

Some experiments relating to artificial immunity in enzootic pneumonia of pigs

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Enzootic pneumonia of pigs is a common disease with a world-wide distribution. In Britain, the majority of pig herds appear to be infected, and the resulting economic loss is very great. Under intensive husbandry conditions, where large numbers of pigs are closely confined in a common air-space, the pneumonia is slow to heal and commonly persists to the normal slaughter age and beyond. Pigs so affected require significantly more food—often an average increase of 20 to 25 %—to make the same live-weight gain.

Because there is no satisfactory way of combating the economic effect of this disease in an infected herd, except by reducing the concentration of animals, most efforts to date have been spent on the problems of eradication. A co-ordinated health-control scheme was initiated in this country for enzootic-pneumonia-free herds in 1959 (Goodwin & Whittlestone, 1960) but various difficulties have arisen, the greatest being the problem created by the high reinfection rate (Goodwin & Whittlestone, 1967). Some alternative or complementary method of control would be very useful, therefore, and the immunological approach is an obvious choice.

There is good reason to believe that a strong natural immunity occurs in the field, and this is supported by the findings of Lannek & Börnfors (1957) who were unable to reinfect pigs which had recovered from the experimentally-induced disease. More recently, Goodwin, Hodgson, Whittlestone & Woodhams (1969) infected hysterectomy-produced, colostrum-deprived pigs with enzootic pneumonia and then challenged them, after recovery, with the same strain of the disease. These animals developed virtually no lung lesions when inoculated with lung suspensions that produced extensive lesions of enzootic pneumonia in control animals; this was so, even when the pigs were as young as 16 days old when first infected and were not challenged until up to 60 weeks later.

Goodwin, Pomeroy & Whittlestone (1965, 1967) established that enzootic pneumonia was caused by a mycoplasma, by inducing the disease with colonies that had been passaged on solid medium; these colonies were named *Mycoplasma suisipneumoniae*. Consequently, the way has been cleared to investigate the possibility of inducing an artificial immunity to this disease, using pure cultures of the causal agent. The present paper describes some preliminary experiments in this direction.

MATERIALS AND METHODS

Antigens

The J strain of *M. suis*pneumoniae and strain 603 of *Mycoplasma hyorhinis* were used (Goodwin *et al.* 1967).

The cloned strains of these two organisms had passed through five consecutive single-colony subcultures on solid medium.

The *M. suis*pneumoniae antigen that was injected into pigs was uncloned and was grown in the liquid medium previously described (Goodwin *et al.* 1969). Each batch of this antigen was the fifth passage from a culture which had induced enzootic pneumonia after being passaged considerably more times in parallel. The antigen was washed three times by being shaken in phosphate-buffered saline (PBS) and then centrifuged for $\frac{1}{2}$ –1 hr. at 30,000–50,000 *g.* on each occasion. The antigen was then resuspended in PBS and the opacity compared with Brown's tubes: on the assumption that two mycoplasma organisms would be equivalent in opacity to one organism of *Brucella abortus*, the antigen suspensions used (before mixing with adjuvant) contained about 10^{11} mycoplasmas/ml. Formalinization was with 1/2000 formaldehyde at 37° C. for 6 hrs. When Freund's complete adjuvant was used, this was mixed with approximately equal quantities of antigen suspension containing 200 units of penicillin and polymyxin and 240 μ g. of streptomycin/ml. All the pigs in Table 1 received 1–2 ml./dose of either antigen suspension alone or of antigen suspension plus adjuvant; the sow in Table 3 received 5 ml. doses.

Serological techniques

Serum samples were stored at about –20° C.

Metabolic inhibition (MI)

This test was performed as previously described (Goodwin *et al.* 1969), using both heated (56° C. for 30 min.) and unheated serum samples, and both cloned and uncloned strains of *M. suis*pneumoniae.

Indirect (passive) haemagglutination (IHA)

This test was performed as before (Goodwin *et al.* 1969).

Complement fixation (CF)

This test was carried out in the same general manner as before (Goodwin *et al.* 1969), but with the following variations. The 4% red-cell suspensions were standardized colorimetrically in a spectrophotometer. Only one series of doubling dilutions was made, starting at 1/10, and the test was discarded if the titre of the standard positive pig serum differed by more than one dilution from the usual titre. The titres quoted represent fixation of 50% or more.

As mentioned in our previous paper (Goodwin *et al.* 1969) this test presents various difficulties. Inactivated (56° C. for 30 min.) and unheated samples of some sera were titrated in parallel: in some cases, the titres were the same but, in others, the fixation in the tests with heated serum was reduced to 50–75% throughout the

dilution range. This suggested that inactivation damaged some stabilizing accessory factor. The short period of fixation (3 hr. at room temperature) used by Roberts (1968) was found to be unsatisfactory, in that false positives and cross reactions, as between *M. suis pneumoniae* and *M. hyorhinae*, for example, were obtained: such discrepancies did not seem to occur with overnight fixation.

Precipitation in agar-gel

The double-diffusion method of Ouchterlony (1964) was used. The *M. suis pneumoniae* and *M. hyorhinae* antigens were prepared as for the IHA and CF tests.

Pigs

The pigs listed in Tables 1 and 2 were all hysterectomy-produced, colostrum-deprived (HPCD) animals. Litter 183 was 14 weeks old and litter 185 was 5 weeks old when first injected. Litter 186 (Table 2) was 17 days younger than litter 185. The sow shown in Table 3 came from a herd established entirely from HPCD pigs and maintained in isolation on the Veterinary School farm: the sow was 18 months old when first injected and had had one litter previously. The lungs of routinely slaughtered pigs from this herd are regularly checked and have been free from lesions of enzootic pneumonia.

All the pig experiments were performed in a specially designed isolation building, as previously described (Goodwin, Pomeroy & Whittlestone, 1968). The injection routes for the antigen are given in the text. Challenge was by intranasal inoculation of suspensions in broth of ground lung affected with the J strain of enzootic pneumonia. This strain has been repeatedly used to infect pigs and the only mycoplasma isolated from it has been *M. suis pneumoniae*. The challenged pigs in Table 1 received about 5 ml. of a 1/10 lung suspension. This dose became the high dose in Table 2, where the pigs receiving the lower dose were given 5 ml. of a 1/100 suspension. The doses of lung suspension used in the sow-and-litter experiment are detailed in Table 3.

The criteria for diagnosing enzootic pneumonia were as previously described (Goodwin *et al.* 1969). In some cases, the diagnosis was confirmed by isolating and identifying *M. suis pneumoniae*. The scoring system for recording the extent of the consolidated lesions was related to the fact that, in enzootic pneumonia, such lesions occur almost entirely in the apical and cardiac lobes of the lung, in the intermediate lobe, and in the leading edges of the diaphragmatic lobes. Ten points were allocated to each apical or cardiac lobe, five points to the intermediate lobe and five points to each leading edge of the diaphragmatic lobes; thus, if all this tissue were totally consolidated (which would be an unusually severe case) the pneumonic score would be 55. The mycoplasma score was based on the numbers of mycoplasmas with the morphology of *M. suis pneumoniae* seen in touch preparations made from pneumonic tissue: 0 = none; 1 = few; 2 = moderate numbers; 3 = large numbers; and 4 = very large numbers.

RESULTS

Stimulation of antibodies in young pigs with formalinized antigen, and the subsequent challenge of these animals

Eleven pigs were injected twice with formalinized antigen, the first injections being given with Freund's complete adjuvant. They were injected intramuscularly on each occasion, or intradermally followed by intravenously, and the interval between the two injections was 18 days. The IHA and CF titres of the sera are shown in Table 1, together with the results of challenging six of these animals plus two controls.

The pigs that had been injected with antigen showed no obvious resistance to challenge. One of the two positive controls (3013) developed only a small area of enzootic pneumonia, as did one of the injected pigs (3016). The average pneumonic score and the average mycoplasma score in these controls was 14 and 2.5, respectively; the comparable figures for the six injected pigs were very similar (12 and 2). Table 1 shows that neither the IHA nor the CF serum titre appeared to be related to protection, in that the two injected pigs with the smallest area of pneumonia had the highest and lowest CF titres, while the pig with the highest IHA titre had almost as much pneumonia as the pig with the lowest IHA titre.

The IHA titres developed slowly, 4 of the 11 still being < 10 by 18 days after the first injection; however, substantial titres were recorded by 14 days after the second injection, five of them being over 20,000.

The CF titres appeared earlier than the IHA titres, in that all but one were 80 or more by 18 days after the first injection. However, they did not increase so dramatically thereafter: the mean CF titre after the first injection was a little over 200, and the mean titre after the second injection was about 930.

No significant change in the CF titres had occurred by 19 days after challenge, but there was a notable increase in most of the IHA titres by this time.

Effect of a lower challenge dose

The second part of this main animal experiment involved the five remaining injected pigs. It seemed possible that some degree of immunity might have been induced but that this had been overwhelmed by a too heavy dose of challenge inoculum. The effect of a smaller challenge was therefore observed in pigs 3031, 3032 and 3030 (Table 2) for which pigs 3027, 3028 and 3029 served as positive controls. The latter three controls all developed lesions of enzootic pneumonia, as did one of the injected pigs (3032); the other two injected pigs (3031, 3030), however, did not develop lesions and no mycoplasmas were found in touch preparations made from the lungs. It is very unusual to obtain complete negatives in such HPCD pigs, and this result might therefore indicate that a sufficient degree of protection had been induced by the injections. These two pigs were also the ones with the highest IHA titres and the ones that had received the double intramuscular injections; one or both of these facts might be relevant to their lack of lesions.

Table 1. IHA and CF titres developed by pigs injected with formalinized suspensions of *M. suis* pneumoniae, and the effect of challenging such animals with enzootic pneumonia

Pig	Litter	Route of injection	Serum titres (reciprocals)												Pneu- monic score*	Mycoplasma score*
			18 days after (and just before 2nd injection)		14 days after 2nd injection		37 days after 2nd injection (when challenged)		56 days after 2nd injection (when killed)							
			IHA	CF	IHA	CF	IHA	CF	IHA	CF	IHA	CF				
3017	183	I/M; I/M	160	80	640	640	1280	640	5120	640	12.5	3				
3019			< 10	160	80	640	640	10,240	1280	22	3					
3031			80	160	2560	2560	ND	ND	Not killed	NA	NA					
3065	183	I/D; I/V	40	20	1280	640	ND	ND	Not killed	NA	NA					
3016			160	640	20,480	1280	10,240	1280	20,480	1280	1	1				
3018			20	640	40,960	1280	10,240	640	81,920	1280	18	2				
3032			20	80	640	1280	ND	ND	Not killed	NA	NA					
3066	185	I/M; I/M	< 10	160	2560	320	ND	ND	Not killed	NA	NA					
3014			40	80	20,480	640	10,240	320	40,960	320	3	1				
3015			< 5	160	40,960	320	40,960	320	40,960	640	17	2				
3030			< 5	160	40,960	640	ND	ND	Not killed	NA	NA					
3012	183	—	—	—	—	—	Uninjected positive controls challenged on same day		< 5	40	26	2				
3013			—	—	—	—	< 5	40	2	3						

Note. The 11 injected pigs were all bled before their first injection: the IHA titres were all < 5, and the CF titres were all < 10. * The scoring systems are described in Materials and Methods. ND = not done; NA = not applicable; I/M = intramuscular; I/D = intradermal; I/V = intravenous.

Table 2. *Low-level challenge of pigs that had previously been injected with formalinized suspensions of M. suis pneumoniae, and a high-level challenge of pigs that had probably recovered from enzootic pneumonia*

Pig	Litter	Route of injection	Dose of first challenge inoculum	Serum titres (reciprocals)											
				Day before challenge		22 days after challenge (when killed)		17 weeks after challenge (when rechallenged)		26 days later (at slaughter)		Pneu- Myco- monic plasma score* score*			
				IHA	CF	IHA	CF	IHA	CF	IHA	CF		IHA	CF	
3030	185	I/M; I/M		81,920	320	81,920	320	—	—	—	—	—	—	0	0
3031	183	I/M; I/M		2560	640	81,920	1280	—	—	—	—	—	—	0	0
3032		I/D; I/V	Low (5 ml. of 1/100)	640	640	40,960	640	—	—	—	—	—	—	23	2
3027	186	None; Positive controls		< 5	< 10	< 5	40	—	—	—	—	—	—	8	2
3028				< 5	< 10	< 5	40	—	—	—	—	—	—	3.5	2
3029				< 5	< 10	< 5	80	—	—	—	—	—	—	8.5	2
3065	183	I/M; I/M		1280	640	—	—	20,480	1280	10,240	1280	10,240	1280	1	0
3066		I/D; I/V		5120	320	—	—	20,480	640	10,240	320	0.5	0	0	0
3022	186	None; positive controls	High (5 ml. of 1/10)	< 5	< 10	< 10	40	—	—	—	—	—	—	4	2
3023				< 5	< 10	< 5	40	—	—	—	—	—	—	7	2
3064	183	None; positive control		—	—	—	—	At challenge	< 5	< 10	20	80	8	3	3

* The scoring systems are described in Materials and Methods.

Response to a superimposed experimental infection

In parallel with the above low-dose challenge experiment, two pigs (3065, 3066) were given the higher challenge dose used earlier (Table 1) with pigs 3022 and 3023 serving as positive controls. The control animals were killed 20 days later to check that the inoculum was capable of inducing enzootic pneumonia and as this was the case, pigs 3065 and 3066 were kept alive, in the hope that they would have already developed enzootic pneumonia, as did their litter-mates (3017, 3019, 3016, 3018) when similarly challenged earlier (Table 1), and thereafter become naturally immune during recovery. At 17 weeks after their first challenge, therefore, they were rechallenged with the higher dose of infective inoculum and killed 26 days later. Both pigs had only very small lesions of a type resembling late enzootic pneumonia and no mycoplasmas were found in the touch preparations, whereas the positive control (3064), challenged and killed at the same time, had active lesions of enzootic pneumonia and large numbers of organisms with the morphology of *M. suis pneumoniae* were present in the touch preparations. It seemed from this that both pigs 3065 and 3066 had strongly resisted their second challenge, almost certainly because they had developed a natural immunity: this immunity, however, was not associated with IHA or CF serum titres that were any higher than those of pigs 3018 and 3015 (Table 1) which did not resist a comparable challenge.

Metabolic-inhibition test

While this work was in progress, it had become apparent in other work (Goodwin *et al.* 1969) that the MI test might be of little value, because non-specific inhibitory substances were present in the sera of some pigs both before and after experimental infection; this non-specific inhibition was sometimes reduced by heating the sera (56° C. for 30 min.), but at other times it was not. Only a limited number (37) of serum samples were examined with this test, therefore, in order to see whether the MI test might be more helpful with serum samples from pigs that had been injected with antigen. The results are not presented in detail; they can be summarized, however, as follows.

Unlike some of the sera in our previous study (Goodwin *et al.* 1969) all the pre-exposure serum samples that were examined had MI titres of < 3. After the pigs had been injected with antigen, however, the sera became positive: the highest titre in the heated serum samples was 1/12 and the highest in the unheated samples was 1/24. Titres of this order, however, were not associated with obvious immunity and there was no increase in the MI titres of the heated serum samples from pigs 3065 and 3066 (Table 2) when these animals became immune following natural infection.

Precipitation in agar-gel

Sera from the 11 pigs in Table 1 that were injected with antigen were examined by this method against *M. suis pneumoniae*. All were negative before injection, 9 out of 11 were positive 18 days after the first injection and all were positive 14 days after the second injection; the longest period between the first injection of antigen and challenge was 11½ weeks and all the five pigs examined after this time were

positive by the agar-gel precipitation test. The sera of two pigs killed 22 and 26 days after infection were negative.

The positive sera usually gave two or three precipitation lines by this method, all of which were continuous with the lines obtained with the serum from a rabbit which had been injected with the same antigen. One pig serum gave a fourth line. No precipitation lines were obtained when these sera were tested against *M. hyorhinis*.

The sera and the colostrum sample from the sow and the sera from the piglets (Table 3) were also examined in this way. The pre-injection serum sample from the sow was negative, but positive results were obtained with all the post-injection sera from this animal, and also with the colostrum. All the piglets had positive sera 7 days after birth, but only two out of the six sera that were collected when the piglets were killed were still positive.

The antibody response was less marked in the sow than in the pigs in Table 1, in that none of the post-injection sera from the sow gave more than one precipitation line, and this was reflected in a single, weakened line in the sera of the piglets. The colostrum sample, however, gave two precipitation lines.

No differences were detected between cloned and uncloned, or between particulate and ultrasonically-disintegrated antigens.

Stimulation of antibodies in a pregnant sow with non-formalinized antigen, with subsequent antibody transfer to the litter via the colostrum

In case the formalin treatment in the earlier experiments had reduced the antigenicity of *M. suis* pneumoniae, the antigen in this experiment was not formalinized; nor was any adjuvant used. Two intramuscular injections were given 22 and 13 days before the sow farrowed seven live pigs. The antibody titres and the results of challenging the sow and her litter are summarized in Table 3. The sow had IHA and CF serum titres when tested 9 days after the first injection and the colostrum taken at farrowing had high titres by these tests. By 7 days of age, when the litter was first tested, the piglets' sera showed good IHA and CF titres. Compared with the colostrum titres, however, the IHA titres were relatively much lower at this time than the CF titres. The sow and litter were challenged with either high or low doses when the litter was 8 days old and killed 19 or 20 days later.

Apart from piglet 3044, which showed slight collapse histologically, the lung lesions in all these animals were typical of enzootic pneumonia. The mean pneumonic score of the three piglets that were given the high dose of inoculum was nearly 15, and the corresponding score for the four piglets that received the low dose was seven: this difference might have been due to the different levels of challenge. In general, however, the litter was not obviously protected by the colostrum antibodies, unless the small lesions in pigs 3049 and 3044 indicated some degree of protection against a low challenge dose. Neither did the injections given to the sow prevent her from developing enzootic pneumonia.

Table 3. IHA and CF titres developed by a pregnant sow injected with non-formalinized suspension of *M. suis* pneumoniae, the titres acquired by the litter from the colostrum, and the effect of challenging the sow and litter with enzootic pneumonia

Pig	Titres (reciprocals)												Pneu- monic score†	Myco- plasma score‡
	Serum, 22 days before farrowing*		Serum, 13 days before farrowing†		Colostrum during farrowing		Serum, 7 days after farrowing, 1 day before challenge		Serum, 19 or 20 days after challenge, when killed		Challenge dose			
	IHA	CF	IHA	CF	IHA	CF	IHA	CF	IHA	CF	ml.	dilution		
Sow 3051	< 10	< 10	80	40	20,480	320	640	80	640	< 10§	8	1/10	6	2
Piglet 3045	—	—	—	—	—	—	1280	160	320	40	5	1/2.5	8	3
3046	—	—	—	—	—	—	640	320	160	80	4	1/2.5	12	2
3048	—	—	—	—	—	—	1280	80	160	< 10§	4	1/2.5	24	3
3044	—	—	—	—	—	—	1280	320	160	< 10§	4.5	1/250	0.5	0
3047	—	—	—	—	—	—	2560	160	160	< 10§	5.5	1/250	9.5	3
3049	—	—	—	—	—	—	320	40	No serum	1	5	1/250	1	3
3050	—	—	—	—	—	—	1280	160	80	40	4	1/250	17	4

* Time of first injection

† Time of second injection

‡ The scoring systems are described in Materials and Methods.

§ Partial fixation occurred with these sera at a titre of 1/40 or 1/80.

DISCUSSION

The first six pigs that were challenged after being given formalinized *M. suis* pneumoniae antigen plus Freund's complete adjuvant (Table 1) showed no clear resistance to the development of enzootic pneumonia. When a lower challenge dose was used, however, some protection might have occurred (pigs 3031, 3032 and 3030 in Table 2), but only experiments involving larger groups of animals could establish whether this was indeed the case. Nevertheless, it is very likely that our usual challenge inoculum (about 5 ml. of a 1/10 suspension of pneumonic tissue given intranasally) is considerably more potent than the initial challenge that would be experienced by most pigs in the field from airborne infection. The mode of natural infection is also different, in that the farm pig is usually continually exposed to the disease, and the initial, possibly small doses of this type might reinforce an artificially-induced immunity rather than overwhelm it. There is an additional complication affecting these conclusions: farm pigs, even when apparently free from enzootic pneumonia, are more difficult to infect with this disease than hysterectomy-produced pigs (R. F. W. Goodwin and P. Whittlestone, unpublished); this natural resistance, coupled with a specific, artificially-induced resistance, might tip the scales in favour of the pig not developing enzootic pneumonia under all but the heaviest weights of natural infection. Certainly, however, any immunity induced in the present experiments was not comparable with that resulting from the experimentally-induced disease, because the challenge dose employed here was generally similar to the challenge doses used in our previous work, where the pigs recovering from enzootic pneumonia were found to be powerfully immune (Goodwin *et al.* 1969).

One further point should be raised concerning the evaluation of protection. Enzootic pneumonia has a marked debilitating effect in the field but it has frequently been observed that this effect does not often correlate with the extent of the pneumonic lesions at slaughter. One possible explanation for this anomaly is that the causal mycoplasma may exert its influence by inducing some extra-pulmonary metabolic derangement. If this were so, the extent of the pneumonic lesions after challenge might not be the best or sole criterion for the effectiveness of artificial immunization—growth rate or conversion ratio should also be taken into account.

Whether any appreciable degree of immunity was induced with killed antigen or not, it seems clear that this immunity did not correlate with either the MI, IHA or CF serum titres; for the higher IHA and CF titres among the challenged pigs in Table 1 appeared to confer no additional benefit, and when pigs 3065 and 3066 (Table 2) became naturally immune they did not develop any higher IHA or CF titres than injected pigs that had not resisted a comparable challenge. Indeed, the MI, IHA and CF titres in these non-immune or poorly immune pigs were generally as high as those in our previous work on the strong immunity following the experimentally-induced disease (Goodwin *et al.* 1969). It seems, therefore, that even if a suitable way of inducing a good immunity by artificial means could be found, the MI, IHA and CF serum titres would not indicate the strength of this immunity.

The immunization of a pregnant sow with non-formalinized antigen did not protect the sow from developing pneumonia; neither was the litter obviously protected by the colostrum transfer of antibodies. These results, coupled with the findings in the pigs given formalinized antigen, are not very encouraging as far as the question of a satisfactory vaccine is concerned but they do not, of course, rule out the eventual production of such a vaccine.

In our earlier study of immunity in experimentally-induced enzootic pneumonia (Goodwin *et al.* 1969) all but one of 21 serum samples from 19 pigs that had not been exposed to infection had an IHA titre of less than 1/5; no higher titres, however, were obtained by 22 days after infection, although substantial titres were obtained in all the pigs that had been infected 16 weeks previously. Likewise, in the work now described, the pre-injection titres were all below 1/10 and the IHA titres were slow to develop: four out of the 11 pigs in Table 1 still had titres of less than 1/10, 18 days after their first injection, but substantial IHA titres were recorded by 14 days after the second injection (32 days after the first injection).

The equivalent situation with the CF titres was that, as in the earlier study, the pre-injection titres were all less than 1/10, but the CF titre in the sow had become 1/40, 9 days after the first injection, and by 18 days after their first injection, all but one of the 11 pigs in Table 1 had CF titres of 1/80 or more. The more rapid development of CF titres compared with IHA titres, which occurred in the experimentally-induced disease, was paralleled, therefore, in pigs which had been injected with antigen.

SUMMARY

Hysterectomy-produced, colostrum-deprived pigs were injected twice with formalinized antigen prepared from the J strain of *Mycoplasma suisipneumoniae*; the first injection was with Freund's adjuvant and the second injection without adjuvant. The immunity of these animals was tested by inoculating them intranasally with different doses of lung suspension prepared from cases of enzootic pneumonia. Two of the pigs were not killed shortly after infection, but were kept and challenged with enzootic pneumonia in order to compare the serology of experimentally-injected animals with the serology of the immune state following the experimentally-induced disease.

In a second main experiment, a pregnant sow was injected twice with non-formalinized antigen without adjuvant, and her litter was subsequently exposed to the disease at 7 days of age after suckling naturally from birth.

There was no evidence to suggest that the injections had protected the pigs in the first experiment against a high dose of infection, but they may have given some protection against low doses. The piglets suckled by the injected sow were not protected against two different doses of infection.

Serum samples taken at different stages were examined by the metabolic-inhibition (MI) test, the indirect-haemagglutination (IHA) test, the complement-fixation (CF) test and the gel-diffusion precipitin test, using *M. suisipneumoniae* as antigen.

Serum samples taken before injection in the first experiment were all negative

in the MI test and they became positive after the injections of antigen. However, the highest MI titres obtained were not associated with obvious immunity; nor was the development of true immunity after experimental infection associated with a change in MI titre.

In the first experiment, substantial IHA titres (over 20,000) were recorded by 14 days after the second injection of antigen. Again, there was no correlation between the IHA titres and the area of pneumonia following experimental infection. In the sow experiment, IHA titres developed after the first injection and increased after the second; a high IHA titre occurred in the colostrum and titres of 320 or more were present in the piglets 7 days after birth.

The CF titres appeared earlier than the IHA titres but did not increase so markedly thereafter. Once more, there was no correlation between the titre before infection and the area of pneumonia afterwards.

In the gel-diffusion test, precipitins were demonstrated in all the post-injection serum samples tested, most of the samples being positive after the first injection. Precipitins were also demonstrated in the colostrum of the injected sow and in her uninjected litter at 7 days of age.

From these experiments it was concluded that, as judged by the development of pneumonic lesions and in marked contrast to the known immunizing effect of the disease itself, injections of antigen given in this manner had little or no protective effect against the dose levels of infection used. Nevertheless, the titres obtained in the MI, IHA and CF tests were comparable with those obtained earlier in pigs that were strongly immune, which provides further evidence for the suggestion that these tests do not measure protective immunity.

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