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THE INTESTINAL FLORA IN PIGS WITH PARAKERATOSIS

III. SEROLOGICAL STUDIES OF BLOOD SERUM AND SOME SKIN TESTS

By

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Pigs fed ad libitum a diet containing 20 per cent fish meal (Icelandic codmeal) acquire an intestinal flora which is dominated by atypical *Clostridium perfringens* and, in conjunction with this, signs of parakeratosis, an increase in the erythrocyte sedimentation rate (ESR), and an increase in the serum γ-globulin. If the pigs are given zinc, the skin changes do not occur, and the ESR and the blood serum proteins remain normal. Zinc supplementation, however, does not prevent the pigs acquiring the abnormal intestinal flora. If the codmeal is replaced by Peruvian sardine meal, the number of atypical *Clostridium perfringens* in the faeces does not increase, no skin changes develop, and the ESR and the serum protein fractions remain normal (*Månsson*, 1964, I and II).

Against this background an obvious question is whether or not the intestinal flora has antigenically stimulated the host. The anaerobic component and particularly the atypical *Clostridium perfringens* which dominates this component is of special interest in this respect. This paper gives an account of a serological study of pigs with parakeratosis — as manifested in the experiments — to see whether they had circulating antibodies against the intestinal anaerobes. Some skin tests were also carried out.

MATERIAL AND METHODS

Three groups of pigs on different diets have been followed. Experimental details have been described in an earlier paper (*Månsson*, 1964, I). Group I, (6 animals) was fed the basal diet. Group II, (6 animals), was also fed the basal diet but three of the animals, pigs 1, 2 and 4, were given a zinc supplement. For group III, (6 animals), the Icelandic codmeal in the basal diet was replaced by Peruvian sardine meal.

Serological techniques

Agglutination

Antigen was prepared from an atypical *Clostridium perfringens* strain, no 968, cultured anaerobically (hydrogen) at 37°C for about 18 hours in a "toxin medium" prepared as follows: Add 1 litre dist. water and 25 ml 1N NaOH solution to 0.5 kg chopped horse meat, pH 7.8. Heat to 90°C with stirring. Let stand to cool. Filter through cotton. Add to the infusion polypeptone 3 %, yeast extract 0.5 %, and NaCl 0.5 %, pH 7.5. Dispense in flasks to which are added a few pieces (about 1 cm^3 in volume) of horse meat. Sterilize. Before inoculation add 1 ml 10 % dextrose solution to 100 ml of the medium.

The culture was decanted to avoid the bits of meat and after dilution 1:4 with saline solution it was used as antigen. Blood serum from the pigs was diluted with saline and added to small tubes containing an equal volume of the antigen. After incubation overnight at 37°C the tubes were examined ocularly and microscopically for agglutination. For microscopy the tubes were shaken very cautiously and a drop taken for examination at 100 and 300 magnifications.

Precipitation

Antigen was prepared from an 18-hours-old culture of strain 968 cultured in the same medium and under the same conditions as for the agglutination antigen. The culture was centrifuged at 3,000 rpm for ten minutes and the supernatant taken as antigen. Precipitation was carried out as a ring test in tubes 3 mm in diameter and the results read after three and thirty minutes.

Reagent for skin tests

Atypical Clostridium perfringens strain 968 was cultured in the same medium and under the same conditions as for the preparation of antigen for agglutination. An 18-hours *culture*, a *sterile filtrate* of the culture (Seitz filter), and *the sediment after centrifugation* at 3,000 rpm for ten minutes, were used for the skin tests. Toxins were neutralised by mixing 0.9 ml sterile filtrate with 0.3 ml of whole or diluted serum. Two serums were used — *Cl. perfringens A serum* ('Wellcome' Cl. welchii Type A diagnostic serum) and a homologous serum obtained

by repeatedly (for several weeks) injecting a horse with the supernatant (3.000 rpm for ten minutes) of an 18-hours culture of strain 968.

A *haemolytic E. coli strain* 0138:K81(B):H14 and two strains isolated from the faeces of a pig with parakeratosis, an *E. coli* and an *enterococcus*, were also tested. Broth cultures 18-hours old were injected intracutaneously. Unlike the other strains the enterococcus was cultured in beef heart broth.

RESULTS

Agglutination and precipitation

The agglutination titres obtained by ocular inspection are given in table 1. For pigs 292 and 299 in *group I*, the animals with the most severe degree of parakeratosis, the agglutination

Table 1.
Agglutination titres.

Pig no.	No of days after the beginning of the exp.								
	1.	8.	20.	26.	34.	41.	49.	63.	77.
Group I.									
292	1/10		1/320	1/1280	1/640	1/640	1/320	1/320	1/160
293	0	1/320							
299	0		1/320	1/1280	1/2560	1/1280	1/640	1/320	1/160
300	1/10		1/320	1/640	1/640	1/320	1/320	1/160	1/80
302	0	1/160							
303	0		1/160	1/320	1/640	1/320	1/320	1/160	1/40

	No of exp. days					
	1.	4.	11.	24.	33.	40.
Group II.						
1	1/10	1/10	1/20	1/10	1/20	1/20
2	1/10	1/10	1/20	1/10	1/10	1/10
4	1/10	1/10	1/20	1/10	1/20	1/20
6	1/10	1/10	1/20	1/40	1/40	1/320
7	0	0	1/20	1/160	1/80	1/640
9	0	0	1/80	1/160	1/640	1/1280

	No of exp. days			
	1.	14.	28.	42.
Group III.				
42	1/10	1/10	1/10	1/10
50	0	0	0	0
51	0	1/10	1/10	1/10
52	0	0	1/10	1/10
53	0	0	1/10	1/10
55	0	0	0	1/10

titres rose from 0—1/10 up to 1/1280 during the first 20 days of the experiment. Later on, the pigs received a zinc supplement and the titres gradually declined.

Much the same results were obtained for pigs 300 and 303.

Except for the samples taken at the beginning of the experiment, positive agglutination could be detected microscopically in tubes representing one or two dilutions more than the macroscopical agglutination titre. Only clumps of at least 15 to 20 bacteria were considered to represent positive agglutination. The toxin medium diluted with saline and/or serums from pigs receiving the control diet (*Månsson & Olsson,* 1961) without any increase in their clostridial intestinal flora, were used as controls.

Macroscopical agglutination was of O-type with distinct clearing of the bacterial suspension in comparison with the control tubes which contained saline solution instead of serum. The sediment was generally granular but sometimes appeared as a film at the bottom of the tubes. Even although complete clearing of the bacterial suspensions was seldom obtained, there were no difficulties in determining the upper limit of the titre.

For *group II* there was a slight titre increase in blood samples taken on day 24 and later on from pigs 6, 7 and 9, see table 1.

There was no detectable increase in antibody titre among the pigs in *group III.* The pigs in this group had been examined at birth and every second week up to the time the experiment was begun as well as throughout the experimental period. The dam of the pigs was also examined. All tests were negative.

The precipitation tests have generally confirmed the agglutination titres. On several occasions, however, the reactions were doubtful.

Both inactivated and unheated serum samples have been used and the reactions verified by using antigen — saline solution and antigen — medium as controls. In some series positive and negative tubes alternated, particularly at the higher serum dilutions. Positive reactions appeared within three minutes.

Skin tests

Group I.

Experiment A

Pigs 293 and 302 were tested on day 10 at the time of onset of the skin changes — erythema laterally on the thighs and on the abdomen, neck and ears.

The pigs were injected intradermally with three inoculates — *sediment* and *sterile filtrates of strain 968* and with *substrate.* The injections were made in macroscopically normal areas of the skin about 5 cm from erythematous areas on the thigh and over the thorax at sides about 6 cm apart in a horizontal line. Enough material was injected to produce a wheal about 5 mm in diameter.

At the site of injection of the *sediment* erythema and swelling developed within two hours and by 24 hours, a central necrotic crater about 1 cm in diameter surrounded by a swollen, erythematous zone. By two hours after injection of the *sterile filtrate* the site was slightly swollen and erythematous and by 24 hours a vesicle surrounded by slight swelling and erythema was formed. There was no reaction at the site of injection of the *medium.*

Experiment B

This test was run on pigs 299 and 300 on day 11 when they developed skin changes of the type described for the pigs in Experiment A. Pig 1 was also used; this pig received a diet containing less protein — the control diet (*Månsson & Olsson,* 1961) — and had no increase in its clostridial intestinal flora.

The intradermal injections were made in the same manner as in Experiment A using a *culture* of strain 968, a *sterile filtrate* of the same culture, and a *sterile filtrate* (Seitz filter) *of jejunum contents* (oral I, and aboral II, portions) obtained from pigs 293 and 302 after slaughter the same day.

In pigs 299 and 300, injection of the *culture* resulted in slight swelling and erythema after two hours and by 24 hours, a necrotic crater about one cm in diameter and surrounded by a swollen and erythematous zone. The necrotic tissue separated after six days to leave a schallow scar. The control, pig 1, developed slight swelling and erythema at the site of injection which subsided completely by 48 hours.

Within 24 hours after the injection of a *sterile filtrate of the culture* pigs 299 and 300 developed local erythema, swelling, and vesiculation but pig 1 showed no reaction. The reactions on pigs 299 and 300 gradually subsided and disappeared by 72 hours.

Injection of *sterile filtrates of jejunum contents* gave no reaction except in pig 300 which developed slight erythema and swelling lasting 48 hours at the site where filtrate I (from the anterior part of the jejunum) was injected.

Experiment C

Pigs 292 and 299 were injected intradermally on the 14th experimental day as in Expts. A and B with a *culture* of strain 968, a *sterile culture filtrate* (of the same strain), and with *culture* and *sterile culture filtrate neutralised with Cl. perfringens A serum* or *with a homologous serum.* The serums were used undiluted and in dilutions of 1:10, 1:100, and 1:1000. The pigs were also injected with *cultures of haemolytic E. coli, non-haemolytic E. coli,* and *enterococci.*

By four hours the sites of injection of *culture,* of *sterile culture filtrate,* and of *culture neutralised with both types of serums* and of *the enterococcus culture* were moderately swollen and erythematous. Both pigs reacted similarly. Only pig 292, however, reacted to the injection of sterile culture filtrate neutralised with Cl. perfringens A serum in a dilution of 1:1000. The pigs did not react at all or only with slight erythema to the other inoculates.

Twenty-four hours after the injection of *strain 968 culture* the reaction was severe and resembled those seen previously — swelling, necrosis etc. Neutralisation with undiluted serums seemed to result in a milder degree of reaction. The reaction to the injection of *sterile filtrates* was similar to that seen previously (Expts. A and B) — swelling, vesiculation etc. Neutralisation with homologous serum in all dilutions and with Cl. perfringens A serum in dilutions up to 1:100, however, abolished the reaction of the sterile filtrate. There was slight swelling and erythema at the site of injection of *the enterococcus strain* and no reaction at all to the other injections.

By 48 hours after injection only the reaction to the *culture of strain 968* persisted and this reaction followed the same pattern as in the previous experiments.

Experiment D

Pigs 2 and 3 in this experiment, like pig 1 in Expt. B, were fed a low protein diet — the control diet (*Månsson & Olsson,* 1961) — and did not acquire increased clostridial counts in the intestinal flora. *A culture* and *a sterile filtrate* of *strain 968* were injected intradermally. Slight swelling and hyperaemia developed within four hours and persisted for 24 hours. More severe changes — necrosis, vesiculation etc. — did not occur.

Pig no 2 was then injected intravenously with 100 ml citrate blood taken from pigs 292 (40 ml) and 299 (60 ml) in the acute

erythematous phase. Pig 3 was injected i. v. with 80 ml citrate blood, 20 ml from each of pigs 292, 299, 300 and 303 taken ten days after the onset of the skin changes.

Twenty-four hours after the injection of blood the pigs were injected intradermally with a *culture* and a *sterile filtrate of the culture* (strain 968), and with *the culture* and *sterile filtrate neutralised with Cl. perfringens A serum or homologous serum* diluted 1:10, 1:100, and 1:1000. Pig 3 was also injected with *haemolytic E. coli, non-haemolytic E. coli,* and *an enterococcus strain.* Moderate erythema and swelling developed within four hours after the injection of *the 968 culture* and after the injection of the *culture neutralised with serums.* By 24 hours there was a central necrosis at least 1 cm in diameter surrounded by strong erythema and swelling. The erythema and swelling regressed somewhat and by 72 hours had practically subsided. The central necrotic area separated from the underlying skin after eight days.

Injection of *the sterile filtrate* resulted in distinct vesiculation by 24 hours. The subsequent course was similar to that in the previous experiments. No reaction occurred after the injection of *filtrate neutralised with serums.* Nor was there a reaction to the injection of the other bacterial cultures.

During the course of the experiment the pigs maintained a normal body temperature, a good appetite, and remained clinically healthy.

Group II

Pigs 6, 7 and 9 developed similar positive reactions to *strain 968* with necrosis, erythema and swelling as did the pigs in Group I when tested at the time of onset of the skin changes. Similar reactions were obtained at the same time with cultures of *Cl. perfringens type B,* milder reactions with *Cl. perfringens type D* and *Cl. septicum,* and no reaction with *Cl. novyi* or *Cl. chauvoei cultures.* The organisms had been cultured in the same manner as strain 968. Intradermal injection of pigs 1, 2 and 4 gave negative results.

Group III

Skin tests were carried out in the same way as on the animals in group I but with negative results.

DISCUSSION

The purpose of these studies was to find out whether or not the atypical *Clostridium perfringens* in the intestinal flora induces the formation of specific antibodies in pigs which develop parakeratosis. Concentration on this particular component in the intestinal flora seems justified in view of the great numerical increase which occurs at the time of onset of the skin changes.

Blood serum from pigs which had developed signs of parakeratosis contained detectable antibody titres against atypical *Clostridium perfringens* strain 968. Animals which remained clinically healthy did not acquire titres. The presence of circulating antibodies was also demonstrated by the series of skin tests. Positive skin reactions were obtained with some other Clostridia species as well as with strain 968. Sterile filtrates of strain 968 gave positive reactions which could be inhibited by antiserum. The negative skin tests obtained with the *E. coli* strains have been complemented by a series of agglutination tests on blood serum. No O-titres greater than 1:20 have been obtained.

Pigs which develop parakeratosis also acquire changes in the composition of the intestinal flora (*Månsson,* 1964, I), the amounts of the serum protein fractions and the erythrocyte sedimentation rates (*Månsson,* 1964, II), and — from the results presented here — from antibodies against the clostridial component of the intestinal flora. Giving a zinc supplement is known to prevent the occurrence of parakeratosis. Nor does parakeratosis occur if the clostridial intestinal flora does not increase in number (group III). The place of zinc in this context is not clear as yet. Aspects of zinc metabolism will be dealt with in a subsequent publication.

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SUMMARY

Pigs which develop parakeratosis acquire agglutinins and precipitins against the atypical *Clostridium perfringens* in the intestinal flora. A strong reaction (oedema, necrosis etc.) also occurs at the site of intradermal inoculation of this organism. No reactions occur after inoculation of animals which lack this component in the intestinal flora or which have received a zinc supplement. The antibodies which seem to govern this reaction can be passively transferred to other pigs which then give a positive reaction to the skin tests.

ZUSAMMENFASSUNG

Die Darmflora bei Schweinen mit Parakeratose.

III. Serologische Untersuchungen von Blutsera und einige Hauttesten.

Bei Schweinen die an Parakeratose erkranken sind sowohl Agglutinine wie Präzipitine gegen die atypischen Clostridium perfringens der Darmflora nachgewiesen worden. Bei solchen Tieren erhält man auch eine starke, lokale Reaktion (Ödem, Nekrose etc) bei intrakutaner Inokulation von solcher Bakterienkultur. Eine negative Reaktion wird von demselben Inokulat bei Tieren erhalten die diesen Komponenten in der Darmflora vermissen oder die einen extra Zuschuses von Zink erhielten. Die Antikörper, die scheinbar die Reaktion bedingen, sind aber überführbar an solche Tiere die danach auch positive Hauttestreaktionen zeigen.

SAMMANFATTNING

Tarmfloran hos grisar med parakeratos.

III. Serologiska undersökningar av blodsera och vissa hudtester.

Hos grisar som insjukna i parakeratos har påvisats såväl agglutininer som precipitiner mot tarmflorans atypiska Clostridium perfringens bakterier. Hos sådana djur erhålles också kraftig lokal reaktion (ödem, nekros etc.) vid intrakutan inokulation av sådan bakteriekultur. Negativ reaktion erhålles av samma inokulat på djur, som sakna denna komponent i tarmfloran eller som erhållit extra tillskott på zink. Antikropparna, som synas betinga reaktionen, är överförbara till sådana djur, vilka därefter likaledes ge positiv hudtestreaktion.

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