 (16)

Figures in parentheses indicate the percentages of sera with type-specific antibody in each group.

tests in three categories of sera. In all these categories the results of the combined test gave percentages of sera with evidence of recent streptococcal infection, 15–25 % greater than either test alone. This was particularly striking among the sera with type-specific antibody to type 5. Only 55·5 % of these had raised ASO titres whereas a combination of the ASO and anti-DNAase B results suggested that 83·3 % had suffered a recent streptococcal infection.

Correlation of ASO with anti-MAP titres

The anti-MAP titres of all the sera were plotted as a scattergram in four ASO categories as shown in Fig. 2. There was good correlation between the two antibody titres, in that there were very few sera (3 of 28) with anti-MAP titres > 20 which had ASO titres of < 50. There were also few sera with ASO titres > 200 which had anti-MAP titres of less than 20, and of these only 2 of 12 had type-specific antibody to types 5, 18 or 58 and only 1 of 12 had antibody to type 5 (see Table 6).

Fig. 2 also shows that there were 34 patients, other than the six with rheumatic fever, who had anti-MAP titres of 60 or greater. Of these 34 patients 22 had type-specific antibody to types 5, 18 or 58.

Correlation of anti-MAP titres with the presence of type-specific antibody

The number of sera with antibody to type 58 (4) and to type 18 (18) were too small for any comparison to be made, but 36 of 142 sera from R.A.F. Halton

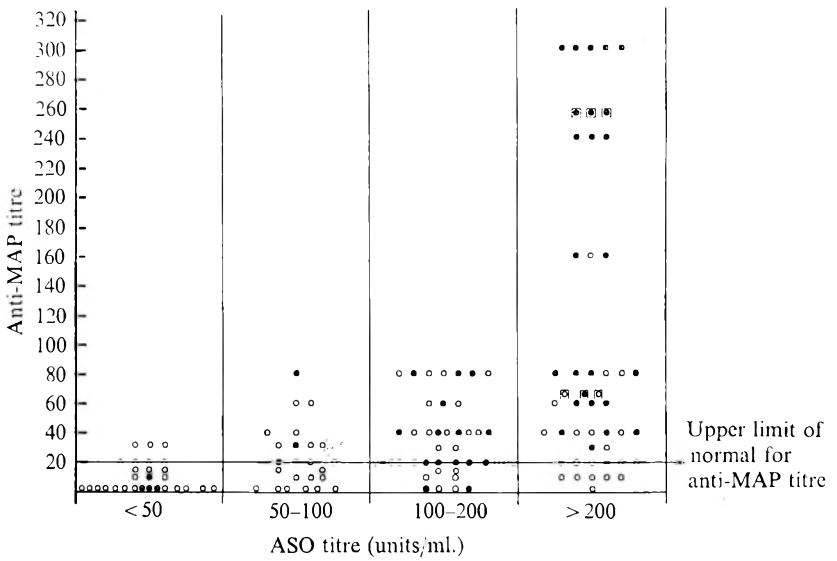

Fig. 2. The relationship of anti-MAP titre with ASO titre and the presence of type-specific antibody. ●, Denotes presence of antibody to M types 5, 18 or 58. ○, denotes absence of type-specific antibody to these types. □, rheumatic fever cases.

had type 5 antibody. Among these 36 sera only 55·5% had raised ASO titres (see Table 5) whereas 75% had elevated anti-MAP titres. The interrelationship between all three antibodies is shown in Table 6, where it is apparent that the highest proportion of sera with type 5 antibody occurs in the group with ASO titres > 200 and anti-MAP titres > 80. There were no anti-MAP titres > 80 among sera with ASO titres < 200.

Persistence of antibody to MAP

Follow-up bleedings were taken one year after the initial outbreak from ten of the cadets who had anti-MAP titres of 80 or greater. Nine out of the ten showed no decrease in anti-MAP titre. One showed a decrease from 80 to 40. The corresponding ASO titres in most cases showed a decrease, although one serum showed a rise from 430 to > 800 probably indicating a new infection.

DISCUSSION

The community at RAF Halton was not under constant surveillance for streptococcal infection before this outbreak. This investigation was begun only after a number of cases of rheumatic fever had been diagnosed. Throat swabs and sera from the rheumatic fever patients and other cadets were taken about 1 month after the start of the sore throat outbreak, so that there was no information about the types of streptococci prevalent at the very beginning. However, the persistence of type 5 in the succeeding 6 weeks, and the presence of type 5 antibody in many of the sera (43% of those with ASO ⩾ 200), including sera

from 4 out of 6 of the rheumatic fever patients, indicated that type 5 was probably responsible for the rheumatic fever, with some minor involvement of types 18 and 58 in the cases of sore throat.

Sera were obtained from a sample of cadets who were symptomless, had negative throat swabs and might have been thought not to have been involved in the outbreak. However, the antibody titres in these 'normal' sera were not significantly different from those in sera from boys who had suffered from sore throat (see Table 3). Only random samples of the group A streptococci isolated from cases of sore throat between 16 April and 13 May were typed, so that relatively few of the sera subsequently obtained could be matched with a throat swab result for the same patient. Also it was not practicable at the time to obtain paired sera from cadets other than those with rheumatic fever. These deficiencies leave considerable gaps in our information about the antibody responses of individual cadets but certain trends were apparent in the antibody responses for the community as a whole. The ASO response was in general rather weak, even among cadets from whose throats type 5 streptoccoci had been isolated, or who had type-specific antibody against this type in the sera. The anti-DNAase B response was in general better. However, it was only when the results of these two antibody tests were combined that a more realistic estimate of the percentage of cadets who had had a recent streptococcal infection was obtained, if the presence of antibody to type 5 could be taken as an indication of infection in this outbreak, where the 'background level' of type specific antibody to type 5 appeared to be between 5 and 10%.

The anti-MAP test is not an established antibody test in the diagnosis of streptococcal diseases. In our first report on the presence of this antibody in human sera (Widdowson *et al.* 1971) we stated that titres were higher in rheumatic fever (range 60–320) than in nephritis (range 0–80) or in sporadic uncomplicated streptococcal infection of the throat (range 0–80). We had not at that stage examined a large number of sera from cases of uncomplicated sore throat in a single outbreak. The result of tests done on sera from R.A.F. Halton showed that high anti-MAP titres were not confined to the sera of the cadets who developed rheumatic fever. Although the anti-MAP titres of the sera from the rheumatic fever patients were all high (> 60), 17 of 38 sera from cadets who had raised ASO titres but no sign of rheumatic fever, also had anti-MAP titres of greater than 60 (see Fig. 2). However, from other outbreaks, in which no cases of rheumatic fever occurred, we have examined sera from over 100 cases of uncomplicated sore throat caused by different streptococcal serotypes (e.g. types 6, 12 and 22) and found that, although the ASO and anti-DNAase B titres were comparable with or higher than those in the type 5 outbreak, the anti-MAP titres were in general much lower. For example only about 10% of sera with ASO titres greater than 200 from an outbreak of sore throat caused by type T12/M12 and T12/M22 strains had anti-MAP titres greater than 60. Moreover, about 70% of these sera had anti-MAP titres below the upper limit of normal (Widdowson, Maxted, Notley & Pinney, *in preparation*).

It therefore seems likely that the anti-MAP response is to a large extent

influenced by the infecting type. Although a high anti-MAP titre may not always be associated with rheumatic fever *per se*, perhaps the generally higher titres seen in rheumatic fever, compared with, for example, nephritis, are a reflexion of the influence of the type of streptococcus, both on the magnitude of the anti-MAP titre and the nature of secondary sequelae of streptococcal infection.

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The effect of preadministration of *Corynebacterium parvum* on the protection afforded by heat-killed and acetone-killed vaccines against experimental mouse typhoid

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(*Received* 27 *April* 1973)

SUMMARY

Mice given an intraperitoneal injection of 0·5 mg. *Corynebacterium parvum* (dry weight) before vaccination with heat-killed (HK) or acetone-killed (AK) *Salmonella typhimurium* vaccine and later challenged intraperitoneally with *S. typhimurium* strain 1566, showed a statistically significant increase in mortality when compared with mice that had received only *C. parvum* but no vaccine. They also showed a higher mortality rate than mice receiving only HK or AK vaccine or mice that had received no vaccine before challenge. Prior administration of *C. parvum* to mice that are vaccinated with HK or AK vaccine appears to make them more susceptible to an intraperitoneal challenge with *S. typhimurium*. This was more apparent with HK vaccine than with AK vaccine.

INTRODUCTION

In the last decade considerable interest had been focused on the ability of heat-killed suspensions of *Corynebacterium parvum* to stimulate the immune defence mechanisms of experimental animals. Halpern *et al.* (1964) demonstrated an intense and prolonged stimulation of phagocyte activity of the reticulo-endothelial system after injecting *C. parvum* into mice by the intravenous or intraperitoneal route. These mice were found to have enlarged livers and spleens and histological studies revealed proliferation of existing elements and lympho-histiocytic infiltration of these organs. The administration of *C. parvum* also leads to an increase in the production of antibodies to specific antigens (Neveu, Branellec & Biozzi, 1964) by increasing not only the number of antibody-producing cells but also the output of antibody by each cell (Biozzi *et al.* 1966). Pinckard, Weir & McBride (1967*a*, *b*) administered *C. parvum* strain 10387 to rabbits 6 days before challenge with a weak antigen and demonstrated a considerable increase in the production of antibodies. The binding capacity of the antisera produced was considerably augmented and there was an increase in the affinity of the antibodies for the antigen. These workers concluded that the adjuvant effect of *C. parvum* was not entirely due to lymphoreticular proliferation and increased phagocytosis but possibly also to stimulation of nonspecific immunity

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by affecting the 'recognition' and 'triggering off' stages of the immune response. Siskind & Howard (1966) showed that *C. parvum* was able to augment protective immunity to pneumococci in mice. They injected *C. parvum* into the animals before administration of pneumococcal polysaccharide and demonstrated a prolonged survival time of the mice upon subsequent challenge with live pneumococci.

Sterne & Trim (1970) investigated the ability of calcium alginate to enhance the protection conferred on mice by heat-killed *Salmonella typhi* vaccine. These workers suggested that the enhanced potency of typhoid vaccine when mixed with calcium alginate and injected intraperitoneally into mice was due to the stimulation of nonspecific immunity in the peritoneum by the calcium alginate rather than a specific immune mechanism. In the present study a heat-killed suspension of *C. parvum* was administered intraperitoneally to mice in an attempt to augment the immunity conferred by heat-killed (HK) and acetone-killed (AK) vaccine against mouse typhoid.

MATERIALS AND METHODS

Animals

Male Swiss white mice weighing 19–22 g. were used.

Bacterial strains

Salmonella typhimurium strain 1566 was used for the preparation of vaccines and for challenge.

Vaccines

Heat-killed (HK) and acetone-killed (AK) *S. typhimurium* vaccines were prepared as described in a previous publication (Cronly-Dillon, 1972*a*). A dose of 10^3 HK or AK organisms was administered to each mouse as a single subcutaneous injection at the root of the tail. This dose was known to be non-protective against an intraperitoneal challenge with 100 *S. typhimurium* strain 1566 (Cronly-Dillon, 1972*b*) and was used in this experiment so that any possible potentiation of vaccine activity would be more apparent.

Challenge inoculum

The challenge organisms were suspended in 0·1 M phosphate buffer at pH 8 made by adding 5·3 ml. of 0·2 M solution of monobasic sodium phosphate to 94·7 ml. of 0·2 M solution of dibasic sodium phosphate and diluting to 200 ml. Preliminary tests had shown that this medium does not impair the viability of the organism. The challenge dose of 100 organisms was administered intraperitoneally in a volume of 0·1 ml. The actual dose administered was determined by a surface viable count (Miles & Misra, 1938).

Preparation of C. parvum

C. parvum strain 10387 was grown and killed by heat at 70° C in a water bath for 1 hr. as described by Pinckard *et al.* (1967*a*). The sterility of the preparation

was tested and strictly confirmed before use. The suspension was diluted in physiological saline and a volume of 0·1 ml. containing 0·5 mg. (dry weight) of the *C. parvum* was injected intraperitoneally into each mouse. This dose appeared to be non-toxic to the animals since mean daily weights recorded over a period of 28 days were not altered when compared with those of controls. Histological studies on the livers and spleens of four of the test mice killed 11 days after injection of *C. parvum* showed no obvious changes and individual weights of livers and spleens of these mice were not significantly altered when compared with those of controls.

Design of the study

Sixty mice were each given an intraperitoneal injection of 0·1 ml. of *C. parvum* suspension containing 0·5 mg. (dry weight) of the organism. Six days later, 20 of these mice and 20 untreated mice were given a single subcutaneous injection of 10^3 HK vaccine organisms. Another group of 20 mice that had received *C. parvum* and 20 untreated mice were given 10^3 AK vaccine organisms subcutaneously. The third group of 20 mice that had received *C. parvum* and 20 untreated mice remained as the control groups. On the 15th day after vaccination, all the animals were challenged intraperitoneally with a dose of 10^2 organism of *S. typhimurium* strain 1566. The mice were then observed daily for 28 days and deaths were recorded every day. The liver and spleen of each mouse that died was cultured in nutrient broth to establish the presence of *S. typhimurium.* On the 28th day after challenge all the survivors were killed and examined in the same way to test for the presence of *S. typhimurium* in their organs.

Statistical analysis

Results were analysed by the χ^2 test of probability using a formula that makes allowances for small numbers. When the value for P was 0·05 or less the result was regarded as statistically significant.

RESULTS

The detailed results are shown in Table 1.

In comparison with mice that did not receive *C. parvum*, those mice that received *C. parvum* 6 days before vaccination with 10^3 HK or AK organisms were not more resistant to intraperitoneal challenge with 100 *S. typhimurium* 15 days after vaccination. In fact there was a statistically significant reduction in protection, i.e. an increased mortality rate, among mice that had received *C. parvum* before vaccination with HK organisms ($0{\cdot}025 > P > 0{\cdot}01$) or AK organisms ($P = 0{\cdot}05$) when compared with the group of mice that had been given *C. parvum* alone. The mortality rates among mice primed with *C. parvum* and subsequently vaccinated with HK or AK organisms were slightly higher than in the untreated control group and in those mice vaccinated with HK or AK vaccine alone, but these results were not statistically significant. Although the mortality among mice primed with *C. parvum* and then vaccinated with

Table 1. *The observed mortality rates, mean times to death, and infectivity rates in groups of* 20 *test mice treated in various ways before intraperitoneal challenge with* 10^2 *organisms of* S. typhimurium *strain* 1566

Immunization procedure		No. of		Mean time to death of	
Initial treatment	Vaccine given s.c. on day 6 after initial treatment	deaths due to *S. typhimurium*	Mortality (%)	fatal cases (days)	Infectivity (%)
C. parvum	Heat-killed vaccine	17/18*	94	13	100
Nil	Heat-killed vaccine	13/20	65	12	100
C. parvum	Acetone-killed vaccine	16/20	80	12	100
Nil	Acetone-killed vaccine	14/20	70	13	100
C. parvum	Nil	9/20	45	12	100
Nil	Nil	14/20	70	12	100

Mice were given 0·5 mg (dry weight) killed *C. parvum* intraperitoneally (IP). Six days later the animals were given a single subcutaneous (SC) injection of 10^3 HK or AK vaccine organisms. All the animals including controls were challenged on the 15th day after vaccination. The experiment was terminated on the 28th day after challenge.

* Two mice died before challenge.

HK organisms was not significantly higher than that of mice receiving only HK organisms, the χ^2 test showed a border-line value ($0{\cdot}1 > P > 0{\cdot}05$).

The dose of 10^3 HK or AK vaccine organisms on their own did not confer any protection against the intraperitoneal challenge with 10^2 *S. typhimurium*. This result was expected.

Mice given only *C. parvum* before challenge showed the lowest mortality rate (45%) in the experiment. However, this result was not statistically significant when compared with the mortality of untreated controls (70%) although it was significantly lower than in mice that had been primed with *C. parvum* before vaccination with HK or AK organisms.

The infectivity rates were 100% in all the groups, and mean survival times of those mice that died were not significantly altered in any of the groups.

DISCUSSION

The adjuvant effect of administering *Corynebacterium parvum* to mice before immunization has been shown to increase the protective potency of the immunogen to a virulent challenge with pneumococci (Siskind & Howard, 1966). The results of the present study with *Salmonella typhimurium* show the reverse. Not only does prior administration of *C. parvum* fail to protect mice vaccinated with HK or AK vaccines against an intraperitoneal challenge with *S. typhimurium*, but it seems to make the challenge more lethal for the vaccinated animals. Thus the mortality was considerably higher in mice primed with *C. parvum* and immunized with HK vaccine than in those mice that had received *C. parvum* alone or HK vaccine alone. This trend was also reflected in the groups of mice receiving AK vaccine in that mice given *C. parvum* and then immunized with AK vaccine showed a significantly increased mortality when compared with mice that had received *C. parvum* alone. This combined form of immunization was definitely less protective against challenge in comparison with the mortality among the

animals given each component on its own. HK vaccine seems to be slightly worse in combination than AK vaccine in combination with *C. parvum* but the difference was not statistically significant.

It is unlikely that an inflammatory reaction resulting from the initial *C. parvum* injection could be responsible for the adverse reactivity to the intraperitoneal challenge injection since those mice given *C. parvum* alone showed significantly fewer deaths after challenge than those given the combined treatment. It is also interesting to note that the group of mice given *C. parvum* alone showed a lower mortality rate than the untreated control group. Although this result was not statistically significant it may indicate that administration of *C. parvum* alone tends to exert a slightly protective rather than adverse effect upon mice challenged intraperitoneally.

Histology of liver and spleen of mice given an intraperitoneal injection of *C. parvum* alone failed to show conclusive evidence of lymphoreticular hyperplasia when studied 11 days after challenge. This is in accord with work done concurrently in this department that demonstrated that the particular strain of *C. parvum* used in the present study was capable of inducing only slight histological changes in the lymphoreticular system 6 days after injection into rabbits (Pinckard, Weir & McBride, 1967*b*, 1968).

The antibody titres of the animals were not followed in the present study since the original object was to achieve protection in terms of survival or a decreased infectivity rate, and it has already been demonstrated that in mouse typhoid antibody titres do not correspond with the degree of immunity (Hobson, 1957*a*, *b*).

The increased mortality noted upon challenge of vaccinated mice that had previously received *C. parvum* is very difficult to explain. A possible explanation worthy of further study is based on the idea of blocking or enhancing antibody (Hellström & Hellström, 1970). Immunity to mouse typhoid is now generally believed to be largely cell-mediated (Mitsuhashi, Sato & Tanaka, 1961; Mitsuhashi & Saito, 1962). It is probable that those animals who survive the experimental infection do so because they develop specific cell-mediated immunity to *S. typhimurium* during the course of the infection. Perhaps the previous administration of *C. parvum* so modifies the humoral response both qualitatively and quantitatively as to result in an 'enhanced' state – the induced humoral factor now blocking the potentially beneficial effects of cell-mediated immunity. In other words, the blocking antibody combines with the organism and prevents direct contact with sensitized lymphocytes, thus enhancing the virulence of the *S. typhimurium* in much the same manner as the enhancement effect noted in studies of tumours and graft versus host reactions (Hellström & Hellström, 1970).

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Toxoplasma antibodies in immigrants from Hong Kong

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SUMMARY

The sera of 200 Chinese adults recently immigrated to Canada from Hong Kong were examined for antibodies to *Toxoplasma gondii* by the indirect fluorescent antibody technique. It was found that the total incidence was 18·0%. This is significantly lower than that of the indigenous population of the same age groups in this area.

INTRODUCTION

Toxoplasmosis is prevalent throughout the world. The prevalence as shown by serological tests is more widespread in the warm climates (Feldman, 1968). A previous report suggested that sera from Hong Kong had a low incidence of positivity according to the Sabin-Feldman dye test (Ludlam, Wong & Field, 1969). The purpose of this communication is to report on the incidence of toxoplasmosis in 200 Chinese recently immigrated to Canada from Hong Kong. The incidence of toxoplasmosis in the general population in this area (Montreal, Quebec, Canada) had previously been determined (Seah, 1973).

MATERIALS AND METHODS

The existence of the Montreal Chinese Hospital, the only hospital for Chinese in Canada, made this study possible. Recent immigrants to this City who presented themselves to this hospital's outpatient clinics for various reasons were asked to submit blood samples. In this survey 200 sera were collected from persons over 20 years of age, all of whom had been in Canada for less than 2 years. All had come from Hong Kong and about half were born there. The rest had spent a number of years in Hong Kong. None of them had symptoms or signs suggestive of toxoplasmosis when seen in the outpatient clinics.

The serological method used was the indirect fluorescent antibody test (IFAT). This method was based on the methods of Kelen, Ayllon-Leidl & Labzoffsky (1962), Fletcher (1965), and the one used in the Center for Disease Control, Atlanta, Georgia, U.S.A. (1970). The antigen was prepared in this laboratory from peritoneal exudate of mice that had been inoculated intraperitoneally with trophozoites of the RH strain of *Toxoplasma gondii* 3 days previously. For the purpose of initial screening, serum dilutions of 1/16 and 1/64 were used.

After appropriate incubation with the unknown serum samples, washing in

Table 1. *Prevalence of positive indirect fluorescent antibody test for toxoplasmosis by age and sex in Chinese immigrants*

Age group	Male			Female			Total		
	No. tested	No. positive	% positive	No. tested	No. positive	% positive	No. tested	No. positive	% positive
20–30	18	2	11·1	20	3	15·0	38	5	13·2
31–40	27	4	14·8	23	6	26·1	50	10	20·0
41–50	21	4	19·0	29	6	20·7	50	10	20·0
51–60	9	2	22·2	21	3	14·3	30	5	16·7
Over 60	8	2	25·0	24	4	16·7	32	6	18·8
Total	83 (634)*	14 (172)	16·9 (27·1)	117 (564)	22 (147)	18·8 (26·1)	200 (1198)	36 (319)	18·0 (26·6)

* The figures in parentheses indicate the prevalence of positive indirect fluorescent antibody test for toxoplasmosis in the general population of the same age groups.

phosphate buffer, incubation with conjugated antihuman globulin, and further washing, the slides were covered with buffered glycerol and cover-slips. The slides were examined with the high dry objective in a Leitz Ortholux fluorescent microscope equipped with BG 12 exciter filter, OG 1 barrier filter and super pressure vapour lamp Osram HBO 200. The reaction was considered positive when greenish yellow fluorescence was seen around the periphery of the *Toxoplasma* trophozoites. A titre of 1/16 was considered positive, and any positives were repeated and diluted to titres.

This indirect fluorescent antibody test for toxoplasmosis had previously been shown to be as sensitive, reproducible, and approximately the same in terms of absolute titres as the long established Sabin-Feldman dye test (Walton, Benchoff & Brooks, 1966).

RESULTS

A total of 200 sera from individuals over 20 years of age was tested. There were 83 men and 117 women in this survey. Table 1 shows the prevalence of positive IFAT for toxoplasmosis by age and sex in these Chinese immigrants. In the male the incidence seems to increase with age, and the total positivity is 16·9 %. In the female the peak incidence is found to be in the 31–40 years group, and the total incidence is 18·8 %. There is no significant difference in the incidence between the male and female. The results of an earlier survey of the general population in Montreal are included in the same table in parentheses (Seah, 1973). It is also seen that while the total positivity of the general population in Montreal is 26·6 % for the age groups indicated, that of the Chinese immigrants is 18·0 %.

DISCUSSION

The prevalence of toxoplasma antibodies varies in various parts of the world (Feldman, 1968). In Britain in a survey of blood donors the incidence was found to increase with age. The incidence reported was 21 %, 30 % and 40 % for 21–30,

31–40 and 41–50 age groups respectively (Fleck, 1969). The prevalence in North America is between 20 and 30% (Feldman, 1968). In Montreal it has been determined that the overall rate of positivity by the indirect fluorescent antibody test is 23·7%, with much lower rate in the paediatric age group, and a peak incidence of 28·4% in the 31–40 years age group (Seah, 1973).

There is very little information on the incidence of toxoplasmosis in Southern Chinese. A literature search revealed no such survey. Ludlam *et al.* (1969) performed the dye test on sera from Hong Kong. There were no positives in 32 adult Hong Kong women; 2 out of 35 sera from men were positive and 5 out of 46 meat workers were positive. This is a very small survey, but it yielded a total positivity of 6·2%. This is very low incidence indeed, especially for a tropical area. The tropical areas are generally believed to have a higher prevalence of toxoplasmic antibodies (Wright, 1957; Roever-Bonnet, 1972). Asians coming to the United Kingdom from East Africa and the Indian subcontinent appear to have the same prevalence of toxoplasmic antibodies as the indigenous population (Dodge, 1972).

Zaman & Goh (1969) reported on the results of 754 sera from Singapore residents using the haemagglutination test. Their results showed that 21·8% of the Chinese, 39·1% of the Malays and 36·6% of the Indians were positive. These sera were from clinically suspected cases of toxoplasmosis or infectious mononucleosis and therefore cannot be considered as representative of the general Singapore population.

The present survey shows that immigrants from Hong Kong have a lower prevalence of toxoplasmic antibodies than the indigenous population of Canada. The exact reason is not clear. Chinese are known to like their meat well cooked. As infected meat is an important source of infection this may account for the relatively lower incidence. In this survey the history of keeping cats as pets was not gone into. In the crowded conditions in Hong Kong very few Chinese keep cats as pets.

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Susceptibility of the VERO line of African green monkey kidney cells to human enteroviruses

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SUMMARY

The relative susceptibility of VERO cells and primary rhesus monkey kidney cells to 47 prototype strains of human enteroviruses is described. Of these strains, types 4, 14, 16, 17, 18, 21, 31 and 34 and Coxsackie virus A 9 failed to cause CPE in the VERO cells whilst only one, echovirus type 34, failed to cause CPE in the monkey kidney cells.

A comparison is given of the efficiency of the two cell cultures for enterovirus isolation from clinical material. Results show that VERO cells are as useful as primary monkey kidney for the isolation of Coxsackie B viruses but less satisfactory for isolating echoviruses. They are satisfactory for the isolation of single types of poliovirus and appear to be more satisfactory than primary monkey kidney cells for the isolation of mixtures of polioviruses. The identification of enteroviruses by neutralization tests in VERO cells is successful.

INTRODUCTION

The isolation and identification of human enteroviruses is done by many laboratories in primary or secondary rhesus monkey kidney cultures. There are however, certain disadvantages associated with these tissues such as their varying sensitivity to viruses and the frequent presence of latent agents. Besides these there are the hazards and expense connected with keeping a monkey colony and the growing concern in the world at the use of wild animals for this purpose. There has thus been an increasing need to find a replacement for monkey kidney cell culture in virus laboratories.

The use of a continuous line of cells would eliminate many of the disadvantages mentioned above. It would be easy to grow and to maintain in any quantity and its sensitivity should be reproducible from week to week and there would be no latent viruses to confuse the results. The problem is to be sure that such a line will produce an adequate cytopathic effect (CPE) to allow the detection of virus in small amounts in clinical specimens and to enable further identification procedures to be done.

With this in mind, the continuous line of African green monkey kidney cells

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(VERO) derived by Yasumura & Kawakita (1963) has been used in a series of experiments to determine whether or not it would be a suitable replacement for primary or secondary rhesus monkey kidney for the isolation and identification of a variety of human enteroviruses. The results of the experiments are given in this report.

MATERIALS AND METHODS

Tissue cultures

VERO Cells

The continuous line of African green monkey cells (*Cercopithecus aethiops*) designated VERO was obtained through Dr A. D. Macrae. Stock cultures were grown in 6 oz. flat bottles on medium 199 containing 2% foetal calf serum, 0·15% sodium bicarbonate and antibiotics. Subcultures were made twice a week when the cell sheet was confluent, by removing the cells from the glass using a mixture of 0·02% sequestric acid (EDTA) and 0·05% trypsin in Dulbecco's phosphate buffered saline solution A (P.B.S.). The cell sheets were first washed in P.B.S. then just covered with the trypsin/EDTA mixture. This was left in contact with the cells for exactly 1 min. at room temperature and then discarded. The bottle was incubated at 36–37° C. for 5 min. after which time 3 ml. of the growth medium was added, the cells were washed off the glass into this and diluted appropriately so that tubes received 1 ml. of cell suspension containing approximately 10^5 cells. The tubes were incubated in stationary racks at 36–37° C. for 48 hr. after which the medium was changed to a maintenance fluid consisting of medium 199 with 1% foetal calf serum, 0·176% sodium bicarbonate and antibiotics, and the cultures were ready for use.

Primary rhesus monkey kidney

A method based on that of Rappaport (1956) was used to trypsinize kidneys from freshly killed monkeys. The growth medium was Hanks's balanced salt solution containing 0·5% lactalbumin hydrolysate, 2% bovine serum, 0·05% sodium bicarbonate and antibiotics. Tubes were seeded with 1 ml. of suspension containing 5×10^4 cells. Maintenance fluid was medium 199 with 0·22% sodium bicarbonate and antibiotics. This replaced the growth medium one day before the cultures were used, usually seven days after trypsinization.

Virus suspensions and specimens

The viruses used were all prototype enteroviruses as listed in Table 1. The clinical specimens consisted of a variety of throat swabs, faeces, cerebro-spinal fluid and urines which were kindly supplied by the Public Health Laboratories at Leeds and Neasden, London.

Virus titrations

Four culture tubes of primary rhesus monkey kidney (p. MK) and four tubes of VERO cells were inoculated each with 0·1 ml. serial 0·5 log. dilutions. Cultures

Table 1. *Infectivity titres of enteroviruses in primary rhesus monkey (p. MK) and VERO cells after three passages in p. MK and VERO cells*

Virus	Strain	Titration of virus grown in p. MK*		Titration of virus grown in VERO cells*	
		In p. MK	In VERO	In p. MK	In VERO
Echo 1	Farouk	7·1	6·9	6·1	6·6
Echo 2	Cornelis	6·2	7·0	5·7	6·0
Echo 3	Morrisey	6·4	7·9	6·5	7·9
Echo 4	Pesascek	5·2	< 1·0	< 1·0	< 1·0
Echo 4	du Toit	7·0	7·7	7·5	7·6
Echo 5	Noyce	7·7	7·5	7·5	8·0
Echo 6	D'Amori	6·9	8·4	7·2	8·4
Echo 7	Wallace	7·4	6·6	7·7	7·5
Echo 8	Bryson	7·2	7·0	8·1	7·5
Echo 9	Hill	6·7	6·1	7·6	7·7
Echo 11	Gregory	7·9	7·5	7·5	7·1
Echo 12	Travis	7·4	6·7	7·1	7·2
Echo 13	11-4D	5·4	6·0	5·9	6·4
Echo 14	Tow	4·5	< 1·0	6·6	7·1
Echo 15	Charleston	5·5	6·1	7·2	7·2
Echo 16	Harrington	3·9	< 1·0	< 1·0	< 1·0
Echo 17	CHHE-29	5·1	< 1·0	< 1·0	< 1·0
Echo 18	Metcalf	3·9	< 1·0	4·5	< 1·0
Echo 19	Burke	5·5	6·1	6·2	6·7
Echo 20	JV-1	4·9	1·7	4·5	5·4
Echo 21	Farina	2·9	< 1·0	< 1·0	< 1·0
Echo 22	Harris	5·4	3·2	5·5	5·6
Echo 23	Williamson	4·1	2·1	< 1·0	< 1·0
Echo 24	DeCamp	5·1	3·4	6·4	6·6
Echo 25	JV-4	6·5	4·0	6·0	6·6
Echo 26	Coronel	5·6	6·1	5·1	6·1
Echo 27	Bacon	6·9	3·2	5·2	1·9
Echo 29	JV-10	5·0	6·2	5·2	4·2
Echo 30	Bastianni	4·4	4·7	5·5	6·9
Echo 31	Caldwell	4·2	< 1·0	4·7	2·4
Echo 32	PR 10	5·6	4·1	6·1	5·9
Echo 33	Toluca-3	5·1	1·0	< 1·0	< 1·0
Echo 34	DN-19	< 1·0	< 1·0	< 1·0	< 1·0
Coxsackie A 7	Dalldorf WP 50140	6·5	6·4	7·6	6·9
Coxsackie A 9	Dalldorf 50546	7·1	< 1·0	< 1·0	< 1·0
Coxsackie B 1	P.O.	5·0	4·9	6·5	6·9
Coxsackie B 2	Ohio (Red)	5·6	6·0	7·1	7·1
Coxsackie B 3	Nancy	6·2	6·1	6·2	6·7
Coxsackie B 4	J.V.B.	6·5	6·0	6·5	6·0
Coxsackie B 5	Faulkner	4·9	3·7	7·0	6·7
Coxsackie B 6	Schmitt	5·1	6·7	5·4	7·0
Poliovirus type 1	Sabin	6·5	6·9	5·7	6·4
Poliovirus type 1	Mahoney	7·1	7·4	7·4	8·0
Poliovirus type 2	Sabin	6·0	6·1	6·3	6·4
Poliovirus type 2	YSK	6·5	6·5	6·7	7·0
Poliovirus type 3	Sabin	5·9	6·0	6·4	6·6
Poliovirus type 3	Saukett	7·0	7·1	7·1	7·2

* Expressed as log TCD 50/0·1 ml.

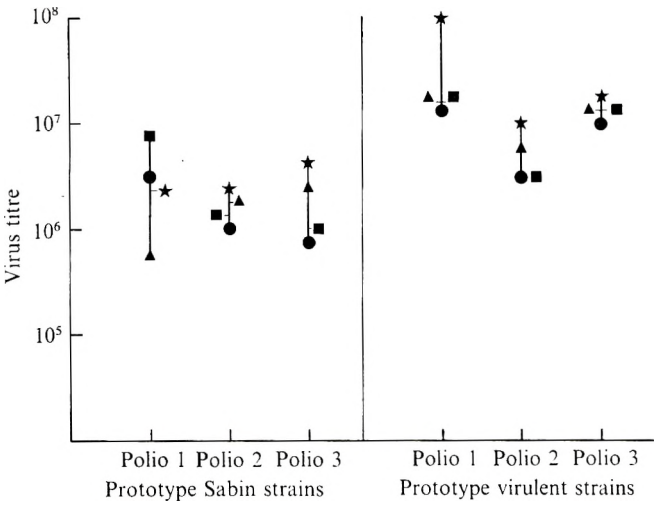

Fig. 1. Titres of the prototype polioviruses after three passages in p. MK and VERO cells. ●, MK_3 pass titrated in p. MK. ■, MK_3 pass titrated in VERO. ▲, $VERO_3$ pass titrated in p. MK. ★, $VERO_3$ pass titrated in VERO.

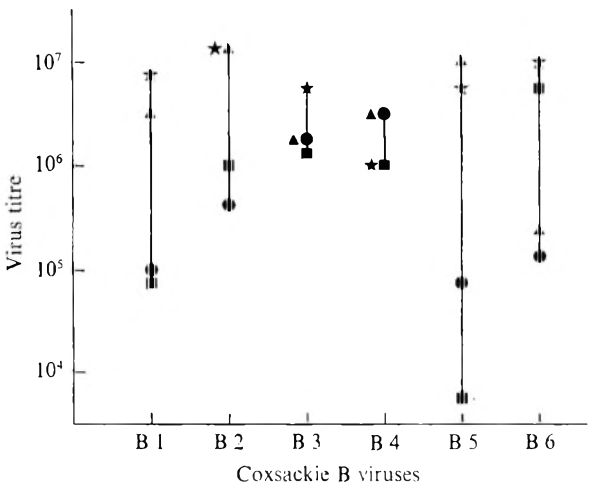

Fig. 2. Titres of the prototype Coxsackie B viruses after three passages in p. MK and VERO cells. ●, MK_3 pass titrated in p. MK. ■, MK_3 pass titrated in VERO. ▲, $VERO_3$ pass titrated in p. MK. ★, $VERO_3$ pass titrated in VERO.

were incubated at 36–37° C. and examined at intervals for CPE, the final reading being made on the sixth day. Any culture showing a distinct CPE was considered to be positive. Kärber's method (1931) was used to estimate the 50% infectivity end-points, which were recorded as log tissue culture infective dose per 0·1 ml. (log TCD50/0·1 ml.) Logarithms are expressed to the base 10.

Virus identification

The agents isolated in p. MK and VERO cells were both typed if they yielded sufficient virus growth. If either tissue did not give sufficient growth at least

Table 2. *Isolation and identification of polioviruses and Coxsackie viruses from clinical specimens in primary monkey kidney (p. MK) and VERO cells*

Viruses isolated	No. isolated and identified in: p. MK and VERO	p. MK only	VERO only	Totals
Poliovirus (types 1 and 2)	6	—	—	6
Poliovirus (3 mixtures of types 1+2 and 2+3)	—	2	4	6
Coxsackie B viruses (types 2, 3, 4 and 5)	36	—	2	38
Coxsackie A virus (type 9)	—	13	—	13
Totals	42	15	6	63

Table 3. *Isolation and identification of echoviruses from 26 clinical specimens in primary monkey kidney (p. MK) and VERO cells*

Viruses isolated	No. isolated and identified in: p. MK and VERO	p. MK only	VERO only	Totals
Echovirus type 1	1	1	—	2
Echovirus type 4	—	10	—	10
Echovirus type 7	—	1	—	1
Echovirus type 9	—	2	—	2
Echovirus type 11	—	1	—	1
Echovirus type 14	2	2	—	4
Echovirus type 20	1	3	—	4
Echovirus type 21	1	—	—	1
Echovirus type 25	—	1	—	1
Totals	5	21	—	26

two passages were made before it was declared unsuitable for use in the identification of the agent. Neutralization tests were done as described by Hambling, Davis & Macrae (1963) using composite antiserum pools.

RESULTS

Stock prototype enteroviruses which had, with the exception of two, been propagated in p. MK were passaged three times in p. MK and VERO cells and then titrated in both cell cultures.

A cytopathic effect was produced in p. MK by 46 of the 47 viruses examined whilst 38 showed an effect in VERO cells. The CPE in all cases was typical of enteroviruses, the cells became round and refractile at first either in foci or all over the cell sheet, shrinking later and eventually falling from the glass. The following viruses failed to cause a distinct CPE in VERO cells: echovirus type 4 Pesascek, 16, 17, 18, 21, 23, 33, 34 and Coxsackie virus type A 9. A comparison of infectivity titres of the viruses tested is given in Table 1.

Out of the 47 viruses titrated, 17 (36%) gave higher titres in VERO cells than

in p. MK even though they had not been adapted to these cells. After three passages in VERO cells 26 out of 47 (55·5%) gave higher titres in VERO cells. With only three echoviruses, types 14, 20 and 31 was there evidence of the virus being adapted to VERO cells, in most of the others three passages produced no great improvement over direct titration in VERO cells from the p. MK pass material although some titres were improved by 1 or 2 log. dilutions. The titre of Coxsackie B 5 virus was increased by 3 log. dilutions. With the exception of Coxsackie B 1 virus the remaining Coxsackie B viruses and the polioviruses showed no significant differences in titre when titrated in both cell cultures (Figs. 1, 2).

The results of attempts at virus isolation from clinical material in both p. MK and VERO cells are shown in Tables 2 and 3. Altogether a virus was isolated in 89 specimens. Twenty-one specimens were negative in both tissues.

All the single type polioviruses were isolated in both cell cultures with no difficulty. However, with the three specimens containing more than one type of poliovirus the p. MK culture in each case did not detect the poliovirus type 2 whereas the VERO cells grew the type 2 and the other virus present in one case but missed the other virus in two specimens. Re-isolation from the original faecal material gave the same results. Of the 38 Coxsackie B viruses isolated all grew in VERO cells but only 36 grew in p. MK; the two which failed were both type B 5. All of the 13 Coxsackie A 9 viruses were isolated in p. MK only. Only 5 of the 26 echoviruses were isolated in both cell cultures; 21 caused CPE in p. MK only (Table 3).

All the agents were typed by neutralization tests in the cells in which they grew, those which grew in both were typed in both. The time for complete CPE to appear from the clinical material varied, taking a little longer in VERO cells than in p. MK, except for the isolation of the Coxsackie B viruses where the time was the same in both cell cultures.

DISCUSSION

A continuous cell line such as VERO has advantages over primary cell culture systems. These include ease of monolayer preparation and freedom from adventitious viruses. Such cell lines are, though, frequently found to be contaminated with mycoplasma organisms; the VERO cells used in this study were known to be contaminated with *M. orale* type I. No premature degeneration of the cell sheets was noted as a result of this contamination.

The results of titrations of the prototype enteroviruses in VERO cells and in p. MK show that, with few exceptions, VERO cells are very suitable for the laboratory manipulation of these well-adapted strains. Such procedures as the preparation of high-titred virus suspensions and the measurement of neutralizing antibody could be done effectively in these cells.

Clinical specimens were obtained from routine diagnostic virus laboratories, and so represented a cross-section of the enteroviruses present in the community during the period of this study. The results of virus isolation show that VERO

cells are not suitable for general 'field' isolation work. The failure of any of the echovirus type 4 strains to grow in VERO cells and the inability of VERO cells to grow the prototype echovirus type 4 (Pesascek) contrasted with the growth of echovirus type 4 (du Toit) provides another example of variation in susceptibility of cell cultures to prime variants of one serotype. Hsuing (1962) found that the prototype echovirus type 6 (D'Amori) failed to cause CPE in HEp 2 cells, yet out of 89 echovirus type 6 isolates reported by Pal, McQuillin & Gardner (1963) all were recovered in HEp 2 cells and only 37 in p. MK.

VERO cells may prove useful in conjunction with p. MK in isolation work as their inability to support the growth of some echoviruses could help in typing procedures by reducing the possible number of identities of a virus. VERO cells are as suitable as p. MK for the isolation of Coxsackie B viruses and may even be superior for poliovirus isolation.

The only isolation of both components of one of the three poliovirus mixtures tested occurred in VERO cells. The failure of p. MK to grow poliovirus type 2 from these mixtures may be due to the higher susceptibility of this virus to interference by adventitious viruses as compared with polioviruses types 1 and 3. Such an effect has been noted during work on polio-marker tests in this laboratory. The identity of the viruses isolated from the mixtures was confirmed by typing in the culture of isolation, and in the other culture (p. MK or VERO cells as appropriate), indicating that selection took place during isolation rather than during the typing of the viruses. In each case also, re-isolation was attempted from the original faecal extract with the same result.

Typing by neutralization tests produced complete agreement of results in both cell cultures. VERO cells had a more stable sensitivity to enteroviruses over the several subcultures of cells which were sometimes necessary during the time taken for the typing process, than that achieved by successive batches of p. MK. This made estimation of the virus dose easier in VERO cells and typing was therefore more accurate and less often frustrated by too few tissue culture doses (TCD_{50}) of virus. VERO cells were therefore useful for typing viruses which could be adapted to them. However, they were found to be less useful for the isolation of certain echoviruses and also Coxsackie virus type A 9.

We wish to thank the Public Health Laboratories at Leeds and Neasden, London, for making the isolation material available to us, also Miss M. Buckley for the preparation of the monkey kidney cell cultures and the technicians in training who have helped with the preparation of VERO cultures. Dr R. H. Leach of the Mycoplasma Reference Laboratory very kindly isolated and identified the *M. orale* type I from the VERO cells for us.

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Biophysical and immunological studies on the differential effect of guanidine hydrochloride on type A and type B influenza viruses

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SUMMARY

Guanidine hydrochloride selectively inactivated both the biological activity and the immunogenicity of the haemagglutinin of influenza A/X-7 (H0N2). The residual neuraminidase was fully active biologically and immunologically. The reverse was observed with influenza B/ROB; with this virus the haemagglutinin was resistant, and was immunogenic; while the neuraminidase was selectively inactivated, and was not immunogenic.

INTRODUCTION

In a previous article a differential effect of guanidine hydrochloride (GH) on over 30 different strains of influenza virus was reported (David-West & Belyavin, 1973). It was shown that generally the neuraminidase (Nase) of the type A viruses, especially the 1957–68 (H2N2) or H3N2 strains, was resistant to GH inactivation, while the haemagglutinin (HA) was sensitive. On the other hand, with the type B viruses the Nase was sensitive, while the HA was relatively resistant. The results of experiments with recombinant strains of influenza viruses suggested the possible application of GH resistance or sensitivity as a marker for the virus-coded surface subunits (David-West, 1973). As a further exploration of the differential effect of GH on the surface subunits of the two groups of influenza viruses some correlative biophysical and immunological studies were conducted. The purpose of these studies is at least twofold; first to provide a method for the selective production of antibodies against either of the subunits, after the selective inactivation by GH. Secondly, GH attacks polar linkages such as hydrogen bonds; thus its effect would elucidate the significance of such bonding in the immunogenically active sites of the Nase and HA. Both of these would add to existing knowledge on the structure and immunogenicity of the Nase and the HA in general, and in particular highlight some other basic differences between influenza virus types A and B.

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MATERIALS AND METHODS

Viruses

Influenza A/X-7 (F1), a recombinant of A/NWS/33 and the A/R15$^+$ strain of Jap/57, having the Nase (N2) of the 1957 parent, and the HA (H0) of the 1933 parent (Kilbourne *et al.* 1967); and influenza B/ROB/55.

Propagation and purification of virus

The methods were as previously described (David-West & Belyavin, 1973). The essential steps were as follows: Virus was propagated in the allantoic cavity of 10- or 11-day old embryonated hens eggs. The infective fluid was harvested after 72 hr. at 35° C. After clarification in a bench centrifuge the virus was pelleted at 90,000*g* for 1 hr. The pellet was taken up in minimum amount of 0·15 M-NaCl + 0·01 M Tris-HCl pH 7·5 (Tris-saline), and after some few minutes on a Vortex mixer in order to disaggregate the virus, a further clarification was carried out as before. Finally the virus was purified by rate zonal centrifugation through a linear (10–50%, w/v) sodium glutamate gradient in Tris-saline at 65,000*g* for 45 min. in a Spinco Model L ultracentrifuge, using rotor SW 39. Fractions were collected and titrated for HA activity. The peak fractions were pooled, dialysed against Tris-saline in the cold, and used as virus stock for subsequent studies.

Isotope-labelled virus was prepared by incorporating [^{14}C]protein hydrolysate (52 mc./m-atom carbon) into the inoculum. Each egg received 5 μc. of the isotope. The ultimate processing of the infective fluid was as described earlier. The isotope was procured from the Radiochemical Centre, Amersham, England. Measurement of radioactivity of purified virus used in the appropriate isopycnic centrifugation experiments was performed in a Packard Tri-Carb scintillation counter. The scintillation fluid consisted of a mixture of toluene-methanol-Cab-O-sil.

Guanidine treatment

A stock solution of guanidine hydrochloride (Grade 1; Sigma) was prepared in Tris-saline. Virus preparation and guanidine stock solution were mixed to give final molar concentration of either 2·0 or 4·0 M. Reaction was at 37° C. for 30 min. In the control Tris-saline, at the same pH, replaced guanidine. A few experiments were also performed with urea, for comparison.

Isopycnic centrifugation of treated or control preparations was carried out at 100,000*g* for 4 hr., through linear 10–50% sodium glutamate gradient in 3 × 5 ml. swing-out rotor in Spinco Model L, using the SW 39 rotor.

Immunization of animals

White New Zealand rabbits were immunized with 2 M guanidine-treated or untreated virus. Each inoculation consisted of 0·4 ml. given by the ear vein. The schedules of inoculation are shown in the appropriate figures (Figs. 2, 3). The starting virus concentration was 40,000 haemagglutinating (HA) units per ml.

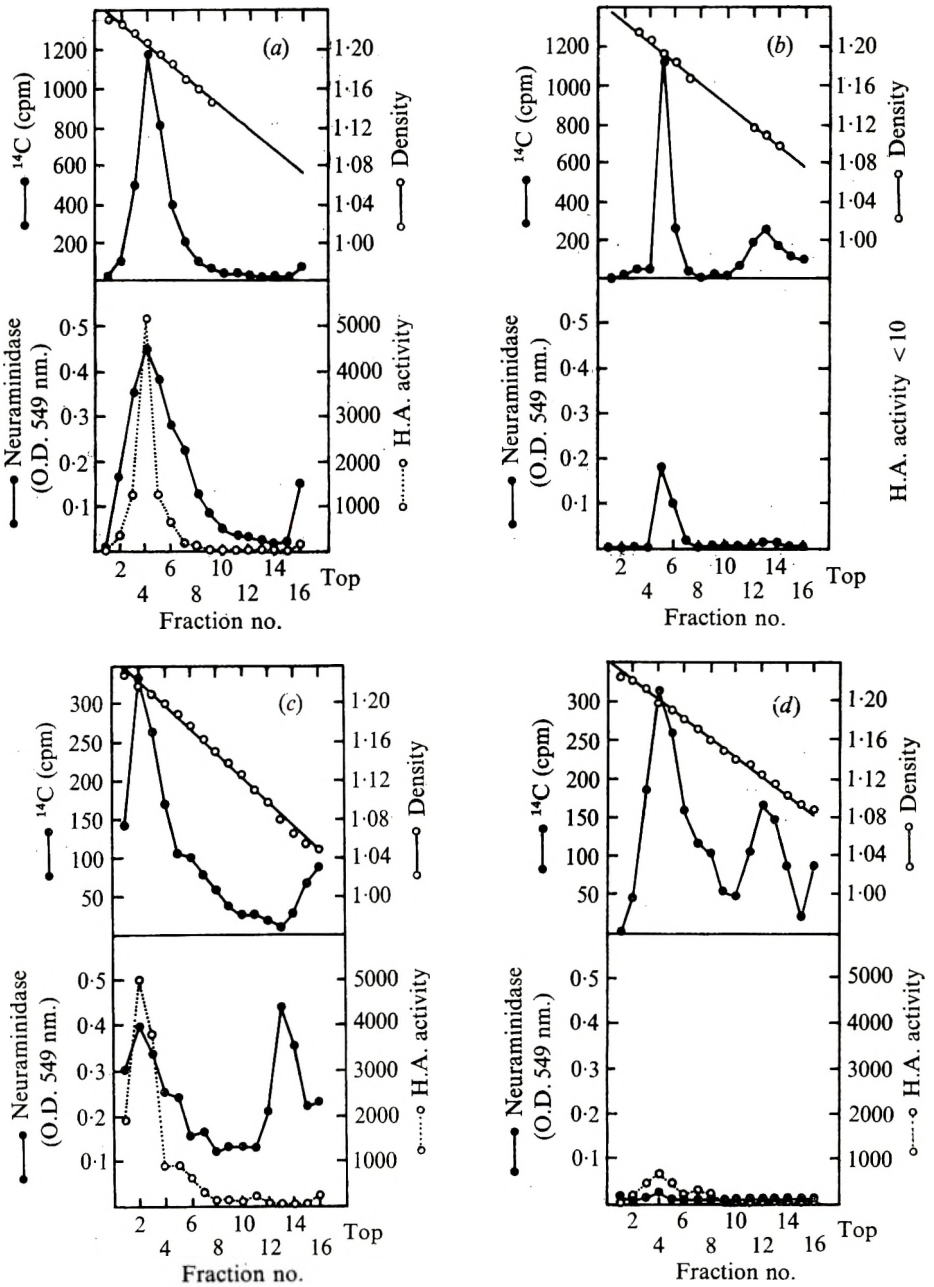


Fig. 1(*a*). Isopycnic centrifugation of untreated A/X-7. (*b*) Isopycnic centrifugation of 4 M guanidine-treated A/X-7. (*c*) Isopycnic centrifugation of untreated B/ROB. (Note that there is some splitting off of Nase.) (*d*) Isopycnic centrifugation of 4 M guanidine treated B/ROB.

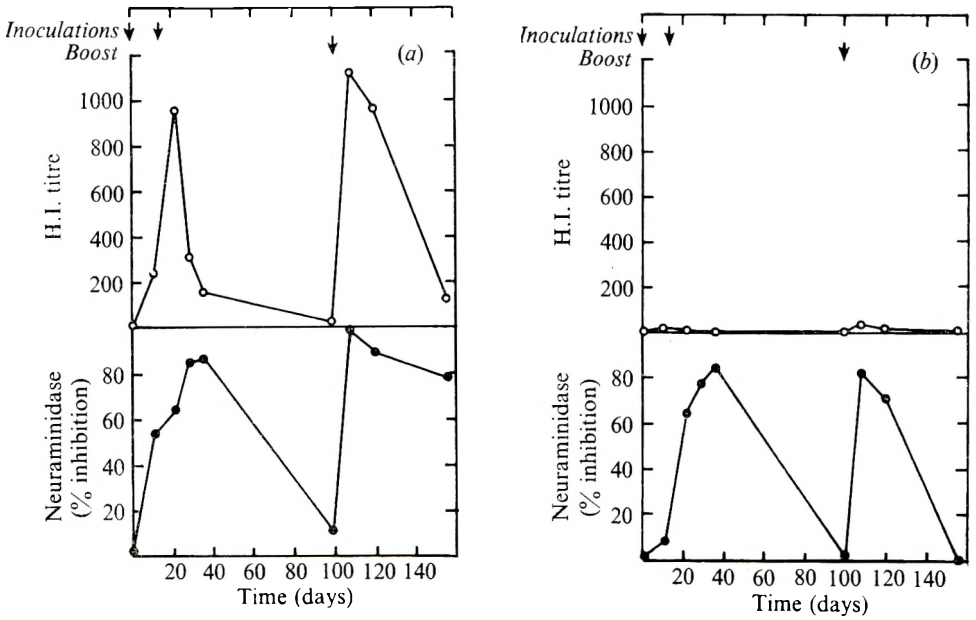


Fig. 2(*a*). Primary and secondary antibody induction for the haemagglutinin or the neuraminidase of untreated A/X-7. (*b*) Selective induction of anti-neuraminidase antibody by 2 M guanidine-treated A/X-7. Immunization schedule: two initial injections spaced 2 weeks apart, followed by a booster dose after 3 months.

for virus X-7, and 20,000 HA units per ml. for virus B/ROB. After treatment with guanidine there was no detectable residual HA activity with the X-7 virus, while the B/ROB virus still had 5000 HA units per ml.

Haemagglutination test

This was done by the micro-titre method using Takátsy loops. Serial twofold dilutions of test material were prepared in Tris-saline, and equal volumes of freshly prepared 0·5% fowl red blood cell suspension were added. The test was read after 45 min. at room temperature.

Haemagglutination-inhibition test

The test sera were inactivated at 56° C. for 30 min. and later periodated. One volume of serum was treated with 3 vols. of 0·9 M sodium periodate in Tris-saline. The reaction was allowed to continue for 15 min. at 4° C. in the dark, and was stopped by the addition of 1 vol. 10% glucose-saline. Four HA units of purified virus was used.

Neutralization test

Serial twofold dilutions of serum were mixed with equal volumes of virus dilution containing 100 EID50. The mixture was incubated at 37° C. for 1 hr. Eleven-day-old embryonated eggs were inoculated with the mixture, using four eggs per dilution. Infectivity was determined by HA after 48 hr. at 37° C.

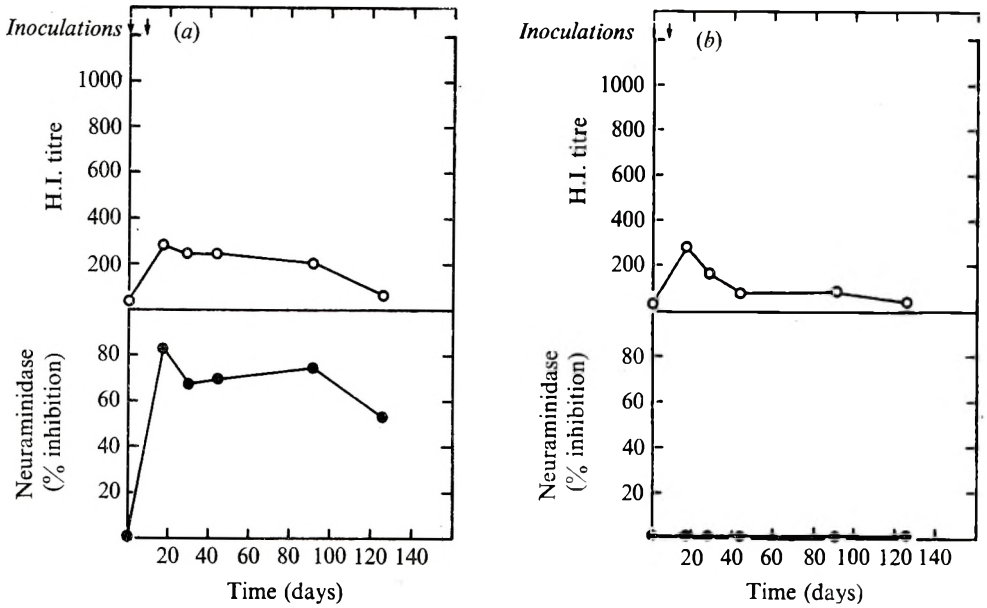


Fig. 3(*a*). Primary antibody induction for the haemagglutinin or the neuraminidase of untreated B/ROB. (*b*) Selective induction of anti-haemagglutinin antibody by 2 M guanidine-treated B/ROB. Immunization schedule: two injections given 1 week apart. No booster dose.

Neuraminidase assay

Test material and collocalia mucoid substrate were mixed and incubated overnight at 37° C. The substrate was prepared in 0·02 M sodium acetate buffer in 0·15 M sodium chloride, pH 6·0 (Kendal, Biddle & Belyavin, 1968). Free sialic acid was determined by the method of Aminoff (1961). All tests were set up in duplicates.

Electron microscopy

Virus preparations (guanidine-treated or control) were fixed in 3·0% glutaraldehyde in phosphate buffer at neutral pH. These were negatively stained with 3·0% ammonium molybdate, pH 6·5, and examined in the Philips EM 300 electron microscope.

RESULTS

Isopycnic banding of labelled virus

Fig. 1(*a*) and (*b*) show the distribution of viral activities in various fractions of control X-7 and 4 M guanidine-treated X-7, after density-gradient centrifugation. In the control preparation there were coincident single peaks of HA, Nase and radioactivity in fraction 4, with a density of 1·207 g. cm.^{3}. In the treated preparation HA activity was reduced to below detection, while a substantial amount of the Nase still remained. The peak of enzyme activity was shifted to fraction 5, with a density of 1·195 g./cm.^{3}. Another set of experiments with 2 M guanidine or 4 M urea showed that with either treatment the HA

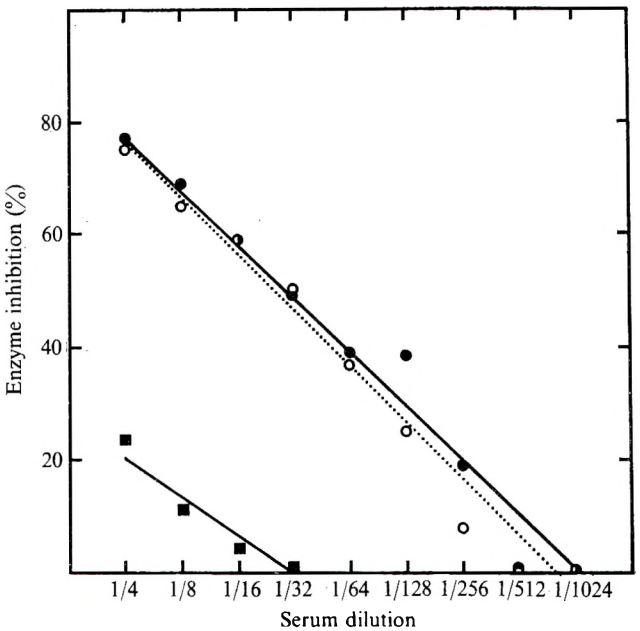


Fig. 4. Comparative neuraminidase-inhibition test with antiserum produced against untreated A/X-7 and antiserum against guanidine-treated A/X-7. Note: serum prepared without adjuvant. X-7: ○.....○, control; ●—●, 2 M guanidine. ■—■, Normal rabbit serum.

activity was completely obliterated, while the residual Nase activity varied between 80% and 90% in independent experiments.

In the B/ROB experiments the control virus preparation had coincident single peaks of HA, Nase and radioactivity in fraction 2, with a density of 1·220 g. cm.^{3}. After 4 M guanidine treatment the Nase activity was completely destroyed while about 13% HA activity remained (Fig. 1*c*, *d*) with a density of 1·198 g. cm.^{3}. The figures also show that there was a certain amount of splitting of B/ROB during the process of ultracentrifugation, as evidenced by the appearance of 'soluble' Nase activity in the top fraction of the gradient (Fig. 1*c*). This split-off enzyme activity is, however, also sensitive to guanidine as was that of the major coincident activities (HA, Nase and radioactivity) peak of fraction 2 (Fig. 1*d*). Treatment with 2 M guanidine also completely destroyed Nase activity, but the residual HA activity was between 25% and 40%, in separate experiments.

Immunization studies

Because the differential inactivation of either HA or Nase in both viruses by either 2 M or 4 M guanidine was similar, the lower molar concentration was selected in the preparation of inoculum for immunization, since this produced greater residual activity. The results obtained with A/X-7 are shown in Fig. 2(*a*) and 2(*b*); those of B/ROB are shown in Fig. 3(*a*) and 3(*b*). Guanidine treated A/X-7 selectively induced anti-Nase antibody (Fig. 2*b*) while guanidine treated B/ROB selectively induced anti-HA antibody (Fig. 3*b*).

The results of neutralization tests conducted with the antiserum produced by both guanidine-treated viruses showed that 1/1000 dilution of the B/ROB antiserum neutralized the infectivity of the virus in eggs. On the other hand, even 1/10 dilution of the A/X-7 antiserum did not reduce the infectivity of A/X-7 in eggs.

A comparison of the potency of the anti-Nase antibody induced by guanidine treated A/X-7 and that of the control preparation was made by testing the antiserum obtained after 35 days immunization, which was the peak of primary antibody induction. Fig. 4 shows that both are equally potent, in enzyme-inhibition titration.

Morphology of guanidine treated virus

These are shown in Plate 1. All micrographs except the last of each set (*c*, *f*) were prepared from virus treated under standard conditions of guanidine inactivation (i.e. 2 M for 30 min. at 37° C.). The last micrographs were prepared from standard preparations that had been at 4° C. for 5 days.

Both viruses retained their basic morphology after standard guanidine treatment (*b*, *e*). However, the surface spikes on these particles were more sparse and somewhat disorganized. There was also some suggestion that the treated particles had lost at least part of their internal components. This was more obvious after prolonged treatment (*c*, *f*). These figures also show that such prolonged treatment did not significantly alter the A/X-7 particles further, but the B/ROB particles, under the same conditions, were virtually 'shaved' of all surface spikes, and the particles are very much bloated.

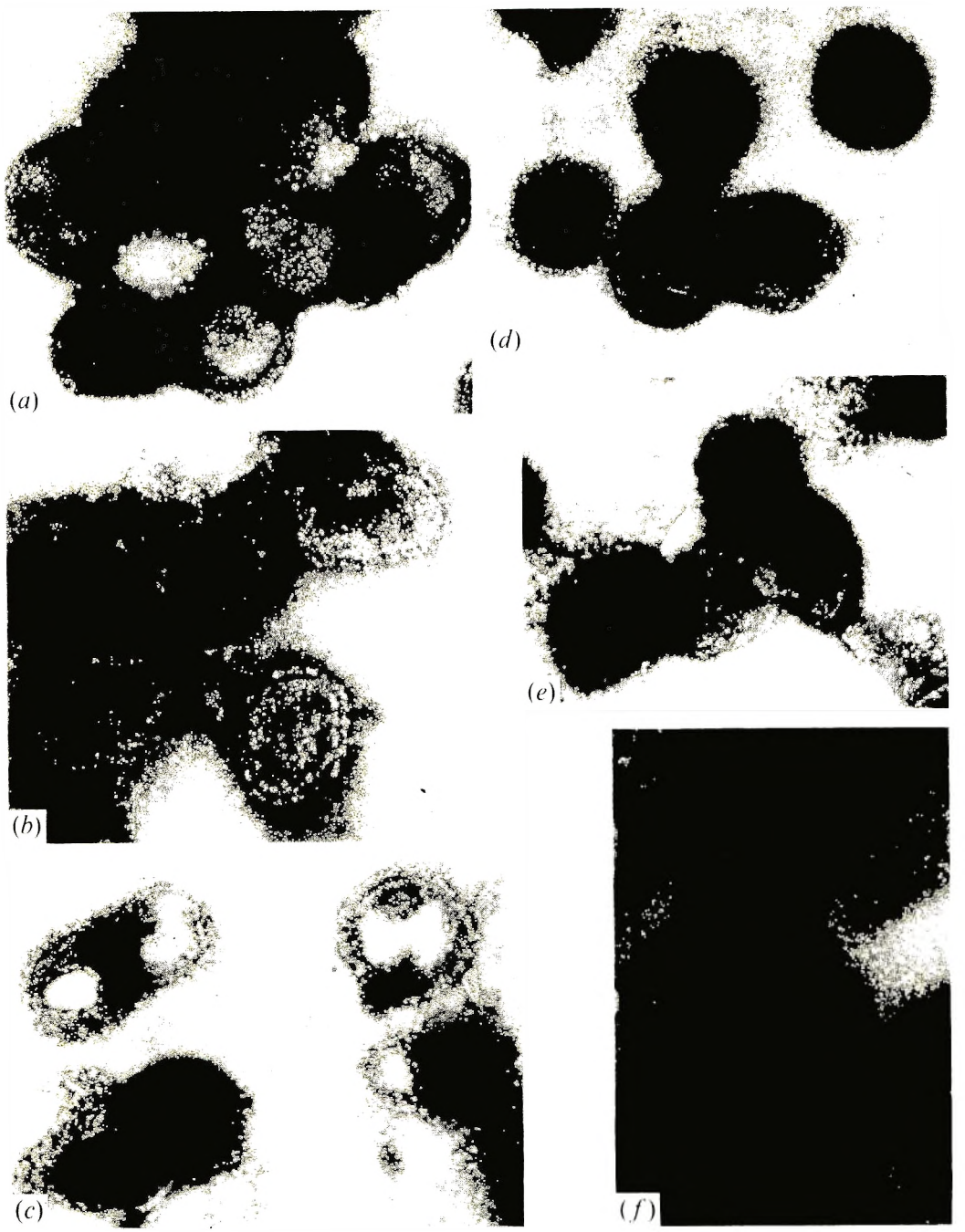
DISCUSSION

Until recently surveillance of influenza virus infection was based primarily on the antibody directed against the haemagglutinin (anti-HA antibody). However, the virus also contains another surface subunit, the neuraminidase (Nase) which also participates in the immune response, by the induction of anti-neuraminidase (anti-Nase) antibody. These two virus-coded surface glycoproteins, the HA and the Nase, are immunologically, functionally and structurally distinct (Laver & Kilbourne, 1966; Laver & Valentine, 1969) and also undergo independent variation (Schulman & Kilbourne, 1969). The new system of nomenclature of the Type A influenza viruses proposed by the World Health Organization Expert Committee (1971) therefore took cognizance of the immunologic and genetic independence of the HA and the Nase. The Nase of the type B influenza viruses, however, did not vary significantly one from the other to justify subgrouping (Chakraverty, 1972). Unlike anti-HA antibody, anti-Nase antibody does not neutralize virus infectivity; but it does modify the course of the infection in both man and animals (Coleman *et al.* 1968; Schulman, Khakpour & Kilbourne, 1968; Schulman & Kilbourne, 1969; Schulman, 1969; Slepushkin *et al.* 1971; Allan, Madeley & Kendal, 1971; Murphy, Julius & Chanock, 1972). The modifying effect of anti-Nase antibody might play an important role in the establishment or spread of the virus in human infection (Coleman *et al.* 1968; Schulman & Kilbourne, 1969).

The maintenance of adequate surveillance of anti-Nase antibody necessarily

requires the use of virus preparations in which the HA activity is obliterated, since it is widely observed that anti-HA antibody can interfere nonspecifically with Nase activity by steric hindrance. Earlier workers have achieved this by working with monovalent Nase prepared by disrupting the virus with various detergents followed by electrophoresis, and ultimate isolation of the enzyme activity (Kilbourne, Laver, Schulman & Webster, 1968). In a previous study (David-West & Belyavin, 1973) it is shown that guanidine hydrochloride selectively inactivated the HA of type A influenza viruses, especially the H2N2 or the H3N2 strains, which also are the most frequent cause of influenza epidemics at present. But the selective inactivation of the biological activity of the HA subunit might not necessarily also reflect a concomitant inactivation of the immunogenity of the molecule, since it is conceivable that the sites of biological activity and immunologic property might occupy different loci on the molecule. Alternatively the molecular groupings for both activities might differ. The results of the present investigation have shown that, with the inactivation of the HA activity of influenza virus A/X-7 by guanidine, the immunogenicity of the molecule is also concurrently destroyed. It should also be mentioned that the guanidine-treated A/X-7 preparation was negative for monovalent or 'soluble' HA in antibody-blocking test (David-West & Belyavin, 1973). This would suggest that the action of guanidine on this surface glycoprotein is not one of solubilization as is widely reported for either detergent or proteolytic enzyme disruption, but one of *in situ* inactivation. This permitted the selective production of Nase fully active biologically and immunologically, and the antiserum prepared against the treated virus was devoid of demonstrable anti-HA activity. The reverse was observed with influenza virus B/ROB; with this virus the Nase was found to be sensitive to guanidine inactivation while the HA was relatively resistant. Furthermore, the guanidine-treated B/ROB induced the production of only anti-HA antibody in rabbits. Ultracentrifugation studies correlated with electron microscopy also showed that the residual Nase or HA in the case of B/ROB was associated with the virus particle, and was thus necessarily polyvalent. The method described here is clearly much simpler than the one involving detergent disruption and electrophoresis, and so provides a simple method of producing anti-HA or anti-Nase antibodies. The guanidine inactivation method also revealed significant basic differences between the HA and the Nase of the type A2 and the type B test viruses. Guanidine uncouples non-covalent bonds (hydrogen bonds); the results therefore emphasize the relative importance of such bonds in the biological and immunological properties of the surface glycoproteins of the two types of influenza viruses.

I am grateful to Professor G. Belyavin for his valuable discussions and continued interest in the investigation. I also wish to thank Dr H. G. Pereira and Dr G. C. Schild for reading the manuscript, and for their helpful suggestions. The co-operation of Dr M. V. Nermut in the preparation of the electron micrographs is also gratefully acknowledged. Mr Alan Lansdell provided enthusiastic technical assistance.



TAM. S. DAVID-WEST

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EXPLANATION OF PLATE

Electron-micrographs of control and guanidine-treated influenza viruses.

(*a*) Control A/X-7.

(*b*) A/X-7 treated with 2 M guanidine for 30 min. at 37° C.

(*c*) A/X-7 treated with 2 M guanidine for 5 days at 4° C.

(*d*) Control B/ROB.

(*e*) B/ROB treated with 2 M guanidine for 30 min. at 37° C.

(*f*) B/ROB treated with 2 M guanidine for 5 days at 4° C.

Magnification of each: × 160,000.

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The sterilization of a new building designed for the breeding of specific-pathogen-free animals

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(*Received* 8 *May* 1973)

SUMMARY

An attempt was made to sterilize a newly erected building of approximately 1200 m.^{3} (43,000 ft.^{3}), specially designed for the breeding of specific-pathogen-free mice, rats and guinea-pigs. Two methods of treatment were used, namely an ampholytic surface acting biocide/formaldehyde aerosol followed after 2 days by formaldehyde vapour. Bacteriological examination was made of 100 sites in the animal rooms, staff quarters and general service area before and after both treatments. Identification of the bacteria isolated was based upon their morphological appearance on laboratory media incubated aerobically and their reaction to Gram's stain. Organisms were isolated from 72/100 sites before treatment, from 50 sites after the first treatment, and from 13 sites after the second treatment. The bacteria that survived both treatments were of several species.

INTRODUCTION

Before stocking a newly erected specific-pathogen-free (S.P.F.) building it was necessary to reduce to a minimum the numbers of micro-organisms present. This need provided a good opportunity to examine the bacterial flora present and to observe the efficiency of conventional methods of fumigation designed to reduce bacterial contamination. Two techniques were adopted, first an ampholytic surface acting biocide with formaldehyde was employed as an aerosol. This was followed 2 days later by fumigation with formaldehyde vapour. The effectiveness of the two methods in reducing the number of contaminated sites is described in this paper.

MATERIALS AND METHODS

The building

The building (see Fig. 1) contains ten animal rooms, each approximately 70 m.^{3} (2500 ft.^{3}) in capacity, a general service area of 350 m.^{3} (12,300 ft.^{3}), a staff room and kitchen of 41 m.^{3} (1450 ft.^{3}), a store of 19 m.^{3} (700 ft.^{3}), an office of 19 m.^{3} (700 ft.^{3}), and two toilet/shower areas of 39 m.^{3} (1400 ft.^{3}). Together these rooms and areas constitute the 'clean' area. Entrance to this area by personnel is via the shower units and for materials via the 'dunk-tank' or autoclave. These portals of entry constitute the 'barrier'. The heating, lighting,

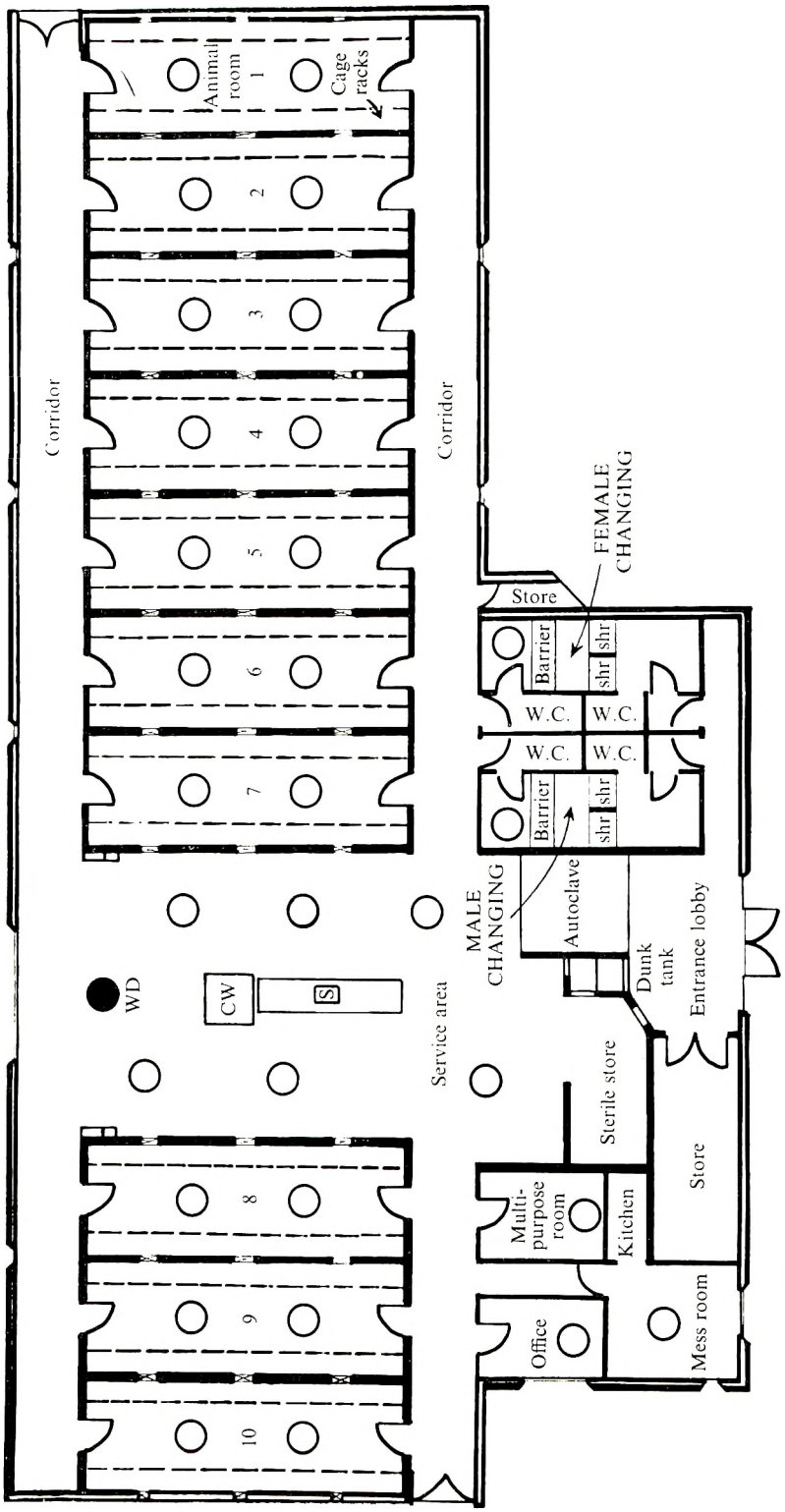

Fig. 1. The SPF animal production building. CW, cage washer; WD, waste disposal unit; S, sink; shr, shower; O, location of formaldehyde generation points.

ventilation and air filtration services are separated from the 'clean' area and are related to it by ducting. A positive air pressure is constantly maintained within the 'clean' area. The building is situated on an open site in the country and is within 46 m. (50 yards) of a farm-animal isolation compound. The period of its construction was from June 1970 to October 1972. During this time there was ample opportunity for a build-up of bacterial flora, including potentially pathogenic bacteria, due to contamination by human contact and from wildlife vectors.

Preparatory cleaning

After completion the building was thoroughly cleaned to remove all gross dirt.

Bacteriological methods

Wet swabs were taken from 100 sites each of which was numbered with adhesive tape for subsequent identification (see Table 1). The swabs were broken into bottles containing 10 ml. Todd-Hewitt broth which was incubated at 37° C. for 36 hr. The cultures obtained were subcultured on 5% ox-blood agar plates which were incubated aerobically for 18 hr. at 37° C. Colonies were examined macroscopically and smears of them were stained by Gram's method and examined microscopically.

Fumigation procedures

First treatment

Before treatment the ventilation system was shut down to reduce the movement of air within the building. A commercially available preparation of an ampholytic surface-acting biocide and formaldehyde* was applied to the whole of the 'clean' area at a recommended concentration of 1% in water. A fogging apparatus was employed to produce a fine spray under pressure. This apparatus was held by an operator wearing a gas mask and suitable protective clothing: all surfaces including ceilings, walls and floors were carefully and systematically saturated with the agent. The doors of each room were closed after treatment; the ducting was also treated. The total time taken for the whole operation was 7 hr. The building was then sealed for 2 days with the air conditioning unit switched off. Two days later swabs were taken, from the sites originally examined, by personnel wearing gas masks and sterile protective clothing. The air conditioning was then switched on.

Second treatment

The temperature of the heating system was increased to approximately 21° C. and the entire area within the 'barrier' was saturated with water to produce conditions of high humidity. The air conditioning plant was then switched off. Measured amounts of potassium permanganate were put into 5 gal. lidless drums and placed in the positions shown in the figure. Measured amounts of formaldehyde, previously dispensed in sealed plastic bags, were poured onto the potassium

* Tegofectol supplied by Messrs Hough & Hoseasons, Manchester.

Table 1. *Results of the bacteriological examination of the building before and after fumigation*

Areas examined	Total no. sites examined	Before fumigation					After 1st fumigation					After 2nd fumigation				
		No. positive sites	Bacteria isolated*				No. positive sites	Bacteria isolated*				No. positive sites	Bacteria isolated*			
			Gm + Sp	Gm + b	Gm − b	Gm + c		Gm + Sp	Gm + b	Gm − b	Gm + c		Gm + Sp	Gm + b	Gm − b	Gm + c
10 animal rooms	50	26	12	10	4	1	25	7	5	3	11	8	2	0	3	3
General service area	20	16	4	5	2	7	8	2	2	0	4	1	0	0	0	1
Office	6	6	4	1	0	3	3	1	0	0	2	0	0	0	0	0
Mess area	6	6	5	1	0	5	5	3	1	0	2	1	1	0	0	1
Store	6	6	2	4	0	3	3	2	0	0	1	0	0	0	0	0
Showers																
Men's	6	6	2	2	1	4	2	2	0	0	0	2	0	0	0	2
Ladies'	6	6	4	2	0	5	4	4	0	0	0	1	0	0	0	1
Totals	100	72	33	25	7	28	50	21	8	3	20	13	3	0	3	8

Gm = Gram, c = cocci, b = bacilli, Sp = spore bearing bacilli.

* Totals of these columns may not coincide with that on the left because one site often yielded more than one organism.

permanganate by personnel wearing gas masks and suitable protective clothing. The amounts of potassium permanganate and formaldehyde used were based on the cubic capacity of each area so that a final concentration of not less than 2 mg./l. of formaldehyde vapour at 20° C. was obtained (Report, 1958). All doors inside the 'clean' area were left open to facilitate adequate fumigation of the corridors. This procedure required approximately 15 min., after which the building was sealed for 2 days. On the third day the air conditioning was switched on but personnel were not allowed to enter the fumigated area for a further 2 days, after which time the final swabs were taken from the sites originally examined.

RESULTS

Results of the three bacteriological examinations are recorded in Table 1.

Bacteria were isolated from 72 of the 100 sites before treatment, from 50 sites after the first treatment and from 13 sites after the second treatment. Nine sites which were originally negative gave positive cultures after the first treatment. This observation indicates the limitations of the swabbing technique, in that, although each site was clearly identified precisely the same site could not be sampled on all three occasions. Bearing in mind the limitations of the simple bacteriological identification methods used, there was no evidence that one type of organism was more resistant to treatment than another. It was apparent that there was less initial contamination in the animal rooms (out of 50 samples 24 were negative) than in the other areas where there had been more human activity (out of 50 samples 4 only were negative).

DISCUSSION

Because the two treatments were used consecutively, the effect of the second cannot be compared with the first. However, it may be said that the first treatment was totally inadequate, and it appears that the second treatment contributed significantly to the reduction in the number of contaminating organisms. The persistence of organisms after both treatments was surprising (13 positive out of 100 sites), in view of the long-standing belief that formaldehyde gas at the correct concentration and under optimum atmospheric conditions is lethal to bacteria. However, this work has indicated that it is probably impossible, with a single fumigation of the type employed, to sterilize completely a building of the size and type described.

I wish to record my gratitude to Mr K. Parsons for his technical assistance and to the staff of the S.P.F. animal building for their willing co-operation.

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Skin scales among airborne particles

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SUMMARY

The air from a house and garden in a rural area has been sampled and the size distributions of the airborne particles have been determined. The particle concentrations are shown to be generally higher indoors. Changes in the particle concentration during various activities in a small room have been shown to be greatest for particles larger than 3 μm. diameter. Stereoscan microscopic observation has shown that many of the airborne particles in rooms and some from those in outside air appear to be scales of desquamated skin. The presence of protein in many of these particles complements the microscopic observations.

INTRODUCTION

Skin scales shed from humans, some having micro-organisms attached to them, are often responsible for contamination and infection in hospital operating theatres and industrial clean rooms (Davies & Noble, 1962). These skin scales are flake-like, about 10–25 μm. across and about 1 μm. thick. They are constantly being shed from the body in great numbers (May & Pomeroy, 1973) by the general movement of the skin surface and by the rubbing actions of clothes and limbs. This, together with the proposition that skin scales shed from the body and dispersed from the human micro-environment could be a link in the chain of airborne infection (Clark & Cox, 1973), prompted the present investigation into the composition of airborne particles. A particle collection and identification system was developed that was portable and could be used in various environments. The apparatus in this system consisted of an Andersen Mini-Sampler, a Casella Cascade Impactor and a Royco Portable Particle Monitor, and these devices have been described and demonstrated elsewhere (Clark, 1973). Complementary to these samplers, Stereoscan electron and conventional light microscopy were used for particle identification in conjunction with protein staining techniques.

METHODS AND RESULTS

The presence of protein in airborne particles

The major portion of hair, nails and epidermal layers of the skin is composed of keratin; consequently a technique that shows the presence of protein in particles recovered from the air complements the microscopic observation that many of the recovered particles appear to be fragments of skin.

Table 1. *The protein content of particles collected from the air of a garden and house in a rural area*

	Percentage of particles containing protein	
	2–6 μm.	6–30 μm.
Indoor air	75%	77%
Outside air	32%	44%

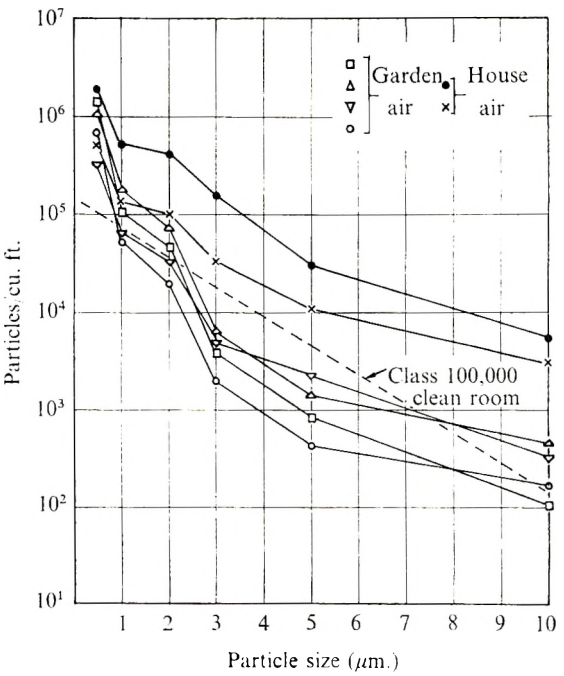


Fig. 1. Particle concentrations in a house and garden in a rural area.

Triketohydrindene hydrate (ninhydrin) solution will indicate the presence of protein in a sample of airborne particles.

If the particles are sprayed with a solution of ninhydrin in acetone and then heated to about 70° C. for some minutes, the particles containing protein show up as pink when viewed under the microscope using incident light. When this technique was applied to particles collected with the Casella Cascade Impactor from the air of a house and garden many particles containing protein were found in both room air and outside air as indicated in Table 1.

Size range of airborne particles

The Royco Portable Particle Monitor was used to size the particles in the air of a rural garden and a house in the same area. The particle monitor determined directly the particle concentration in six size ranges from 0·5 to 10 μm. diameter. Fig. 1 shows the size distributions of the particles in garden air on 4 days contrasted with particle counts made in the house on 2 days.

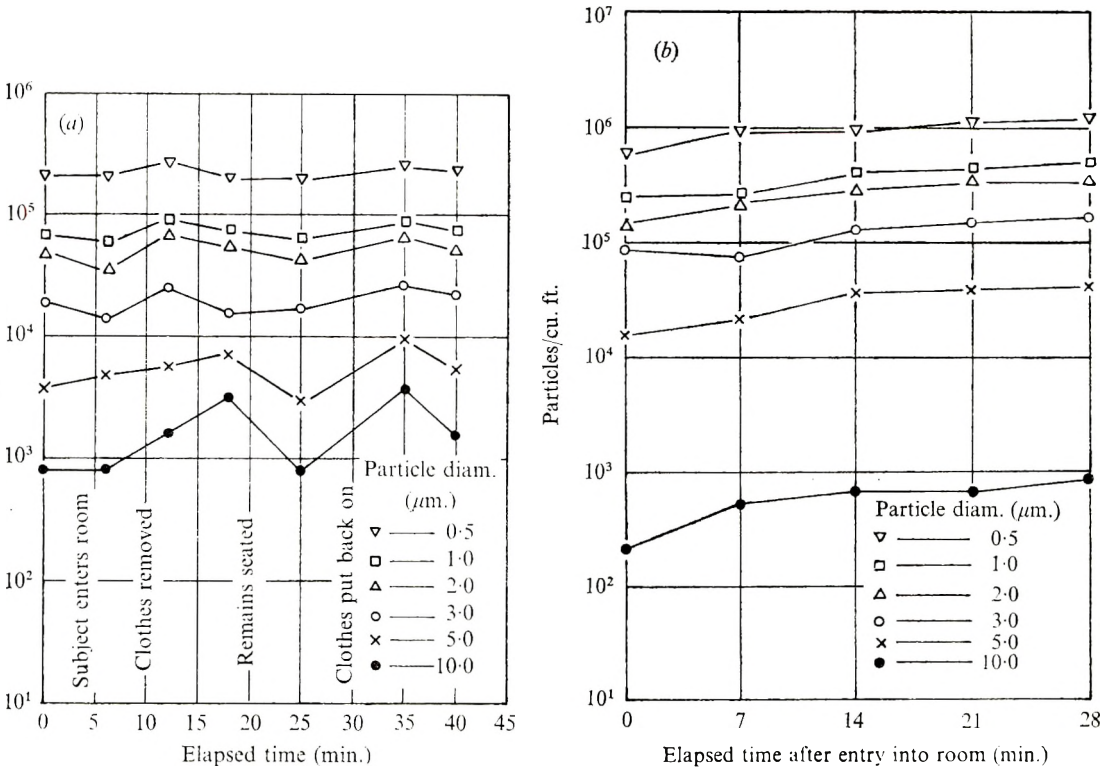


Fig. 2(*a*) Variation of particle concentrations during various activities in a room of 450 ft.³. (*b*) The increase in particle concentration in a room of 450 ft.³ after a subject enters and slowly walks around.

The concentrations in the house air were consistently about 10 times as high as those in garden air for particles larger than 3 μm.

In these observations the garden air was found to be cleaner than a class 100,000 clean room (U.S. Fed. Stand.).

Particle counts within a small room

Particle concentration variations during various activities within a room having a volume of 450 ft.³ (12,800 l.) were recorded. The doors and windows of the room were closed for several hours before each test to allow the background particle counts to stabilize. Fig. 2(*a*) shows the changes in particle concentration as the subject entered the room, removed his clothes and then remained seated quietly. After 23 min. the subject replaced his clothes and again remained seated. The result is that the concentration of the larger particles varies much more than that of the smaller sizes. The concentration of particles greater than 3 μm. diameter is seen to rise considerably with the activities of removing and replacing the clothes. Fig. 2(*b*) shows the result when the subject entered the room and slowly walked around; once again the greatest change in the concentration occurred for the larger particle sizes.

Microscopic examination of the airborne particles

The Andersen Personal Sampler was used to collect samples from various environments at an air-flow rate of 1·4 l./min. (0·05 ft.^{3}/min.). When used with a battery-operated pump the sampling nozzle may be attached to the clothing to sample the air from the subject's micro-environment. The samples deposit on a series of four anodized aluminium disks and the smallest particles (less than 0·3 μm. diameter) are collected on a paper filter. The particles collected on the sampling disks were observed with the Stereoscan electron microscope and were prepared for microscopy by vacuum coatings, first of carbon and then gold.

Plate 1A shows particles recovered from room air and the sample is seen to contain a large number of particles which closely resemble skin scales.

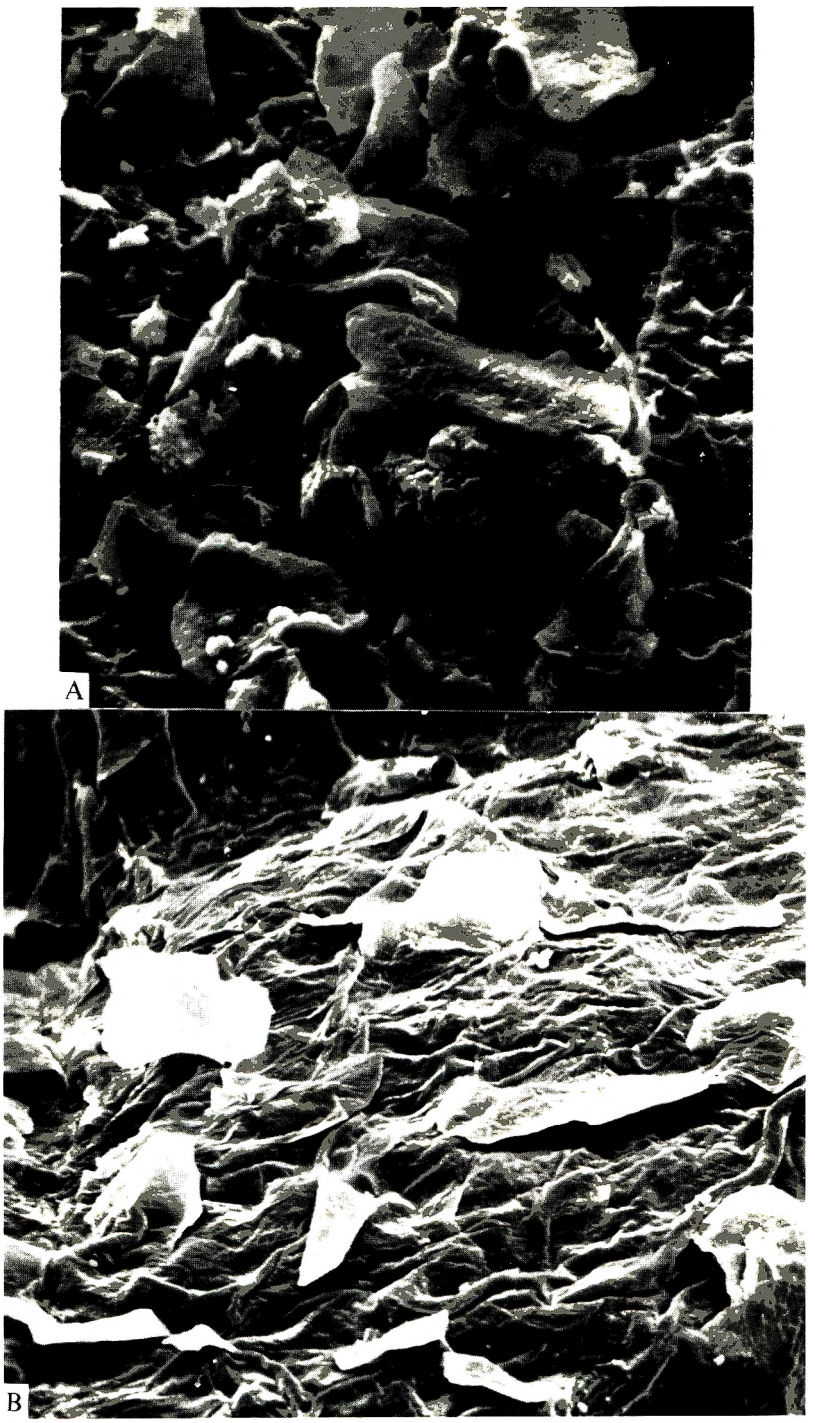
Plate 1B shows some skin scales attached to the skin surface and the similarity between these and the airborne particles is clearly seen.

Samples from the outside air contain some particles which have the appearance of skin scales mixed in with many other kinds of particles, many of which are very difficult to identify microscopically. Some particles which appear as skin scales seem to be more fragmented than those collected from room air.

In view of the prevalence of particles very similar in appearance to skin scales it is not surprising to find that 'house dust' is largely composed of these skin-like particles. Stereoscan pictures of house dust confirm this and examination of vacuum cleaner dust also reveals a great number of particles resembling skin scales. It is interesting to note that few small fibres are collected in the room air. They appear to sediment fairly quickly and many more are recovered from house and vacuum-cleaner dust. This observation contrasts with experiments by Pressley (1958), who found that the airborne dust in hospitals consisted essentially of cellulose fibres. Plate 2A shows some vacuum-cleaner dust which contains some flat and twisted cotton fibres as well as many scale-like particles.

The Mini Sampler was used on a journey on the London Underground system, Northern Line. The stations on this line are heavily contaminated as evidenced by the hazy appearance of the air, and the concentrations of all the particle sizes on the sampler disks were at least 10 times greater than in samples from room air. Plate 2B shows some of these collected particles; many are scale-like and appear similar to the skin particles. Under the light microscope all these particles appeared black and this could have been due to carbon dust and other small particles deposited on the larger sizes. However, in the case of these samples as with other heavily contaminated particles, the protein-staining technique using ninhydrin is ineffective. This is probably because the coating on the particles prevents the penetration of the ninhydrin to any protein.

The author wishes to thank Dr J. M. Hirst and Mr R. H. Turner of Rothamsted Experimental Station, Harpenden, for the preparation of the Stereoscan micrographs.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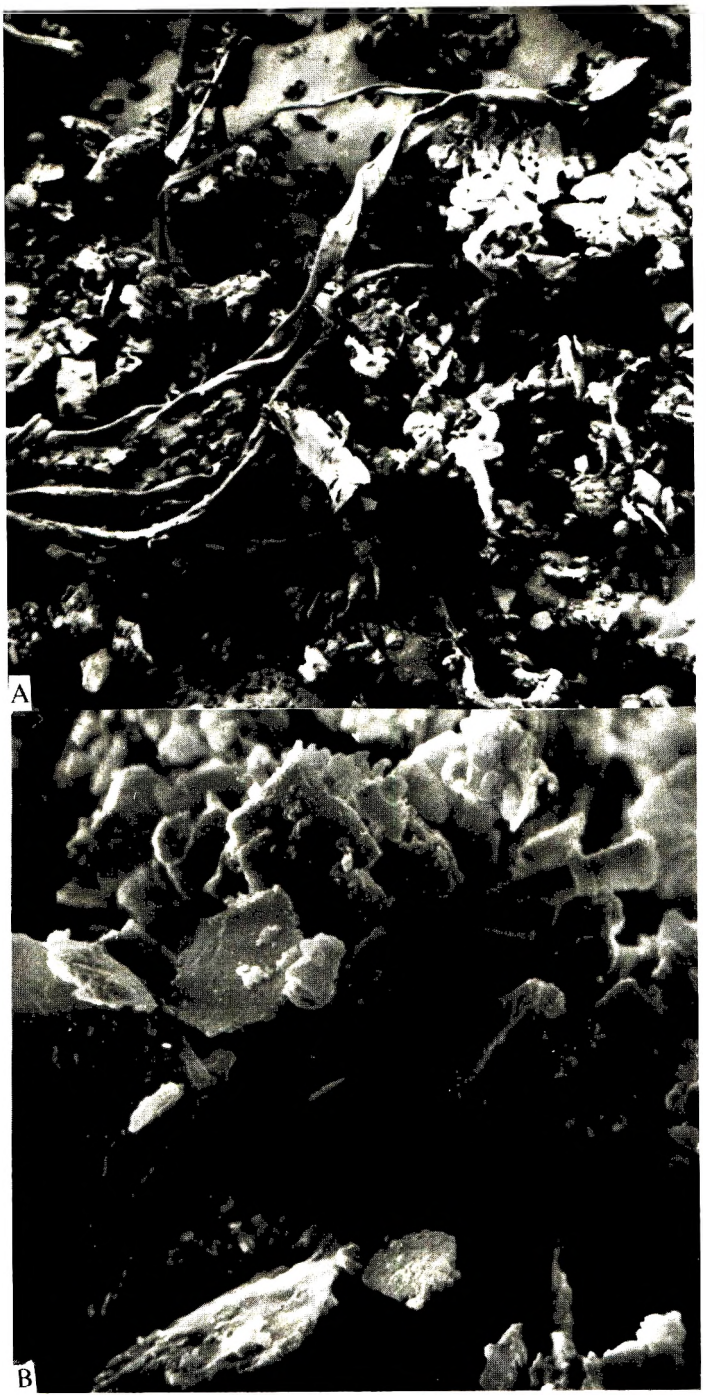
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EXPLANATION OF PLATES

PLATE 1

(A) Skin scales recovered from room air. × 819.

(B) Skin scales on the body surface which are partly detached. × 2410.

PLATE 2

(A) A sample of vacuum-cleaner 'dust', showing many flaky particles and cotton fibres. × 115.

(B) A sample of the heavily contaminated particles from the air of the London Underground system. × 2520.

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Open-air factors in enclosed systems

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SUMMARY

Ventilation of vessels varying widely in size was found to preserve the toxic effect of open-air factor(s). There was a correlation between the minimum rates of ventilation required and the ratios of the surface area of the vessels to their volumes. The data obtained allowed an estimate to be made of the diffusion coefficient of open-air factor(s) and gave an indication that the molecular weight range of the open air factor(s) was from 50 to 150.

INTRODUCTION

Open-air factors (OAF) which decrease microbial survival usually disappear when the air is enclosed (Druett & May, 1968; Hood, 1971). Their effect therefore cannot be studied in conventional laboratory apparatus. To overcome this limitation the ventilated sphere system was developed (Hood, 1971). Success with this system suggested that it might be worth while examining the ephemeral character of OAF further in different vessels covering a wide range of sizes. The experimental vessels could be ventilated at rates that enabled the toxic properties of open air to be fully preserved. The minimum ventilation rate was found to be proportional to the ratio of the surface area of the vessel to its volume. The data obtained allowed an estimate to be made of the diffusion coefficient of OAF and hence gave an indication of its molecular weight.

MATERIALS AND METHODS

Air toxicity was measured biologically by observing the survival of *E. coli* MRE 162 (EC) exposed in small particles ($< 3\,\mu$m. diam.) attached to microthreads (May & Druett, 1968) as described previously (Hood, 1971). Conditions of temperature and relative humidity were chosen for which, in 'clean' air in enclosed systems, the *E. coli* was relatively unaffected for at least 2 hr., i.e. within the relative humidity range of 70–100% at ambient temperatures. Thus the decrease in viability of the organisms exposed concurrently to open air and to the air in ventilated systems could be attributed solely to OAF. The mean survival of *E. coli* (the arithmetic mean of the viabilities measured at several 15 or 20 min. intervals during the total exposure period) was obtained in open air and in the ventilated systems. The ratio of the survival of the bacteria in open air to its survival in air in ventilated systems was used as a relative potency index to compare the toxicity

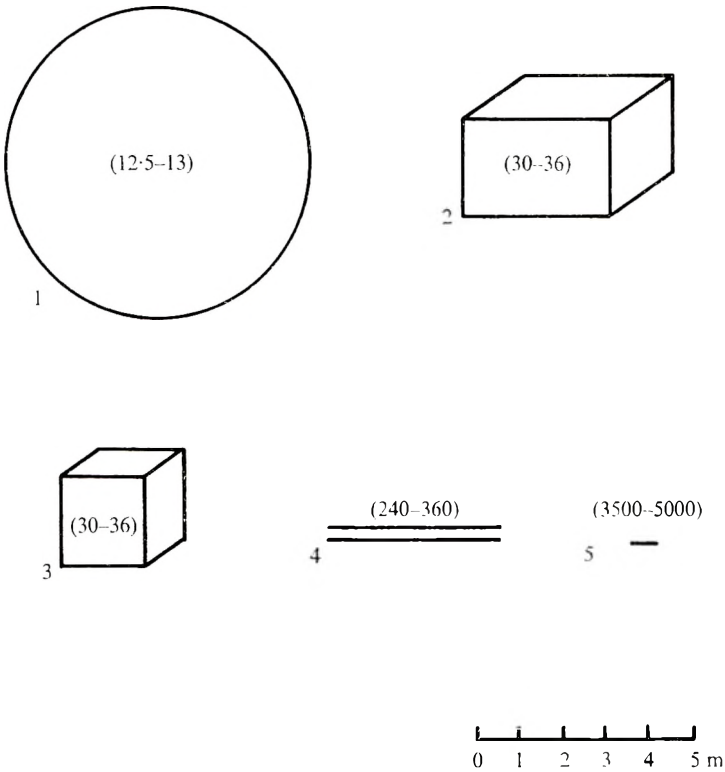

Fig. 1. Ventilated vessels used. 1, Sphere, 7 m. diameter; 2, cuboid, 3·43 × 2·28 × 2·28 m.; 3, cube, 2 × 2 × 2 m.; 4, tube, 4·6 m. × 0·35 m. diameter; 5, tube ('sow') 0·4 × 0·06 × 0·04 m. Figures in parentheses indicate minimum ventilation rate ranges (air changes/hr.) to obtain fully bactericidal effect of open-air factors.

of air in vessels ventilated at various rates and at various times with the toxicity of open air (which varied from day to day).

A maximum of $1\frac{1}{2}$ hr. exposure was used. In order to reflect the most significant data, only results obtained from those occasions when open air caused over 80% loss of viability in this time are presented.

The known effects of daylight and air velocity on viability were minimized by exposing the open-air microthreads in a 'roundabout' (Druett & May, 1969) on the lee-side of a large building.

The minimum ventilation rate which apparently fully preserved the toxicity of open air was determined for each of five vessels: a sphere (7 m. diam.), a cuboid (3·43 × 2·28 × 2·28 m.), a cube (2·2 m.), a tube (4·6 × 0·35 m. diam.), and a microthread loading 'sow' (May & Druett, 1968) about 0·4 m. in length and 24 cm.^{2} cross-section. They were made of mild steel, aluminium, mild steel painted, stainless steel and brass respectively and are shown to scale in Fig. 1.

Since the *E. coli* were being exposed concurrently on microthreads in situations in which the air velocity was not always the same, preliminary tests were made to determine the degree to which air velocity would affect the comparisons.

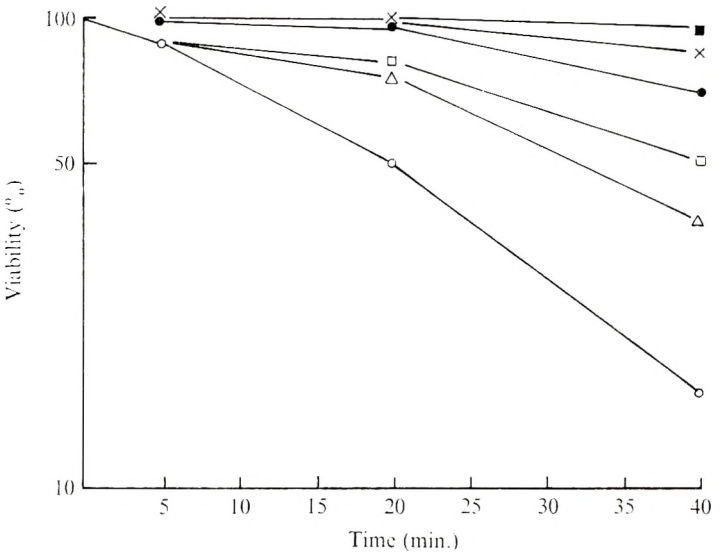

Fig. 2. Air velocity effect on viability of *Escherichia coli* held on microthread. ■, Indoor air at 0 to 1·4 m./sec.; ×, indoor air at 2·8 m./sec.; ●, indoor air at 5·7 m./sec.; □, open air at 1·4 m./sec.; △, open air at 2·8 m./sec.; ○, open air at 5·7 m./sec.

RESULTS

Air velocity effect on viability of E. coli *exposed on microthreads*

The effect of air velocity was tested using a whirling arm similar to that described by May & Druett (1968). The suspending medium and relative humidity range, however, were different. Tests were made at *ca.* 0, 1·4, 2·8 and 5·7 m.sec.$^{-1}$ air velocities in enclosed ('clean') air and in open air. In clean air no adverse effect on viability was apparent at air velocities below 5·7 m.sec.$^{-1}$. At 5·7 m.sec.$^{-1}$ there was a small – possibly significant – effect on survival. In OAF conditions there was a slight air velocity effect at 2·8 m.sec.$^{-1}$ and this increased considerably at 5·7 m.sec.$^{-1}$ (Fig. 2).

The results indicated that the contribution made by air velocity to the viable decay of *E. coli* would not be significant in ventilated vessels in which the air velocity did not exceed 2·8 m.sec.$^{-1}$ when the air was 'clean'. In air containing OAF, velocities in excess of 1·4 m.sec.$^{-1}$ could make a significant contribution to the death-rate. It was not found necessary to exceed these limits in the ventilated vessels and unlikely under the chosen site conditions for exposure in open air. Erroneous comparisons between air in the ventilated systems and open air due to air velocity effect were thus considered unlikely and in any event would be small.

OAF persistence in ventilated vessels

The results obtained at various rates of ventilation in each of the five vessels are shown in Fig. 3. The minimum ventilation rates (air changes/hr.) which apparently preserved open-air toxicity in full were as follows: sphere, 12·5–13; cuboid and cube, 30–36; tube, 240–360; and 'sow', 3500–5000. Consistent results

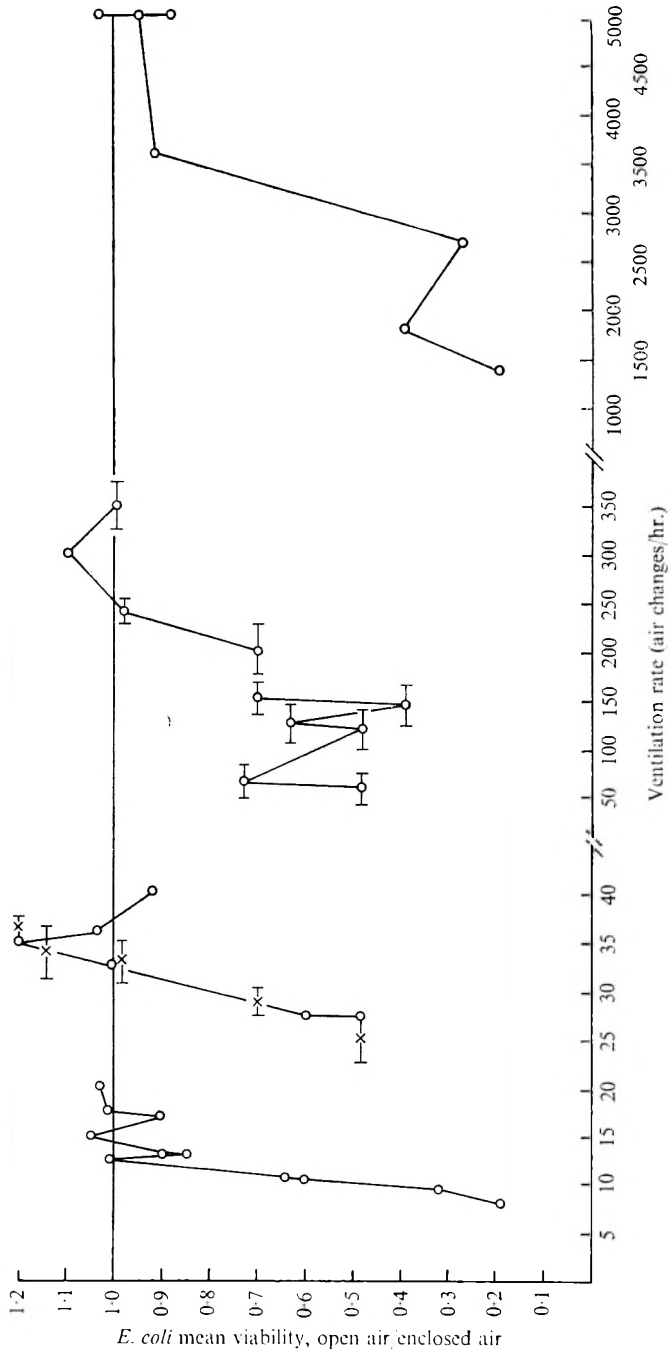

Fig. 3. Open-air factors in vessels ventilated at various rates. 1, Sphere; 2, (○) cuboid; 3, (×) cube; 4, tube; 5, 'sow'.

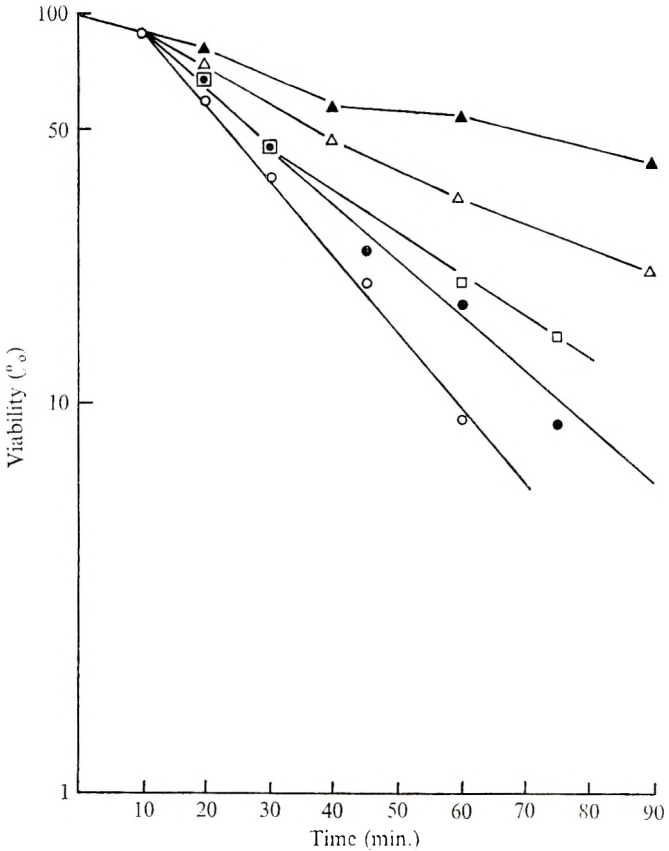

Fig. 4. *Escherichia coli* response to various concentrations of open air. ○, 100% open air; ●, 95%; □, 90%; △, 75%; ▲, 50%.

could only be obtained in the larger vessels when fans were used to ensure adequate mixing of the air within them. The tube was examined by comparing air at the extract end with that at the open (air intake) end. It was not possible to obtain narrower limits of ventilation rates because of fluctuation of air flow caused by ambient winds with this tube when used at the low flow rates found to be required to maintain the OAF throughout its length. The 'sow' was ventilated by application of a negative pressure through critical orifices of a range to give the desired flow and hence ventilation rates.

Biological response to OAF

The results described above show that each vessel can be ventilated at such a rate that there is no apparent loss of the potency of open air. In order to establish a quantitative correlation, however, it is necessary to determine the relationship between the biological response and the OAF concentration. The results obtained with the 'sow' suggested a system that could be used for such a study. Sows were ventilated with open air, or various concentrations of it, simply by adding a twin fitting to their air intake to allow admission of a given proportion of 'clean' air to displace some of the open air. 'Clean' air was obtained from a compressed air

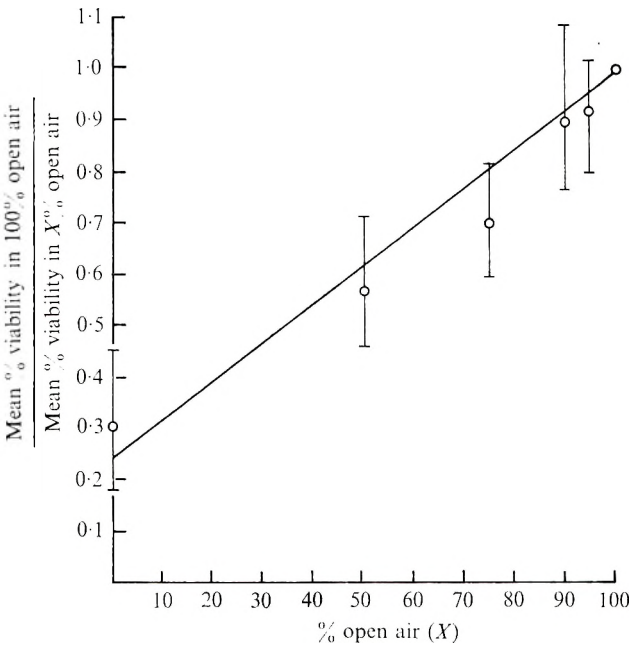

Fig. 5. Biological response of *Escherichia coli* to concentration of open air.

supply and suitably adjusted to ambient conditions of temperature and relative humidity to provide a metered supply to the sow. Such a supply was tested and found to be 'clean' in the sense that the viability of *E. coli* was not diminished when they were exposed to it for periods of 2 hr.

Using several sows it was possible to determine simultaneously the effect of various concentrations of 'open' air on viability. The results (Fig. 4) indicated that a 95 % concentration or less was significantly less harmful to the *E. coli* than 100 % open air.

At a given concentration of open air the variations in the mean viabilities were greater than those obtained in control experiments in the absence of OAF. These differences could be a reflexion of the day-to-day variation in the nature and concentration of OAF in open air in addition to the difficulties in maintaining constant ratios of open to clean air over the 1 hr. period of experiment. Nevertheless, a reasonably linear relationship was found between the ratio of mean viability and OAF concentration as shown in Fig. 5. When the biological response of *E. coli* in a ventilated system is similar to that observed concurrently in the open air then it may be concluded that similar concentrations of OAF are present.

DISCUSSION

It was thought possible that the volume/surface area ratios of the ventilated vessels might correlate with the observed rate of loss of OAF. When the respective ratios were plotted against the maximum air dwell times (calculated from measured ventilation rates) for which OAF persisted in full concentration, a linear

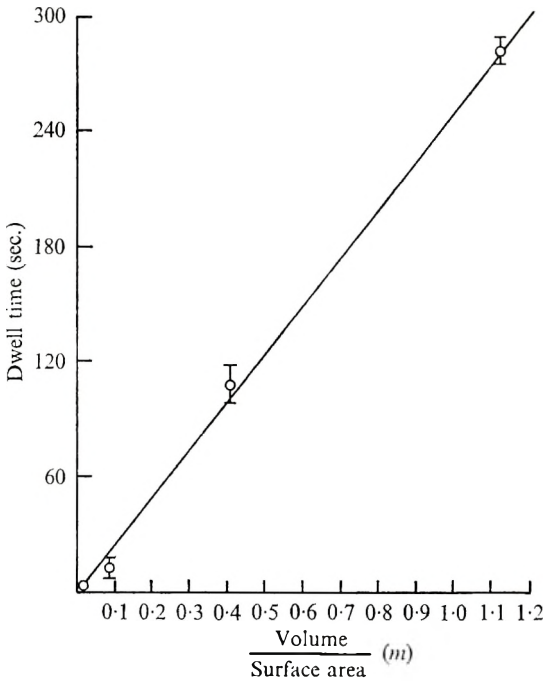

Fig. 6. Open-air factor (OAF) dwell time in vessels in relation to volume/surface area.

relationship was observed (Fig. 6). Furthermore, the curve produced showed an origin close to zero. The similar situation which obtains for the cuboid and cube is of special interest because although the cuboid is about twice the volume of the cube their volume/surface area ratios are similar. The results indicate a direct correlation of volume/surface area ratio with a rate of loss of OAF.

The effect of ambient wind on the air flow in some of the systems examined widened the limits of accuracy possible in this study. The data, however, indicate a near constant factor that can be used to calculate the maximum air dwell time for full preservation of OAF in enclosed systems. Multiplication of the volume/surface ratio (m) by 275 would give a close approximation to this time in seconds.

One explanation for the correlation obtained can be offered from Fick's first law of diffusion if it is assumed that loss of OAF in a vessel occurs by inactivation at the vessel wall. Rate of loss to walls is proportional to (i) the surface area of the wall, A, (ii) the diffusion coefficient for OAF, D, and (iii) the concentration gradient across the boundary layer, c/b, where c is the concentration in the vessel used and b is the thickness of the boundary layer. The total amount of OAF in a vessel of volume V is cV, therefore the rate of loss, $d(cV)/dt$, is given by:

$$V\frac{dc}{dt} = -AD\frac{c}{b}. \tag{1}$$

On integration this gives $c = c_0 \exp\left[-\frac{DA}{bV}t\right]$,

where c_0 is the concentration at zero time. Hence the characteristic time for OAF to be lost is proportional to V/A provided that the thickness of the boundary layer, b, is constant. This is, in fact, approximately true since in fully developed turbulent conditions the thickness of the laminar boundary layer in a ventilated vessel is not very sensitive to the rate of dissipation of turbulent energy in the vessel (Landau & Lifshitz, 1959).

The experiments with OAF indicate that when the concentration is reduced by about 90% little bactericidal effect is apparent. To obtain this situation in the sphere a ventilation rate of approximately 5 air changes/hr. is required, thus indicating that 90% loss of OAF occurs in about 12 min.

Hence from equation (1):

$$\frac{D}{b}\frac{A}{V} = 3{\cdot}2 \times 10^{-3} \text{ sec.}^{-1}.$$

From Chamberlain's work (1967) with loss of ^{132}I vapour in large vessels b can be calculated (since D is known) to be 0·21 cm. The sphere V/A is 112 cm. Hence D for OAF is *ca.* 0·0752 cm.^{2} sec.$^{-1}$.

From Graham's law,

$$M_1 = \frac{M_2 D_2^2}{D_1^2},$$

where M is the molecular weight. Thus M for OAF can be calculated by comparison, for example, with ethylbenzene (D = 0·075 cm.^{2} sec.$^{-1}$; Lugg, 1968) to be about 100 using the D estimate for OAF. The accuracy of the experimental data obtainable is such that a molecular weight range of 50–150 is indicated. It is of interest that this would be within the molecular weight range of the ozone–olefin complexes previously suggested, on other grounds (Druett & May, 1969; Dark & Nash, 1970), as candidates for OAF. The molecular weight suggests that it is the initial complex of ozone and olefin that is bactericidal and not a breakdown product.

I wish to thank Drs J. T. Bartlett and C. S. Cox for their helpful discussion, Mrs H. I. Willis, J. Ching and C. Moody for technical assistance, I. Myles of the Chemical Defence Establishment, Porton, for the engineering requirements and R. D. Lynch of the Chemical Defence Establishment for providing use of the cube vessel.

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The airborne excretion by pigs of swine vesicular disease virus

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SUMMARY

The air of loose-boxes holding pigs affected with swine vesicular disease was sampled for virus. In the multistage impinger virus to a titre of $10^{2.6}$ TCID50 was associated with particles greater than 6 μm., $10^{1.6}$ with particles 3–6 μm. and $10^{1.4}$ or less with particles less than 3 μm. In the noses of workers in contact with the pigs for periods not less than 5 min., virus to a titre of $10^{2.4}$ TCID50 was found. Virus was recovered from the air for 2–3 days during the disease and maximum titre in pigs infected by injection or by contact occurred on the second to third day after generalization of the lesions. The amounts of virus were about 160-fold less than those recovered from pigs affected with foot-and-mouth disease, and the quantity and time of excretion suggest that the source of swine vesicular disease virus in the aerosol may be from the lesions and skin rather than from the respiratory tract.

INTRODUCTION

Outbreaks of swine vesicular disease due to a virus have been described in Italy (Nardelli *et al.* 1968), Hong Kong (Mowat, Darbyshire & Huntley, 1972) and Great Britain (Dawe, Forman & Smale, 1973). At the time of the first outbreaks in Great Britain in December 1972 it was important to determine the method of spread of the disease and investigations were carried out to measure the amounts of swine vesicular disease virus present in the air of loose-boxes containing infected pigs. This paper records the results of two experiments, where pigs were infected by inoculation or by contact.

MATERIALS AND METHODS

Animals

Large white pigs, weight 30–40 kg., were housed in loose-boxes.

Virus

The strain of swine vesicular disease used came from a field case and was used either as the original field material (vesicular fluid and extract of vesicular epithelium – Dawe *et al.* 1973) or after one passage in pigs.

Infection of animals

In one experiment eight pigs housed in a loose-box were inoculated on the bulbs of the heel of both fore feet with virus fluid diluted 1/10 in phosphate buffered

Table 1. *Extent of lesions and recovery of virus from pigs inoculated intradermally with swine vesicular disease virus*

Days after infection	No. of animals affected*		Sites affected†	Virus recovery		
	Primary	Generalised		Multistage impinger	Collector	Examiner
1	No pigs affected				No recovery	
2	6/8	3/8	12/40	1·8‡	⩽ 1·2	2·4§
				⩽ 1·2		
				⩽ 1·2		
3	7/8	7/8	22/40	⩽ 1·2 (*a*)	⩽ 1·2	⩽ 1·2
				⩽ 1·2 (*b*)		
				⩽ 1·2 (*c*)		
4	7/8	7/8	29/40	1·6	⩽ 1·2	⩽ 1·2
				⩽ 1·2		
				1·4		
5	8/8	7/8	32/40	2·6	1·8	1·4
				1·6		2·4
				⩽ 1·2		
6	8/8	8/8	33/40	1·8	1·6	2·0
				⩽ 1·2		1·6
				⩽ 1·2		
7	8/8	8/8	34/40	⩽ 1·2	1·6	1·8
				⩽ 1·2		2·0
				⩽ 1·2		

* Numerator: number of animals affected or number of sites affected. Denominator: number of animals inoculated or number of sites available.

(*a*), (*b*), (*c*): top, middle and bottom stages of multistage impinger (> 6μm., 3–6 μm., < 3 μm.).

† Sites: 4 feet and/or legs. Snout, tongue and/or lips.

‡ Total virus (log TCID 50) recovered over 30 min. from multistage impinger.

§ Total virus (log TICD 50) recovered from nasal swab.

saline. In the other experiment four pigs were placed in contact with two pigs showing lesions of swine vesicular disease. Both groups of animals were observed daily and the extent of lesions recorded.

Air sampling

The air inlet and outlet in the box were blocked and the walls sprayed with water to maintain a high relative humidity. The air was sampled for 30 min. with a multistage impinger (May, 1966). In addition nasal swabs were taken from people operating the multistage impinger (collectors) and from those examining the animals (examiners) as previously described (Sellers, Donaldson & Herniman, 1970). The large volume sampler was also used initially, but it was not possible to disinfect it adequately after use owing to the resistance of swine vesicular disease virus to the cleaning process that could be used.

Table 2. *Extent of lesions and recovery of virus from pigs exposed to pigs suffering from swine vesicular disease*

Days after exposure	No. of animals affected*	Sites affected†	Virus recovery, multistage impinger
1–3		No pigs affected	
4	3/4	5/20	⩽ 1·2 (*a*)
			⩽ 1·2 (*b*)
			⩽ 1·2 (*c*)
5	4/4	15/20	1·4‡
			1·25
			⩽ 1·2
6	4/4	16/20	2·4
			1·25
			⩽ 1·2
7	4/4	18/20	⩽ 1·2
			⩽ 1·2
			⩽ 1·2

* Numerator: number of animals showing lesions or number of sites affected. Denominator: number of animals exposed or number of sites available.

† Sites: 4 feet and/or legs. Snout, tongue and/or lips.

(*a*), (*b*), (*c*): top, middle and bottom stages of multistage impinger (> 6 μm., 3–6μm., < 3 μm.).

‡ Total virus (log TCID 50) in stage of multistage impinger.

Virus assay

The virus present in the stages of the multistage impinger and in the nasal swabs (suspended in a volume of 10 ml.) was assayed in tissue-culture tubes of IB-RS-2 cells (de Castro, 1964). The results were expressed as tissue culture ID 50 per sample.

RESULTS

In the experiment where pigs were infected by inoculation, primary lesions were not observed until the second day, at which time some had ruptured and in three animals generalization had occurred. Further lesions appeared over the next 5 days involving the coronary band, the interdigital spaces, supernumerary digits, snout, lips and the skin over the hocks. Two peaks of virus recovery were found – on the second day at the time of development of primary lesions and on the fifth day 2–3 days after development of secondary lesions (Table 1). On average there was more virus in the nose of the examiners than in the nose of the collectors, although after the fifth day the difference was not great.

In the other experiment, when pigs were exposed to contact infection, the lesions first appeared on the fourth day, at one site on the feet in two animals, and at three sites in the third. On subsequent days lesions were found at sites on all feet and also on the lips and tongue. Maximum virus recovery was on the sixth day, 2 days after lesions were first observed (Table 2).

The greatest infectivity was associated with particles in the top stage (> 6 μm.), a mean of 83 % compared with a mean of 11 % and 6% in the middle and bottom stages respectively.

DISCUSSION

The virus of swine vesicular disease was found to be present in the air surrounding affected pigs. The amounts of virus recovered were less than those found in loose-boxes containing pigs affected with foot-and-mouth disease by a factor of 160-fold or more (Sellers & Herniman, 1972) and virus was detectable for 2–3 days compared to 5 days for foot-and-mouth disease (Sellers & Parker, 1969). In swine vesicular disease the maximum amount was recovered 2–3 days after generalization had occurred; in contrast, in pigs affected with foot-and-mouth disease the maximum amount of virus was recovered at the time of generalization of the disease. These results suggest that the source of airborne virus may be different in the two diseases. In foot-and-mouth disease (Sellers, Herniman & Donaldson, 1971) it appears that the upper respiratory tract may be the source and in more recent work infection of the lung of pigs may be involved (Terpstra, 1972). Present investigations (Dawe *et al.* 1973; Burrows & Mann, personal communication, 1973) indicate that the virus in high titre is present in the vesicles and in the skin at the time of disease, and it might be that the main source of virus in swine vesicular disease is virus shed into the surroundings as the result of rupture of the lesions. That the source of airborne virus may be different between the two diseases is also reinforced by the finding that the partition of infectivity associated with particle size is different (foot-and-mouth disease – 63%, 27%, 10% – Sellers & Herniman, 1972).

With the finding that smaller amounts of swine vesicular disease virus were excreted by infected pigs over a shorter period, spread by the airborne route would not be expected to the same extent as in foot-and-mouth disease. In investigations in the field during the present epidemic, most of the spread was attributed to movement of pigs or feeding of infected swill. Only 2 of the 103 outbreaks could be attributed to local spread. The large size of the particle associated with infectivity would require strong and turbulent winds to lift the infected particles and maintain them in the airborne state. In addition, pigs are not as efficient samplers of virus as are cattle exposed to foot-and-mouth disease virus and recent experiments have shown that a large dose is required to infect the pig by the nasal or oral routes (Burrows & Mann, personal communication, 1973).

The technical assistance of Misses L. M. Isaac and W. E. Reynolds and Mr I. D. Gumm is gratefully acknowledged. Mr N. P. Ferris helped to collect some of the air samples. Messrs P. S. Dawe and A. J. Forman provided the clinical observations of the in-contact experiment.

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The origin of O serotypes of *Escherichia coli* in babies after normal delivery

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SUMMARY

A total of 2525 strains of *Escherichia coli* were isolated from the faeces of 33 mothers, the faeces of their babies and the mucus extracted from the babies' mouths after delivery. Of these strains 1832 could be O-serotyped with 150 O antisera. *E. coli* were isolated from 28 babies and the same serotypes as were found in their mothers were found in 22 of them. *E. coli* was isolated from only 14 of the mucus specimens but in 12 of these at least one of the serotypes present was subsequently found in the babies' stools.

INTRODUCTION

Babies have *Escherichia coli* in their stools within a few days of birth. Some of these organisms are thought to come from the mother's bowel at the time of delivery but their appearance in babies delivered by Caesarean section and the spread of entero-pathogenic strains of *E. coli* make it clear that intestinal organisms of this group can be acquired from other sources.

MATERIALS AND METHODS

Full clinical details of mothers and babies were recorded. An attempt was made to obtain stool specimens from mothers before delivery and from the baby on each day after delivery. Mucus was also sucked from each baby's mouth immediately after birth. Stools and mucus were sown directly on MacConkey's medium. A second culture on a MacConkey plate was made from the mucus after it had been incubated overnight with broth.

All plates were incubated overnight and from each at least ten colonies of *E. coli* were isolated and were O-serotyped using 150 antisera by the method described by Bettelheim & Taylor (1969).

RESULTS

Thirty-three babies and their mothers were studied. All the babies were delivered normally and Table 1 shows the origin of the 2525 strains of *E. coli* that were isolated from them and their mothers.

Table 1. *Distribution of strains of* Escherichia coli

Strains	Number of strains from Mothers' stools	Babies' stools	Babies' mucus	All three
Typable with 150 O sera	622	987	223	1832
Not typable	154	151	2	307
Rough	145	215	26	386
Total	921	1353	251	2525

The babies were observed for an average of 7 days, and in this time 28 were found to have one or more strains of *E. coli* in the stool. In 22 the same serotype had been found in the mother's stool either before delivery (19 cases) or after delivery (21 cases). For the present study rough or non-typable strains were considered similar if they were found in specimens from one mother and her baby. Mucus was examined from 30 of the 33 babies and 14 of these samples contained *E. coli*. In 12 of these at least one of the serotypes present was found subsequently in the babies' stools, and in 11 of these 12 at least one of the serotypes was also found in the mother's stools (Table 2).

More serotypes were identified in mothers' stools than in babies' stools, 119 serotypes being identified from 32 mothers and 60 from the 28 babies whose stools were positive. The number of serotypes present in the babies appeared to be related to the presence or absence of *E. coli* in the baby's mucus. For the 14 babies with *E. coli* present in the mucus, 40 serotypes were identified in the stools compared with 19 serotypes for the 16 babies with no *E. coli* in the mucus. Only one of the five babies from whose stools no *E. coli* were isolated had the organism present in the mucus.

All the babies were given supplementary bottles and eight were breast fed, but this did not appear to be related. Of more interest was the finding that in the 11 babies from whom maternal serotypes were not isolated, the mean time from rupture of the membranes to birth was 157 min., as compared with 284 min. for the 22 babies from whom maternal serotypes were isolated.

DISCUSSION

Since Escherich (1885) first observed that, although sterile at birth, babies' stools soon contained bacteria, including *E. coli*, there have been many studies of the babies' faecal flora, and in recent years particular attention has been paid to the presence of entero-pathogenic strains.

The use of an almost complete set of *E. coli* O-sera has enabled us to identify almost certainly the same serotype in mother and baby in 22 of the 28 babies from whose stools we isolated *E. coli*. Our results therefore support and extend those of others (Nejedlá, Šrajbr & Lanc, 1967; Gareau *et al.* 1959; Rosner, 1966; Ironside, Brennand, Mandal & Heyworth, 1971) who have claimed that at least a proportion of the baby's strains come from the mother, probably at the time of delivery, and additional support for this view is given by the frequency with which we found

Table 2. *O-serotypes in mothers and babies*

Pt. no.	Mothers' stools	Babies' Mucus	Babies' Stools
2	O 3, O 7, O 8, O 19, O 78, O 81, NT	O 3, O 78, O 81	O 3, O 81
17	O 18, O 71, O 81, R	O 81, O 147, R	O 71, O 81
21	O 10, O 18, O 58, R	O 10, O 18, O 58, R	O 18, O 58, R
27	O 27, O 40, O 108, R, NT	O 19, O 27, O 41, O 108, O 116, O 129, R	O 27, O 79, O 108, O 116, R, NT
35	O 11, R	O 11, R	O 1, O 11, R
1	O 1, O 8, O 102, NT	No specimen	O 1, R
7	O 42, O 96, R	No *E. coli*	O 20, O 42, R
15	O 4, O 12, R	No *E. coli*	R
19	O 1, R	No *E. coli*	R
26	O 1, O 46	No *E. coli*	O 1
31	O 9, O 16, O 129, NT	No *E. coli*	O 9
8	O 19, O 82, O 129, NT	No. *E coli*	No *E. coli*
20	O 6, R	O 6	No *E. coli*
28	O 22	No *E. coli*	No *E. coli*
12	O 145, R	No *E. coli*	NT
16	O 6, O 30	No *E. coli*	O 3
32	O 21, O 1	No *E. coli*	O 3
5	O 18, O 42, O 78, R	O 42, O 75, O 78,	O 19, O 42, O 75, O 78, O 117
18	O 1, O 25, O 48, O 71, NT	O 48, R	O 48, R
23	O 7, O 19, O 46, R, NT	O 19, O 75, R	R, NT
29	O 11, O 42, O 79, O 108, R, NT	O 86, O 108, R	O 42, O 79, O 86, O 34,/ O 41, R, NT
33	O 27, O 84, O 153, NT	O 2, O 27, NT	O 3, O 84, R, NT
37	O 25, R, NT	O 25	O 25, NT
6	O 7, O 8, O 38, O 184, R	No *E. coli*	O 38
10	NT	No specimen	NT
13	O 5, O 7, O 8, O 82, O 148, NT	No specimen	O 7, O 82
22	O 6, O 52, R	No *E. coli*	O 6
30	O 46, O 141, R, NT	O 15	O 141, NT
4	O 18, O 26, O 37, O 75, NT	No *E. coli*	No *E. coli*
14	O 4, O 50, O 52, O 147, R	No *E. coli*	No *E. coli*
3	O 3, O 9, O 16, O 18, O 79, O 106, NT	No *E. coli*	O 7, O 102
24	O 9, O 106, NT	No *E. coli*	O 3
25	No specimen	O 10	O 10

matching of the maternal faecal serotypes with those found in the mucus from the baby's mouth immediately after delivery.

Nine of our babies had serotypes in their stools that we did not find in the mucus in their mouths at birth or in their mothers' stools and on six occasions serotypes were found in the baby's mucus that we did not identify in the mother's stools. These may represent strains acquired in some other way but present methods do

not permit the identification of all serotypes in a stool (Bettelheim, Faiers & Shooter, 1972) and these strains may have been in the mother's bowel.

The study of babies delivered by Caesarean section appears to offer a chance to investigate some of the other sources from which newborn babies acquire their intestinal *E. coli*, and work on this is in progress.

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The prevalence of salmonellas in mink

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SUMMARY

The mean isolation rates of salmonellas from the mesenteric lymph nodes and faeces of healthy mink were 16·7% of 618 animals from three farms and 3·9% of 772 animals from four farms respectively. *Salmonella senftenberg* was the most commonly isolated serotype. *S. typhimurium*, *S. dublin*, *S. livingstone*, *S. menston*, *S. enteritidis*, *S. bredeney* and *S. infantis* were also seen.

The prevalence of salmonellosis in 316 dead mink from 12 farms was 0·6%.

The epidemiological aspects are discussed.

INTRODUCTION

The wide distribution of salmonellas makes their prevalence in mink of interest not only to the mink farmer but also to other agriculturalists and those concerned with public health. Previous surveys have shown a low incidence varying from 0·6% of 3079 autopsies (Momberg-Jorgensen, 1949) to 1·6% of 1602 German mink (Loliger, 1956). These figures are supported by more general surveys where the number of isolations of salmonellas from different species has been reported (Bigland, Wilton, Vance & Carlson, 1962; Karlsson, Rutqvist & Thal, 1963; Hurvell, Lagerquist, Rutqvist & Thal, 1969). The isolations from mink have been low compared with those from cattle, pigs and poultry. *S. typhimurium* was the most commonly isolated serotype.

Outbreaks of salmonellosis among mink are not often reported. During pregnancy, infection with *S. choleraesuis* var. *kunzendorf* causes abortion, metritis and the occasional death (Hartsough, 1946–7). Secondary salmonellosis in mink dying from distemper has been observed by Zimmerman (1962) and by Head (1959) who, in the same paper, also recorded sudden deaths in weaned kits associated with *S. dublin*. He commented that probably salmonellosis only becomes a problem when mink are stressed. This was demonstrated experimentally by Gorham, Cordy & Quortrup (1949), who found that the oral administration of *S. choleraesuis* var. *kunzendorf*, *S. enteritidis* and *S. newport* had no effect on normal animals. However, when the mink were stressed by starving them before dosing with *S. newport*, an occasional animal died. Despite these observations many mink farmers and their advisers still think that salmonellas commonly cause disease in mink. The present work has attempted to assess this impression by determining the prevalence of salmonellas in healthy mink and the prevalence of salmonellosis

Table 1. *Number of animals examined during survey*

Farm	Period 1 Nov. 1970–Jan 1971	Period 2 March 1971	Period 3 Nov. 1972	Total
A	188	—	5	193
B	203	—	—	203
C	174	25	100	299
D	200	—	100	300
Total	765	25	205	995

in the mink received for pathological examination at the Veterinary Investigation Centre, Leeds.

MATERIAL AND METHODS

Carcass survey

Details of the numbers of healthy mink and when they were examined are given in Table 1. A total of 706 fresh and 289 deep frozen carcasses were examined after pelting in November, December, January and March. Although all animals were clinically healthy before slaughter, one batch on farm C had previously failed the iodine agglutination test and could therefore be expected to be suffering from the early stages of Aleutian disease. The survey included male and female kits (6–7 months old) and adults.

Cultures were made from the stomach contents, rectum, gall bladder, mesenteric and gastro-hepatic lymph nodes, but not all sites were cultured in any one batch of animals. This is made clear in the text and Table 2.

The mink received at the Veterinary Investigation Centre, Leeds, during 1969–72 for pathological examination, comprised 73 1-day-old kits from five farms and 316 fully grown animals (known hereafter as 'dead mink') from twelve farms. All were examined bacteriologically and salmonellosis was diagnosed where the infection was septicaemic or where salmonella was isolated from a diseased tissue.

Feeding

During period 1, farms B and C fed the same constituents from the same sources, i.e. broiler offal, day-old chicks, bovine rumens, fish offal and cereals. During period 2, the ration on farm C included bovine livers and excluded the broiler offal. In period 3, the diet was the same as period 1 but the bovine rumens had been replaced by ovine and the sources of the broiler offal, fish and cereal had changed.

Farm A fed a similar diet to farms B and C but the sources were different.

Farm D's sources also differed from the rest and during period 1 did not include any chicken, i.e. diet consisted of ovine rumens, heads, fish offal and cereals. A change had been made by period 3 and the ration included hen offal.

Table 2. *Isolation of salmonellas from clinically normal mink on four farms*

Batch	Date	Sex and age	No. of carcasses examined	Percentage of isolations from: Mesenteric lymph node	Gall bladder	Stomach contents	Rectal contents	Serotype
Farm A								
1	Nov. 70	M, kit	49	6·1	0	0	0	1
2	Nov. 70	M, adult	16	43·75	0	6·25	6·25	1, 2, 3, 4
3	Nov. 70	F, adult	23	34·8	ND	ND	ND	1, 2, 3, 4, 7
4	Nov. 70	F, adult	50	26·0	2·0	2·0	4·0	2, 4
5	Dec. 70	F, adult	50	0	0	0	6	1
6	Nov. 72	F, kit	5	40·0	0	ND	40·0	4, 8
	Sub-total		193	17·1	0·6	1·2	4·7	
Farm B								
7	Nov. 70	F, kit	50	ND	2	ND	6·0	5
8	Nov. 70	M, kit	50	ND	0	ND	4·0	5
9	Nov. 70	M, adult	33	ND	0	ND	3·0	5
10	Nov. 70	F, adult	70	ND	0	ND	1·4	1
	Sub-total		203	ND	0·5	ND	3·4	
Farm C								
11	Nov. 70	M, kit	74	ND	5·4	ND	13·5	1, 5
12	Dec. 70	F, adult*	40	0	0	15	0	1
13	Dec. 70	F, kit	25	12·0	8·0	16·0	4·0	1
14	Dec. 70	F, adult	35	8·6	0	17·1	2·9	1
15	Mar. 71	M, adult	25	72	32	28	8	3
16	Nov. 72	Both, kit	50	18	2	6	ND	2, 6
17	Nov. 72	M, kit	50	26	ND	ND	ND	2, 6
	Sub-total		299	20·4	6·0	14·9	7·0	
Farm D								
18	Nov. 70	Both, both	100	ND	0	ND	1	3
19	Jan. 71	F, adult	100	1	ND	ND	0	2
30	Nov. 72	Both, both	100	23	ND	1	ND	2
	Sub-total		300	12	0	1	0·5	
	Total		995	16·7	2·4	6·6	3·9	

Abbreviations: F = female; M = male; * = batch which failed the iodine agglutination test.
Numbers in the serotype column refer to the numbers indicating the serotypes in Table 5, e.g. 3 = *S. dublin*.
ND = not done.

Bacteriological examinations

Alginate swabs were inserted into the stomach, rectum and gall-bladder and transferred to 10 ml. of selenite F broth. Lymph nodes could not be cultured this way and instead a portion (0·5 cm.^{3}) was excised from each node and transferred to selenite F broth. In one experiment with twenty-three cadavers, a portion was placed directly in broth, whilst a further portion was homogenized in the broth before incubation. Selenite F broths were incubated at 43° C. for 18–24 hr. and subcultured on brilliant green agar plates. After a further 24 hr. incubation at 37° C., non-lactose fermenting colonies were selected for serological and biochemical identification. Confirmatory serological typing was carried out by the Central Veterinary Laboratory, Weybridge, and phage typing by the Enteric Reference Laboratory, Colindale.

RESULTS

Isolation from different sites in healthy animals

The observed values for each batch, the mean values for each farm and the overall means are given in Table 2. Isolations from the mesenteric lymph nodes were significantly greater than from any other site ($P < 0{\cdot}001$, Table 3), whilst those from the stomach contents were significantly greater than from the gall bladder and rectal contents ($P < 0{\cdot}01$). There was no significant difference between the recovery rates from the gall bladder and rectal contents. These observations were valid overall but not for the individual batch since between-batch variations were considerable, even where consecutive batches came from the same farm (Table 2). The gastro-hepatic lymph nodes were cultured in two batches (Table 3). The isolation rate (3·0%) was significantly lower than from comparable mesenteric lymph nodes (22·0%).

The statistical analyses in Table 3 have grouped all serotypes and assumed that their prevalence in each site would rank the same. This was true for most but not for *S. senftenberg*, where there was no significant difference between the recovery rate from the mesenteric lymph nodes, stomach contents and rectum (Table 4. $\chi^2 = 2{\cdot}6$ for 2 degrees of freedom).

It was found that homogenizing the mesenteric lymph nodes resulted in an increase in the rate of isolation of salmonellas. Eight isolations were made from 23 nodes in the control group and 13 from 23 homogenized glands. However, since only two isolates were duplicated, it was impossible to make any direct comparison of the two methods. In two animals, different serotypes were isolated from the same node; *S. senftenberg* and *S. dublin* from one and *S. senftenberg* and *S. livingstone* from the other. In all, 19/23 nodes (82·6%) were positive.

In the 290 animals where four sites were cultured, the same serotype was isolated on thirteen occasions from more than one site. The numbers were too small to analyse statistically but there was no suggestion of any correlation between infection of the mesenteric lymph nodes and excretion in the faeces. Occasionally, more than one serotype was isolated from the same animal. Two examples of dual infection of the lymph node have been given above. Others were *S. senftenberg* and

Table 3. *A comparison of the isolation rates from different sites*

	Cultured site	No. of positive isolations	Percentage positive	*F*	χ^2	*P*
290 mink (eight batches)	Mesenteric lymph node	47	16·2	3	41·90	< 0·001
	Stomach contents	25	8·6	2	9·76	< 0·01
	Gall bladder	11	3·8	.	.	n.s.
	Rectal contents	10	3·4	.	.	n.s.
100 mink (two batches)	Mesenteric lymph node	22	22	1	13·9	< 0·001
	Gastro-hepatic lymph node	3	3	.	.	.

F = degrees of freedom. n.s. = not significant.

Table 4. *Prevalence of individual serotypes in those batches in which the serotype occurred and where four sites were cultured*

Serotype	No. of batches	No. of animals examined	Percentage of isolations from: Mesenteric lymph node	Gall bladder	Stomach contents	Rectal contents
S. senftenberg	7	265	4·5	0·75	6·0	3·0
S. typhimurium	3	116	7·8	0	1·7	0
S. dublin	2	41	46·3	19·5	17·1	4·9
S. livingstone	2	66	10·6	1·5	1·5	0
S. enteritidis	1	50	18·0	2·0	4·0	0

S. typhimurium from the mesenteric lymph node, *S. typhimurium* from the mesenteric lymph node *S. senftenberg* from the rectum, and in a third, *S. typhimurium* from the mesenteric lymph node and *S. livingstone* from the stomach contents.

At the beginning of the survey it was thought that the isolation rate from the mesenteric lymph nodes might be increased in kits and in animals suffering from early Aleutian disease. In fact, where animals were examined at comparable times, isolations from kits (14·1% of 99) and adults (15·6% of 289) were similar, and it was clear that any such influences were insignificant compared with between-batch variations.

Salmonellas on the individual farm

On farm A during period 1, *S. typhimurium* (phage type 9), *S. senftenberg*, *S. livingstone*, *S. dublin* and *S. bredeney* were isolated, and from a small batch of five mink during period 3, *S. livingstone* and *S. infantis* were found.

During period 1, farms B and C shared the same serotypes, *S. menston* and *S. senftenberg*, and the same feed supplies. Thereafter, only farm C was examined. A single batch during period 2 revealed *S. dublin* and in period 3 *S. typhimurium* (phage type 1) and *S. enteritidis* (phage type 8) were found.

The prevalence on farm D was low during period 1 but high during period 3. *S. typhimurium* (phage type 3a) and *S. dublin* were isolated in period 1 and *S. typhimurium* (phage type 12a) during period 3.

When batches were examined during consecutive weeks (Table 2, e.g. batches 1–5, 7–9, 11–14), it was usual to find the same serotype in each batch; this was not

Table 5. *Isolation of serotypes from four mink farms*

Serotype	No. of times serotype isolated from 20 batches	Farms A	B	C	D	No. of farms from which serotype was isolated
1. *S. senftenberg*	9	+	+	+	.	3
2. *S. typhimurium**	7	+	+	+	+	4
3. *S. dublin*	4	+	.	+	+	3
4. *S. livingstone*	4	+	.	.	.	1
5. *S. menston*	4	.	+	+	.	2
6. *S. enteritidis*	2	.	.	+	.	1
7. *S. bredeney*	1	+	.	.	.	1
8. *S. infantis*	1	+	.	.	.	1
Number	.	6	3	5	2	.

+ = isolation; * Not always the same phage type.

necessarily true if several months separated the sampling periods. On farm C, *S. senftenberg* and *S. menston* were isolated during period 1, *S. dublin* during period 2 and *S. enteritidis* and *S. typhimurium* during period 3. On the other hand, on farm A, *S. livingstone* was seen 2 years after the first isolation. Eight serotypes were isolated during the survey, *S. senftenberg* being the most common (Table 5). All these serotypes, with the exception of *S. menston*, were isolated from the mesenteric lymph nodes indicating that infection and not passive carriage had occurred.

The overall prevalence of individual serotypes from each site has not been given since a high recovery rate from a batch (e.g. *S. dublin* from batch 15, Table 2) unduly weights the means and this could be misleading. The method used in Table 5 (i.e. the number of serotypes in 20 batches) is considered to be more valid.

Salmonellas as a cause of disease

Salmonellosis was diagnosed in 0·6% of the 316 dead mink. *S. typhimurium* (*phage* type 1a) was septicaemic in a 6-month-old male that had died suddenly on farm B and *S. dublin* in a mink suffering from urolithiasis on farm C. In addition, five isolations (three of *S. typhimurium* (untypable), one of *S. typhimurium* (phage type 29) and one of *S. dublin*) were made after enrichment cultures, but these were not causing disease.

No salmonellas were isolated from the 73 1-day-old kits.

DISCUSSION

Salmonellas are prevalent in mink but clinical salmonellosis is uncommon. These conclusions were expected since mink thrive on a diet of raw offals which often contains salmonellas. The assumption that infection originates mainly from the food is supported by our observations. Farms B and C shared the same feed during period 1 and also the same serotypes, and on farm C when the diet changed so did the serotypes. The contaminated constituents have not yet been identified, but since *S. senftenberg*, *S. livingstone*, *S. menston*, *S. enteritidis*, *S. bredeney* and

S. infantis are all more commonly found in birds than animals (Sojka & Field, 1970), broiler offal and, in the case of *S. menston*, day-old chicks were the probable sources of infection for these serotypes. The flocks from which the day-old chicks came were known to be infected with *S. menston*. *S. dublin* probably originated from ruminant offals, but the origin of the ubiquitous *S. typhimurium* must remain uncertain. The contribution of the cereals and fish is thought to have been insignificant, although Hobbs & Hugh-Jones (1969) suspected that white fish meal was the source of the outbreak of *S. senftenberg* in domestic animals and humans which they investigated. Wild birds might be a further source of infection, but surveys have shown neither the number nor the prevalence of serotypes found in mink (Goodchild & Tucker, 1968).

The survey measured the prevalence of salmonellas in healthy mink on four farms during the autumn and winter. Observations were not made at other times as carcasses were unavailable, but this was not a serious drawback since on all farms the dietary constituents and their sources tend to remain the same throughout the year; it is the proportions which vary. The incidence of *Salmonella* infection therefore depends on the frequency and degree of contamination in feedstuffs; the amounts that are collected; how long they are fed; the proportion of contaminated constituents in the total diet; the invasiveness of the salmonellas and the length of time for which mink remain carriers after infection. The incidence of avian serotypes in mink might be fairly constant since the poultry industry is becoming vertically integrated and individual organizations can often be identified by their salmonellas. Mink receiving poultry offal tend to be regularly challenged by the same serotypes.

On comparing the prevalence in different sites it was found that the recovery rate from the mesenteric lymph nodes was significantly higher than from any other site. This suggests that mink do become carriers but for how long is not known. The absence of correlation between the recovery rate from the mesenteric lymph nodes and the faeces suggests that the excretion rate from carriers is low or non-existent. The increased recovery rate from the mesenteric lymph nodes was true for most serotypes but not for *S. senftenberg*, a serotype which does not appear to be particularly invasive in mink.

The prevalence in the gall bladder and gastro-hepatic lymph nodes was low, whilst the recovery rate from the faeces was significantly lower than from the stomach contents or mesenteric lymph nodes but still high compared with other domestic species. Williams-Smith (1971) has listed the frequency with which salmonellas have been isolated from the faeces of adult healthy domestic animals in Britain. The highest figures of 2·5 and 2·0% were for turkeys and geese respectively but the list did not include mink. The faecal recovery rate from these mink was 3·9%. The reason for the lower recovery rate from the rectum than from the stomach contents is not known.

Comparisons with other surveys are not easy since it is not always clear from accounts which sites were cultured and what methods were used but the prevalence of 0·6% for clinical salmonellosis in the present survey is similar to that quoted by Momberg-Jorgensen (1949). The effect of stress in precipitating salmonellosis

may have been over emphasized. Two common causes of death in mink are chronic Aleutian disease and the stress/starvation syndrome. Prolonged stress occurs in both but secondary salmonellosis is uncommon and other factors may be more important. For example, the association between distemper and secondary salmonellosis (Head, 1959; Zimmerman, 1962) is probably due to the viral leucopenia reducing the resistance of the animal to bacterial infection and we have concluded that, with the exception of *S. choleraesuis* var. *kunzendorf*, the pathogenicity of salmonellas for even young mink is doubtful.

Whether mink are an important epidemiological source of salmonellas for people and livestock is still uncertain, but clearly their carcasses and excreta are potentially dangerous. Mink carcasses are usually disposed of at meat rendering plants and certainly if the hygiene in any of these plants was poor, they could be an important source of contamination for meat meals. The excreta lie in the mink sheds for up to 1 year and are then usually spread on agricultural land. To our knowledge, this has not been associated with subsequent outbreaks of salmonellosis in grazing animals but there would appear to be a potential danger and this will depend on whether salmonellas can survive for long periods in mink faeces. This we are investigating.

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Antibody responses in patients with farmer's lung disease to antigens from *Micropolyspora faeni*

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SUMMARY

Three purified cell-wall glycoprotein antigens, *a*, *b* and *c*, sensitive to sodium periodate and resistant to pronase, were extracted from mycelium of *Micropolyspora faeni* with aqueous phenol or trichloracetic acid. Pronase-sensitive, sodium periodate-resistant protein antigens were extracted from mycelium with aqueous phenol. Immunoelectrophoresis was a critical method of assessing purity of preparation.

Antibodies to glycoprotein antigens only occurred in clinically defined cases of farmer's lung disease, whereas antibodies to protein antigens also occurred in symptomless farmers. Precipitins to *a* occurred in all cases of farmer's lung disease, and this antigen was isolated and purified.

Fractionation of pooled sera from cases of farmer's lung disease showed that precipitins were IgG globulins, whereas latex-agglutinating antibodies were IgG, IgA and especially IgM globulins. It is suggested that farmer's lung may involve a cytotoxic type II reaction, in which glycoproteins adsorbed to tissue cells react with IgG, IgA and IgM immunoglobulins in the presence of complement, causing cellular damage.

INTRODUCTION

Sera from most patients with farmer's lung disease (FLD) contain precipitating antibodies to the thermophilic actinomycete *Micropolyspora faeni* and less frequently to *Thermoactinomyces vulgaris* (Pepys *et al.* 1963). However, about 20% of farmers exposed to mouldy hay, but without overt clinical FLD, also have precipitating antibodies to *M. faeni* (Pepys & Jenkins, 1965). Fletcher, Rondle & Murray (1970) showed that precipitating antibodies to two antigens of *M. faeni* appeared more frequently than others. These antigens were later shown to be cell wall components, extractable from *M. faeni* mycelium by trichloracetic acid (TCA), and containing arabinose, galactose and glucosamine (Fletcher & Rondle, 1973). Kobayashi, Stahmann, Rankin & Dickie (1963) found that a similar TCA extract of mouldy hay gave precipitin reactions with sera of all patients with symptoms and 17 of 29 symptomless farmers, some of whom had completely recovered, and no reaction with 38 controls. Moreover, Barbee, Dickin & Rankin (1965) found that aerosol inhalation of a TCA extract of mouldy hay produced symptoms in six FLD patients identical with those of acute FLD. Pepys & Jenkins (1965),

however, showed that both the TCA extract and a TCA precipitate of *M. faeni* culture filtrate provoked systemic and pulmonary reactions in affected subjects on inhalation.

The present study was undertaken to identify and purify glycoprotein and protein antigens of *M. faeni*, and to measure the rate of incidence of serum antibodies to such purified antigens in farmers with clinical FLD and suitable controls. Both gel diffusion and latex agglutination methods were used and an attempt was made to identify the class of reactive antibody.

MATERIALS AND METHODS

Cultures

The three strains of *M. faeni* examined were 1156 (Fletcher *et al.* 1970), and 5280 and 9355, isolated from the sputa of patients with FLD by Dr B. Moore, Public Health Laboratory, Exeter.

The liquid growth medium was that described by Fletcher *et al.* (1970). One volume of a 5-day culture was added to each 100 vol. of medium, and grown for 48 hr. at 50° C. with vigorous stirring. Determination of the microbial nitrogen present by the micro-kjeldahl method showed that the logarithmic phase of growth ended at 48 hr. (Fletcher, 1971). Cells were harvested at 20,000 *g* in an MSE continuous-action rotor, washed three times in 0·02 M phosphate-buffered saline, pH 7·2 (PBS), and stored at −30° C. until used.

Preparation of M. faeni *antigens by physical methods*

Culture supernatant (CS) antigens

Antigens from the supernatant after centrifugation of culture medium used for growth of *M. faeni* were prepared as described by Fletcher *et al.* (1970).

Mycelial (MU) antigen

Washed mycelium was disrupted by ultrasonic treatment as described by Fletcher & Rondle (1973), except that sonication was for 20 min., when examination by microscopy showed that no intact mycelium was present.

Cell walls

Cell walls were prepared from the deposit of the preparation of MU antigen according to Fletcher & Rondle (1973).

Preparation of M. faeni *antigens by chemical extraction*

Phenol

Mycelium or cell walls, suspended in water, were shaken vigorously with an equal volume of aqueous phenol (90 %, w/w) for 2 hr. at 4° C. (Westphal, Luderitz & Bister, 1952; Gierer & Schramm, 1956). After separation the aqueous and phenolic layers were dialysed against water to remove phenol. The opalescent aqueous phase was lyophilized (extract PA). The insoluble material which separated

from the phenolic phase was also lyophilized and extracted for 30 min. at 37° C. with PBS, centrifuged at 10,000 *g* for 1 min. and the supernatant used in serological tests (extract PP).

Trichloracetic acid

Washed mycelium (20 mg./ml.) in PBS was stirred in 5% (w/w) TCA at 4° C. for 24 hr. The insoluble residue, deposited at 10,000 *g*, was re-extracted in the same way. After dialysis against PBS, ethanol was added to the extract to a final concentration of 90% (v/v), held for 24 hr. at 4° C. and centrifuged at 10,000 *g* for 20 min. The deposit was resuspended and dialysed against distilled water and lyophilized (extract TE). The supernatant from the ethanol precipitation was further precipitated with 3 vol. of acetone containing a trace of sodium acetate for 24 hr. at 4° C., centrifuged at 10,000 *g* for 20 min., the deposit dialysed against distilled water and lyophilized (extract TEA).

Alternatively, some crude TCA extracts of *M. faeni* were precipitated directly with 3 vol. of acetone and a trace of sodium acetate, the deposits from centrifugation dialysed against distilled water and lyophilized (extract TA).

Antisera

For antigenic analysis, strongly reacting sera from 25 patients with clinically defined FLD were pooled and lyophilized in small volumes. This serum, H2, was identical in gel diffusion tests with the serum H1 used by Fletcher *et al.* (1970).

For a survey of the incidence of antibodies to selected antigens, sera received in this laboratory for routine serological testing were used. Each serum was initially tested for precipitating antibodies to *M. faeni* MU antigens.

Group A (76 patients) had clinically defined FLD and precipitins to *M. faeni*. Group B (66 patients) had pulmonary diseases other than FLD and precipitins to *M. faeni*. Group C (75 subjects) were healthy urban dwellers with no history of exposure to mouldy farm produce. Each group was tested in gel diffusion with *M. faeni* PA, TA and PP antigens, and in latex agglutination tests with *M. faeni* MU and TA antigens.

Serological tests

Gel diffusion

The method was that of Fletcher *et al.* (1970), except that 1·2% Difco Noble agar was used. MU and PP antigens were tested at 25 mg./ml. and other extracts at 2 mg./ml. In the survey of human sera, these and 1/10 dilutions were used.

Immunoelectrophoresis

The method of Pepys & Jenkins (1965) was used. MU and PP antigens were tested at 40 mg./ml. and other extracts at 5 mg./ml.

Latex agglutination

Latex (Difco polystyrene, 0·81 μm. particle size) was sensitized as follows: 1 volume of antigen (10 mg./ml. in PBS) was added to 4 vol. of latex solution and

mixed gently for 2 min. This mixture was diluted with 95 vol. of PBS and shaken for 2 hr. at room temperature. Such sensitized latex suspensions remained active for at least 3 months stored at 4° C. Control sensitized latex was prepared similarly, but excluding antigen.

Doubling dilutions of sera in PBS containing 0·2 % (w/v) bovine serum albumin were prepared in 0·4 ml. volumes in perspex WHO pattern plates containing eight rows of ten cups. The dilutions ranged from 1/8 to 1/2048. Two 0·02 ml. drops of sensitized latex suspension were added to each dilution. The plates were held for 2 hr. at 37° C. and 48 hr. at room temperature. The degree of agglutination was recorded on a scale from 4 + to negative, and the end-point taken as 2 + or greater to help exclude non-specific agglutination. In all tests the last cup of each row of dilutions contained buffer and sensitized latex particles only, and each serum was titrated against control sensitized latex. Known positive (H 2) and negative human sera were always included.

For absorption of latex agglutinating (LA) antibody, 0·15 ml. of H 2 serum was held for 72 hr. at 4° C. with 1·5 mg. of antigen, and the precipitate was removed by centrifugation. Control serum was held in the same way with PBS.

Treatments of antigens

Pronase

Five volumes of MU or PP antigens at 35 mg./ml. or other extracts at 3 mg./ml. were held with 1 volume of pronase (Sigma Chemical Company, St Louis, Missouri, U.S.A.) at 10 mg./ml. for 6 hr. at 37° C. A second volume of pronase was added and the mixture further held for 18 hr. at 37° C. Control antigens were held with PBS under the same conditions.

Sodium periodate

Five volumes of MU or PP antigens at 30 mg./ml. or other extracts at 3 mg./ml. were held with 1 volume of 0·02 M sodium periodate for 24 hr. at room temperature (21° C.) in the dark. Control suspensions were similarly held with PBS.

Preparation of antibody fractions

Mercaptoethanol

H 2 serum was held with an equal volume of 0·2 M 2-mercaptoethanol as described by Strannegård & Yurchision (1969).

Absorption with specific antisera

One volume of H 2 serum was held with 1 volume each of all paired combinations of goat anti-human IgG, IgA, or IgM globulins (Nordic Pharmaceuticals and Diagnostics, Tillburg, Holland) for 48 hr. at 4° C. and the precipitates removed by centrifugation.

Fractionation of serum

Crude gamma-globulin was separated from 2 ml. of H 2 serum with 50 % saturated ammonium sulphate, and the precipitate resuspended in 2 ml. of 0·1 M

Table 1. *Properties of* Micropolyspora faeni *antigens*

	MU antigen			
	a	*b*	*c*	13 antigens
CS antigen	*a*	–	–	16 antigens
Periodate sensitivity	+	+	+	–
Pronase sensitivity	–	–	–	+
Phenol extract				
PA	+	+	+	–
PP	–	–	–	+
TCA-acetone extract	+	+	+	–
TCA-acetone ethanol extract	+	–	–	–
I.E. region	C	C	C	A, B

+, Presence or sensitivity; –, absence or resistance, of antigens.
PA, PP, Aqueous or phenol phases respectively after phenol extraction.

Tris-HCl buffer, pH 7·5. Samples of 1 ml. were applied to Bio-Gel A-5m (Bio-Rad Laboratories, Richmond, Calif., U.S.A.), equilibrated in the same buffer packed into a column (45 × 2·5 cm.). Elution was performed using the same buffer, and 3 ml. fractions collected and the absorption at 280 nm. recorded in an Optica CF 4 Spectrophotometer. Each fraction was tested in gel diffusion with goat anti-human IgG, IgA and IgM globulins, and those containing each antibody were pooled and concentrated to 1 ml. by dialysis against polyethylene glycol.

RESULTS

Extraction of antigens

Gel diffusion

In gel diffusion tests with H2 serum, two groups of antigens in strain 1156 MU and CS preparations were distinguished by their sensitivity to sodium periodate and pronase respectively, and their presence in chemical extracts. The results are summarized in Table 1. MU contained three antigens designated *a*, *b* and *c*, each sensitive to sodium periodate, with *a* closest to the antiserum well, whereas only *a* was present in CS antigen (Pl. 1, fig. 1). Of a total of 17 pronase-sensitive antigens detected in both preparations, 12 were present in both MU and CS antigens, 4 only in CS antigen and 1 only in MU antigen. A similar distribution of sensitive antigens was observed in strains 5280 and 9535.

Separation of sodium periodate and pronase sensitive antigens was achieved with aqueous phenol extraction of mycelium or cell walls. Gel diffusion tests with H2 serum showed that antigens *a*, *b* and *c* passed into the aqueous phase (PA extract, Pl. 1, fig. 2), gave a positive result with Molisch reagent, remained sensitive to sodium periodate and resistant to pronase. The 13 pronase-sensitive antigens were recovered from the phenol phase, remained sensitive to pronase and resistant to sodium periodate. Similar PA and PP extracts prepared from *M. faeni* strains 5280 and 9535 gave reactions of complete identity with those of strain 1156.

Extraction of *M. faeni* 1156, 5280 and 9535 mycelium or cell walls with TCA

Table 2. *Absorption of latex agglutinating (LA) antibody from H2 serum (titre* 1024) *by extracts of* Micropolyspora faeni

(Sera titrated against latex coated with MU antigen at 10 mg./ml.)

H2 serum absorbed with	Factor by which titre decreased
Nil (control)	1
PBS	0
MU antigen	⩾ 128
CS antigen	32
MU + periodate	4
MU + pronase	64
PP extract	2
PA extract	32
TA extract	32
TE extract	32
TEA extract	16

followed by acetone precipitation (TA extract) yielded the three sodium periodate-sensitive antigens which remained sensitive to sodium periodate and resistant to pronase, and which all gave reactions of complete identity with the PA extract in gel diffusion tests with HS serum (Pl. 1, fig. 2). These antigens were also precipitated with 90% (v/v) ethanol (TE extract, Pl. 1, fig. 2), but further precipitation of the ethanol supernatant with acetone yielded only antigen *a* (TEA extract, Pl. 1, fig. 2).

Serial dilutions of the TA or TEA extracts showed that in gel diffusion tests with H2 serum, antigen *c* reacted at concentrations of 400 μg./ml. or above, *b* at 100 μg. per ml. or above, but *a* reacted even at 2 μg./ml.

Immunoelectrophoresis

The immunoelectrophoretic patterns of PA, TA, TE, TEA and PP extracts with H 2 serum are shown in Pl. 1, figs. 3 and 4. Both the PA and TA extracts gave three arcs of precipitation adjacent to the antiserum well (the 'C' region of Pepys & Jenkins, 1965). The TE extract gave in addition to three 'C' region arcs, several arcs towards the anode (A and B regions of Pepys & Jenkins, 1965), as did the supernatant of the TA (TA-SN) precipitation at high concentrations. Crude TCA extracts before ethanol or acetone precipitation also gave 'A', 'B' and 'C' region arcs. After treatment of the TE extract with pronase, these 'A' and 'B' region arcs disappeared. As in gel diffusion, the TEA extract gave only one line of precipitation, in the 'C' region. The pronase-sensitive antigens in the PP extract gave arcs in only the A and B regions.

Latex agglutination

The results of absorbing LA antibodies are shown in Table 2. LA antibody was fully absorbed with MU antigen and mostly with CS antigen. The absorbing ability of MU antigen was significantly decreased after treatment with sodium periodate, but little altered after pronase treatment. The significant role of sodium

Table 3. *Agglutination, by H 2 serum, of latex coated with extracts of* Micropolyspora faeni

Latex coated with	Titre H 2 serum
H_2O control	< 8
MU extract	1024
PP extract	< 8
PA extract	64
TA extract	512
TE extract	256
TEA extract	128

Table 4. *Agglutination of latex coated with* Micropolyspora faeni *MU antigen, and precipitin activity against* M. faeni *MU or TA antigens, by fractions of H 2 serum*

H 2 absorbed with	Titre of latex agglutinating antibody	Precipitins against	
		MU	TA
PBS control	1024	+	+
mercaptoethanol	< 8	±	±
IgA + IgM (= IgG)	256	+	+
IgG + IgM (= IgA)	512	–	–
IgG + IgA (= IgM)	1024	–	–
H 2 serum fractions			
(IgG)	128	+	+
(IgA)	64	–	–
(IgM)	1024	–	–

periodate antigens was confirmed by the strong absorption of LA antibody with PA, TA, TE and TEA extracts, and not PP extract.

The results of coating latex with PP, PA, TA, TE and TEA extracts are shown in Table 3. PP extracts were not active. However, extracts containing the sodium periodate-sensitive antigens effectively coated latex, with TA extract being the most active.

Activity of antibody fractions

H 2 serum treated with 2-mercaptoethanol was completely inactive in LA tests although its precipitating activity was reduced in gel diffusion with MU and TA antigens (Table 4).

After absorption of H 2 serum with goat antiserum to IgG, IgA and IgM globulins, only the IgG fraction possessed precipitin activity whereas the IgG, IgA and IgM all possessed significant LA activity (Table 4).

Fractionation of H 2 serum on Bio-Gel A-5m is shown in Fig. 1. Again, only IgG possessed precipitating activity, although all antibody fractions contained LA activity (Table 4).

Survey of human sera

The incidence of precipitins and LA antibodies in group A and B sera is given in Table 5. No antibodies were detected in any test in group C sera. The average LA titre of group C sera was 1/8, and none was above 1/16. A positive LA titre, therefore, was taken as 1/32 or higher.

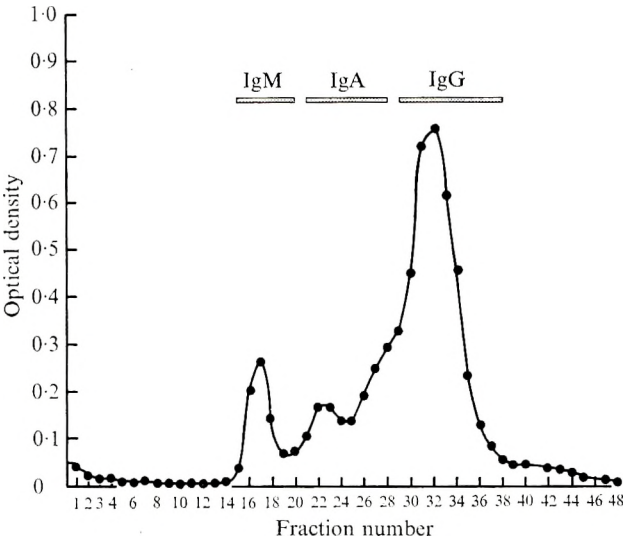


Fig. 1. Fractionation of H 2 serum on Bio-Gel A-5m.

Table 5. *Number of sera in Groups A and B reacting in precipitin and latex agglutination (LA) tests with* Micropolyspora faeni *antigens*

	Group A	Group B
Total number ...	76	66
Sera with precipitins to		
PP antigens	73	62
PA antigens	74	3
TA antigens	74	3
a antigen	74	0
b antigen	36	2
c antigen	6	1
Sera with LA antibodies to		
MU antigens	76	2
TA antigens	76	2

Almost all group A (73/76) and group B (62/66) sera with precipitins to *M. faeni* MU antigen contained precipitins to pronase sensitive (PP) antigens. However, precipitins to *M. faeni* sodium-periodate-sensitive (PA and TA) antigens, occurred in 74/76 sera from group A and in only 3/66 group B sera. Of these 74 positive group A sera, 74 (100%) were positive to *a*, 36 (49%) were positive to *b*, and 6 (8%) were positive to *c*; of the 3 positive group B sera, 2 were positive to *b* and 1 was positive to *c*.

Similarly LA antibodies to MU and TA antigens occurred in all group A sera and only the 2 group B sera with precipitins to *b*.

DISCUSSION

The results obtained by chemical extraction of *M. faeni* mycelium and treatment of sonicated mycelial antigen, (MU) antigen, with sodium periodate and

pronase, show that this thermophilic actinomycete possesses two chemically distinct groups of antigens. The three individual antigens in the first group are sodium periodate-sensitive and pronase-resistant, pass into the aqueous phase of phenol extraction, and are soluble in TCA. Their predominantly carbohydrate nature and the insensitivity to pronase of the small amounts of bound protein identifies them as glycoproteins. Antigens in the second group are sodium periodate-resistant, pronase-sensitive proteins, and are soluble in phenol. The three glycoprotein antigens are derived from the cell wall. Only one (*a*) is released into the culture medium during the period of growth used, although extending the period of incubation may well release other glycoproteins into the growth medium through autolysis. Both groups of antigens appear to be identical in each of the three strains of *M. faeni* examined.

It appears that glycoproteins prepared by phenol or TCA extraction followed by precipitation with acetone do not migrate towards the anode during immunoelectrophoresis, but remain adjacent to the antigen well, i.e. in the 'C' region as described by Pepys & Jenkins (1965). Other workers have found that cruder TCA extracts of *M. faeni* mycelium or mouldy hay (Pepys & Jenkins, 1965; Fletcher & Rondle, 1973) or ethanol precipitates of such extracts (Kobayashi *et al.* 1963; LaBerge & Stahmann, 1966*a*, *b*) give reactions not only in the 'C' region, but also in the 'A' and 'B' regions as well. This study has shown that arcs reacting in the 'A' and 'B' regions in such TCA extracts are formed by small amounts of pronase-sensitive proteins. It appears therefore essential that in any critical study the purity of extracted glycoproteins should be confirmed by immunoelectrophoresis when arcs should be formed only in the 'C' region.

In gel diffusion of the three glycoprotein antigens detected, *a* appears to be the most reactive. A combination of ethanol and acetone precipitations separated some of *a* present in the crude TCA extract from the other antigens. Preliminary studies on the inhibition of glycoprotein antigen *a* in quantitative precipitation have shown that arabinose and glucosamine are involved in the precipitation with human immune serum.

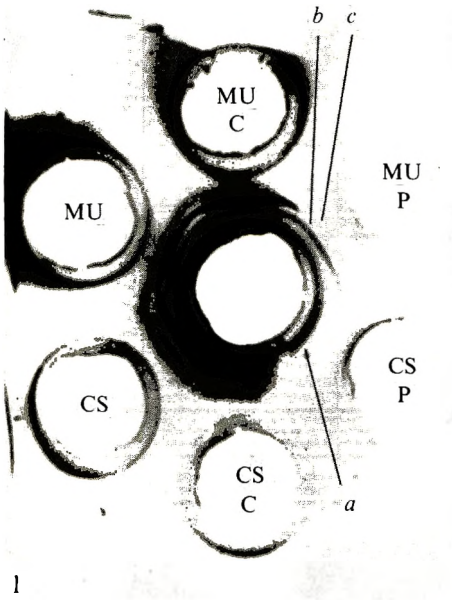
Gel diffusion tests have previously shown that sera from some subjects without clinical FLD but with exposure to mouldy hay contain precipitins to *M. faeni* antigens (Pepys & Jenkins, 1965; Fletcher *et al.* 1970). The results of this survey of human sera using purified antigens have show that precipitins to the three glycoproteins occurred only in sera from patients with FLD, whereas precipitins to protein antigens were present and presumably occurred as a result of exposure to mouldy hay, both in FLD cases and in patients with other respiratory illnesses. This extends the findings of Fletcher *et al.* (1970) that antibodies to glycoproteins occurred more frequently than antibodies to other types of antigen in sera from patients with FLD and rarely in sera from patients without FLD; however, the antigen used in this earlier study was sonicated mycelial supernatant (MU) and not purified glycoproteins. Of interest in this respect is the finding by Pepys & Jenkins (1965) that less than 50 % of their patients with FLD contained antibodies precipitating in the 'C' region; their antigen, however, appeared to contain only one arc in the 'C' region.

The occurrence of antibodies to glycoproteins only in cases of FLD was also confirmed by using latex coated with purified glycoproteins or whole MU antigen. In the latter case, absorption studies showed that only antibodies to glycoproteins appear to agglutinate the latex particles. Latex with *M. faeni* glycoproteins, therefore, appears to be a more specific test than gel diffusion for the detection of antibodies only involved in FLD.

Isolation of the three major classes of immunoglobulins of human serum has shown that precipitins to *M. faeni* antigens are IgG globulins and confirms the suggestion made by Pike (1967) that IgG are effective precipitins. In contrast, LA antibodies were found mostly in the IgM globulins, although IgG and IgA were also active. Thus, the LA test estimates the three classes of antibodies, and the findings are consistent with Pike's suggestion that smaller amounts of IgM than IgG are required for agglutination. The higher LA titres of sera from patients in the acute phase of FLD may, therefore, represent a higher concentration of IgM than IgG or IgA globulins in these patients. It is suggested that lower LA titres in the subacute or chronic cases may represent a fall in the concentration of IgM globulins and their replacement by IgG.

The immunological processes involved in the pathogenesis of FLD have yet to be fully elucidated. Pepys & Jenkins (1965) and Pepys (1969) have suggested the role of precipitins in an Arthus type III reaction. However, Wenzel, Emanuel & Gray (1971) studied the walls of bronchioles in patients with acute FLD stained with fluorescein-labelled IgG, IgA and IgM globulins, and showed that the three globulins were found in plasma cells and histiocytes. In addition, the C 3 component of complement was fixed in the histiocytes, suggesting that the pathogenesis of FLD may involve a cytotoxic type II reaction, at least in the acute phase. It was suggested that the absorption of antigen evidently renders tissue cells susceptible to cytotoxic antibody and complement. The present survey of human sera has shown that precipitating and agglutinating antibodies (i.e. IgG, IgA and IgM globulins) to glycoproteins occur only in those patients with FLD. It is possible, therefore, that *M. faeni* glycoproteins fix onto tissue cells and bring about a cytotoxic type II reaction in the presence of specific antibody. It is noteworthy that bacterial polysaccharides have a tendency to become fixed onto red cells (Coombs & Gell, 1968).

Until further work has revealed whether either or both type II and type III, or indeed the delayed type IV, reactions are involved in FLD, it would seem advisable that all three classes of immunoglobulins should be measured in the serological diagnosis of FLD. It is possible that some patients with FLD but without detectable precipitins (Pepys & Jenkins, 1965), and therefore without IgG immunoglobulins, may possess IgA or IgM globulins active in the type II reaction and only detectable by agglutination tests. Such studies are planned.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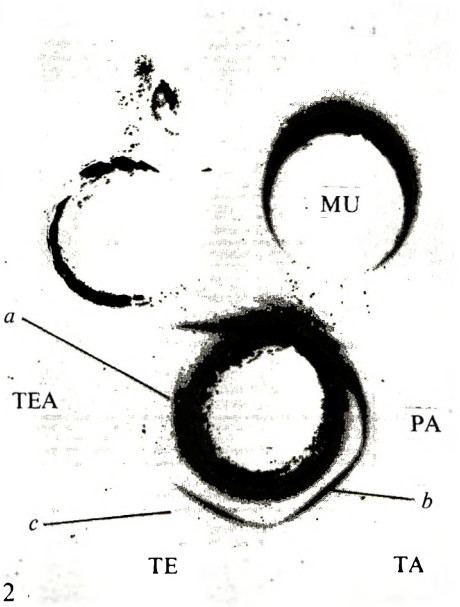
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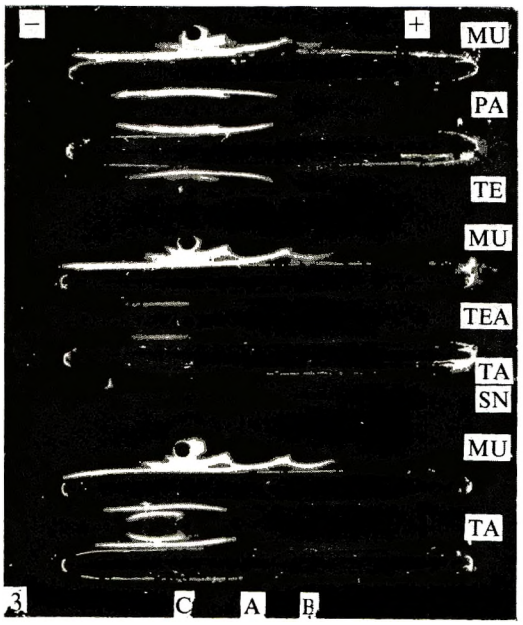
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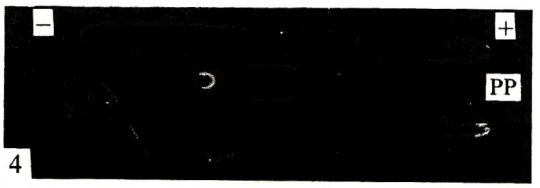
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4

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EXPLANATION OF PLATE

Fig. 1. Precipitin reactions given by MU and CS preparations and after treatment with pronase (MU.P and CS.P), and controls (MU.C and CS.C), revealing *a*, *b* and *c* antigens.

Fig. 2. Precipitin reactions given by MU preparation compared with PA, TA, TE and TEA extracts, showing, *a*, *b* and *c* antigens, and isolated *a* antigen.

Fig. 3. Immunoelectrophoresis of MU, PA, TA, TE, TEA and TA.SN extracts. Letters refer to C, A and B regions in text.

Fig. 4. Immunoelectrophoresis of PP extract, showing arcs in 'A' and 'B' regions.

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Immunity to influenza in ferrets

VII. Effect of previous infection with heterotypic and heterologous influenza viruses on the response of ferrets to inactivated influenza virus vaccines

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SUMMARY

Normal ferrets did not produce serum antibody following immunization with 200 i.u. of inactivated A/Hong Kong/68 influenza virus vaccine and were found to be susceptible to subsequent challenge infection with A/Hong Kong/68 virus. High titres of virus were recovered from nasal washings collected 3 days after infection, serum antibody was produced, increased nasal protein was detected and HI antibody was detected in nasal washings. Ferrets infected with influenza virus A/PR/8/34 7 weeks before immunization with inactivated A/HK/68 virus did, however, produce serum HI antibody to A/HK/68 virus. This antibody conferred partial immunity to challenge infection with A/HK/68 virus, as shown by decreased titres of virus in nasal washings and reduced levels of nasal protein. Previous infection of ferrets with influenza virus B/Ann Arbor/66 did not result in the production of serum antibody to A/HK/68 virus following immunization with A/HK/68 vaccine and the animals were completely susceptible to subsequent challenge infection with A/HK/68 virus. Differences in the amount of nasal protein and nasal antibody produced after A/HK/68 infection were also found in ferrets previously infected with either A/PR/8/34 or B/AA/66 virus, compared with normal ferrets.

INTRODUCTION

Influenza in ferrets closely resembles that in man, and the animals are therefore useful as an experimental model in which to study the disease (Smith, Andrews & Laidlaw, 1933; Haff, Schriver, Engle & Stewart, 1966). Attempts to immunize ferrets with inactivated influenza virus vaccines against a challenge infection have, however, been unsuccessful (Potter *et al.* 1972). Antibody was only produced in the serum of animals given vaccine together with adjuvant, but this antibody failed to give the same degree of resistance to challenge infection as equal titres of antibody produced as a result of live, homologous infection (Potter, McLaren & Shore, 1973).

The ferrets used in the above studies differed from man in that they had no history of previous infection with influenza viruses. In the present study we report the effect of previous infection with either an influenza B virus or a heterotypic

influenza A virus on the response of ferrets to subsequent immunization with inactivated influenza virus A/Hong Kong/68 vaccine. In addition, the response of the ferrets to subsequent infection with influenza A/Hong Kong/68 virus is also reported.

MATERIALS AND METHODS

Viruses

Influenza viruses B/Victoria/98926/70, A/PR/8/34 (H0N1) and A/Hong Kong/68 (H3N2) were obtained from Dr G. C. Schild, World Influenza Centre, Mill Hill, London.

Viruses were grown in 10-day embryonated hen's eggs incubated for 72 hr. at 33° C, and stored at −70° C. A pool of A/HK/68 virus was also grown in duck eggs in a similar manner.

A pool of a cold adapted strain of B/Ann Arbor/66 virus grown in hen's eggs, was obtained from the Wellcome Research Laboratories, Beckenham.

Vaccines

Inactivated influenza virus A/HK/X31/68 vaccine containing 200 International Units (i.u.) per 0·5 ml. was kindly supplied by Dr D. Breeze, Evans Medical Ltd., Speke. This virus is antigenically similar to A/HK/68 (Kilbourne *et al.* 1971).

Animals

Young albino ferrets were kindly supplied by Wellcome Research Laboratories, Beckenham. The animals were immunized against canine distemper virus a number of weeks before the beginning of the experiment.

Experimental design

Groups of ferrets were infected intranasally with either A/PR/8/34 or B/AA/66 virus. Blood samples were taken by cardiac puncture before and 5–7 weeks after infection. Half of each group of convalescent animals were then inoculated intramuscularly with 200 i.u. of A/HK/X31/68 vaccine; a group of normal ferrets was also inoculated with the same vaccine.

Blood samples were taken from some of the ferrets 6 days after immunization, and 5 weeks after immunization all animals were bled and a nasal wash specimen was collected. The ferrets, together with a group of non-infected, non-vaccinated animals, were then challenged with approximately $10^{7\cdot0}$ EID50 of A/HK/1/68 influenza virus, which had had two passages in monkey kidney cells followed by four passes in eggs, inoculated intranasally under light ether anaesthesia. Nasal washings for virus isolation were collected 3 days after challenge infection, and further specimens were collected for protein determination and antibody studies on subsequent alternate days, as described previously (Potter *et al.* 1972). A further blood sample was taken three weeks after challenge.

Table 1. *Response of ferrets to immunization with* 200 *i.u. of A/HK/68 vaccine*

Ferret no.	Primary infection	Change in serum HI titre after immunization*		
		A/HK/68	A/PR/8/34	B/Vic
326	B/AA/66	—†	—	30–30
327		—	—	40–< 10
328		—	—	60–30
330		—	—	60–20
324	A/PR/8/34	< 10–160	960–240	—
329		< 10–120	1920–480	—
331		< 10–60	640–240	—
333		< 10–120	960–480	—
368	Nil	—	—	—
370		—	—	—
371		—	—	—

* Titre before immunization – titre 5 weeks after immunization.
† < 10–< 10.

Virus isolation

The titre of virus in unconcentrated nasal washings collected three days after infection was determined by infectivity titrations in 10-day eggs as described previously (Potter *et al.* 1972).

Protein estimations

The protein content of ten-fold concentrated nasal washings was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Serological tests

Haemagglutination inhibition (HI) tests. HI tests on sera and ten-fold concentrated nasal washings were carried out as described previously (Potter *et al.* 1972), but with an incubation period of 40–60 min between the addition of virus and erythrocytes.

Neutralization tests. The titre of neutralizing antibody in ten-fold concentrated nasal washings was measured using the allantois-on-shell technique (Fazekas de St Groth, Withell & Lafferty, 1958).

RESULTS

Response to immunization

None of the normal ferrets nor those animals infected with B/AA/66 5 weeks previously produced detectable titres of serum HI antibody to A/HK/68 virus after inoculation with 200 i.u. of A/HK/68 vaccine (Table 1).

In contrast, all of the ferrets which had been infected with A/PR/8/34 influenza virus seven weeks previously produced serum HI antibody in response to immunization with A/HK/68 vaccine (Table 1). The titres of antibody ranged from 60 to 160 5 weeks after immunization, although sera taken from two of these animals 6 days after immunization showed nearly the same titre of antibody as at 5 weeks.

Table 2. *Response of ferrets to infection with A/HK/68 virus after infection with heterotypic and heterologous influenza viruses*

Ferret no.	Primary infection	Virus yield (log_{10}EID50/ml)‡	Response to A/HK/68 infection: Change in serum HI titre* A/HK/68	Change in serum HI titre* A/PR/8/34	Change in serum HI titre* B/Vic	Change in nasal HI titre† A/HK/68	Change in nasal HI titre† A/PR/8/34	Change in nasal HI titre† B/Vic
317	B/AA/66	5·5	< 10–640	—‖	40–< 10	< 5–80	—‖	—
318		5·16 (5·41)§	< 10–640	—	120–40	< 5–120	—	—
322		5·5	< 10–480	—	10–< 10	< 5–40	—	—
325		5·5	< 10–960	—	30–20	< 5–60	—	—
319	A/PR/8/34	4·16	< 10–960	320–320¶	—	—	—	—
320		4·50	< 10–1280	480–640	—	< 5–7·5	—	—
321		4·83 (4·41)	< 10–640	240–240	—	—	—	—
323		4·16	< 10–480	960–240	—	—	—	—
352	Nil	5·16	< 10–> 5120	—	—	< 5–30	—	—
359		4·50	< 10–960	—	—	< 5–80	—	—
363		5·16 (4·91)	< 10–1920	—	—	< 5–80	—	—
367		4·83	< 10–960	—	—	< 5–30	—	—

* Titre before infection – titre 3 weeks after infection.
† Titre before infection – peak titre after infection.
‡ Titre of virus in nasal washings collected 3 days after challenge infection.
§ Mean titre of group.
‖ < 10–< 10 (serum); < 5–< 5 (nasal washings).
¶ Died 7 days after challenge.

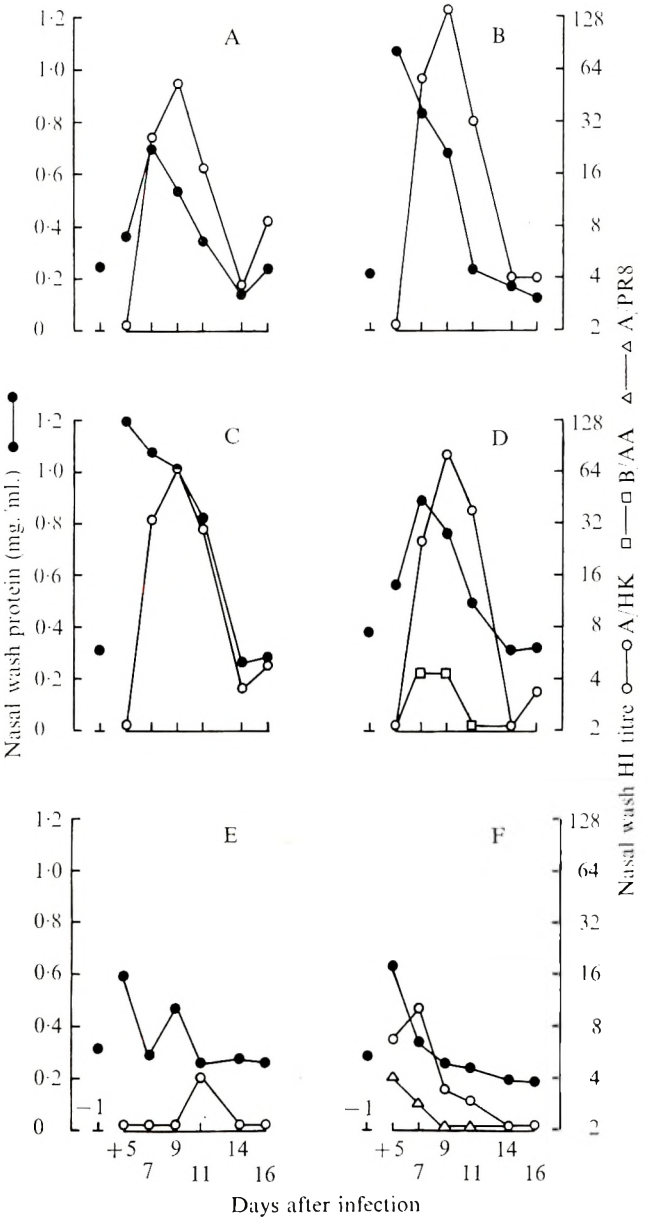

Fig. 1. Protein concentration and HI antibody titres in tenfold concentrated ferret nasal washings collected before and 5–16 days after intranasal infection with influenza virus A/Kong Kong/1/68. A. Normal ferrets. B. Normal ferrets immunized with 200 i.u. of inactivated A/HK/68 virus vaccine five weeks before challenge infection. C. Ferrets infected with B/Ann Arbor/66 influenza virus 10 weeks before challenge infection. D. Ferrets infected with B/AA/66, immunized with 200 i.u. of inactivated A/HK/68 virus vaccine 5 weeks later and then challenged with A/HK/68 virus after a further 5 weeks. E. Ferrets infected with A/PR/8/34 influenza virus 12 weeks before challenge infection. F. Ferrets infected with A/PR/8/34, immunized with 200 i.u. of inactivated A/HK/68 virus vaccine 7 weeks later and then challenged with A/HK/68 virus after a further 5 weeks.

Similar antibody titres were measured using both hen and duck-egg grown A/HK/68 virus. No increase in antibody titres to A/PR/8/34 virus was observed after immunization with A/HK/68 vaccine.

Response to challenge infection with A/Hong Kong/68 virus

Normal ferrets

Four normal ferrets were infected by intranasal inoculation of approximately 10^7 EID 50 of A/HK/1/68 virus. Virus was recovered from nasal washings collected on day 3 after infection from all animals in the group (titres $10^{4.50}$–$10^{5.16}$ EID 50/ml., geometric mean titre (gmt) = $10^{4.9}$ EID 50/ml.) and the animals all produced serum HI antibody (Table 2). After infection, a 2–3-fold increase in protein concentration was detected in nasal washings, with maximum concentrations usually present on day 7 (mean = 0·7 mg/ml.). HI antibody was also found in nasal wash specimens, but the maximum titres of HI antibody (1/30–1/80) occurred on day 9, with only one out of four ferrets producing nasal antibody after day 11 (Fig. 1).

Ferrets previously infected with influenza virus B/AA/66

The effect of previous infection with influenza virus B/AA/66 on the response to A/HK/68 challenge infection 10 weeks later was studied in four ferrets. After A/HK/68 infection, virus was recovered from nasal washings collected on day 3 from all animals in the group (gmt = $10^{5.41}$ EID 50/ml.) and serum HI antibody to A/HK/68 virus was produced by all animals (Table 2). No boost in the serum HI titre to B/AA/66 virus was observed. The animals produced threefold increased amounts of protein in their nasal washings, with highest levels on day 5 (mean = 1·2 mg/ml.). Antibody to A/HK/68 was detected in nasal washings collected 7 days and later after infection, with maximum titres on day 9 (Fig. 1).

Ferrets previously infected with influenza virus A/PR/8/34

Ferrets infected with A/PR/8/34 virus were challenged with heterotypic A/HK/68 virus 12 weeks later. The animals were susceptible to the second infection, as shown by recovery of virus from nasal washings (gmt = $10^{4.4}$ EID 50/ml.) and the production of serum HI antibody to A/HK/68 virus (Table 2). However, after A/HK/68 infection, the concentration of protein in nasal washings increased only twofold, to a maximum mean concentration of 0·6 mg./ml. on day 5 (Fig. 1). HI antibody to A/HK/68 virus was detected in the nasal washing of only one ferret (F. 320), and on only one occasion, in this group of animals (Table 2, Fig. 1).

Response to challenge infection with A/Hong Kong/68 virus following virus infection and immunization

Normal ferrets immunized with A/HK/68 vaccine

None of the three ferrets produced serum HI antibody after inoculation with 200 i.u. of A/HK/68 vaccine and all were susceptible to challenge infection with A/HK/68 virus. Thus, virus was recovered from nasal washings collected 3 days after infection (gmt = $10^{5.05}$ EID 50/ml.) and all the animals produced serum HI antibody to A/HK/68 virus (Table 3). The animals also responded by producing

Table 3. *Response of ferrets to infection with A/HK/68 virus after immunization with* 200 *i.u. of A/HK/68 vaccine*

Ferret no.	Primary infection	Response to A/HK/68 infection: Virus yield (log_{10}EID50/ml)	Change in serum HI titre*: A/HK/68	A/PR/8/34	B/Vic	Change in nasal HI titre†: A/HK/68	A/PR/8/34	B/Vic
326	B/AA/66	5·16	< 10–1280	—§	30–< 10	< 5–30	—§	—
327		4·83	< 10–960	—	< 10–10	< 5–60	—	—
328		4·16 (4·83)‡	< 10–640	—	30–60	< 5–80	—	< 5–10
330		5·16	< 10–960	—	20–15	< 5–160	—	—
324	A/PR/8/34	4·50 (3·25)	160–640	240–240	—	< 5–7·5	—	—
329		2·83	120–960	480–240	—	—	—	—
331		3·50	60–1280	240–240	—	< 5–30	< 5–7·5	—
333		2·16	120–480	480–240	—	< 5–7·5	< 5–5	—
368	Nil	5·5 (5·05)	< 10–640	—	—	< 5–30	—	—
370		5·5	< 10–2560	—	—	< 5–320	—	—
371		4·16	< 10–960	—	—	< 5–60	—	—

* Titre before infection – titre 3 weeks after infection.
† Titre before infection – peak titre after infection.
‡ Mean titre of group.
§ < 10–< 10 (serum); < 5–< 5 (nasal washings).

a fivefold increase in nasal wash protein concentration, with a maximum mean concentration of 1·06 mg./ml. on day 5, and high titres of nasal HI antibody to A/HK/68 (Table 3, Fig. 1). The nasal antibody was first detectable on day 7, with maximum titres (1/30–1/320) on day 9, and was usually absent after day 11.

Ferrets immunized with A/HK/68 vaccine after infection with B/AA/66 virus

Ferrets previously infected with B/AA/66 virus failed to produce serum HI antibody to A/HK/68 after immunization with 200 i.u. of A/HK/68 vaccine and were found to be susceptible to challenge infection with A/HK/68 virus. Virus was recovered from nasal washings from all the animals in this group (gmt = $10^{4\cdot83}$ EID50/ml.) and they all produced serum antibody to A/HK/68 virus (Table 3). No increases in B/AA/66 serum HI titres were observed after the challenge infection. The concentration of protein in nasal washings increased threefold after infection, with maximum values seven days after infection (Fig. 1). HI antibody to A/HK/68 virus was found in nasal washings from all the ferrets in this group, with highest titres (1/30 to 1/160) on day 9 (Fig. 1, Table 3). Antibody to B/AA/66 virus was also detected in nasal washings collected on days 7 and 9 from one animal (F. 328).

Ferrets immunized with A/HK/68 vaccine after infection with A/PR/8/34 virus

All four ferrets previously infected with A/PR/8/34 virus produced serum HI antibody to A/HK/68 when immunized with 200 i.u. of A/HK/68 virus vaccine. The animals were, however, infected after challenge with A/HK/68 virus. Thus, reduced titres of virus were recovered from nasal washings (range = $10^{2\cdot16}$–$10^{4\cdot50}$ EID50/ml., gmt = $10^{3\cdot25}$ EID50/ml.) and fourfold or greater rises in serum HI titres to A/HK/68 were measured. No increases in A/PR/8/34 serum HI titres were observed after challenge infection. A twofold increase in protein concentration of nasal washings was measured on day 5, but the concentration fell to pre-infection values by nine days after infection (Fig. 1). Only three out of four ferrets produced detectable titres of A/HK/68 antibody in nasal washings collected after A/HK/68 infection, with peak titres on day 5 for two animals and day 7 for the other ferret (F. 331). Two of the ferrets also had low titres (1/5 and 1/7·5) of A/PR/8/34 HI antibody in nasal washings collected 5 and 7 days after A/HK/68 challenge infection (Table 3).

DISCUSSION

Ferrets previously infected with either influenza virus A/PR/8/34 or B/AA/66 were as susceptible to challenge infection with A/HK/68 virus as normal ferrets, as measured by titre of virus recovered from nasal washings and production of serum HI antibody. The heterotypic protection produced by live influenza virus infection of mice, as measured by reduction in lung consolidation (Schulman & Kilbourne, 1965), was not observed in the experiment. However, the interval between the two infections in mice was only 4 weeks; in the present study the interval was 10–12 weeks. Other observations in this laboratory suggest that heterotypic immunity can be demonstrated in ferrets infected with a second virus 3 weeks after the first infection but that it disappears by 10 weeks after the first infection.

The local responses to challenge infection of the ferrets were found to be altered by previous infection in the present study. Thus, ferrets previously infected with B/AA/66 produced more nasal protein; an average peak concentration of 1·2 mg./ml. of protein was found in nasal washings from these animals after A/HK/68 infection, compared with a peak concentration of 0·7 mg./ml. produced by normal ferrets after infection. The cause and significance of this increased amount of protein is not known, nor is it clear whether it is associated with more severe local symptoms, such as increased nasal congestion. The protein found in nasal washings from B/AA/66 infected ferrets is probably produced locally and does not arise by transudation of serum proteins across the nasal epithelium, since the titres of HI antibody in nasal washings were similar to those in normal animals infected with A/HK/68. Similarly, no B/AA/66 antibody was detected in the nasal washings of B/AA/66-convalescent ferrets after A/HK/68 challenge infection, although high titres were present in their sera. In contrast, heterotypic infection with A/PR/8/34 virus resulted in the almost complete absence of HI antibody to A/HK/68 virus in nasal washings collected after the challenge infection. The protein concentration of nasal washings was, however, similar in A/PR/8/34-immune ferrets compared with normal animals, although the peak was reached earlier.

A striking result of previous infection of ferrets with A/PR/8/34 virus was the response of the animals to immunization with 200 i.u. of inactivated A/HK/68 virus vaccine. These animals produced serum HI antibody to A/HK/68, in contrast to normal or B/AA/66-infected ferrets which did not produce detectable serum HI antibody after immunization. Challenge infection of these ferrets showed that immunization had produced partial immunity to A/HK/68 infection, as shown by a 10–1000-fold reduction in the titres of virus recovered from nasal washings. The titre of A/HK/68 HI antibody in nasal washings was also reduced in these ferrets; however, this may have been a result of the previous A/PR/8/34 infection, since non-vaccinated A/PR/8/34-convalescent animals also had lower titres of nasal wash antibody than normal ferrets infected with A/HK/68 virus. The source of the low titres of A/PR/8/34 HI antibody in the nasal washings from vaccinated A/PR/8/34-infected ferrets is not known. Although all nasal wash specimens were negative when tested for occult blood, transudation of some antibody from the serum cannot be completely discounted (Shore, Potter & McLaren, 1972).

The results indicate that previous infection with a heterotypic influenza A virus has 'primed' the immunologic system of the animal to respond to subsequent immunization with A/HK/68 vaccine. Other studies in ferrets and hamsters have shown that an HI antibody response to inactivated influenza virus vaccine can be potentiated by previous infection with a wide range of heterotypic influenza A viruses (McLaren & Potter, 1973; Potter, Jennings, Marine & McLaren, 1973; Jennings & Potter, 1973). The mechanism involved in this priming is not known, but may involve the trapping of common carrier proteins by cells primed by the initial infection (Fazekas de St Groth & Webster, 1966; Webster, 1966; Dixon & Maurer, 1955). The nature of the hypothetical carrier protein is not yet known, but it may be one of the antigens common to influenza A viruses, such as the matrix

antigen or the ribonucleoprotein. Alternatively part of the haemagglutinin molecules, other than the haemagglutinating site, might be acting as a carrier protein.

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A field study of some swimming-pool waters with regard to bacteria, available chlorine and redox potential

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SUMMARY

The waters of one indoor bath and three outdoor baths were examined once an hour during 3 days (bath 1) or 6 days, for available chlorine, redox potential, permanganate number, ammonium, nitrate and total nitrogen, total bacterial count at 22° C., total bacterial count at 37° C. and faecal coliform bacteria. The weather, number of swimmers and the chlorine gas addition were continuously registered, and the pH was checked a few times at each bath. In bath 1, an indoor pool with aluminium sulphate precipitation about once a week and with sand filters back-washed every 2 days, less than 10 bacteria/ml. were found in all samples. In bath 2, an outdoor pool with aluminium sulphate precipitation twice a week and with sand filters back-washed twice a week, also few bacteria were found. In bath 3, an outdoor pool with only filtering through sand filters back-washed about every 14 days, high bacterial counts were found every day except the first, when the filters had been newly back-washed. In bath 4, an outdoor pool with only filtering through sand filters back-washed about once a week, high bacterial counts were found now and then during the first 4 days when the weather was warm, but few bacteria were found the last 2 days when the weather was cold and windy, and there were few swimmers.

Values from different analyses on the same sample showed relatively good correlation between the redox potential and the free available chlorine. In bath 3 both the redox potential and the available chlorine were weakly correlated to the bacterial count, but in bath 4 there was no such correlation. No other factors were well correlated with the bacterial count either.

The bacterial counts at 22° and 37° C. were of the same order. No faecal coliforms were ever found. Use of these bacteria as indicator organism in swimming pools is criticized.

The method of using certain minimum values of the free available chlorine as guarantee for a satisfactory bacteriological quality of the swimming pool water is also questioned. The degree of purity of the water is fundamentally connected with the disinfecting power of the available chlorine.

Use of certain minimum values of the redox potential, according to these investigations, seems to be a method of somewhat greater accuracy. Provided that the methods of precipitation are performed correctly and filters are being back-washed often enough, then an automatically registering redox potential device,

perhaps connected to the chlorine gas pump, ought to constitute a good control of the hygienic quality of a swimming-pool water. This must, however, always be completed by bacteriological examinations, preferably made at high bathing load.

INTRODUCTION

The importance of available chlorine and redox potential as indicators of the kill of *Escherichia coli* has previously been studied in laboratory experiments (Victorin, Hellström & Rylander, 1972). Different chlorine compounds were tested in chlorine demand free water. The reduction of bacteria within 3 min. was relatively well correlated with both available chlorine and redox potential for each pure chlorine compound, but the available chlorine needed for total kill was about 10 times higher for inorganic chloramines than for free available chlorine (hypochlorite). The organic chlorine compound chloramine T in its turn needed about 10 times the amount of available chlorine than did mono- or dichloramine. The variations in redox potential for the same degree of reduction of bacteria with different chlorine compounds were much smaller. The conclusion was drawn that in water containing unknown proportions of free, inorganic combined, and organic combined residual chlorine, the measuring of the redox potential of the water ought to be a much surer way of establishing the disinfecting properties of the water than measuring of total available chlorine.

An examination of redox potential and bacterial count in an indoor swimming pool has been made (Carlson, Hässelbarth & Mecke, 1968). They tested the water once an hour during 1 day, and also once a day during 5 weeks. During the day test the redox potential was held on 700 mV. by controlled chlorine gas addition, whereupon a hygienically excellent water was obtained. During the 5-week test it was stated that every impairment in the water's disinfecting capacity was reflected in a lowering of the redox potential. The indoor pool had excellent water purification by continuous aluminium sulphate addition and daily back-washings of the sand filters, and the bacterial counts were always very low. In an outdoor bath the variations in the chlorine demand of the water are much greater than in an indoor bath. Systems of water purification with differing efficiencies also give different amounts of residues. It was therefore decided to test whether the conclusions from earlier laboratory experiments and from Carlson's (Carlson *et al.* 1968) work hold true even of outdoor baths with less efficient purification. Repeated samplings during a rather long period of time were undertaken in order to obtain variations in weather and bathing load.

THE SCOPE OF THE INVESTIGATION

Four different swimming pools were investigated, one indoor bath and three outdoor baths of different sizes. In each bath samples were taken once an hour from the water leaving the pool before entering the purification plant. The samples were analysed with regard to available residual chlorine, redox potential, permanganate number, ammonia, nitrate, total nitrogen, total bacterial count at 22° C., total bacterial count at 37° C. and faecal coliform bacteria at 44° C. The

Table 1. *Description of the swimming pools*

	Bath 1 (indoor)	Bath 2 (outdoor)	Bath 3 (outdoor)	Bath 4 (outdoor)
Size ...	33·3 × 12 m	50 × 21 m	37 × 12 m	25 × 12·5 m
Depth	0·4–4·5 m	1·2–1·8 m	0·4–4·5 m	0·9–1·4 m
Mode of water circulation	In from both short sides. Out through hole in the bottom and over skiboards	In from one short side. Out at opposite side + ski-boards	In from one long side + 'cascade' at one short side. Out over ski-boards	In from one short side. Out at opposite side
Total volume of water*	1800 m³	5500 m³	1200 m³	380 m³
Theoretical water circulation time	3 times per 24 hr.	4 times per 24 hr.	3 times per 24 hr.	3 times per 24 hr.
Sand filter area	30 m²	120 m²	14 m²	12 m²
Filter washings	Every other day	Twice a week	Every other week	Once a week
Aluminium sulphate precipitation	2–8 times a month	Twice a week	None	None
pH	7·2–7·5	7·2–7·5	7·4–7·8	7·5–8·0
Filters washed	On day 1	3 days before and on days 3 and 6	One day before	One day before
Treated with aluminium sulphate	On day 1	2 days before and on day 4	—	—

* This includes water in the bath and in the purification plant.

pH was checked a few times at every bath. The chlorine gas addition was continuously reported. The weather was noted, as was the total number of bathers every day. The indoor bath was analysed for 3 days, and the outdoor baths for 6 days. All baths were continuously filtered and chlorinated, with regular cleansing of the filters by backwashing. Water in two baths was subjected at intervals to treatment with aluminium sulphate: two baths were not so treated.

Table 1 describes the baths and the schedules of water treatment.

METHODS

The available chlorine was analysed both with orthotolidine and with DPD ferrous titrimetric method according to Palin (Taras, Greenberg, Hoak & Rand, 1971). In this laboratory it has been shown earlier that these methods do not give equal results in waters containing inorganic and organic interfering substances, but the DPD method and amperometric titration give about the same values (K. Victorin, unpublished data). Amperometric titration is considered more accurate by the author, but as the DPD titrimetric method is faster and easier to perform in field operation, that method was used. It was shown in this investigation that the orthotolidine method gave values from 30 to 100 % of the values obtained with the DPD method both for free and combined available chlorine. The values measured with DPD are shown in the figures.

The redox potential was measured on a Radiometer pH meter with a platinum electrode and against a calomel electrode as standard, which has a normal potential of +244 mV. at 25° C. The value was measured after the stabilization of the electrode, 5–10 min. The platinum electrode was stored overnight in ascorbic acid.

Ammonium was measured with direct nesslerization without prior treatment of the samples because analysis of ammonia in chlorinated water must be performed immediately. The values are therefore somewhat unreliable.

Total nitrogen was measured by oxidation to NO_3^- with aluminium persulphate under pressure, reduction to NO_2^- through a cadmium–mercury column and spectrophotometry analysis of the nitrite.

Nitrate was measured both directly with brucine and by reduction through a cadmium–mercury column and measurement of the formed nitrite. The method of reduction by cadmium gave 30–100 % of the values obtained with brucine, with the greatest differences in baths 3 and 4. The cadmium reduction method was regarded as more reliable than the brucine method by the author for swimming-pool water. Analysis of total nitrogen and nitrate by the cadmium reduction method was performed 0–2 months after the collection. The samples had been treated with 5 ml. of 4 M-H_2SO_4 per 100 ml., but the composition of the samples stored for the longest period of time might have changed somewhat. The nitrogen analyses are thus subject to a certain degree of uncertainty, but the potential error is not so large as to prevent the use of the values obtained as background material for discussion. They are presented as means for each pool, with the range of variation included.

Table 2. *Mean values and limits of variations for values of ammonium-nitrogen, nitrate-nitrogen and total nitrogen*

	Bath 1	$\bar{x}$	Bath 2	$\bar{x}$	Bath 3	$\bar{x}$	Bath 4	$\bar{x}$
NH_4^+-N	0·1–0·3	0·15	0–1·8	0·5	0–1·6	0·3	0·8–2·2	1·4
NO_3^--N	3·1–3·8	3·2	2·6–4·6	3·4	2·1–3·2	2·6	4·4–5·2	4·7
Tot-N	2·8–5·0	3·2	2·6–10·6	4·9	2·1–10·6	3·8	4·4–7·8	5·6

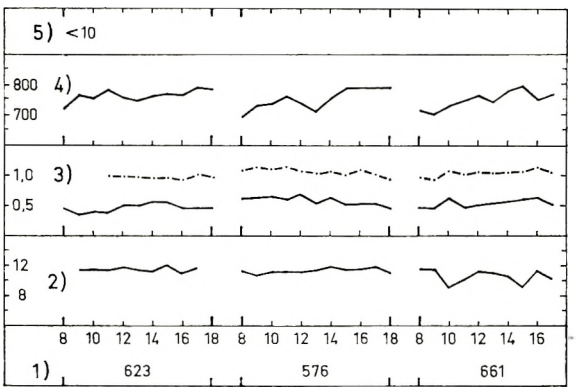

Fig. 1. Analytical data from bath no. 1 during 3 days. (1) Number of swimmers during 1 day. (2) Permanganate number, mg./l. (3) Free (—) and total (–·–·) available chlorine, mg./l. (4) Redox potential, mV. (5) Bacteria/ml., 37° C.

Collection of the samples was made from a drain cock before the water entered the purification plant in all baths except bath number 4, where the samples were taken directly from the pool at the outflow side.

RESULTS

The nitrogen values are shown in Table 2 and other results in Figs. 1–4.

In Figs. 1–4 the total bacterial count at 37° C. is shown. Less than 10 bacteria per ml is considered equal to 0. The standard plate count at 22° C. was about 75–125 % of the standard plate count at 37° C. No faecal coliforms at 44° C. were ever found.

The variations in the number of swimmers are not shown in the figures. In sunny weather the number was greatest between about 1 and 2 p.m. When the weather was cloudy and not so many people came to the pool, the number was usually highest in the morning and in the late afternoon.

The correlation between some variables analysed is shown in Table 3, in which it can be seen that the redox potential is relatively well correlated to the free available chlorine except in bath 1, where the variations were small. The redox potential and the free available chlorine were weakly correlated to the bacterial count in bath 3, but not at all in bath 4. The total nitrogen was weakly correlated to the bacterial count, but the permanganate number was not. Total nitrogen and permanganate number were not correlated to each other within each bath, but calculated for the values from all baths, a weak correlation was achieved.

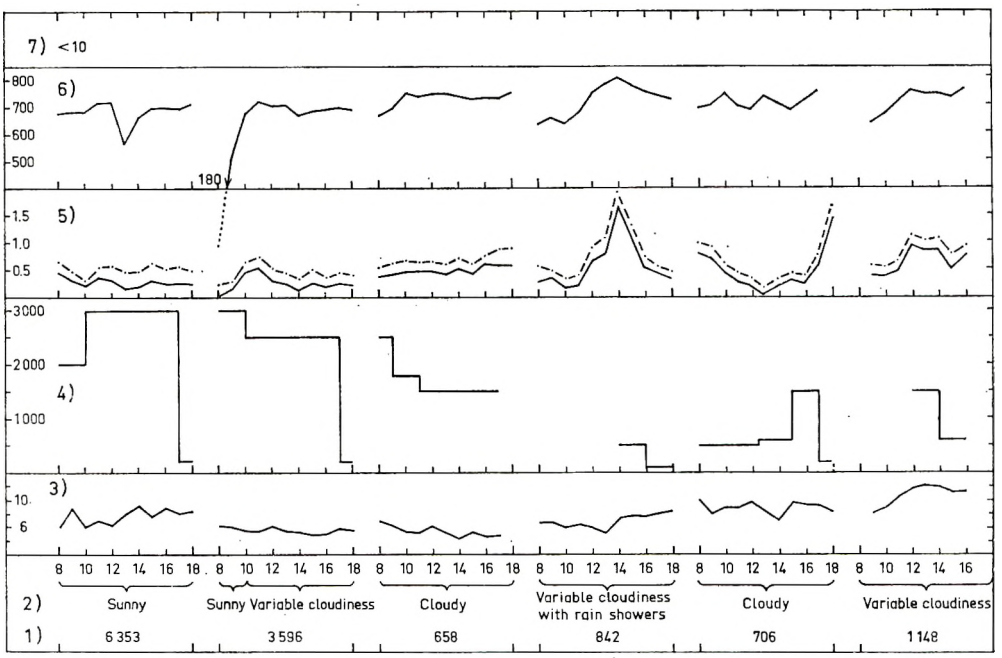

Fig. 2. Analytical data from bath no. 2 during 6 days. (1) Number of swimmers during one day. (2) Weather. (3) Permanganate number, mg./l. (4) Chlorine gas addition, g./hr. (5) Free (—) and total (–·–·) available chlorine, mg./l. (6) Redox potential, mV. (7) Bacteria/ml., 37° C.

DISCUSSION

Probably in most countries the hygienic quality of public swimming-pool water is controlled by bacteriological examinations performed by health authorities. These can be made very infrequently, however. The amount of disinfectant in the water is checked by the pool manager every day. When using chlorine as a disinfectant effort is made to attain certain minimum values of the free available chlorine. In Sweden it is professed that the hygienic control of so called break-point chlorinated water can be made exclusively with continuous measurement of the free available chlorine. This should then be $\geqslant 0{\cdot}4$ mg./l. at pH 6·5–7·5, $\geqslant 0{\cdot}8$ at pH 7·5–8·5 and $\geqslant 1{\cdot}5$ at pH > 8·5. Whether any of the four swimming-pool waters examined can be called break-point chlorinated is however doubtful. The water in bath number 3 is closest to this theoretical designation, but whether water is really break-point chlorinated or not ought not to be very interesting from a theoretical hygienic point of view, as long as the free available part of the total available chlorine can be measured. In bath number 1 the free available chlorine was $\geqslant 0{\cdot}4$ mg./l. in 91 % of the samples, and no bacteria were found. In bath number 2 the free available chlorine was less than 0·4 mg./l. in 53 % of the samples, but still no bacteria were found. In bath number 3 the free available chlorine was higher than 0·8 mg./l. in 82 % of the samples, but more than 100 bacteria/ml., 37° C. were still found in 55 % of the samples. (In Sweden a swimming-pool water with more than 100 bacteria/ml., 35° C. is not desirable.) In bath number 4 the

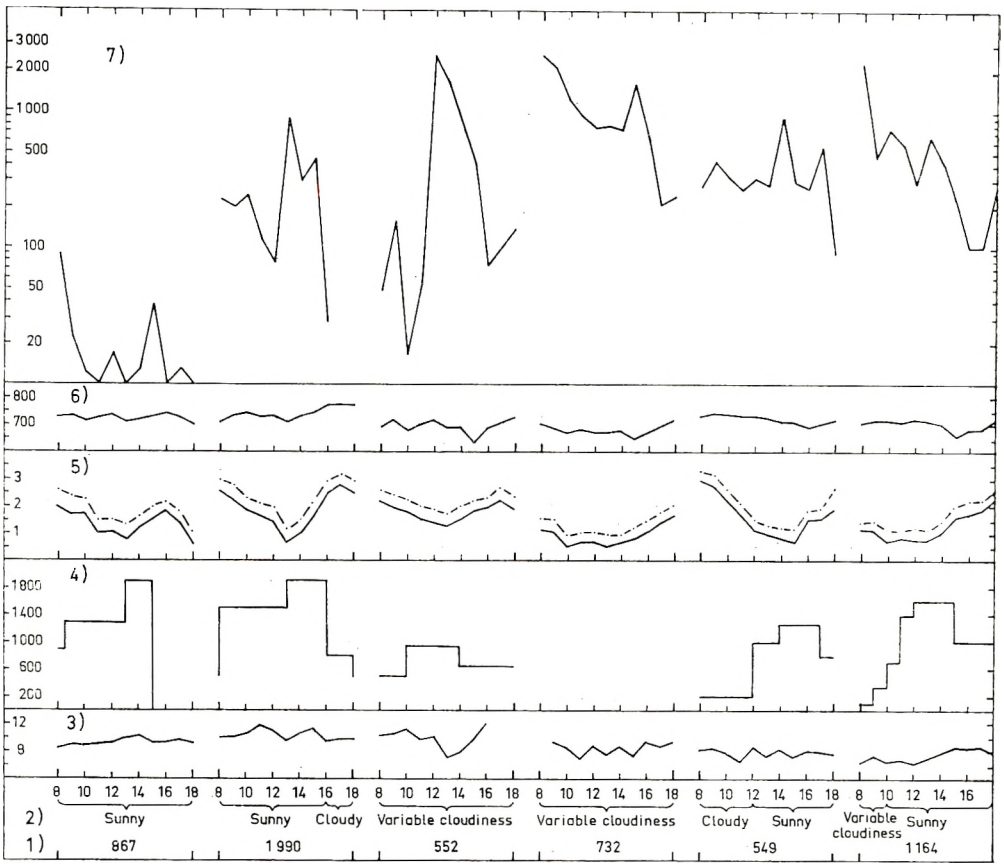

Fig. 3. Analytical data from bath no. 3 during 6 days. (1) Number of swimmers during one day. (2) Weather. (3) Permanganate number, mg./l. (4) Chlorine gas addition, g./hr. (5) Free (——) and total (–·–·) available chlorine, mg./l. (6) Redox potential, mV. (7) Bacteria/ml. 37° C.

free available chlorine was lower than 0·8 mg./l. in 95 % of the samples, and in 16 % of the samples more than 100 bacteria/ml., 37° C. were found.

Thus according to these examinations the free available chlorine gives a poor estimation of the number of bacteria in the water. The minimum values of the free available chlorine practised in Sweden are not satisfactory for all baths.

In Germany there are now official recommendations that a swimming-pool water should have a redox potential of $\geqslant$ 700 mV. at pH $<$ 7·5 and $\geqslant$ 730 mV. at pH $>$ 7·5. The same values have been proposed in Sweden. In the present investigation in bath 1, 97 % of the samples had a redox potential of $\geqslant$ 700 mV., and no bacteria were found. In bath, 2, 60 % of the samples were $\geqslant$ 700 mV., and no bacteria were found. In bath 3, 24 % of the samples were $\geqslant$ 730 mV, and, of these 16 samples, 8 contained $\geqslant$ 100 bacteria/ml., 37° C. In bath 4 the redox potential was never as high as 730 mV.

In short, it cannot be claimed that a high redox potential could function as an absolutely sure yardstick of the disinfecting power of swimming-pool water. But according to the results of this investigation, the error is less than that experienced when using free available chlorine as a standard.

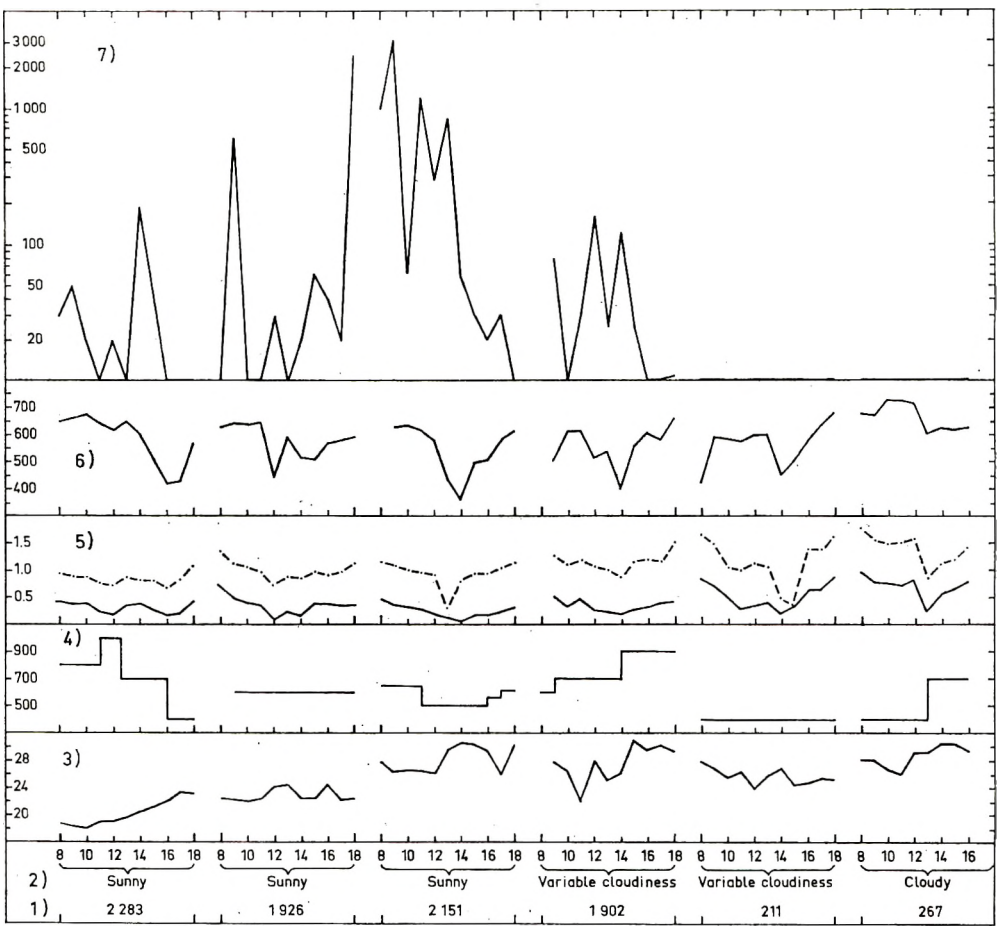

Fig. 4. Analytical data from bath no. 4 during 6 days. (1) Number of swimmers during 1 day. (2) Weather. (3) Permanganate number, mg./l. (4) Chlorine gas addition, g./hr. (5) Free (——) and total (-·-·-) available chlorine, mg./l. (6) Redox potential, mV. (7) Bacteria/ml. (37° C.)

Table 3. *Coefficient of correlation* (r) *for some values simultaneously analysed*

	$\frac{\text{Free } Cl_2}{\text{redox}}$	$\frac{\text{Tot. } Cl_2}{\text{redox}}$	$\frac{\text{Free } Cl_2}{\text{tot. bact. 22° C.}}$	$\frac{\text{Tot. } Cl_2}{\text{tot. bact. 22° C.}}$	$\frac{\text{Redox,}}{\text{tot. bact. 22° C.}}$	$\frac{KMnO_4,}{\text{tot. bact. 22° C.}}$	$\frac{\text{Tot. N}}{\text{tot. bact. 22° C.}}$	$\frac{KMnO_4,}{\text{tot N}}$
Bath 1	−0·28	−0·16	—	—	—	—	—	+0·01
Bath 2	+0·49	+0·47	—	—	—	—	—	−0·58
Bath 3	+0·49	+0·47	−0·39	−0·45	−0·32	−0·31	+0·20	−0·19
Bath 4, days 1–4	—	—	+0·09	+0·08	+0·11	+0·01	+0·39	—
Bath 4, days 1–6	+0·54	+0·52	—	—	—	—	—	+0·09
Baths 3–4, day 4	—	—	−0·08	−0·13	+0·12	−0·13	+0·15	—
All baths	+0·39	+0·26	—	—	—	—	—	+0·24

The values from days 1 and 2 in bath 2 are interesting. The free available chlorine was less than 0·4 mg./l. in 19 samples out of 22, and the redox potential less than 700 mV. in 15 samples out of 22. The number of swimmers was very high, and the weather sunny and warm. During the night between days 1 and 2 the chlorine tube ran out of gas and the available chlorine and the redox potential of the water were not normal again until 10 o'clock on day 2. Despite this, less than 5 bacteria/ml. were found in the water. This can be compared with bath 3, where relatively few bacteria were found on day 1, but high bacterial counts were found on the other days, although the available chlorine was considerably higher than in the other baths. In bath 4 no bacteria were found the 2 last days when the weather was cloudy and the number of swimmers low.

No definite conclusions can be drawn from an investigation of this restricted volume, but there seems to be a difference between those baths (1 and 2) using aluminium sulphate precipitations + sand filter and those (3 and 4) using only sand filters. In both baths 3 and 4 the filters had been back-washed on the day before the investigation, and the bacterial count was lower on day 1 than on the following days. This indicates that the efficiency of the water purification system is of as great an importance for the bacteriological state of the water as is the mere disinfection process. The nitrogen values did not increase significantly during the week in any baths. The permanganate number did not increase either during the week in baths 1, 2 or 3, but it increased in bath 4, where the permanganate number from the start was already significantly higher than in the other baths. This probably depends on a more efficient water purification in baths 1 and 2, and an oxidative destruction of organic compounds by the high available chlorine in bath 3.

If the efficiency of the water purification is of such importance as the present investigation indicates, the high bacterial counts probably can be explained by the formation of particle clusters containing bacteria surrounded by a protective coat, so that the chlorine cannot reach the bacteria.

No classification of the bacteria was made. It is interesting though that the bacterial count at 22° C. was about the same as that at 37° C. This contradicts the possibility that 'normal flora' exist in the swimming pools.

It is further noted that faecal coliforms were not found in baths 3 and 4 even when high counts of bacteria growing at 37° C. occurred. This suggests that faecal contamination was effectively controlled, but reinforces the criticisms raised by various experts that faecal coliform organisms may not be the best indicators of the numbers of all organisms derived from the human body during swimming.

The main reason for performing this investigation was to test whether the redox potential could be used instead of measurement of the available chlorine to estimate the bacteriological quality of swimming-pool water. Available chlorine is obviously not reliable in all waters, and in some cases not even a high amount of free available chlorine necessarily guarantees a germ-free water (bath 3). Considering that in most swimming pools probably only the total available chlorine is being measured (Black *et al.* 1970), the reliability may be very low if repeated bacteriological examinations are not performed. The proposed method of using

certain minimum values of the redox potential of the water as guarantee for hygienic safety, however, seems to involve a lesser degree of uncertainty than the available chlorine does. Neither does a high redox potential necessarily mean a germ-free water in all cases, however, according to the present investigation. The efficiency of the water purification system is probably decisive for the disinfective effect of the available chlorine. If filters are back-washed often enough and coagulants are dosed correctly, then an automatic, continuously registering redox potential device, perhaps connected to the chlorine dosing pump, ought to constitute a good control of the hygienic quality of the water. Even so, bacteriological examinations of the water must be performed. These should preferably be made at high bathing load.

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The local antibody response to R.S. virus infection in the respiratory tract

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SUMMARY

Nasopharyngeal secretions were taken during the acute phase of illness from 66 infants and children admitted to hospital with lower respiratory tract infections. Second secretions were taken, after an interval of 7 days, from 33 of these patients. A significant increase in neutralizing activity to R.S. virus was demonstrated in the nasopharyngeal secretions of patients in response to severe R.S. virus infection. Seventeen out of 25 patients (68%) with R.S. virus infections developed a rise in secretory neutralizing titre, compared with only 1 out of 8 patients (13%) with respiratory infections not involving R.S. virus.

A high titre of secretory neutralizing activity was found more often in the acute phase of illness in patients with R.S. virus infections, especially bronchiolitis, than in patients with respiratory infections not involving R.S. virus. Fifteen out of 34 patients (44%) with R.S. virus bronchiolitis were found to possess a neutralizing titre of 1/4 or more in their first secretions, compared with 4 out of 12 patients (33%) with R.S. virus infections other than bronchiolitis and 3 out of 20 patients (15%) with respiratory infections not involving R.S. virus.

A quantitative analysis of the immunoglobulins present in the secretions indicated that IgA was the only immunoglobulin consistently present at a detectable concentration. The geometric mean values of IgA, IgM and IgG in the secretions examined were found to be 22·3 ,4·3 and 5·3 mg./100 ml. respectively.

The neutralizing activity against R.S. virus, present in the secretions, was shown to be due to specific IgA antibody. This was accomplished by removing the neutralizing activity in two secretions by absorption with anti-IgA serum.

INTRODUCTION

Respiratory syncytial (R.S.) virus has been shown to be the chief viral pathogen in respiratory infections of infancy and the one most frequently associated with bronchiolitis (Chanock *et al.* 1961; Gardner, 1968). Severe infection with this virus occurs predominantly in infants between 2 and 6 months of age. The moderate to high levels of maternally derived serum antibody, present in the sera of infants of this age, does not appear to protect against infection. In spite of attempts to produce an inactivated vaccine (Kapikian *et al.* 1969; Kim *et al.* 1969*a*) the problem of prevention of infection with this virus remains unsolved. In fact these studies

with inactivated vaccine have shown that those who have been given vaccine fare worse than those without, on natural infection with R.S. virus, despite the production of humoral antibody.

Many workers' attention is now turning to studies on the importance of local secretory antibody in resistance to infection by respiratory viruses; these include studies on influenza A_2 (Alford, Rossen, Butler & Kasel, 1967), rhinovirus type 13 (Perkins *et al.* 1969) and parainfluenza type 1 (Smith, Purcell, Bellanti & Chanock, 1966).

The present study was initiated to investigate the role of local antibodies in R.S. virus infection of infancy. In a recent study of experimental R.S. virus infection of adult volunteers, Mills, Van Kirk, Wright & Chanock (1971) reported that resistance to infection with R.S. virus was correlated with high titres of nasal wash antibody, but not with the titre of serum antibody. It is not known, as yet, whether the same is true for natural R.S. virus infection of infants and young children. However, the development of local neutralizing response to R.S. virus in nasal secretions of infants with natural infection has been demonstrated by workers both in the U.S.A. (Kim *et al.* 1969*b*) and in this country (Scott & Gardner, 1970).

An important aim of the present study was to confirm and expand the previous results and to correlate the different categories of acute respiratory illness with local antibody response. This may be of particular importance with respect to the various views expressed on the pathogenesis of R.S. virus (Chanock *et al.* 1970; Gardner, McQuillin & Court, 1970).

Another aim was to study the immunoglobulin content of nasopharyngeal secretions of infants with severe respiratory infection. A number of investigations have shown that the predominant immunoglobulin in adult nasal secretions is IgA (Remington, Vosti, Lietze & Zimmerman, 1964; Rossen, Schade, Butler & Kasel, 1966). Moreover, antibody activity in adult nasal secretions, following various rhinovirus, influenza and parainfluenza infections, has been predominantly in the IgA fraction (Cate *et al.* 1966; Alford *et al.* 1967; Smith, Bellanti & Chanock, 1967; Perkins *et al.* 1969). There have been few parallel studies for the immunoglobulins of nasal secretions of infants and young children (Haworth & Dilling, 1966; Cohen, Goldberg & London, 1970). Cohen, Goldberg and London reported that the predominant immunoglobulin in the nasal secretions of 34 healthy infants was IgG, whereas IgA was found to be predominant in the nasal secretions of 19 infants with acute respiratory infections.

A further aim of the present study was to obtain a quantitative estimate of the concentrations of immunoglobulins in the secretions of infants with severe respiratory infections. We also wished to determine whether the neutralizing activity in the secretions was due to antibody and, if so, which particular class of immunoglobulins was responsible.

MATERIALS AND METHODS

Patients

The patients included in this study were children between 2 weeks and 2 years of age, who were admitted to hospital with acute lower respiratory tract infections

The categories of clinical illness used have been previously defined (Gardner *et al.* 1960). A nasopharyngeal secretion was obtained from the patient within 24 hr. of admission to hospital; the onset of illness was rarely more than 48 hr. before the first specimens were taken. Whenever possible, a second secretion was obtained from the patient after an interval of approximately 7 days. The method of collection and preparation of secretions has been fully described elsewhere (Sturdy, McQuillin & Gardner, 1969; Scott & Gardner, 1970).

Estimation of neutralizing activity

The neutralizing activity in nasopharyngeal secretions was measured by a modification of the R.S. virus plaque reduction technique, first described by Coates, Alling & Chanock in 1966 and described in detail elsewhere (Scott & Gardner, 1970). The protein content of the secretions was measured by a microtechnique based on the original method of Lowry, Rosebrough, Farr & Randall (1951), and the neutralizing titres of the secretions were adjusted to a protein level of 10 mg./ml.

Estimation of immunoglobulin content

The concentrations of IgA, IgM and IgG in the secretions were determined quantitatively by radial immunodiffusion in agar (Mancini, Carbonara & Heremans, 1965). The assay of each immunoglobulin was carried out in Hyland Immunoplates. It must be emphasized that sera, and not secretions, of known immunoglobulin content were used as standards in all tests.

Removal of IgA by precipitation with specific antiserum

The method used for the removal of IgA from the secretions was based on the technique employed by Fireman, Vannier & Goodman (1963). Antiserum against human IgA produced in goats (Hyland Laboratories) was added to an equal volume of secretion. The mixture was incubated at 37° C. for 2 hr., and subsequently at 4° C., for 18 hr.; it was then centrifuged at 2000 rev./min. for 2 hr., after which the supernatants were tested for the presence of IgA.

RESULTS

Antibody response in nasopharyngeal secretions

Nasopharyngeal secretions taken from 66 patients, within 24 hr. of admission to hospital, were examined for the presence of neutralizing activity to R.S. virus. Second secretions were taken approximately 7 days after admission from 33 of these patients, of whom 19 had R.S. virus bronchiolitis, 6 had R.S. virus infections other than bronchiolitis (bronchitis and pneumonia), and 8 had acute respiratory infections (bronchiolitis, bronchitis and pneumonia) not associated with R.S. virus (Table 1). Eleven of the 19 patients (58 %) with R.S. virus bronchiolitis developed a rise in titre of neutralizing activity in their second secretions compared with their first (both adjusted to 10 mg./ml. protein). All 6 patients with R.S. virus infections other than bronchiolitis developed a rise in titre. A rise in nasal secretory neutralizing activity was detected, therefore, in 17 out of 25 patients (68 %)

Table 1. *Neutralizing activity to R.S. virus in paired nasopharyngeal secretions of infants and children with severe respiratory infection*

Clinical category	No. in group	Average age (weeks)	No. of rises in titre of neutralizing activity to R.S. virus	No. of 4-fold rises in titre of neutralizing activity to R.S. virus
R.S. virus bronchiolitis	19	14	11 (58%)	7 (37%)
R.S. virus infection other than bronchiolitis	6	23	6 (100%)	2 (33%)
Respiratory infections not involving R.S. virus	8	25	1 (13%)	0

Table 2. *Neutralising activity to R.S. virus in first nasopharyngeal secretions of infants and children with severe respiratory infection*

Clinical category	No. in group	Average age (weeks)	No. of secretions with neutralizing titres of 1/4 or greater
R.S. virus bronchiolitis	34	15	15 (44%)
R.S. virus infections other than bronchiolitis	12	23	4 (33%)
Respiratory infections not involving R.S. virus	20	36	3 (15%)

with R.S. virus infections, 9 of these rises were 4-fold or greater, and a further 4 were 3-fold. In the group of 8 patients with respiratory infections not associated with R.S. virus, 1 patient (13%) developed a rise in neutralizing activity, which was 2-fold.

Antibody in first secretions

A neutralizing titre of 1/4 or greater was detected in the first secretions of 15 out of 34 patients (44%) admitted with R.S. virus bronchiolitis (Table 2). This compared with 4 out of 12 patients (33%) in the group of patients with R.S. virus infections other than bronchiolitis (bronchitis, pneumonia and croup). Only 3 of the 20 patients (15%) with respiratory infections not associated with R.S. virus (bronchiolitis, bronchitis, pneumonia and croup) were found to possess a neutralizing titre of 1/4 or greater in their first secretions. The differences between these three groups of patients were further emphasized by comparing the geometric mean reciprocal neutralizing titres in their first secretions. These were found to be 4·1, 2·8 and 2·7, respectively. The average ages of the three groups were 15, 23 and 36 weeks (Table 2), which makes these findings even more surprising; it might be assumed that older age groups have had an increased chance of exposure to R.S. virus antigen.

Table 3. *Geometric mean immunoglobulin content of nasopharyngeal secretions of infants and children with severe respiratory infections*

	Geometric mean immunoglobulin content (mg./100 ml.)					
	First secretion			Second secretion		
Clinical category	IgA	IgM	IgG	IgA	IgM	IgG
R.S. virus bronchiolitis	23·1	3·1	4·5	27·4	4·2	3·4
R.S. virus infections other than bronchiolitis	19·7	4·1	7·7	25·7	3·8	5·5
Respiratory infections not involving R.S. virus	27·7	7·9	10·7	9·3	4·7	2·6
All respiratory infections	23·2	4·4	6·5	20·6	4·2	3·5

Table 4. *Relationship of age to the geometric mean concentrations of immunoglobulins in the first nasopharyngeal secretions of infants and children with severe respiratory infections*

Age (months)	No. in group	Geometric mean concentrations of immunoglobulins (mg./100 ml.)		
		IgA	IgM	IgG
0–2	17	19·0	4·5	6·2
2–4	19	27·3	4·2	4·0
4–6	4	15·1	5·4	4·5
6–12	19	23·8	4·9	10·7
> 12	5	30·8	4·3	13·1

Standardization by IgA

In the present study, protein content was used to standardize the neutralizing titres of the secretions; others (Kim *et al.* 1969*b*) have used IgA content (10 mg./100 ml.). When IgA content (20 mg./100 ml.) was used for standardization in the present study the results were found to be similar to those obtained using protein standardization. Of patients with R.S. virus infections 73%, compared with 68%, developed a rise in secretory neutralizing activity in response to infection. After IgA adjustment, 30% of patients with R.S. virus bronchiolitis, 9% of patients with R.S. virus infections other than bronchiolitis, and 10% of patients with respiratory infections not associated with R.S. virus, were found to possess a neutralizing titre of 1/4 or greater in their first secretions. The corresponding figures obtained for these three categories using protein standardization were 44%, 33%, and 15%.

Immunoglobin content

The 66 first and 33 second secretions, previously examined for neutralizing activity to R.S. virus, were also quantitatively examined by radial immunodiffusion for concentration of IgA, IgM and IgG. The geometric mean values of immunoglobulins in the first secretions were 23·2 mg. IgA/100 ml., 4·4 mg. IgM/100 ml. and 6·5 mg. IgG/100 ml. (Table 3). Values of 20·6, 4·2 and 3·5 mg./100 ml. were obtained for the means of the three immunoglobulins in the second secretions (Table 3). No correlation was found between the clinical categories of illness,

whether caused by R.S. virus or not, and the concentration of immunoglobulins in either first or second secretions. The only exception to this was the abnormally low value obtained for the mean IgA concentration (9·3 mg./100 ml.) in the second secretions of patients with respiratory infections not associated with R.S. virus. The difference between IgA in first compared with second secretions was not considered to be significant, owing to the small number of readings obtained in this category. When the three groups of patients (Table 3) were considered separately, however, the geometric mean concentration of IgA was found to be slightly higher in second compared with first secretions, whereas the reverse was true for IgG.

IgA was detectable in 94·8% of secretions, compared with 58·5% for IgM and 65·5% for IgG, which further emphasized the predominance of IgA in secretions. Moreover, the range of values obtained for IgA was 0–64 mg./100 ml. (S.D. 1·49) compared with 0–47 mg. IgM/100 ml. (S.D. 3·77) and 0–188 mg. IgG/100 ml. (S.D. 4·54). The geometric mean concentrations of IgA and IgG, but not IgM, in the first secretions rose gradually with age (Table 4).

Neutralizing activity and immunoglobulins

Two first secretions, with known neutralizing activity, were absorbed with anti-IgA serum in order to establish whether the neutralizing activity was due to specific IgA antibody. The quantity of secretions available permitted absorption of only one immunoglobulin. IgA absorption was chosen, because previous immunoglobulin analysis had shown that IgA was the predominant immunoglobulin in the secretions. Each secretion was divided into two equal parts, one of which was treated with anti-IgA serum and the other with phosphate buffered saline (unabsorbed control): both were otherwise treated in exactly the same way. After absorption, both parts were tested for the presence of neutralizing activity to R.S. virus. Neutralizing titres of 1/16 and 1/4 were detected in the unabsorbed secretions, compared with titres of less than 1/4 in the two corresponding absorbed secretions. This indicates that the neutralizing activity to R.S. virus in the two secretions was due to specific IgA antibody.

DISCUSSION

In view of the ability of R.S. virus to cause severe illness in infancy in the presence of either natural or actively produced humoral antibody a rational approach to immune prophylaxis against infection would appear to be by way of local immunity. It has been shown in a study of experimental R.S. virus infection of adults (Mills *et al.* 1971), that resistance to infection is more closely related to high titres of nasal wash antibody than to serum antibody. The development of R.S. virus neutralizing activity in nasal secretions has been demonstrated in 11 out of 17 infants and children (65%) with natural R.S. virus infection of the lower respiratory tract (Kim *et al.* 1969*b*). Recent studies have shown that it is possible to induce a local immune response to R.S. virus infection in infants by means of a live attenuated vaccine (Kim *et al.* 1971). A 3-fold or greater rise in nasal secretory neutralizing activity to R.S. virus was induced in 15 out of 39 infants and children (38%).

The present study has also demonstrated a specific local immune response to

natural R.S. virus infection of infants and young children (Table 1). Seventeen out of 25 patients (68%) with R.S. virus infections developed a rise in secretory neutralizing titre to R.S. virus, compared with only 1 out of 8 patients (13%) with respiratory infections not associated with R.S. virus ($\chi^2 = 7{\cdot}53$; $0{\cdot}01 > P > 0{\cdot}005$). Nine of these 17 rises in titre were 4-fold or greater; a further 4 of the 17 rises were 3-fold. The only rise in titre in the group of patients with respiratory infections not associated with R.S. virus was a 2-fold rise. The results obtained for the development of secretory response were comparable with those of Kim and her colleagues (1969*b*). This was surprising as the interval between secretions in that particular study was 3 weeks, compared with approximately 7 days in the present study.

An important observation made in the present study was that neutralizing activity is more prevalent, during the acute phase of illness, in patients with R.S. virus infections, especially bronchiolitis, than in the patients with respiratory infections not involving R.S. virus (Table 2). There is a significant difference between the figures for patients with R.S. virus bronchiolitis compared with patients with respiratory infections not associated with R.S. virus ($\chi^2 = 4{\cdot}80$; $0{\cdot}05 > P > 0{\cdot}025$). The difference between these two groups was further emphasized by the fact that the average age of the second group was more than twice that of the R.S. virus bronchiolitis group (Table 2). The R.S. virus bronchiolitis group also showed the highest geometric mean reciprocal titres for first secretions.

The presence of neutralizing activity in the first secretions of patients with R.S. virus infections, especially bronchiolitis, could be a manifestation of the presence of non-specific viral inhibitors, the rapid development of nasal secretory antibody, or prior infection with R.S. virus. The first explanation would seem unlikely, as the results have shown a higher incidence of neutralizing activity in the first secretions of patients with R.S. virus bronchiolitis, than in patients with R.S. virus infections other than bronchiolitis and with respiratory infections not associated with R.S. virus. Furthermore, the results of the absorption experiments have indicated that the secretory neutralizing activity is due to IgA antibody. Rapid development of antibody in secretions can be proved only after a longitudinal study of antibody in secretions, collected before exposure to R.S. virus and frequently thereafter. This was not, however, within the scope of the present investigation. The acute onset of R.S. virus illness did not support the hypothesis of a rapid primary antibody response. It is possible, therefore, that these results are an indication of prior infection with R.S. virus. A hypothesis of prior infection would tend to support the theory of a type 1 allergic reaction (Gell & Coombs, 1968), which has been postulated to explain the pathogenesis of R.S. virus bronchiolitis in infants (Gardner *et al.* 1970).

The results of the immunoglobulin analysis indicated that IgA was the only immunoglobulin consistently present in the nasopharyngeal secretions. The geometric mean concentrations of IgA, IgM and IgG in the 99 secretions (first and second) examined were 22·3, 4·3, and 5·3 mg./100 ml. Similar values for the three immunoglobulins were found in both first and second secretions (Table 3). The geometric mean concentrations of IgA and IgG, but not IgM, tended to gradually increase with age (Table 4).

The IgA concentrations found in the present study were of the same order as those reported in two other studies, in which nasal secretions from infants and children were examined (Haworth & Dilling, 1966; Cohen *et al.* 1970). The same did not apply, however, to the concentrations of IgG in the secretions. Haworth and Dilling reported mean IgA values of 14·5 and 11·7 mg./ 100 ml. in two groups of patients with respiratory infections. Cohen, Goldberg and London showed that the mean concentrations of IgA and IgG in nasal secretions of 34 healthy infants were 22·8 and 55·7 mg./100 ml., compared with 46·6 mg. IgA/100 ml. and 42·5 mg. IgG/100 ml., in the secretions of a group of 19 infants with respiratory infections.

The same technique was used in all three studies for the estimation of immunoglobulin concentrations, although the method of obtaining secretions from patients differed. In addition, secretions of infants and children without respiratory infections were not included in the present study, because the method used for obtaining secretions was impracticable for these patients.

The final part of the present investigation was concerned with determining the nature of the neutralizing activity to R.S. virus detected in the secretions. It is very difficult to explain the results obtained (Tables 1, 2) on the basis of non-specific viral inhibitors, as the secretory neutralizing activity was distributed unevenly among the three groups of patients. It is equally improbable that the neutralizing activity is due to interferon, as the rises in neutralizing titre occurred at a time when antibody and not interferon would be increasing.

The secretory neutralizing activity, therefore, is probably due to specific R.S. virus antibody. In order to test this hypothesis the IgA content of two secretions was removed by absorption with anti-IgA serum and the secretions subsequently examined for neutralizing activity to R.S. virus. The choice of secretions was dependent on the amount available, and in both cases first secretions, with known neutralizing titres, were chosen. It was originally intended that all three immunoglobulins would be absorbed, but this proved impracticable owing to insufficient volume of the secretions. The present study has shown IgA to be the predominant immunoglobulin in the secretions (Table 3), as in a number of other investigations which have indicated that antiviral activity in adult nasal secretions resides in the IgA fraction (Cate *et al.* 1966; Alford *et al.* 1967; Smith *et al.* 1967). Therefore, the absorption studies were for IgA only, and it proved possible to remove the R.S. virus neutralizing activity completely from both secretions by absorption with anti-IgA serum. Further confirmation of this specificity has been provided by fluorescent antibody studies and reported elsewhere (Gardner, McQuillin & Scott, 1973).

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Cultivation of TRIC agents: a comparison between the use of BHK-21 and irradiated McCoy cells

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SUMMARY

BHK-21 cells and HeLa cells were as sensitive as irradiated McCoy cells for titrating both fast- and slow-growing strains of TRIC agents. BHK-21 cells were as efficient as irradiated McCoy cells for isolating TRIC agents from trachoma and from the genital tract of patients with non-gonococcal urethritis. More inclusions were formed in BHK-21 than in irradiated McCoy cells.

INTRODUCTION

Like some other *Chlamydia*, the agents of trachoma and inclusion conjunctivitis (TRIC agents) were first isolated in the chick embryo yolk sac, and this host is still used both for isolation and for growing the organisms in the laboratory. Many of the disadvantages of chick embryos can be avoided by using cell cultures. For isolation work, Gordon, Magruder, Quan & Arm (1963) pioneered the use of cell cultures by demonstrating that trachoma organisms from the conjunctiva of experimentally infected monkeys formed inclusions in McCoy cell cultures. Later the agent was isolated from patients with trachoma in McCoy cells previously exposed to gamma irradiation (Gordon & Quan, 1965). Irradiated McCoy cells were shown to be as sensitive as chick embryos to several TRIC agents that had been grown in yolk sacs or McCoy cell cultures (Gordon, Dressler & Quan, 1967); subsequently these irradiated cultures were reported to be more sensitive than eggs for the isolation of TRIC organisms from both human conjunctival and genital specimens (Gordon *et al.* 1969; Darougar *et al.* 1971; Gordon *et al.* 1972*b*), although Ford and McCandlish (1971) found that the isolation rates from urethral specimens were similar in eggs and in irradiated McCoy cells.

In the first direct comparison of unirradiated and irradiated cultures, Gordon *et al.* (1972*a*) showed that, from a given yolk sac suspension of a TRIC agent, more and bigger inclusions developed in irradiated than in unirradiated cultures of McCoy cells. By contrast, in the only reported comparison with a different cell line, unirradiated cultures of HeLa-229 cells proved to be as efficient as irradiated

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McCoy cells for isolation of TRIC agents (Kuo, Wang, Wentworth & Grayston, 1972); both sorts of culture were, however, treated with DEAE-dextran. We report here a comparison of the sensitivity of BHK-21 cells and McCoy cells, both irradiated and unirradiated, for isolating TRIC agents from clinical specimens and for titrating laboratory strains.

METHODS

TRIC agents

The following strains were used; the suffix *f* denotes a fast-killing variant (Taverne, Blyth & Reeve, 1964): yolk-sac (YS) pools of MRC-4 (Jones, 1961) and MRC-4*f* purified with M-KCl (Taverne & Blyth, 1971), and a 20 % YS suspension from the 25th egg passage of TW 3 (Wang & Grayston, 1963) kindly provided by Dr F. B. Gordon, Naval Medical Research Institute, Bethesda, Maryland.

Conjunctival specimens

Specimens were collected from Gambian children by Dr S. Sowa of this Unit, who also provided the relevant clinical and laboratory information. Scrapings, suspended in 1 ml. of phosphate-buffered saline (PBS), pH 7·4, containing 0·25 M sucrose, 10 % calf serum and 1000 μg. of streptomycin, were stored at $-70°$ C. Before inoculation of cell cultures the suspensions were treated in an ultrasonic bath at 0° C. for 30 sec. on stage 8 (Soniprobe Cleaning Tank, Type 1130/2A, Dawe Instruments Ltd.) and diluted with 3 ml. of the maintenance medium used for BHK-21 cells.

Urethral specimens

Specimens were taken from men with non-gonococcal urethritis attending St Thomas's Hospital, London, S.E.1. They were stored at 4° C., each in 1 ml. of PBS containing 0·25 M sucrose, 1 % bovine serum albumen, 500 μg. streptomycin and 25 units neomycin; if not used within 24 hr. they were stored at $-70°$ C. Before inoculation they were shaken vigorously for 30 sec. with glass beads in a mechanical shaker and then diluted with 3 ml. of McCoy cell maintenance medium.

Cell cultures

BHK-21 cells (Stoker & Macpherson, 1964) and HeLa cells (bought from Flow Laboratories Ltd.) were grown in 20 oz. glass bottles from Friday to Monday, when they were stripped off the glass, suspended in fresh growth medium and stored at 4° C. for the rest of the week. For use, cells were suspended in warm growth medium and seeded, 2×10^5 cells per tube, in flat-bottomed plastic tubes containing glass cover-slips 12 mm. in diameter. The tubes were incubated at 35° C. overnight in air with a 5 % CO_2 concentration maintained by Pardee's buffer (Bellett, 1960).

McCoy cells were obtained through the courtesy of Dr J. Treharne (Institute of Ophthalmology, London) and Mrs Phillipa Powis (St Thomas's Hospital, London, S.E.1.). They were shown to be mouse cells by chromosome analysis, for which we are grateful to Dr A. Whitaker, Wellcome Laboratories, Beckenham, Kent; this confirms the finding of Gordon *et al.* (1972*a*). Confluent cell layers in plastic

bottles (Falcon Plastics) were subjected to 4000 R. from a ^{60}Co source at the Imperial College of Science and Technology; after irradiation the medium was changed. Next day 2×10^5 cells were seeded onto cover-slips in flat-bottomed plastic tubes. These were then incubated at 35° C. in 5% CO_2 in air for 3–5 days. Cultures of unirradiated McCoy cells were used after incubation overnight.

Growth medium for BHK-21, HeLa and unirradiated McCoy cells used in titrations was Eagle's tissue culture medium (BHK) (Wellcome Reagents Ltd.) supplemented with 0·035% $NaHCO_3$, 10% tryptose phosphate broth (Difco Laboratories) and 10% calf serum (Flow Laboratories Ltd.).

Maintenance medium (MM) was growth medium without serum; the pH was adjusted to 7·5 with tris and HCl to a final concentration of 0·05 M tris. It contained streptomycin 200 μg./ml. and, when used with human specimens, neomycin 25 units/ml.

Growth and maintenance medium for irradiated McCoy cells used for isolation of TRIC agents was Eagle's MEM tissue culture medium (Wellcome Reagents Ltd.) supplemented with 0·15% $NaHCO_3$, 10% foetal bovine serum, 1% vitamins (MEM vitamins, Flow Laboratories Ltd.), 0·03% *l*-glutamine and 200 μg./ml. streptomycin. Neomycin 25 units/ml. was added after inoculation of clinical material.

Infectivity titrations in cell cultures

Yolk sac suspensions were diluted in the appropriate maintenance medium (MM) and 1 ml. volumes were centrifuged onto the cells in tubes in a swing-out head at 600 ***g*** for 30 min. at 35° C. Concentrations of organisms, when known, were adjusted to infect no more than 10% of the cells (less than 5 inclusions per high power field of the microscope) (Blyth & Taverne, 1972). For isolation from clinical specimens three cultures of BHK-21 and three of irradiated McCoy cells were inoculated each with 0·4 ml. volumes and centrifuged at 3500 ***g*** for 1 hr. at 35° C. After centrifugation, the medium was replaced by fresh MM and the cultures were incubated in 5% CO_2 in air at 35° C. for 3 days, or for 2 days when *f* strains were titrated. Cultures were then fixed and stained with iodine and the inclusions were counted; titres are expressed as the number of inclusion-forming units (IFU) per ml. of original suspension. With clinical specimens, if no inclusions were found in 100 microscope fields chosen at random, the whole cover-slip was examined before being recorded as negative.

RESULTS

Our preliminary experiments with McCoy cell cultures confirmed the observations of Gordon *et al.* (1972*a*) on the effects of irradiation; by the 4th or 5th day after irradiation many giant cells, often with bizarre nuclei, had formed and the cultures slowly degenerated by the 8th or 9th day. The greatest numbers of inclusions and the most reproducible titrations were obtained with cultures which had been irradiated with 4000 R. and infected 4–5 days later; this procedure was therefore used as a routine. The numbers of inclusions and their morphology were about the same whether we grew and maintained the McCoy cells in BHK-21

Table 1. *Infectivity titrations of MRC-4 and MRC-4f strains of TRIC agent in unirradiated and irradiated McCoy cell cultures: duplicate titrations of two yolk sac suspensions*

TRIC agent	Infectivity titre (inclusion-forming units/ml.)	
	Unirradiated	Irradiated
MRC-4	$5{\cdot}5 \times 10^6$	$3{\cdot}7 \times 10^6$
	$1{\cdot}2 \times 10^7$	$7{\cdot}4 \times 10^6$
	Mean $8{\cdot}8 \times 10^6$	$5{\cdot}5 \times 10^6$
MRC-4*f*	$5{\cdot}8 \times 10^7$	$6{\cdot}0 \times 10^7$
	$1{\cdot}2 \times 10^8$	$5{\cdot}4 \times 10^7$
	Mean $8{\cdot}9 \times 10^7$	$5{\cdot}7 \times 10^7$

medium or in the medium provided by Dr Gordon (Gordon *et al.* 1972*a*). Likewise, there was no obvious difference whether we used 0·22 % $NaHCO_3$ in the medium (as have others) or 0·035 %. When irradiated McCoy cells were used for isolation of TRIC agents, we grew and maintained them on Eagle's MEM – rather than the BHK medium – since this has generally been used by others for isolation work.

Infectivity titrations in irradiated and unirradiated McCoy cells

Yolk sac suspensions of MRC-4 and MRC-4*f* were titrated simultaneously in unirradiated and irradiated McCoy cells; Table 1 shows the results of two representative tests. Although the infectivity titres obtained in the two types of culture were similar the morphology of the inclusions was very different. In unirradiated cells, they were often smaller than the cell nuclei, stained almost black with iodine and contained few elementary bodies. In irradiated cells, the inclusions were 5- to 10-fold greater in diameter; they stained reddish brown with iodine and contained many elementary bodies.

Infectivity titrations in BHK-21 cells, HeLa cells and irradiated McCoy cells

Yolk sac suspensions of MRC-4, MRC-4*f* and TW3 were titrated in parallel in BHK-21 cells and irradiated McCoy cells or in BHK-21 cells and HeLa cells. No significant differences were found between the three types of cell culture; the minor variations reflected the state of health of a particular series of cultures rather than any inherent differences in susceptibility to TRIC agents. (Table 2).

Some investigations were also made of BHK-21 cells irradiated and treated like McCoy cells. The cells did not enlarge after irradiation but, instead, gradually degenerated. After infection with TRIC agents, fewer inclusions developed in them than in control unirradiated cultures.

Isolation of TRIC agents from conjunctival specimens using BHK-21 cells and irradiated McCoy cells.

Conjunctival scrapings were taken from children with active trachoma (stage II or III) from whom TRIC agents had previously been isolated in eggs. The specimens were inoculated in parallel into cultures of BHK-21 cells and irradiated

Table 2. *Infectivity titrations of MRC-4, MRC-4f, and TW3 strains of TRIC agents in irradiated McCoy, BHK-21 and HeLa cells*

TRIC agent	Pool	Infectivity titres (inclusion-forming units/ml.) Irradiated McCoy	BHK-21	HeLa
MRC-4*f*	a	$1{\cdot}6 \times 10^8$	$2{\cdot}1 \times 10^8$	ND
	b	ND	$1{\cdot}4 \times 10^9$	$1{\cdot}5 \times 10^9$
MRC-4	a	$5{\cdot}4 \times 10^7$	$1{\cdot}0 \times 10^8$	ND
	b	ND	$1{\cdot}9 \times 10^7$	$2{\cdot}7 \times 10^7$
TW3		$7{\cdot}3 \times 10^4$	$1{\cdot}1 \times 10^5$	ND

ND = not done.

McCoy cells. Both here and with urethral specimens, a control titration of a yolk sac suspension of MRC-4 of known infectivity titre was always included as a check on the health of the cultures and their sensitivity to infection.

None of the specimens tested gave sufficient numbers of inclusions for valid comparisons to be made between the cell systems on this basis. However, if the observation of at least one iodine-staining inclusion per cover-slip is accepted as evidence of isolation, there were no significant differences; of the seven specimens examined, six were positive in BHK-21 cells, five of which were also positive in irradiated McCoy cells.

Isolation of TRIC agents from urethral scrapings using BHK-21 cells and irradiated McCoy cells

For preliminary work, Mrs Phillipa Powis provided material from three specimens which had already been shown to contain TRIC agents and, in our comparative tests, inclusions developed in both culture systems from all of them. A further 74 specimens (not previously screened) were subsequently tested in parallel in BHK-21 cells and irradiated McCoy cells. Inclusions developed in both cell types from six specimens; a further two were positive only in BHK-21 cells. In eight of the total of nine specimens positive in both cell types more inclusions developed in BHK-21 cells (a mean of 23 per 100 microscope fields, with a range of 0·8–91) than in irradiated McCoy cells (a mean of nine per 100 fields, with a range of 0·01–30).

DISCUSSION

A virtually unsubstantiated belief that irradiated McCoy cells have some special advantage for the isolation of TRIC agents has become widespread among workers investigating the incidence of these organisms in the genital tract. Indeed, a considerable number of isolations were made in various laboratories with this culture system before any evidence was published to suggest that irradiation increased the susceptibility of McCoy cells to infection by TRIC agents, or that such irradiated cells were more efficient than other cell lines used unirradiated. Although there is no doubt that irradiation of McCoy cells has a remarkable effect

on the morphology of inclusions that form in them, inclusions of similar morphology form in other cells that have not been irradiated; it thus seems that the choice of McCoy cells for work with TRIC agents was unfortunate. Our work with other cell lines shows that irradiation of the host cell is unnecessary for efficient multiplication of TRIC agents, whether they are laboratory strains grown in the chick embryo yolk sac or wild-type strains obtained directly from their human host. Indeed, a consideration of the particle/infectivity ratios of TRIC agents makes such a finding likely. When fast-growing strains of TRIC agents (*f* variants) are titrated by centrifugation onto BHK-21 cells, the number of infective organisms measured as inclusion-forming units is almost as great as the total number of organisms in the inoculum, counted by dark-ground illumination (Taverne & Blyth, 1971). It is thus impossible for another cell culture system to yield substantially more inclusions when inoculated with the same suspension. By contrast, the ratio of particles to infectivity for BHK-21 cells of yolk sac suspensions of slow-growing strains is usually between 100:1 and 1000:1. These organisms may be potentially infective, and their viability might be demonstrated by adaptation to another host cell, but so far no other system has been reported that supports the multiplication of the apparently uninfective organisms. Our comparative titrations were done with organisms passaged in the chick embryo; strains that had been selectively passaged in particular cell lines were deliberately avoided.

In our hands, no more inclusions were formed from a given inoculum of slow-growing TRIC agents titrated in irradiated McCoy cells than in BHK-21 cells. Similarly the yields of new infective elementary bodies from such inclusions were at best approximately the same: 52 hr. after infection with strain MRC-4 in conditions designed to avoid multiple infection, cultures of BHK-21 cells yielded 1 IFU per inclusion; irradiated McCoy cells maintained in medium containing 0·035 % $NaHCO_3$ yielded 0·05 IFU per inclusion, which was increased to 1 IFU per inclusion in medium containing 0·22 % $NaHCO_3$ (unpublished results).

When unirradiated and irradiated cultures of the same cell line – McCoy cells – are compared, our results conflict with those of Gordon and his co-workers (1972*a*), who found 4–8 times more inclusions in irradiated cultures. However, we used different criteria to identify inclusions in the two sorts of culture. In irradiated cells inclusions were large and resembled those commonly obtained in BHK-21 cells, but in unirradiated cells they were often extremely small and stained black with iodine, an appearance sometimes seen in conditions unfavourable to normal development of TRIC agents. Thus, although the numbers counted were similar in both instances, the cycle of development in terms of morphology was complete only in irradiated cells. Moreover, not only the morphology but the number of inclusions obtained from a given inoculum is well known to vary with the condition of the cultures, including, among other factors, their age and the pH of the medium. The higher yields of infective organisms from irradiated cultures reported by Gordon *et al.* (1972*a*) were expressed not per inclusion but per volume of culture harvest. This result would be expected, irrespective of whether development was complete in the unirradiated cells, since more inclusions were present in each irradiated culture.

Apart from results obtained with irradiated McCoy cells, there is evidence that TRIC agents grow best in cells that are not multiplying. The conditions used in our routine work with BHK-21 cells produce cultures that contain very few dividing cells and are parasynchronous (Newton & Wildy, 1959). Suspended cells taken from storage at 4° C. were cultured overnight, during which time their number increased but failed to double. The cultures were then maintained in medium without serum, which prevented further multiplication. By contrast, a transformed line of BHK-21 cells that continued to multiply even after the cultures were infected, yielded at least ten times fewer inclusions than cultures of the same line in which cell multiplication was inhibited, either by exposing the cultures to γ-irradiation or to medium containing a low concentration of glutamine. (In cultures deprived of glutamine the number of inclusions were similar to those in irradiated cultures but the growth cycle of the agent was not complete.) (T. Rota & W. A. Blyth, unpublished results.)

That irradiation does not confer a unique advantage is plain from our results both with TRIC agents cultivated in the chick embryo yolk sac and with organisms from human tissues. We emphasize that our work with material from men with non-gonococcal urethritis does not give a measure of the rate of isolation of TRIC organisms; it merely demonstrates that isolations from human tissues can be made equally well in BHK-21 or in irradiated McCoy cells.

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Neutralization of TRIC organisms by antibody: enhancement by antisera prepared against immunoglobulins

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SUMMARY

The neutralizing activities of fowl, rabbit and mouse antisera prepared against TRIC agents were enhanced, in some cases over a 100-fold, by the addition of antiserum against the appropriate IgG. Significant non-specific inactivation of the organisms was observed in the absence of antiserum against TRIC agents, in reaction mixtures containing normal serum and antiserum against IgG. Since the extent of this inactivation showed a prozone, control tests with dilutions of normal serum equivalent to the full range of dilutions of the antiserum tested are necessary to show that enhancement of neutralization is specific.

INTRODUCTION

Antibodies that neutralize *Chlamydia* can be found in serum from naturally infected hosts and from immunized animals, but usually their titres are so low as to make the neutralization test of little use either in laboratory work or in diagnosis. Williams & Hahon (1970) reported, however, that the titre of neutralizing antibody in serum from roosters immunized against psittacosis was enhanced significantly in the presence of antiserum against rooster immunoglobulins. They also obtained higher titres of neutralizing antibodies in the sera of patients suffering from subclinical or overt psittacosis when antiserum against human immunoglobulins was included in the neutralization test.

We report here that although the neutralizing activity of antisera against TRIC agents can be significantly enhanced by adding antiserum against IgG, commensurate inactivation was sometimes obtained when normal serum was substituted for antiserum against the TRIC agent.

MATERIALS AND METHODS

TRIC agents

The following strains were used: PK-2*f* (T'ang, Chang, Huang & Wang, 1957), MRC-4 (Jones, 1961) and MRC-4*f*. The suffix *f* indicates a fast-killing variant (Taverne, Blyth & Reeve, 1964).

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Preparation of pools

Pools were made from infected chick embryo yolk sacs; they were treated with M-KCl and stored in 0·25 M sucrose in buffer (Taverne & Blyth, 1971).

BHK-21 cells

Methods and media for culture of these cells, and for infectivity titrations of TRIC agents in them, have been described (Blyth & Taverne, 1974). Cultures were inoculated with MRC-4 by centrifugation at 2000 ***g*** for 30 min at 35° C.; they were fixed with methyl alcohol 3 days later. For inoculation with PK-2*f* and MRC-4*f*, cultures were centrifuged at 600 ***g*** for 30 min. at 35° C. and fixed 2 days later. Iodine stained inclusions were counted in 30 microscope fields chosen at random from each of three replicate cultures and the mean number per field was calculated.

Antisera

Rabbits were injected at monthly intervals with strain PK-2*f* grown in BHK-21 cells. They were given six intravenous injections of 1 ml. each, containing about 5×10^9 infective organisms, and were bled 1 week after the last injection.

Mice received equal parts of a yolk sac suspension of PK-2*f* containing 7×10^9 infective organisms/ml. and Freund's complete adjuvant. They were injected intramuscularly with 0·2 ml. into each hind leg; a month later they received by the same route a second dose of suspension without adjuvant. They were bled a fortnight later and the serum was pooled.

Fowl. A cockerel was given six intraperitoneal injections of a suspension of MRC-4*f*, each equivalent to one infected yolk sac, at weekly intervals. It was bled 8 days after the last injection.

Baboons. The conjunctivae of six baboons were inoculated with a yolk sac suspension of MRC-4. All animals developed an infection and the mean score (Collier & Blyth, 1966) for the group was 42·6; they were bled 6 weeks after inoculation and the serum was pooled.

Antisera against immunoglobulins

'Species precipitating sera' against hen, mouse and rabbit serum globulins were obtained from Wellcome Reagents Ltd. Antisera prepared in rabbits against hen IgG and in goats against rabbit IgG (Cappel Laboratories, Downington, Pa., U.S.A.) were also tested. Goat antiserum against human IgG was kindly given to us by Dr T. Phillips, MRC Blood Group Reference Laboratory, London. Although the activity of the antisera differed quantitatively, precipitation and non-specific inactivation were observed with those prepared against purified IgG as well as with the 'species precipitating sera'.

Neutralization tests

Dilutions of antisera, none of which contained complement, were made in maintenance medium containing 50% calf serum. Reaction mixtures containing 0·5 ml. of an appropriate concentration of TRIC organisms and 0·5 ml. of a

Table 1. *Enhancement of neutralization by antisera against IgG*

TRIC agent	Source of antiserum *v.* TRIC agent	Dilution of antiserum *v.* TRIC agent*	Dilution of antiserum *v.* IgG* 2	4	8	16	32	128
MRC-4*f*	Fowl							
	Batch 1	10	.	.	>93†	.	>93	4·2
	Batch 2	16	.	>300	.	50	.	.
PK-2*f*	Rabbit	8	10	.	3	.	1·5	1·1
PK-2*f*	Mouse	4	.	.	203	.	65	8·5

* Reciprocal.

† Enhancement of neutralization: $\frac{\text{no. of inclusions/field with antiserum v TRIC alone}}{\text{no. of inclusions/field with antiserum v TRIC and antiserum v IgG.}}$

Table 2. *Neutralization titres of antisera against TRIC agents in the presence and absence of antisera against IgG*

TRIC agent	Source of antiserum	Dilution of antiserum *v.* IgG*	Titre of antiserum *v.* TRIC* Alone	With antiserum *v.* IgG
MRC-4*f*	Fowl	15	12	1280
PK-2*f*	Rabbit	2	32	2560

* Reciprocal.

dilution of homologous antiserum were incubated for 30 min. at 35° C. Each mixture then received 0·5 ml. of a dilution of antiserum against the IgG of the species in which the antiserum against the TRIC agent had been prepared (or 0·5 ml. of medium with 50% calf serum), and was incubated for a further 15 min. Final dilutions were therefore 3-fold higher than those quoted. From each reaction mixture, 0·4 ml. volumes were added to each of three cultures of BHK-21 cells which were then centrifuged at 35° C. The end-point of neutralization was calculated as the reciprocal of that dilution of test serum which decreased the number of inclusions by 50% compared with the controls containing calf serum.

RESULTS

Titration of antisera prepared against IgG

The enhancing effect of antiserum against IgG was tested on organisms sensitized by incubation with specific antisera prepared in various animals against TRIC agents. For each experiment a dilution of sensitizing antiserum was chosen which decreased the number of infective organisms by about half during the initial incubation. Dilutions of antiserum against the appropriate IgG were then added to the mixtures, which were incubated for a further 15 min. A control containing one dilution of antiserum against IgG was included in each test with a dilution of normal serum from the same animal species substituted for the sensitizing serum. There were no significant differences between the numbers of inclusions in these controls and in others containing 50% calf serum.

Table 3. *Non-specific inactivation of TRIC organisms in the presence of normal serum and antiserum against IgG*

Source of normal serum	TRIC agent	Dilution of normal serum*	Dilution of antiserum against IgG*	Inactivation (%)†	Precipitation
Fowl	MRC-4*f*	8	.	5	−
		.	4	27	−
		8	4	17	+
		16	4	46	+ +
		32	4	32	+ + +
Baboon	MRC-4	8	.	0	−
		.	2‡	0	−
		8	8	0	+
		16	8	17	+ +
		32	8	94	+ + +
		64	8	64	+ + +
		128	8	27	+ +
Rabbit	MRC-4*f*	10	.	0	−
		.	8	0	−
		8	8	43	−

* Reciprocal.
† Compared with control containing 50 % calf serum.
‡ Antiserum against human IgG.

Antisera against fowl, rabbit and mouse IgG all enhanced neutralization to some extent (Table 1); that against rabbit IgG was weak and another batch showed no activity. On the basis of these results, a dilution of each antiserum against IgG was chosen for use in subsequent experiments.

Titration in the presence of antiserum against IgG of specific antisera prepared against TRIC agents

The neutralizing activities of two antisera against TRIC agents were enhanced about a 100-fold in the presence of the selected dilution of antiserum against the appropriate IgG (Table 2). By itself, a 1/4 dilution of pooled serum from a group of baboons infected conjunctivally with strain MRC-4 did not neutralize, but even higher dilutions did so when a 1/8 dilution of antiserum against human IgG was added: after a prozone, neutralization rose to a maximum of 64 % in the reaction mixture containing a 1/64 dilution of baboon serum. In this titration, however, a precipitate which also showed a prozone was observed in some reaction mixtures. This finding suggested that the decrease in infectivity might not have been caused by specific antibody but be related to the precipitate, since the two effects occurred in parallel. TRIC organisms were therefore incubated with a range of dilutions of normal serum from different animal species and the appropriate antiserum against IgG (Table 3). Some inactivation occurred in all these mixtures which with baboon serum reached 94 %. A precipitate formed in some reaction mixtures of the series containing fowl or baboon serum and prozones occurred in both precipitation and inactivation.

Table 4. *Neutralization of strain MRC-4f by homologous antiserum prepared in a cockerel; enhancement by antiserum against fowl IgG*

	Normal serum			Antiserum v TRIC			
Antiserum *v.* IgG	Dilution*	Inactivation (%)	Precipitation	Dilution*	Neutralization (%)	Precipitation	Titre†
None	8	5	—	8	85	–	12
				10	76	–	
				16	21	–	
				40	18	–	
				160	20	–	
1/4	8	17	+	8	>99	+	2560
	16	46	+ +	16	>99	+ +	
	32	32	+ + +	32	>99	+ + +	
	160	13	+ +	160	99	+ +	
	640	13	+	640	97	+	
	2560	12	–	2560	47	–	

* Reciprocal.

† Reciprocal of dilution giving 50 % neutralization.

In the light of these findings the fowl antiserum was titrated again, using as controls one dilution of antiserum against fowl IgG and the complete range of dilutions of normal fowl serum (Table 4). Normal serum alone at a dilution of 1/8 did not diminish infectivity significantly but with antiserum against fowl IgG some inactivation occurred; it reached a maximum of 46 % with a 1/16 dilution of normal serum. By itself the antiserum against TRIC agent had a titre of 1/12, which was increased to 1/2560 in the presence of antiserum against IgG, an enhancement of about 200-fold. It is apparent that this enhancement is specific since mixtures containing dilutions of normal serum above 1/32 did not inactivate the organisms significantly. Precipitation was greatest at a dilution of 1/32 both with normal serum and with antiserum against TRIC agent.

DISCUSSION

Our demonstration that specific neutralization of TRIC agents can be enhanced by antiserum against IgG confirms with *Chlamydia* group A the findings of Williams & Hahon (1970) with *Chlamydia* group B. Specific neutralization was significantly greater than the extensive non-specific inactivation produced by normal serum in the presence of antiserum against IgG. With viruses, non-specific inactivation of this kind does not appear to have been a major problem, although Brown, Elsner, Zebovitz & Allen (1969), who studied Group A arboviruses, reported 'a rare case of non-specific inhibition in mouse fluids up to 1:5 dilution'. It is not possible to determine from most reports whether sufficient controls were included to cover the range of dilutions of normal serum equivalent to that of antiserum. Although it is likely that gross precipitation would have been noticed, we emphasize that non-specific inactivation sometimes occurs in the absence of visible precipitation.

It is well known that not all specific antibodies that combine with virus particles

neutralize infectivity, and that particles may sometimes become sensitized so that subsequent reaction with antibody against immunoglobulin renders them uninfective. Results obtained with chlamydiae may, however, be only superficially similar; nothing is known about the mechanism of their neutralization and there is no reason to believe that it is the same as for viruses. Neutralization of chlamydiae may not involve a specific alteration of the organism, or the blocking of a particular site on its surface, but rather an altered reaction of the host cell to an organism coated with antibody.

Infective elementary bodies are phagocytosed selectively by the host cell and the vacuole in which they multiply does not fuse with lysosomes (Lawn, Blyth & Taverne, 1973). By contrast, in the macrophage – a cell in which the organism fails to multiply – elementary bodies enter lysosomes and are inactivated. An organism coated with antibody is perhaps not recognized by the host cell and so fails to enter, or on entry is immediately transferred to a lysosome; in either case no chlamydial inclusion would develop and the organism would apparently be 'neutralized'. Furthermore, if the host cell fails to recognize elementary bodies coated non-specifically with globulin combined with antibody against globulin, inactivation in the absence of specific antibody would be explained. If this hypothesis is correct, then measurements of neutralizing antibodies against chlamydiae and enhancement of their titre are of no special value, and merely provide an insensitive way of assaying antibodies that are more easily measured by complement fixation or immunofluorescence.

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Swine vesicular disease: virological studies of experimental infections produced by the England/72 virus

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SUMMARY

Pigs exposed to relatively small amounts of virus by intradermal inoculation of the feet or by skin scarification developed clinical disease. Large amounts of virus were recovered from samples taken from the nose, mouth, pharynx, rectum and the prepuce or vagina during the first week of infection and smaller amounts during the second week. Virus was recovered from the faeces of most animals 16 days after infection and from one animal for 23 days. Pigs in contact with inoculated animals were killed at intervals before the appearance of clinical disease. The distribution and amounts of virus in various tissues indicated that infection had most likely gained entry through the skin or the epithelia and mucosae of the digestive tract. Some pigs acquired subclinical infections in which no virus excretion was detected and no transmission of infection to susceptible pigs took place over a period of 5 weeks.

INTRODUCTION

The outbreak of swine vesicular disease (SVD) which began in the West Midlands of England in December 1972 (Dawe, Forman & Smale, 1973) was the fourth to be identified. Previously the disease had been recognized in Italy in 1966 (Nardelli *et al.* 1968), in Hong Kong in 1971 (Mowat, Darbyshire & Huntley, 1972) and in Italy in 1972 (L. Nardelli, personal communication). In January 1973 further cases of disease were reported in Austria and Poland (Draft report of the *ad hoc* committee consultation on swine vesicular disease and the foot-and-mouth disease position in Eastern and Southeastern Europe, F.A.O. Rome, 9 January, 1973) and in France (Dhennin & Dhennin, 1973).

Epidemiological investigations of outbreaks of disease in Great Britain (R. S. Hedger, R. F. Sellers & G. N. Mowat, personal communications) have indicated that, although infection spreads rapidly within groups of pigs housed in the same pen, transmission of infection from pen to pen or from building to building occurred less readily than in many outbreaks of foot-and-mouth disease (FMD). This difference in epidemiological behaviour could be due to differences in the susceptibility of pigs to particular routes of infection, to differences in virus excretion from diseased animals or to differences in the resistance of the virus to environmental conditions.

These questions are studied in this paper, which is concerned with the responses

of pigs exposed to virus by different routes, and with the identification of regions of virus multiplication and excretion during the course of disease.

MATERIALS AND METHODS

Virus

The England/72 virus (Dawe *et al.* 1973) was used as a suspension of infected pig foot epithelium or as a tissue culture harvest from the first or second passage in the pig kidney cell line IB-RS-2 (de Castro, 1964).

Experimental pigs – inoculation and sampling procedures

Groups of large white pigs ranging in weight from 30–40 kg. were exposed to virus by one of the following methods: the intradermal inoculation of approximately 0·1 ml. of a virus suspension in the bulbs of the heel, the coronary band or the skin of the thorax and abdomen; the application of approximately 0·1 ml. of virus suspension to areas of scarification on the rostrum of the snout or on the skin of the thorax and abdomen; the instillation of approximately 0·5 ml. of virus suspension into the nasal or oral cavities, or by direct contact with inoculated pigs. All animals were examined daily and rectal temperatures recorded. In some experiments nasal, oral, rectal and preputial or vaginal swabs, blood and pharyngeal/tonsillar samples (Burrows, Greig & Goodridge, 1973) were taken daily for varying periods after exposure. Seven pigs exposed to infection by contact with inoculated pigs were killed 2–5 days later by the intravenous inoculation of thiopentone sodium (BPC) and a range of tissues were taken for virus assay. No attempt was made to collect the tissues aseptically but each tissue or mucous membrane was washed thoroughly in running water after collection.

Assay of virus and neutralizing antibody

All swabs and tissue samples were stored over liquid nitrogen before examination. Swabs were processed by immersion and shaking in 3 ml. diluent and tissues were prepared as 1/10 (w/v) suspensions. The virus content was estimated by counts of plaque forming units (p.f.u.) after 48 hr. incubation on IB-RS-2 monolayer cultures. The identity of the plaque forming virus was monitored periodically by neutralization tests using an antiserum prepared in guinea pigs against the Italy/66 virus. Serum neutralization tests were performed as described by Burrows *et al.* 1973, using a plaque reduction procedure in which residual virus was determined after 48 hr. incubation.

RESULTS

Response to inoculation by different routes

Table 1 lists the concentrations of virus used for each method of inoculation or exposure and the numbers and responses of pigs used in the different experiments. The clinical signs of natural and experimental SVD have been described by Nardelli *et al.* (1968), Mowat *et al.* (1972) and by Dawe *et al.* (1973).

Foot inoculation. Inoculation of the bulbs of the heel with $10^{5\cdot9}$ p.f.u./site resulted in the development of vesicles within 48 hr. at 22 of 36 sites in nine pigs.

Table 1. *Numbers and responses of pigs exposed to various concentrations of virus by different routes*

	Intradermal inoculation			Skin scarification		Instillation	
Virus dose*	Heel	Coronary band	Abdominal skin	Snout	Abdomen	Oral	Nasal
1·5	—	—	0/8†	—	1/8	0/4	0/4
2·5	2/8	5/8	0/8	0/2	1/8	0/4	1/4‡
3·5	5/8	3/8	0/8	0/2	2/8	0/4	2/4‡
4·5	5/8	6/8	0/8	0/2	3/8	—	—
5·5	—	—	0/8	0/2	2/8	—	—
5·9	22/36	—	—	—	—	—	—
6·5	—	—	0/8	—	1/8	—	—
No. of pigs	13	4	4	8	4	12	12

* Log_{10} p.f.u./site or pig.
† No. of sites or pigs reacting / No. of sites or pigs exposed.
‡ Subclinical infection.
—, Not tested.

Table 2. *Virus content of samples taken from pigs after heel inoculation – England/72 virus*

Days after inoculation	Nasal swabs	Mouth swabs	Pharyngeal samples	Rectal swabs	Preputial/ vaginal swabs	Serum
1	0*	0	0·74 (0–5·9)	1·25 (0–2·5)	1·05 (0–2·1)	2·56 (1·0–5·2)
2	3·85 (3·2–4·5)	4·0 (3·5–4·5)	4·88 (3·3–5·9)	4·75 (4·5–5·0)	3·05 (2·2–3·9)	4·61 (2·7–6·0)
3	4·3†	6·3 (6·1–6·5)	5·73 (4·7–6·3)	5·1†	3·5†	3·84 (2·9–5·3)
4	5·22 (3·5–6·5)	6·0 (4·5–7·0)	5·40 (4·1–6·5)	4·59 (3·0–5·7)	4·24 (2·3–4·8)	0·68 (0–3·2)
5	5·06 (2·5–6·7)	5·14 (4·0–7·3)	5·07 (4·3–6·8)	4·85 (4·3–6·3)	3·41 (2·5–5·0)	0
6	3·55 (0–5·0)	4·19 (1·5–6·3)	3·72 (2·3–4·7)	3·75 (2·7–4·7)	2·50 (1·7–4·4)	0
7	2·52 (0–3·9)	0·99 (0–2·7)	2·97 (1·5–4·5)	2·29 (0–4·0)	2·34 (0–5·7)	0
8	0·71 (0–2·2)	0	1·59 (0–2·5)	1·15 (0–3·0)	0·70 (0–1·7)	—
9	0·45 (0–2·1)	0	0·69 (0–3·1)	1·24 (0–2·9)	0·89 (0–3·1)	—
10	0	0	0·21 (0–1·7)	0	0	—
11	0	0	0	0·87 (0–2·5)	0	—
12	0	0	0·27 (0–2·2)	0·19 (0–1·5)	0	—
14	0	0	0	0	0	—

* Log_{10} p.f.u./swab/sample/ml. (serum) – geometric mean and range of 8 pigs.
† Result for one pig.
0 No virus recovered i.e. < 1·0/swab/sample, < 0·7/ml serum.
—, Not tested.

Secondary lesions were evident in six of the nine pigs within 72 hr. Comparative titrations by the bulb of the heel and the coronary band procedures in groups of four pigs (Burrows, 1966) indicated that both regions were of similar sensitivity to virus. Approximate concentrations of virus estimated to produce lesions at 50% of sites inoculated were $10^{3·4}$ p.f.u. (heel) and $10^{3·3}$ p.f.u. (coronary band).

Skin scarification and inoculation. Eight pigs were exposed by the application of single virus concentrations ranging from $10^{2·5}$ to $10^{5·5}$ p.f.u. to scarified areas of the

Table 3. *Virus content of samples taken from pigs exposed to contact infection*

	Days after inoculation of donor pigs				
	1	2	3	4	5
No. of pigs ...	7	7	6	4	2
Nasal swab	0·3* (0–2·0)	2·4 (0–3·3)	4·0 (3·3–5·0)	4·7 (3·3–5·8)	3·9 (3·8–4·0)
Oral swab	0	2·8 (1·7–3·5)	3·7 (3·0–4·7)	5·3 (4·0–7·0)	4·5 (4·5)
Pharyngeal swab	0	2·4 (1·7–3·1)	3·5 (2·9–4·5)	5·0 (3·3–5·8)	3·9 (3·5–4·3)
Rectal swab	0	2·6 (0–3·7)	4·3 (3·7–5·0)	4·0 (3·9–4·2)	4·0 (3·8–4·2)
Preputial/vaginal swab	0	1·0 (0–2·2)	3·0 (1·5–5·0)	3·3 (2·3–4·2)	4·1 (3·4–4·8)
Serum	0	0	0·8 (0–2·8)	2·2 (0–4·6)	2·0 (1·0–3·0)

* Log_{10} p.f.u./swab/sample/ml (serum) – geometric mean and range.

rostrum of the snout. No lesions developed at the sites of virus application but 2 of the 8 pigs developed signs of generalized disease 6 days later. Four pigs were exposed to multiple concentrations of virus ($10^{1·5}$–$10^{6·5}$ p.f.u.) applied to scarified areas of the abdominal skin lateral to each teat. Erythematous plaques approximately 1·5 cm. in diameter and 2–3 mm. in height appeared within 48 hr. at 10 of the 48 sites exposed but these lesions were distributed in random fashion and were not obviously related to the concentrations of virus applied to the area. Biopsy material from one lesion contained $10^{1·8}$ p.f.u./g. whereas only traces of virus were recovered from an adjacent area of normal skin. Two of the four pigs developed signs of generalized disease 4 days after exposure. Intradermal inoculation of the abdominal skin with the same concentrations of virus produced no obvious lesions and no signs of disease appeared within a 4-day period.

Oral and nasal exposure. No clinical signs were seen in pigs given virus concentrations up to $10^{3·5}$ p.f.u. as a single dose. However, serological studies showed that one of the four pigs given $10^{2·5}$ p.f.u. and two of the four pigs given $10^{3·5}$ p.f.u. by intranasal instillation had acquired a subclinical infection.

Virus concentrations in the secretions and excretions of inoculated pigs

Table 2 lists the geometric mean amounts and ranges of virus measured in nasal, oral, rectal and preputial or vaginal swabs, and from pharyngeal/tonsillar and serum samples collected from eight pigs infected by heel inoculation (four sites at $10^{5·9}$ p.f.u./site). Peak concentrations of virus were found in the serum on the second day and in other samples on the third to the fifth day after inoculation. These periods coincided with the development of primary and secondary lesions. The infectivity of samples declined after the fifth day and no virus was recovered from the mouth after the seventh day or from swabs taken from the nose and the urogenital orifices after the ninth day. Although only 5 of 32 rectal swabs taken between the 10th and 14th days yielded virus, no difficulty was experienced in demonstrating virus in the faeces for longer periods. Twenty-eight of 30 samples of faeces collected from pigs 14–16 days after infection contained virus (mean infectivity

Table 4. *Virus content* of samples and tissues taken from pigs exposed to contact infection.*

	Pig identification						
KE:	3	4	5	6	7	8	9
Days after inoculation of donors	2	3	3	4	4	5	5
Ante-mortem samples							
Nasal swab	2·5	3·7	3·9	4·9	5·1	3·8	4·0
Oral swab	3·3	4·0	3·0	7·0	6·5	4·5	4·5
Pharyngeal sample	2·8	4·5	3·3	5·8	4·7	3·5	4·3
Rectal swab	2·5	5·0	4·0	4·1	4·2	4·2	3·8
Serum	0	0	0	4·6	0	1·0	3·0
Post-mortem samples							
Nasal septum	0	0	0	3·3	0	2·2	—
Turbinate mucosae	0	1·7	0	4·0	0	2·5	—
Dorsal surface of soft palate	0	0	0	3·5	0	0	—
Tracheal mucosa	0	0	0	3·9	0	0	—
Lung: Apical lobe	0	0	0	5·2	0	2·2	—
Cardiac lobe	0	0	0	5·3	0	0	—
Intermediate lobe	0	1·7	0	5·2	2·7	3·9	—
Diaphragmatic lobe	0	2·0	1·7	5·0	2·2	0	—
Tongue	0	4·0	2·2	3·9	1·7	1·7	—
Glosso-pharyngeal area	0	0	0	3·0	2·2	2·5	—
Tonsils (2)	0	1·5	0·7	5·2	0·9	3·2	—
Pharynx	0	3·0	1·7	4·4	2·2	3·5	—
Mandibular salivary gland	0	0	1·4	2·5	0	2·8	—
Parotid salivary gland	0	1·7	2·3	2·8	2·7	4·2	—
Duodenum	1·7	0	0	3·0	2·9	0	—
Small intestine	0	0	0	3·7	3·8	2·7	—
Caecum	0	2·0	0	3·0	3·7	1·7	—
Small colon	0	1·4	0	2·0	3·9	3·2	—
Large colon	0	2·0	0	2·5	3·0	0	—
Rectum	0	2·8	2·1	3·5	—	2·3	—
Spleen	0	0	0	4·5	2·3	2·7	—
Liver	0	0	0	5·0	2·3	2·2	—
Pancreas	0	0	0	3·5	2·3	2·0	—
Kidney	0	2·1	0	3·7	1·7	0	—
Cerebrum	0	0	—	2·9	0	0	—
Medulla	0	1·4	—	2·8	1·7	2·0	—
Spinal cord	0	2·0	0	2·8	0	0	—
Leg muscle	0	2·3	2·4	3·0	2·5	2·9	4·3
Heart muscle	0	0	0	4·2	3·9	2·3	—
Bone marrow	0	0	—	3·6	0	1·7	—
Skin: Rostrum of snout	0	2·8	4·0	2·7	2·7	7·3	—
Interdigital	3·4	2·5	2·6	4·0	2·4	2·7	8·6
Hairy (thorax)	0	2·3	3·0	4·2	3·2	3·5	3·8
Lymph nodes: Pharyngeal (2)	0	0	0	3·0	0	2·5	3·2
Mandibular	0	2·0	0	5·1	0	3·3	4·7
Parotid	0	0	0	2·8	2·7	3·8	—
Bronchial	0	2·1	0	4·9	0	2·2	3·8
Mediastinal	0	0	0	4·3	2·2	0	—
Mesenteric	0	0	0	5·0	3·0	2·6	—
Prescapular	0	0	0	3·9	0	2·3	—
Popliteal	0	1·4	2·0	2·6	3·0	0	6·7
Inguinal	0	2·8	0	5·7	3·0	—	7·0

* Log_{10} p.f.u./g/ml. or sample.

0 = < 0·7 per ml. of serum. < 1·0 per specimen (ante-mortem). < 1·4 per specimen (post-mortem). — = Not tested.

Table 5. *Serum neutralizing-antibody responses of pigs following inoculation or exposure to England/72 virus*

No. of pigs	Method of exposure	Disease	Days after exposure				
			0	7	14	28–35	42–49
12	Inoculation or contact	Clinical	< 0·5*	1·8	—	4·1	3·9
3	Intranasal	Subclinical	< 0·5	0·8	2·0	2·7†	2·5†

* Geometric mean of the log reciprocal of the initial serum dilution which neutralized 90 % of the test virus.
† Two pigs.

$10^{2·85}$ p.f.u./g., range $10^{1·7}$–$10^{3·6}$) and one of five samples collected 23 days after infection contained $10^{2·5}$ p.f.u./g.

Virus concentrations in the secretions, excretions and tissues of pigs exposed to contact infection

The mean amounts of virus in samples taken daily from recipient pigs after the inoculation of two donor pigs are detailed in Table 3. Virus was recovered from the nose of only one of seven recipient pigs 24 hr. after inoculation of the donors, which indicated that little virus was excreted by the donor animals during this period. Virus was recovered from most of the samples (except serum) collected from the recipients from 48 hr. onwards. Comparisons of the virus content of samples taken before and after death (Table 4) from pigs killed on the second and third day indicated that most of the virus in the samples taken before slaughter was in the surface film overlying the epithelia and mucosae and was not related to virus growth in those regions. It was apparent that most of this virus was derived from the donor animals and had been acquired passively from the environment. However, some evidence of active infection and dissemination of virus was obtained in all recipient pigs (Table 4). Pig KE 3, killed on the second day had considerable amounts of virus in a sample of interdigital skin. In pigs killed on the third and subsequent days there was consistent evidence of virus growth in the epithelia and mucosae of the upper and lower digestive tracts, in salivary glands and in all regions of skin examined. Variable concentrations of virus were found in other tissues, in some pigs these were obviously related to amounts of virus in the blood or to drainage from regions of virus growth or to virus passively acquired from the environment. Only one of seven pigs (KE 9) had developed clinical signs of disease (an early foot lesion) by the time of killing.

Subclinical infection following a limited exposure to virus

Twenty-four pigs were exposed to infection by the instillation of small amounts of virus into the nose or into the mouth. Oral and rectal swabs were taken daily for a period of 14 days from all pigs but no evidence of virus growth or excretion was obtained. Serological tests revealed that 3 of the 24 pigs acquired a subclinical infection and in Table 5 the neutralizing antibody response of these pigs is com-

pared with that of pigs which had experienced clinical disease as a result of inoculation or contact infection. No transmission of infection took place from subclinically infected pigs to susceptible pigs housed in the same room over a period of 5 weeks.

DISCUSSION

The response of pigs to graded amounts of virus was irregular and did not permit accurate determinations of the 50 % infective doses. However, the results showed that pigs were highly susceptible to small amounts of England/72 virus by foot inoculation or by skin scarification. With FMDV the coronary band route of inoculation is approximately 300 fold less sensitive than the bulb of the heel route (Burrows, 1966), but with SVDV the coronary band was found to be of equal or greater sensitivity. This may be a reflexion of general skin susceptibility to virus as it has been observed that vesicles on the coronary band are not restricted to the skin/horn junction as in FMD, but may extend for 1 or 2 cm. up the limb. Skin lesions may also appear on the lower and upper regions of the limb and occasionally on the thorax and abdomen.

A number of outbreaks in the field were attributed to the movement of pigs in contaminated lorries (Ministry of Agriculture, Fisheries and Food, 1973). The probable route of infection in these circumstances would be by viral contamination of the minor wounds and abrasions which occur frequently during transport.

In inoculated pigs it was found that the amounts of virus in the secretions, excretions and tissues differed to some degree from those measured in comparable experiments with FMDV. In FMD maximum concentrations of virus were found in samples collected 2–4 days after inoculation; the amounts of virus were less than those found in SVD and rarely was virus recovered from samples (other than vesicular epithelium) for longer than 5 days (unpublished data). In SVD peak virus concentrations were measured in samples collected 2–5 days after inoculation and virus was recovered from swabs and pharyngeal samples for periods of 7–12 days. Faeces collected 16 days after infection contained considerable quantities of virus and evidence of faecal excretion was obtained for 23 days from one pig. An important source of virus is vesicular fluid and epithelium; in FMD infective virus is no longer detectable in lesions over 10 days old (unpublished data) whereas in SVD remnants of vesicular epithelium collected from a 10 day old lesion contained $10^{6.9}$ p.f.u./g. (unpublished data).

Susceptible pigs housed with inoculated pigs acquired virus rapidly and evidence of active infection and dissemination of virus was found in all pigs killed after the second day. Unfortunately the results do not identify any particular region as the initial site of virus entry and multiplication. No consistent evidence was obtained of early virus growth in respiratory tract mucosae and it would seem likely that infection took place through areas of damaged skin or through the epithelia or mucosae of the digestive tract.

Observations in the laboratory and the field indicate that SVD spreads less readily than FMD between groups of pigs and this has been related to the amounts of airborne virus produced in the 2 diseases. In SVD Sellers & Herniman (1974)

recovered smaller amounts of airborne virus for shorter periods of time than in comparable experiments with FMD. In addition a greater proportion of airborne SVDV was associated with a large particle aerosol which would tend to remain airborne for short periods only.

These studies of SVD show that large amounts of virus are present in the immediate vicinity of infected pigs for a considerable period of time. Airborne transmission of virus is unlikely (Sellers & Herniman, 1974) and the spread of disease depends mainly on the movement of infected pigs and their products. The stability of the virus is such that it is not inactivated by the acid changes which occur in the musculature after death, and the virus can be expected to withstand the various processes used in the production of sausages and salami. Little or no reduction in infectivity occurs in cold storage and so uncooked pork and pork products will remain a hazard indefinitely. Several outbreaks of disease in Great Britain were attributed to this recycling of virus during the spring and summer of 1973.

The numbers of outbreaks reported in France and Italy have been considerably fewer than those reported in Britain. This apparent difference in epidemiology may be due to regional differences in the methods of husbandry and marketing of pigs or it may be that cases of infection have gone unrecognized or have been subclinical in nature. In both the present and earlier experiments with SVD some pigs exposed to small amounts of virus acquired subclinical infections. An interesting finding was that these infections were not accompanied by virus excretion which was detectable by the methods employed or by the transmission of infection to susceptible animals over periods of 7 weeks (Italy/66 – Burrows *et al.* 1973) or 5 weeks (England/72). The pigs which acquired subclinical infection and had no contact with clinical disease developed lower titres of neutralizing antibody than did pigs which experienced or were in contact with clinical disease. This fact should be borne in mind in the interpretation of the results of serological surveys for evidence of subclinical and past infection.

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