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Immunity in experimentally induced enzootic pneumonia of pigs

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For many years the main research objective in the study of enzootic pneumonia of pigs was to establish the nature of the causal agent. Now that this has been shown to be a mycoplasma (Goodwin, Pomeroy & Whittlestone, 1965) differing from a wide range of other mycoplasmas (Goodwin, Pomeroy & Whittlestone, 1967), work in several laboratories is being concentrated on the serological and immunological aspects of this disease. Various tests are available for studying mycoplasmal antibodies in pig sera, and some of these have already been examined: indirect haemagglutination (Ross & Switzer, 1963), agar-gel precipitation and immuno-fluorescence (Goodwin *et al.* 1967), metabolic inhibition (Goiš, 1968) and complement fixation (Roberts, 1968). The broad history of serological tests, however, shows that the mere demonstration of titres is of limited value; too often, such tests have been shown later to be non-specific, or to have such a high rate of falsely positive or falsely negative results that they are of little practical use in diagnosis or for the measurement of immune status. Before investigating sera from the field, therefore, we decided to study the merits of different serological tests using the sera from hysterectomy-produced pigs that had been infected at known times with enzootic pneumonia. A proportion of these pigs were subsequently challenged with the infection and killed to observe their immune status; sera collected before and after challenge in these animals were similarly evaluated.

Although Betts, Whittlestone & Beveridge (1955) said that there was no field evidence to show that a natural attack of enzootic pneumonia produced any appreciable degree of immunity, Whittlestone (1957) subsequently commented on the fact that young pigs were generally more obviously affected than older stock, partly because the latter had usually experienced the infection previously. The general weight of field evidence, however, suggests that a good immunity develops in this disease: clinical signs are rare in adult stock, and are usually seen in such animals only when the disease first enters one of the few herds that are free from enzootic pneumonia. Goodwin (1965) observed that the disease was clinically suppressed in the litters from older sows, even though the causal agent was probably present; he felt that one explanation for this suppression could be that passive immunity was transferred in the colostrum, and he postulated that, because they did not themselves develop active, substantial lung lesions of

pneumonia, litters protected in this way might not develop a sufficiently strong immunity to protect their own first litters via the colostrum in due course.

Lannek & Börnfors (1957) showed that a strong immunity developed in experimentally induced enzootic pneumonia. Pigs were infected, allowed to recover (as judged by radiological examinations) and then challenged 118 days after the primary infection: all the challenged pigs were free from enzootic pneumonia at slaughter, whereas all the positive controls, which were the same age, had pneumonia. Some of the challenged animals were housed with the positive controls for 28 days, and were thus probably exposed to a natural challenge in addition to the artificial challenge. Although the authors wrote that neutralizing antibodies had not been demonstrated by then in pig sera, it seems that they did not examine the sera of these particular immunized pigs.

MATERIALS AND METHODS

Pig inoculations

All the pigs were hysterectomy-produced, colostrum-deprived animals, kept under conditions of strict isolation as previously described (Goodwin *et al.* 1967). They were inoculated intranasally with ground suspensions in broth of lung affected with enzootic pneumonia, the dose varying between 5 and 15 ml. of dilutions that ranged from 1/5 to 1/10. The term 'challenge' applies only to the attempts to re-infect pigs that had previously been inoculated in this way. All the pigs that started as a group remained as a group while they were subject to the same procedures; because of this, the inoculated pigs were, on some occasions, also incidentally exposed to their infected litter-mates. However, whenever a pig was treated differently from the other animals in its group it was removed to a separate isolation cubicle beforehand.

The diagnosis of enzootic pneumonia was based on the examination of touch preparations for organisms with the morphology of *Mycoplasma suipneumoniae* (Whittlestone, 1967) and on the nature of the gross lesions and the histological picture. The lesions described in the text as early ones were pale creamy-pink in colour with ill-defined edges; when cut, they exuded oedematous or slightly cloudy fluid. Histologically, they showed alveolar-cell pneumonia, catarrhal or purulent bronchiolitis, perivascular and peribronchiolar mononuclear-cell accumulations, and varying degrees of peribronchiolar lympho-reticular hyperplasia. The lesions referred to as late ones had the gross appearance of small areas of collapse with pin-head lymphoid-like nodules; when cut, the tissue was dry. Histologically, there was hyperplasia of the peribronchiolar lympho-reticular tissue which sometimes invaded the lamina propria, causing partial obliteration of the bronchiolar lumina, and was associated with compression collapse of the surrounding alveoli; apart from an occasional area of alveolar-cell pneumonia, affecting a few alveoli, there was usually no cellular exudate. Where lungs were recorded as being free from abnormalities, there were no macroscopic or microscopic changes, and no mycoplasmas were seen in the touch preparations.

Culture medium

The liquid medium consisted of Hanks's balanced salt solution (39 %), Hartley's broth (30 %), inactivated pig serum (20 %), 5 % lactalbumin hydrolysate (10 %), penicillin solution containing 40,000 units/ml. (0·5 %), yeast extract (0·5 %), and thallium acetate (1/8000). The pig serum was obtained from a herd free from enzootic pneumonia. The yeast extract was prepared as described by Herderscheê (1963). The Hanks solution, the Hartley's broth and the lactalbumin hydrolysate were autoclaved; the pig serum, the yeast extract and the thallium acetate were sterilized by Millipore filtration. The complete medium was stored at about −20° C.

Strains of Mycoplasma

Two strains of *M. suipneumoniae* were used: our type strain (J) and strain CZ (Goodwin, Pomeroy & Whittlestone, 1968). The source of *Mycoplasma hyorhinis* was mycoplasma 603 (Goodwin *et al.* 1967) and the sources of *Mycoplasma pneumoniae*, mycoplasma B 3, and *Mycoplasma granularum* were as shown in the same publication; *Mycoplasma gallisepticum* was supplied by Dr D. Taylor-Robinson. All these mycoplasmas were checked with specific antisera when used: the serum for *M. pneumoniae* was supplied by Dr R. H. Leach (the Wellcome Research Laboratories) and the serum (514) for *M. gallisepticum* was supplied by Dr H. P. Chu; the remaining mycoplasmas were checked before use against rabbit sera prepared in this laboratory against the same strains of antigen as used in this work.

Serological techniques

Serum samples were stored at about −20° C.

Metabolic inhibition (MI)

The metabolic-inhibition test for acid-producing mycoplasmas (Taylor-Robinson, Purcell, Wong & Chanock, 1966) was performed in 3 × ½ in. glass tubes with metal caps (Oxoid) sealed with Parafilm (Gallenkamp). The serum dilutions were made in two series in standard liquid medium, and 0·5 ml. of mycoplasma culture, diluted as required in the same medium, was added to 0·5 ml. of each serum dilution, to give final serum dilutions of 1/3, 1/6, 1/12, etc., and also 1/4, 1/8, 1/16, etc. Medium and organism controls were included with each test, and the tests were read when the pH of the organism control was 0·5 lower than the medium control. The culture of *M. suipneumoniae* normally used in this test had been cloned by five consecutive single-colony subcultures on solid medium, but a few comparisons were made using both cloned and uncloned antigen. The serum samples that were heat-treated were held at 56° C. for 30 min.

In fifty consecutive tests where various serum samples were examined in duplicate, 50 % showed no difference in the titre of the samples, 30 % differed by only one dilution, and a further 16 % (to make a total of 96 %) did not vary by more than two dilutions. (As two series of dilutions were made, a difference of one dilution in this work is less than the more conventional difference of one doubling dilution.) The reproducibility of the test was then observed by examining one

serum sample on six different days, the same bottle of liquid medium being used throughout: the greatest difference in all the titres was two dilutions. This test was then repeated six times in duplicate on three different days, using six different batches of liquid medium: the maximum variation in titre was two dilutions, except for one result which showed a difference of three dilutions.

The effect of varying the mycoplasma concentration was also observed. Six sera were tested, using standard ampoules of the organism: the ampoule contents were diluted in tenfold stages from 10^{-2} to 10^{-6}, and between the dilutions of 10^{-3} and 10^{-5}, inclusive, the titres obtained for each serum did not vary by more than two dilutions, but at either end of the scale (that is, at 10^{-2} and 10^{-6}) there was sometimes a greater variability. The organism dilution most usually used in the work described later was 10^{-3}.

Indirect (*passive*) *haemagglutination* (*IHA*)

The stabilizing solution, referred to as 1 % protein, was normal rabbit serum, inactivated at 56° C. for 30 min., and diluted 1/100 in phosphate buffered saline (PBS) pH 7·2, after being absorbed at 37° C. for 1 hr. with an equal volume of washed, packed, sheep erythrocytes. Pig sera for testing were inactivated and absorbed in the same way.

Sheep erythrocytes in Alsever's solution were washed three times with PBS, pH 7·2, adjusted to a final concentration of 2·5 % and mixed with an equal volume of freshly prepared 1/120,000 tannic acid in PBS, pH 7·2. After 15 min. at 4° C., the cells were centrifuged, washed twice in PBS, pH 7·2, and resuspended to the original volume and concentration in PBS, pH 6·4.

The mycoplasma antigens (uncloned) were prepared by centrifuging liquid-medium cultures, previously incubated until the pH had changed from 7·4 to 6·9, at 53,700*g* for 30 min. The deposit was washed twice in PBS, pH 7·2, resuspended in one-hundredth of the original volume, and deep frozen until required. The opacity of the *M. suipneumoniae* antigen suspension corresponded to Brown's tube no. 4.

To sensitize the tanned red cells, the appropriate dilution of antigen (1/30 in PBS, pH 6·4) was mixed with an equal volume of cells and incubated at 37° C. for 15 min. in a water bath. The red cells were then centrifuged, washed three times in 1 % protein and resuspended in the same solution to the original volume of antigen. The optimum concentration of antigen for sensitization had been found by block titration with specific rabbit antiserum. In case these sera contained antibodies against liquid-medium constituents contaminating the antigens used to immunize the rabbits, the rabbit sera were absorbed with 100 mg./ml. of freeze-dried liquid medium, in addition to being absorbed with washed, packed, sheep erythrocytes.

The tests were made in 12 × 75 mm. Pyrex tubes. Doubling serum dilutions, starting at 1/5, were made in 1 % protein in 0·5 ml. volumes. To each tube 0·05 ml. of sensitized cells was added. After thorough shaking, the tubes were incubated at 37° C. for 2 hr.; the results were then noted, the tubes re-shaken and left at room temperature overnight before making the final reading. The end-point was taken as the highest serum dilution to give a clearly positive agglutination; red-cell

patterns intermediate between this and the controls were taken as negative. All tests on unknown sera had the following controls: the first four dilutions of each test serum with tanned, unsensitized, red cells; sensitized cells plus diluent (antigen control); a positive control consisting of a complete titration of a specific rabbit antiserum; and a negative control using a pig serum that was known to be free from antibody by this test. In reproducibility experiments the titres for the same serum samples tested on different days did not vary by more than one doubling dilution.

The specificity of the test was demonstrated by adding 0·05 ml. of antigen to one set of paired serum dilutions, and 0·05 ml. of 1 % protein to the other. After being held overnight at 4° C., 0·05 ml. of sensitized cells was added. After the usual incubation, the tubes were read for inhibition of the haemagglutination. Both the rabbit serum and high-titred pig serum were inhibited by dilutions of the *M. suipneumoniae* antigen, but not by an antigen prepared in similar fashion from *M. hyorhinis*. Also, significant titres were not obtained when the rabbit and pig sera were titrated against tanned red cells sensitized with *M. hyorhinis*, mycoplasma strain B 3, *M. granularum* or *M. gallisepticum* antigen. Non-specificity of a low order, however, was observed with a few sera, but in no instance did this extend beyond a dilution of 1/40.

Complement fixation (CF)

The technique of Bradstreet & Taylor (1962) was used, except that the haemolytic system was prepared just before use and, as suggested by Roberts (1968), the sera were not inactivated. The tests were made in 12 × 75 mm. Pyrex tubes. The optimum concentration of antigen (uncloned J strain of *M. suipneumoniae*) was established by block titration against a positive control serum: it was found that the antigen concentration was rather critical, and the highest concentration that was not anti-complementary was used. Two series of doubling dilutions of each pig serum were made, starting at 1/10 and 1/15, respectively. The test was read visually immediately after incubation and the titres quoted refer to fixation of 75 % or more. A standard, known positive pig serum was included with each test and, when the titre for this serum varied by more than half of one doubling dilution, the results were discarded.

A few serum samples were exchanged with another laboratory (Pfizer Ltd) and these were titrated blind in each case: the sera yielding titres of less than 1/10 in each laboratory gave the same result in the other; but the titres for positive sera were notably higher in this laboratory.

RESULTS

Infection and challenge of pigs

Four main immunization experiments were made.

Experiment 1. Three of four litter-mates aged 24½ weeks were infected. After 18 days one of the infected pigs (2803) was killed, when it had extensive, early lesions of enzootic pneumonia; from this it was presumed that all three infected

animals had probably developed lesions of enzootic pneumonia. The uninfected control (2806) was killed at the same time and had no abnormalities in the lung.

The two surviving pigs (2858 and 2859) were left until 16 weeks after their initial infection; they were then challenged with a lung suspension which was made from their litter-mate (2803) killed earlier and which, as described below, was shown to be capable of inducing enzootic pneumonia. After a further 20 days both pigs were killed and in each there were only extremely small, late lesions of enzootic pneumonia at the tips of the cardiac lobes; no mycoplasmas were seen in the touch preparations prepared from these lesions.

The same dose of the challenge inoculum was given to two pigs (2856 and 2857) aged 17½ weeks, two of their litter-mates remaining as uninfected controls (2854 and 2855). These four pigs were killed on the same day as the two challenged pigs: the two infected animals had moderate and extensive early lesions, respectively, of enzootic pneumonia, while the uninfected controls showed no abnormalities in the lungs.

Experiment 2. Four of eight litter-mates aged 24 weeks were infected. After 18 days one of the infected group (2805) was killed: it had moderately extensive, early lesions of enzootic pneumonia and from this it was presumed that the whole group had been satisfactorily infected. Two uninfected controls (2807 and 2809) killed at the same time showed histological changes not suggestive of enzootic pneumonia, and no mycoplasmas were seen in touch preparations made from their lungs.

As in Expt. 1, the survivors were left until 16 weeks after the initial infection, when all three infected animals were challenged and one of the two remaining controls received the same dose of the challenge inoculum. When killed 22 days later the control pig (2860) had extensive, early lesions of enzootic pneumonia, whereas a challenged pig (2861), killed at the same time, had only a very small, late lesion of enzootic pneumonia in the right cardiac lobe; two organisms that were probably mycoplasmas were seen in one area of the touch preparation made from this lesion.

The two remaining challenged pigs in the infected group were re-challenged twice more, 20 weeks 3 days and 21 weeks 2 days after the initial infection. The inoculum used for the second of these challenges was given in parallel to the remaining uninfected control, and all three animals were killed 20 days later. The control animal (2878) showed extensive, early lesions of enzootic pneumonia, whereas one of the challenged pigs (2877) had no abnormalities in the lung, apart from some increased cellularity around the bronchial tree, and the other (2876) showed only fairly small, late lesions of enzootic pneumonia. No mycoplasmas were seen in the touch preparations made from the latter lesions. All the lung material used to provide the challenge inocula in this experiment came from the pig that was killed first in the infected group.

Expts. 1 and 2 were started together, but in separate accommodation, in November 1965; both experiments were completed by April 1966. They indicated a strong natural immunity to challenge, but the longest time between initial

infection and killing after a single challenge was 19 weeks, at which time there were very small lesions in the lungs of the challenged animals. To establish whether such lesions were residual ones from the initial infection, and to observe whether a naturally induced immunity might still protect pigs from a substantial subsequent challenge after a much longer interval than 19 weeks, two further experiments (3 and 4) were made.

Experiments 3 *and* 4

In these later experiments the pigs were under 1 month old when infected; this was to see whether young pigs would develop as protective an immunity as the older ones in Expts. 1 and 2. A single inoculum was used to initiate both Expts. 3 and 4: as it was made from a combination of lesions from one positive control from each of the first two experiments, there was a connexion between the first two and the last two experiments. But as the latter were not begun until July 1966 there was a break of 3 months in the use of the animal accommodation.

The general plan of Expts. 3 and 4 was identical, and all infections, challenges, bleedings and killings were performed in parallel on the same dates. The only difference between the experiments was that in Expt. 3 the pigs were 16 days old, while in Expt. 4 they were 29 days old, when first infected.

Experiment 3. Five pigs were infected and three litter-mates were kept as controls. After 12 days one of the infected group (2895) was killed: it had extensive, early lesions of enzootic pneumonia, and from this it was assumed that the whole group had been satisfactorily infected. Four months after the primary infection a second pig (2928) was killed, in order to see whether the pneumonic lesions had resolved: this animal had virtually normal lungs macroscopically; microscopically, there was some evidence of late enzootic pneumonia but no mycoplasmas were seen in the touch preparations.

At 19 weeks after infection one of the three survivors (2943) was challenged and one control animal (2944) received the same dose of the challenge inoculum: both pigs were killed after a further 20 days. The control had moderately extensive, early lesions of enzootic pneumonia, whereas the challenged pig had only minute, late lesions and no mycoplasmas were seen in the touch preparations.

A second, similar challenge was made 39 weeks after primary infection and, as before, the two pigs were killed 20 days later. The control (2958) had extensive, early lesions of enzootic pneumonia, whereas the challenged pig (2960) showed only histological evidence of enzootic pneumonia and no mycoplasmas were found in the touch preparations.

A third, similar challenge was made 60 weeks after primary infection, the pigs again being killed 20 days later. The control (3024) had extensive, early lesions of enzootic pneumonia, whereas the challenged pig (3025) showed only minimal histological evidence of late enzootic pneumonia and no mycoplasmas were seen in the touch preparations.

Experiment 4. Five pigs were infected as in Expt. 3, but only two litter-mates were available as controls. The pig that was killed after 12 days in the infected group (2896) had moderately extensive lesions of enzootic pneumonia. The pig

that was killed after 4 months (2929) had almost healed, late lesions and no mycoplasmas were found in the touch preparations.

When the first challenge was made at 19 weeks, the control pig (2945) and the challenged pig (2942) showed essentially the same picture as the corresponding animals in Expt. 3.

This was likewise the case after the second challenge, in which the control was pig 2959 and the challenged animal was pig 2961.

None of the control pigs remained by the time of the third challenge, but as the inoculum was the same for both Expts. 3 and 4, the control pig for Expt. 3 (3024) indicated that the challenge dose was capable of initiating the disease in a non-immune animal. The challenged pig (3026) had very small consolidated areas which, histologically, proved to be a macrophage and giant-cell pneumonia with giant-cell granulomata; no mycoplasmas were seen in the touch preparations.

Examination of sera by different methods

In Expts. 1 and 2 all the 16 pigs involved were bled for serum samples just before entering the experiment, but the pigs in Expts. 3 and 4 were not. In all four experiments, serum was collected when each pig was killed. Various other serum samples were taken at intervening stages, as follows. In Expt. 1, pig 2858 was bled before it was challenged. In Expt. 2, pigs 2861, 2876 and 2877 were bled before their first challenge. In Expt. 3, pigs 2960 and 3025 were bled before challenge, and their respective positive controls (2958 and 3024) were bled before being infected. In Expt. 4, pigs 2961 and 3026 were likewise bled before challenge, and their respective positive controls (2959, and 3024 from Expt. 3) were also bled before being infected. Whenever antibody titres are quoted in the Tables in this section, the essential data about the sera are given; there is thus no need to deduce this information from the previous section on infection and challenge.

Metabolic-inhibition test

All the serum samples referred to above were examined using the MI test. The highest titre obtained was 1/24, and nearly all the titres were less than 1/12. It became apparent, however, that the titres did not correlate with the immune status of the pigs; for some of the animals that were shown by challenge to be strongly immune had low titres, while titres of up to 1/16 were obtained with over one third of the serum samples (10 out of 23) taken from uninfected controls or from pigs that had not yet been exposed to infection. For this reason, the results are not presented in detail. We wished to know, however, what the MI test might be measuring in these sera, and to study this question further the following experiments were undertaken. Before proceeding to these, however, the sera that appeared to give falsely positive titres were re-tested: in every case they continued to give positive results.

The sera from four pigs that had not been exposed to infection but which nevertheless had titres against *M. suipneumoniae*, the sera from five pigs that had been infected with ground lung, and the sera from two pigs (3014 and 3016) that had been injected with *M. suipneumoniae* in other work, were examined by the

MI test against various mycoplasmas; the results are summarized in Table 1. Whenever a comparison was made between unheated and inactivated serum, the titrations were performed in parallel on the same day; in addition, the titrations for each serum against *M. suipneumoniae* and *M. hyorhinis* were performed in parallel.

It can be seen that, apart from pig 3014, whenever an unheated serum gave an MI titre against *M. suipneumoniae*, it also gave a titre against the other mycoplasmas against which it was tested (*M. hyorhinis*, *M. pneumoniae* and *M. gallisepticum*). Sometimes these titres were reduced by the inactivation method, but often they were not. From these results it appears that many of the pig sera listed contained one or more metabolic-inhibitory substances that were non-specific (as judged by tests using four different mycoplasmas of porcine, human and avian origin) and were still active after heating at 56° C. for 30 min. Secondly, some of the pig sera contained one or more metabolic–inhibitory substances that were likewise non-specific, but were either inactivated or partly inactivated by the same heat treatment. Because of the different combinations of these substances that could be present in the various sera, and because the metabolic–inhibitory substances appeared to operate at different levels against the different mycoplasmas, it is difficult to analyse these results further.

In order to see whether the titre obtained might vary with the strain of organism used, sera from six pigs were titrated against the cloned J strain, the uncloned J strain and the CZ strain of *M. suipneumoniae*: with each serum, there was no significant difference between the results.

Indirect-haemagglutination test

A total of 21 serum samples were examined from 19 pigs that had not been exposed to infection in the four immunization experiments. All but one of these had an IHA titre of less than 1/5; the exception was the pre-infection sample from pig 2858 in Expt. 1, where the titre was 1/160.

The sera from 12 pigs in the four experiments that were killed either 12 (one), 18 (two), 20 (eight) or 22 (one) days after infection were tested. All these animals had substantial lesions of enzootic pneumonia, but all the sera had IHA titres of less than 1/5.

However, in constrast to the above findings, high IHA titres were obtained with sera from all the pigs infected 16 or more weeks previously, and with sera from all the challenged animals, as shown in Table 2. The second main conclusion from this Table is that the titres were usually as high before challenge as after challenge; most of the animals were killed so soon after challenge, however, that a slow increase in titre, as occurred after the primary infections would not have been detected. It is nevertheless possible that the IHA titre did not increase much further after challenge, because the titres in pigs 2876 and 2877, 20 days after their third challenge were no higher than in pig 2861, 22 days after its single challenge. With pig 2858, however, the titre had increased by 20 days after challenge, although at least part of this variation might be a reflexion of the variability of the test.

Table 1. *Titres obtained in the metabolic-inhibition test with various pig sera against different mycoplasmas*

Pig	Status when sampled	Organism used in titration: *M. suipneumoniae* Unheated serum	*M. suipneumoniae* Inactivated serum	*M. hyorhinis* Unheated serum	*M. hyorhinis* Inactivated serum	*M. pneumoniae* Unheated serum	*M. pneumoniae* Inactivated serum	*M. gallisepticum* Unheated serum	*M. gallisepticum* Inactivated serum
2807	Control	8 16	4 4	8 12	3 6	—	—	—	—
2854	Control	8 16	8 12	4 6	< 3 < 3	—	—	—	—
2958N	Control	4 4	4 4	4 6	3 3	—	—	—	—
3080*	Control	4	6	8	6	24	6	⩾ 32	3
3014	Doubly injected†	12 16	3 4	< 3 < 3	< 3 < 3	—	—	—	—
3016	Doubly injected†	8 12	6 8	6 12	< 3 < 3	—	—	—	—
2928	18 weeks after infection	⩾ 32 ⩾ 32	8 12	12 12	12 12	—	—	—	—
2861‡	22 days after challenge (immune)	6	6	8	6	⩾ 32	12	12	4
2860‡	22 days after infection (not immune)	3	4	4	3	⩾ 32	8	8	< 3
2858‡	22 days after challenge (immune)	12 12	12 16	8 12	12 16	⩾ 32 ⩾ 32	16 16	16 16	6 6
2803‡	18 days after infection (not immune)	4 4	3 4	8 8	3 3	8 12	4 4	8 8	< 3 < 3

NOTE. The figures given are the reciprocal of the serum dilution. The paired figures are the results of duplicate tests on the same day.

* This pig was in an experiment not described in this paper.

† These pigs were injected with *M. suipneumoniae*, either twice intramuscularly (3014), or intradermally and then intravenously (3016).

‡ Pigs 2861 and 2860 had pre-infection titres of < 3 against *M. suipneumoniae*, whereas pigs 2858 and 2803 had pre-infection titres of 3 and 6 respectively against the same organism.

Complement-fixation test

In general, we have not found the test easy to work with. Different batches of antigen varied both in their activity and anti-complementary effect; even with an apparently satisfactory batch of antigen, the test was occasionally nullified by an unexpected anti-complementary effect.

Table 2. *Titres obtained in the indirect-haemagglutination (IHA) test and the complement-fixation (CF) test with pig sera taken after infection and after challenge with enzootic pneumonia*

	Pre-challenge			Post-challenge*		
	Weeks after	Titre†		Days after	Titre†	
Pig no.	infection	IHA	CF	challenge	IHA	CF
2858	16	5,120	80	20	20,480	160
2859	16	N.D.	N.D.	20	10,240	120
2861	16	2,560	640	22	5,120	480
2876	16	1,280	160	20‡	1,280	40
2877	16	1,280	160	20‡	1,280	60
2928	18	20,480	640		Pig not challenged	
2929	18	40,960	N.D.		Pig not challenged	
2942	—	N.D.	N.D.	20§	20,480	60
2943	—	N.D.	N.D.	20§	10,240	480
2960	39	10,240	240	20	10,240	160
2961	39	20,480	320	20	20,480	240
3025	60	10,240	20	20	10,240	< 10
3026	60	10,240	20	20	20,480	30

* All the challenged pigs were shown to be immune.
† The figures given are the reciprocal of the serum dilution.
‡ This was the third challenge.
§ Challenged 19 weeks after initial infection. N.D. = Not done.

Nine sera from uninfected pigs were examined by the CF test: all had a titre of less than 1/10. Ten sera, taken 12–22 days after infection, were likewise examined: four of these had titres of less than 1/10, one had a titre of 1/20 and the remainder (50%) had titres ranging from 1/40 to 1/120. There was no correlation between either the extent of the pneumonic lesions or the time of sampling and the titre obtained; thus, the pig sampled earliest (12 days after infection) had a titre of 1/40. The remaining titres are shown in Table 2: these refer to serum samples taken at longer times after infection and also after challenge, and they can be directly compared with the IHA titres for the same serum samples. It can be seen that all the seven samples taken 16–39 weeks after infection had titres between 1/80 and 1/640, but the two pigs bled 60 weeks after infection had low titres.

In general, there was no increase in CF titre after challenge: the mean titres for the eight pigs sampled both before and after challenge were about 1/200 and 1/150, respectively, which indicates that possibly, on the contrary, the titre was falling with time, and this idea is supported by the low titres obtained 60 weeks after infection.

DISCUSSION

There is reason to believe that we were working with a relatively pure system microbiologically in the animal experiments; for the pigs had been produced by hysterectomy and kept in strict isolation throughout the whole course of the experiments and, also, the J strain of *M. suipneumoniae* used to infect the pigs was one link in a long chain of serial passages in pigs, during which there has been no evidence to suggest that any other mycoplasma, and in particular *M. hyorhinis*, was concurrently present. More specifically, the pneumonias from the following pigs in these experiments were cultured on solid medium and in liquid medium: 2856 and 2857 (Expt. 1), 2805, 2860 and 2878 (Expt. 2), and 2895, 2896, 2958 and 2959 (Expts. 3 and 4). No isolations of *M. hyorhinis* were made and *M. suipneumoniae*, which was the only mycoplasma recovered, was isolated from every case (Goodwin *et al.* 1968, table 1). It is known that this solid medium is able to support the growth of a wide range of mycoplasmas (Goodwin *et al.* 1967). Therefore, although the primary infections and challenges in these experiments were not made with cultures of *M. suipneumoniae*, it seems very likely that no other mycoplasma was involved.

It has been said earlier that, because one pig in each of the four main infected groups in the pig experiments had enzootic pneumonia when killed 12 or 18 days after infection, it was concluded that all the litter-mates in these groups had probably developed the disease when first infected. This conclusion is supported by the fact that from the 11 pigs that remained, nine were challenged once and killed: all these nine were found to be solidly immune. Secondly, there were nine positive controls in these experiments, and 18 similar pigs in other experiments immediately before or after the four described here: all these 27 animals developed enzootic pneumonia when inoculated in the same way. Thus, altogether, 31 pigs were inoculated about this time and killed shortly afterwards; as all these animals developed enzootic pneumonia, it seems highly probable that the remaining 13 in the present experiments, which were not killed early, did so also.

The animal experiments showed that all but one of the 11 challenged pigs had virtually no lung lesions at slaughter; the exception was pig 2876 in Expt. 2, which had been challenged three times, but even in this animal the lesions were fairly small in extent, dry, recovering macroscopically, and late histologically. As these animals resisted challenges that produced extensive, early lesions in their respective controls, they were—as judged by pulmonary evidence—strongly immune. This was the case when the pigs were as young as 16 days at first infection, and over a year old (60 weeks) at first challenge. It is interesting that such a strong, long-lasting immunity should develop in first-generation, hysterectomy-produced pigs, and it is unlikely that the immunity after natural infection would be any less in normally born pigs in the field.

Taylor-Robinson, Shirai, Soběslavský & Chanock (1966) concluded that the titres given by the tetrazolium-reduction inhibition test in man correlated with resistance to febrile illness caused by *M. pneumoniae*; they also believed that this test was measuring antibody, as the proportion of positive sera in a population increased with age. On the other hand, Davies & Hudson (1968) could find no

correlation between antibodies to *Mycoplasma mycoides*, as measured by growth inhibition in liquid medium, and immunity to contagious bovine pleuro-pneumonia. In our work there was likewise no such correlation; furthermore, the test frequently measured non-specific inhibitory substances. These substances might not have been specifically associated with the sera because, from the nature of the MI test, coupled with the fact that *M. suipneumoniae* does not grow as readily as some other mycoplasmas in our present media, errors in technique will tend to give a falsely positive result. But this seems unlikely, however, as we would not expect such errors to be reproducible; also, some sera have recently been re-examined by another worker in this laboratory who obtained results similar to the original ones. It is more probable, therefore, that the inhibitory substances were actually in the pig sera but, if so, they were not at constant levels; indeed, a high titre was sometimes obtained with one sample and a negative result with a subsequent sample from the same animal. At the moment, therefore, we can see no way in which the MI test in our hands can give intelligible results. Nor can much light be thrown on the nature of the non-specific inhibitory substances: they could be dietary in origin, however, and vary in type and concentration in the serum according to the nature of the diet and the time interval between feeding and bleeding.

The indirect-haemagglutination test gave much more coherent results: apart from one pig, all the pre-infection serum samples, or sera from uninfected pigs, had a titre of less than 1/5, and in every case high titres eventually developed after infection. It is not known how soon after infection they would be readily apparent, however, because no serum samples were taken between the first month and 16 weeks after infection, by which time the titres were substantial. Although all the pigs that were immune to challenge had high IHA titres, it cannot be assumed that such titres correlate with the degree of immunity. Only further work, in which there is variability in immunity among the animals, might elucidate this point further. However, these experiments have shown that paired sera taken before and after experimental infection with enzootic pneumonia show a marked change in reaction in the IHA test.

The results obtained with the complement-fixation test showed a consistent change from a titre of less than 1/10 before infection to a titre of 1/80 or more by 16–39 weeks after infection. In this sense, the results of the CF test were comparable with those of the IHA test. CF titres of 1/40 or more, however, had commonly appeared by 12–22 days after infection, whereas the IHA titres obtained at this stage of the disease were always less than 1/5. On the other hand, it seems likely that the CF titres declined earlier because they were very low at 60 weeks after infection, whereas the IHA titres were still high at this time. It could be, of course, that the two pigs bled as late as this were peculiar, and had never developed substantial CF titres, but it seems more probable, because their littermates had high titres at 39 weeks, that they themselves had had higher titres earlier. If the CF titre does decline almost to extinction point around one year of age, then this test would be of little use in the field for detecting whether older animals might have been exposed to the disease. Further work is clearly necessary

on this point, and also to see for how long the IHA titres are detectable in pigs that have had the disease.

It has already been mentioned that the IHA titres may not be a measure of immunity, but it seems more certain that there was little correlation between the CF titres and immune status; for pigs 3025 and 3026, which were powerfully immune 60 weeks after infection, both had CF titres of only 1/20. Davies & Hudson (1968) described a similar situation in contagious bovine pleuro-pneumonia. It is not known what aspect of the post-infection response these two serological tests were assessing but it is clear that they were measuring different factors, because the CF titres appeared before the IHA titres and, secondly, the two pigs just mentioned had at one stage high IHA titres and very low CF titres.

SUMMARY

Hysterectomy-produced, colostrum-deprived pigs, reared in special isolation accommodation, were infected with enzootic pneumonia and later challenged with the same strain of the disease. Both the original infections and the subsequent challenges were made with intranasal inoculations of suspensions of ground pneumonic lung, but there was no evidence to suggest that any mycoplasma other than the J strain of *Mycoplasma suipneumoniae* was involved.

Pigs that had recovered from the disease were strongly immune to challenge, in that they developed virtually no lung lesions when inoculated with lung suspensions that produced extensive lesions of enzootic pneumonia in control animals. This was the case, even when the pigs were as young as 16 days old when first infected and were not challenged until up to 60 weeks later.

Sera from these pigs taken before infection, about 2–3 weeks after infection, at various times after natural recovery, and before and after challenge were examined using the metabolic-inhibition test, the indirect-haemagglutination test and the complement-fixation test.

The metabolic-inhibition test proved of little value, because non-specific inhibitory substances were present in the sera of some pigs both before and after infection: these substances inhibited the growth of *Mycoplasma hyorhinis*, *Mycoplasma pneumoniae* and *Mycoplasma gallisepticum* as effectively as *M. suipneumoniae*. Sometimes the non-specific inhibition was reduced by heating the sera at 56° C. for 30 min., but at other times it was not, which suggests that at least two types of non-specific inhibitors were present.

Apart from one pig, all the sera that were expected to be negative for antibodies against *M. suipneumoniae* proved to be so by the indirect-haemagglutination test. Titres of less than 1/5 were obtained in this test using the sera from pigs killed 12–22 days after infection, but high titres were obtained 16–60 weeks after infection. It was not possible to say whether these titres correlated with immunity.

All the pre-infection sera when examined by the complement-fixation test had titres of less than 1/10, but by 12–22 days after infection over half the serum samples had titres of 1/40 or more, and titres of 1/80–1/640 were obtained at 4 and 9 months after infection. There was some evidence to show that these titres

declined more rapidly than the titres obtained in the indirect-haemagglutination test; for they were very low at 60 weeks after infection, at which time the indirect-haemagglutination titres were still high.

It seemed, therefore, that these two serological tests were measuring different aspects of the post-infection response. Also, because the complement-fixation titres were very low in two pigs that were shown to be powerfully immune, these titres did not appear to correlate with immunity.

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ADDENDUM

The results of Boulanger & L'Ecuyer (1968) appeared while this paper was in the Press. In the complement-fixation test for enzootic pneumonia, these authors inactivated their pig sera and used unheated calf serum as a supplementary factor; by this method, they obtained a rise and fall of post-infection CF titres broadly similar to those published here.

More recently, Takatori Huhn & Switzer (1968), using essentially the technique of Boulanger & L'Ecuyer (1968), likewise found that CF titres first appeared 2–3 weeks after the experimental production of enzootic pneumonia.

REFERENCES

Betts, A. O., Whittlestone, P. & Beveridge, W. I. B. (1955). Investigations on the control of virus pneumonia of pigs (v.p.p.) in the field. *Vet. Rec.* **67**, 685.

Boulanger, P. & L'Ecuyer, C. (1968). Enzootic pneumonia of pigs: complement-fixation tests for the detection of mycoplasma antibodies in the serum of immunized rabbits and infected swine. *Can. J. comp. Med.* **32**, 547.

Bradstreet, C. M. P. & Taylor, C. E. D. (1962). Technique of complement-fixation test applicable to the diagnosis of virus diseases. *Mon. Bull. Minist. Hlth* **21**, 96.

Davies, G. & Hudson, J. R. (1968). The relationship between growth-inhibition and immunity in contagious bovine pleuro-pneumonia. *Vet. Rec.* **83**, 256.

Goiš, M. (1968). Serological response of pigs to infection with mycoplasma. Demonstration of growth and metabolism-inhibiting antibodies in sera of pigs inoculated intranasally with *Mycoplasma hyorhinis*. *Zentbl. VetMed.* **15** (B), 382.

Goodwin, R. F. W. (1965). The phenomenon of suppressed respiratory disease in the litters of older sows. *Vet. Rec.* **77**, 383.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1965). Production of enzootic pneumonia in pigs with a mycoplasma. *Vet. Rec.* **77**, 1247.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1967). Characterization of *Mycoplasma suipneumoniae:* a mycoplasma causing enzootic pneumonia of pigs. *J. Hyg., Camb.* **65**, 85.

Goodwin, R. F. W., Pomeroy, A. P. & Whittlestone, P. (1968). Attempts to recover *Mycoplasma suipneumoniae* from experimental and natural cases of enzootic pneumonia in pigs. *J. Hyg., Camb.* **66**, 595.

HERDERSCHEE, D. (1963). An improved medium for the cultivation of the Eaton agent. *Antonie van Leeuwenhoek* **29**, 154.

LANNEK, N. & BÖRNFORS, S. (1957). Immunity to enzootic pneumonia in pigs following recovery from the disease. *Nord. VetMed.* **9**, 91.

ROBERTS, D. H. (1968). Serological diagnosis of *Mycoplasma hyopneumoniae* infection in pigs. *Vet. Rec.* **82**, 362.

ROSS, R. F. & SWITZER, W. P. (1963). Comparison of isolates of *Mycoplasma hyorhinis* by indirect haemagglutination. *Am. J. vet. Res.* **24**, 622.

TAKATORI, I., HUHN, R. G. & SWITZER, W. P. (1968). Demonstration of complement-fixation antibody against *Mycoplasma hyopneumoniae* in the sera of pigs infected with swine enzootic pneumonia. *Natn. Inst. Anim. Hlth Q., Tokyo* **8**, 195.

TAYLOR-ROBINSON, D., PURCELL, R. H., WONG, D. C. & CHANOCK, R. M. (1966). A colour test for the measurement of antibody to certain mycoplasma species based upon the inhibition of acid production. *J. Hyg., Camb.* **64**, 91.

TAYLOR-ROBINSON, D., SHIRAI, A., SOBĚSLAVSKÝ, O. & CHANOCK, R. M. (1966). Serological response to *Mycoplasma pneumoniae* infection II. Significance of antibody measured by different techniques. *Am. J. Epidem.* **84**, 301.

WHITTLESTONE, P. (1957). Some respiratory diseases of pigs. *Vet. Rec.* **69**, 1354.

WHITTLESTONE, P. (1967). Mycoplasma in enzootic pneumonia of pigs. *Ann. N.Y. Acad. Sci.* **143**, Art. 1, 271.