ENZOOTIC PNEUMONIA OF PIGS:

GROWTH AND BEHAVIOUR OF THE CAUSAL MYCOPLASMA IN LIQUID MEDIA

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VARIOUS authors have claimed to have isolated the causal agent of enzootic pneumonia of pigs, but only rarely have these isolates been passed outside the the pig to a point that excludes the possibility of carrying over other agents. Furthermore, most isolates from enzootic pneumonia have not been shown to be capable of inducing the disease in pigs, which gives rise to the possibility that in many cases they may not be a primary causal agent of this condition.

Lannek and Wesslén (1957) and Betts and Whittlestone (1963) might have grown a causal agent of enzootic pneumonia in tissue cultures, but their results were not clear cut. Goodwin and Whittlestone (1963) passed a bacteria-free isolate from the J strain of enzootic pneumonia twice in plasma-clot cultures and then five times in pig-lung-monolayer cultures. After maintaining the agent for 54 days in tissue culture, a pig inoculum was prepared, in which the final dilution of the original pneumonia was at least 10^{-16}. This inoculum induced enzootic pneumonia in experimental pigs, from which the disease was transmitted serially four times in further pigs. The same authors (1964), in a preliminary report, showed that the J agent of enzootic pneumonia could be grown in an acellular liquid medium. This medium was used by Mare and Switzer (1965), who induced enzootic pneumonia with fluids containing small, coccoid and ring-form organisms; from these fluids they isolated a mycoplasma. Consequent upon our own work with liquid media, a solid medium was prepared which grew the J agent as a mycoplasma; after passing on solid medium, this mycoplasma induced enzootic pneumonia (Goodwin, Pomeroy and Whittlestone, 1965). Because, for most purposes, it is more convenient to work with the mycoplasma in liquid medium, this paper presents our experiences with 2 types of liquid medium in more detail.

MATERIALS AND METHODS

Source of infective agent.—The J strain of enzootic pneumonia was used (see Goodwin and Whittlestone, 1963, for its general history). Pig inocula A and C derived from the pneumonic lung of pig 2206(expt 9, Goodwin and Whittlestone, 1965); pig inoculum B derived from the tissue-culture fluids used to infect this pig and these fluids had, in turn, arisen from the pneumonic lung of pig 1857 (Gocdwin and Whittlestone, 1963). Pig inocula D and E derived from the pneumonic lung of pig 2262 (expt 11, Goodwin and Whittlestone, 1965), which had been directly infected from the pneumonic lung of pig 1857.

Preparation of liquid media.—The boiled-tissue-culture medium was prepared from pig-lung-monolayer cultures, the feeding fluid being 20 per cent inactivated pig serum (from an enzootic-pneumonia-free herd), 10 per cent of 5 per cent lactalbumin hydrolysate in phosphate-buffered saline, and 70 per cent Hanks' balanced salt solution, with Difco yeast extract

(0·01 per cent), penicillin (200 u. per ml.) and nystatin (50 u. per ml.). The fluids of growing cultures were removed and replaced with phosphate-buffered saline; the tubes were then almost totally immersed in boiling water for 30 min., after which the buffered saline was replaced with normal feeding fluid.

The acellular liquid medium was the feeding fluid described above, the pH being about 7·4. This medium was made in batches and deep frozen, aliquots being used as required.

The methods of inoculation and incubation were as previously described (Goodwin and Whittlestone, 1963).

Assessment and passing of cultures.—Cultures were assessed initially by comparing the degree of acidity in the infected and control tubes. Coverslips were then removed, stained immediately with May-Grünwald-Giemsa and examined at low power (×125) for micro-colonies, followed by a high-power examination of the individual organisms; they were scored arbitrarily from 0–5, the highest score indicating large numbers of organisms in every area of the coverslip. The scores obtained and the appearance of the organisms determined which cultures were chosen for passing and, unless frozen (about—60°), the fluids were always passed the same day.

Infection of pigs.—Two types of pig were used (Goodwin and Whittlestone, 1963): primary hysterectomy-produced colostrum-deprived (HPCD) animals and naturally-born animals (MH) from a herd established from HPCD pigs. Except in one indicated experiment, where 20 ml. of lung suspension was injected intra-tracheally under light pentobarbitone anaesthesia, all pig inoculations were made intra-nasally without anaesthesia. The dose of lung suspension (1 : 10–1 : 25 in broth) was 4–8 ml. All pigs were killed by bleeding under pentobarbitone-sodium anaesthesia, and the tissues freshly harvested under aseptic conditions. Tissues for histological examination were fixed in Zenker's fluid and stained with haematoxylin and eosin. The touch preparations from lungs were made with the freshly-cut surfaces of about eight different areas of pneumonia. They were air-dried, fixed in methyl alcohol for 2 min. and stained for 3 hr. with a 1 : 20 dilution of Gurr's improved Giemsa in phosphate buffer, pH 7·2. If no macroscopic lesions were present, the touch preparations and histological sections were made from the predeliction areas.

The diagnosis of enzootic pneumonia was based on the presence of consolidated lesions (mainly in the anterior lobes of the lung), the histological picture (alveolar-cell pneumonia, catarrhal bronchiolitis, perivascular and peribronchiolar mononuclear-cell accumulations, followed by the progressive development of lympho-reticular hyperplasia with resolution of the alveolar-cell pneumonia) and the presence of pleomorphic organisms in touch preparations (see Goodwin and Whittlestone, 1962).

RESULTS

Growth of the J Mycoplasma in Liquid Media

Boiled-tissue-culture medium

The pneumonias induced in pigs with inoculum A show that the causal mycoplasma was grown in this culture system (see later).

In the earlier passes in the boiled-cell medium, the organisms tended to be associated closely with cellular remnants; this was particularly striking in some cultures. In later passes, however, this association was lost—the organisms being more evenly scattered and in areas of the coverslip devoid of cellular remnants. In general, the organism in this medium resembled morphologically the organism seen in the acellular medium, except that beaded-ring forms predominated (Fig. 1).

The agent grew more readily in this medium than in our previous living cultures (Goodwin and Whittlestone, 1963) and in the acellular medium described below: better scores could be obtained more consistently and the growth conditions seemed less critical; for samples of a batch of acellular medium taken from the deep freeze might support growth on some occasions but not on others—an unaccountable variability not observed with the boiled-cell medium.

Acellular medium

The pneumonias induced in pigs with inocula C and D show that the causal mycoplasma was grown in this medium (see later). Although such growth was achieved, and sometimes readily, it is important to stress that passing the agent in this medium could be, in some cases, particularly difficult. Thus, on some occasions, the agent could not be passed for long, even in several parallel experiments; it was then necessary to return to deep-frozen material and start again. More often, the organism would pass, but fitfully, and it was a constant struggle to prevent it fading out. Furthermore, tubes inoculated in parallel with the same material at the same time often showed very variable growth. In this general context, 2 critical points were observed.

First, there was only a short period of time when the cultures were ready for passing: if the coverslips were removed too early, organisms were very difficult to find; if they were removed a few hours too late, the organisms were partially or completely degenerate and took the stain poorly. Such cultures passed with difficulty or not at all. Conversely, the most suitable cultures for passing were those with distinct organisms in high numbers.

Secondly, the dose of the inoculum was important: if it were too concentrated, the cultures rapidly became too acid and degenerate organisms were seen in the coverslips; if the inoculum were only slightly too dilute, no organisms could be seen in the next pass. Because of these 2 points, a number of passes were always kept going together and different dilutions of inoculum were used in parallel within each branch of a pass; in this way, a proportion of the passes were usually successful.

Because the scores obtained in this medium were lower than in the boiled-cell medium, some attempts to produce a pig inoculum were abandoned, even though the agent had been passed a sufficient number of times; it was felt, from the experience with inoculum E, that the few visible organisms present were unlikely to induce pneumonia in pigs. Once low scores were obtained throughout the different branches of one experiment, it was usually impossible to improve the scores in any subsequent passes.

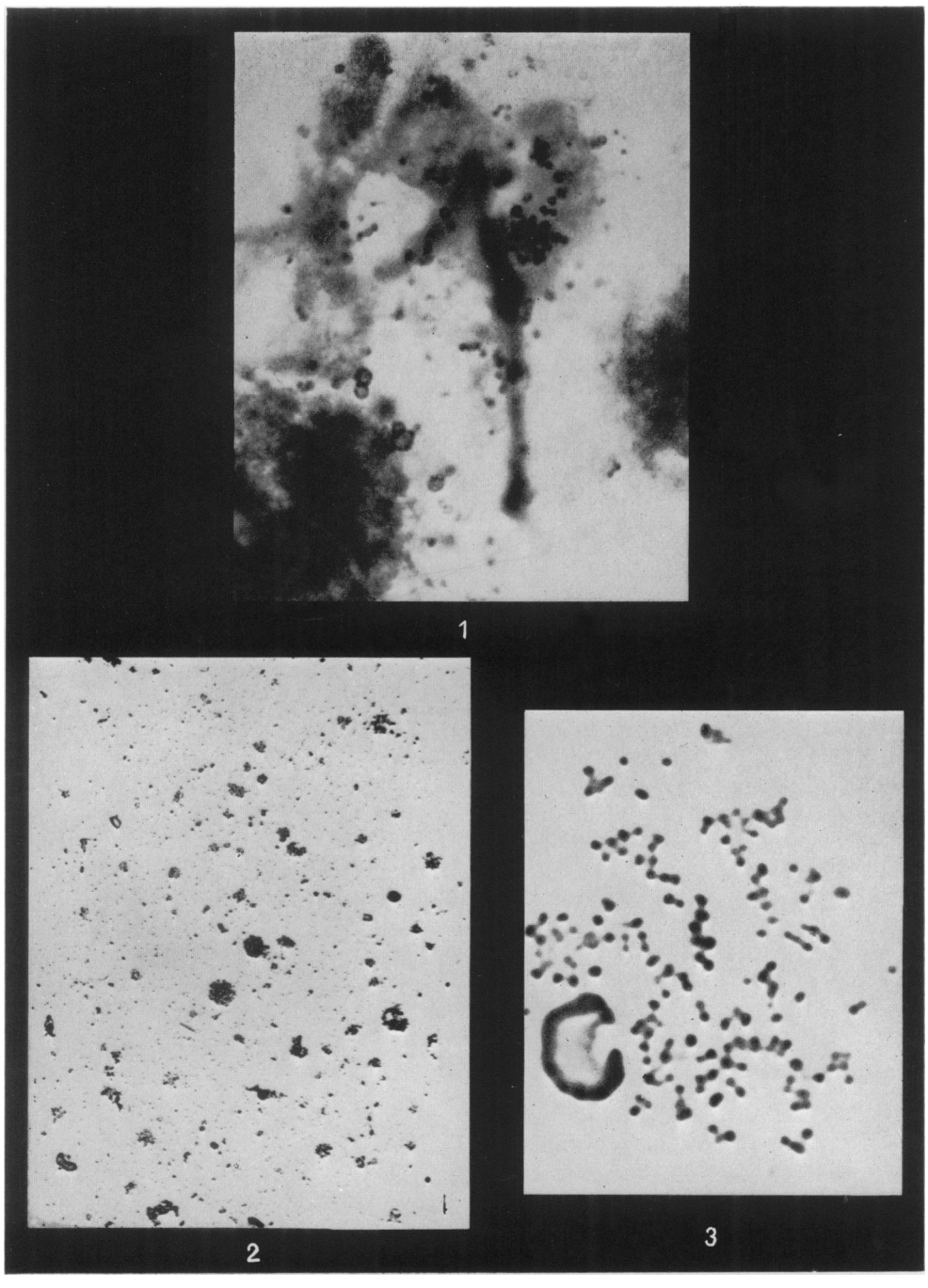
Morphologically, the organisms seen in this medium, and in the boiled-cell medium above, were similar to those described in living cultures (Goodwin and Whittlestone, 1963). In the present liquid medium, however, groups of organisms tended to grow in colonies; these reached an average diameter of 10–25μ (Fig. 2). Sometimes, very much larger colonies were seen. Short chains of cocci had been seen in living cultures but now these chains were much longer, the cocci (0·5μ diam.) being strung on fine filaments (about 0·1–0·2μ diam.) like branching necklaces (Fig. 3). Individually, the organisms, when in the form of globular structures, reached a maximum diameter of about 6μ (Fig. 3). In young cultures, the coccal forms predominated and they were then solid and intensely staining.

EXPLANATION OF PLATE

FIG. 1.—The J mycoplasma on boiled-cell remannts. Beaded ring forms predominate. May-Grünwald-Giemsa ×1500.

FIG. 2.—The J mycoplasma in acellular medium, showing groups and colonies. May-Grünwald-Giemsa ×130.

FIG. 3.—The J mycoplasma in acellular medium. Colony showing cocci strung on fine filaments and a globular structure. May-Grünwald-Giemsa ×2700.



Goodwin and Whittlestone

In more acid conditions, however, more faintly-staining ring forms were seen. It was found that the densely staining cocci were more suitable for obtaining successful passes.

Experimental Infection of Pigs

Inocula prepared from boiled-tissue-culture medium

Pig inoculum A induced enzootic pneumonia in pigs, but pig inoculum B did not.

Pig inoculum A.—Pneumonic material from pig 2206 was first inoculated into normal-lung plasma-clot tissue cultures. Thereafter, the agent was passed in boiled-culture medium (Table).

Four MH pigs, aged 33 days, were inoculated: when killed 19 and 20 days later, 3 of them had pneumonia. Two of these cases provided the intra-tracheal inoculum for 2 further MH pigs, aged about 4 months. When killed 15 days later, both had pneumonia. In both experiments the pneumonias resembled enzootic pneumonia completely. In each experiment there were three control pigs, none of which showed signs of enzootic pneumonia.

Pig inoculum B.—The agent in the tissue-culture fluids that had been used to induce the pneumonia in pigs 2003, etc. (Goodwin and Whittlestone, 1963) was passed 7 more times in pig-lung-monolayer cultures and then passed in boiled-culture medium (Table).

Three MH pigs, aged 13 days, were inoculated: when killed 19 days later, none had pneumonia. Histologically, no abnormalities were detected and only one pleomorphic organism was found.

Inocula prepared from acellular medium

Pig inocula C and D induced enzootic pneumonia in pigs, whereas inoculum E was only partially successful.

Pig inoculum C.—Pneumonic material from pig 2206 was first inoculated into normal-lung plasma-clot cultures, the fluids from which were passed once in boiled-culture medium; thereafter, the agent was passed in acellular medium (Table).

Three MH pigs, aged 29 days, were inoculated: when killed 20 days after the first inoculation, 2 of them had pneumonia. These pneumonias were jointly passed to 3 HPCD pigs, aged 55 days: when killed 20 days later, all 3 had pneumonia. Two of these cases provided the inoculum for a second pass in 3 HPCD pigs, aged 14 days: when killed 20 days later, all 3 had pneumonia. In all these experiments the pneumonias resembled enzootic pneumonia completely. In the first and third experiment there were 2 control pigs; in the second experiment there were 3 controls. The 7 control animals were killed at or about the same time as the infected pigs and none showed signs of enzootic pneumonia.

Pig inoculum D.—The object of this experiment was to confirm that enzootic pneumonia could be produced by the J mycoplasma grown in acellular medium. The starting point chosen, therefore, was a different pneumonia (pig 2262), which was inoculated directly into acellular medium; thereafter, the agent was passed in the same medium (Table).

Two HPCD pigs, aged 59 days, were inoculated: when killed 20 days after

TABLE.—*Details of Pig Inocula in Five Attempts to Induce Enzootic Pneumonia*

Pig inoculum	Culture medium	Seed pneumonia	Number of passes (a) in final medium	Number of passes (b) from seed	Days cultured in final medium	Minimum dilution (a) in final medium	Minimum dilution (b) of seed	Number of doses per pig	Total dose per pig (ml.)	Average score of visible organisms	Experimental result (pneumonia)
A	Boiled tissue cultures	2206	6	7	18 and 19	10^{-15}	10^{-17}	3	20	5—	Positive
B	Boiled tissue cultures	1857	6	20	22	10^{-7}	10^{-31}	1	4	4½	Negative
C		2206	8 and 9	10 and 11	19—21	10^{-15} to 10^{-17}	10^{-18} to 10^{-20}	2	10	4½	Positive
D	Acellular	2262	12 and 13		44—47	10^{-15} to 10^{-18}		4	29	2½	Positive
E		2262	12		28	10^{-15}		1	4	3	Marginal

the first inoculation, both had pneumonia. These pneumonias were jointly passed to 2 further HPCD pigs, aged 15 weeks: when killed after 17 days, both had pneumonia. The latter pneumonias jointly provided the inoculum for a second pass to a single HPCD pig, aged 24½ weeks; when killed 18 days later, this animal had an extensive pneumonia. All these cases of pneumonia resembled enzootic pneumonia completely. In each experiment, litter mates were used as controls; these were killed at the same time as the infected pigs, the first controls receiving control tissue-culture fluids and the subsequent controls receiving lung suspensions from the control animals in the previous pass. None of these control pigs had pneumonia.

Pig inoculum E.—This inoculum was initiated in the same way as pig inoculum D but only a single, small dose of the agent was given (Table).

Three HPCD pigs, aged 5 weeks, were inoculated: 2 were killed after 20 days and the third after 28 days; none had gross pneumonia but all showed slight catarrhal bronchitis and this was associated with minute areas of pneumonia histologically. In the first 2 pigs, pleomorphic organisms were readily found in touch preparations made from these areas and also from bronchial exudate, but no such organisms were found in the 2 control animals which had received uninfected culture fluids. No attempt was made to pass this material further in pigs.

DISCUSSION

Because the J mycoplasma was passed in both media to a dilution of at least 10^{-15} of the original culture seed, and because these final fluids induced a disease indistinguishable from enzootic pneumonia (which retained all the characteristics of enzootic pneumonia on further serial passes in pigs), it is clear that both media are capable of growing the causal agent of this disease. However, better growth occurred in the medium containing cellular remnants. This may be due either to the presence of additional nutritional or mechanical factors, or to the blocking of inhibitory components, and it is difficult to choose between these explanations on the information available.

The morphology of the agent was extremely variable but certain forms predominated in the different media: thus, in the boiled-cell medium there were many beaded rings; in the liquid medium, dots and filaments predominated; while, in touch preparations from pneumonic lung, bipolar organisms were commonly seen. The filamentous forms were seen best in high-contrast photographic enlargements; they then resembled the darkfield photographs of the comparable bovine agent, *Mycoplasma mycoides* (Turner, 1935).

From the Table, it can be seen that not all the inocula containing the agent induced enzootic pneumonia. There could be many reasons for this, but they are unlikely to lie on the animal side: very many successful transmissions of enzootic pneumonia have been made in this laboratory over several years using pigs of these ages and types. It seems more likely, therefore, that the variation was due to the pig inocula and, although one should not draw firm conclusions from only five experiments, there is a strong suggestion that the number of organisms given to the pigs was the determining factor. Thus, in the 3 fully successful experiments, the product of the total dose multiplied by the mean score of organisms was conspicuously greater than in the other 2 experiments.

SUMMARY

The J mycoplasma from enzootic pneumonia of pigs was passed in both a boiled-tissue-culture medium and an acellular medium until the final dilution of the original seed in each of these culture systems was at least 10^{-15}. These final fluids induced enzootic pneumonia in pigs.

The mycoplasma grew more readily in the boiled-cell medium than in the acellular medium. In the latter medium, the organism tended to form colonies consisting of cocci strung on fine filaments.

The problems associated with growing this mycoplasma in acellular medium, with special reference to the difficulty of obtaining a sufficiently heavy growth to induce the disease, are described.

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