Study of the Effect of Antibodies in the Intestinal Tract of Germ-free Baby Pigs

J. REJNEK, J. TRÁVNÍČEK, J. KOSTKA, J. ŠTERZL and A. LANC

Department of Immunology, Institute of Microbiology,
Czechoslovak Academy of Sciences, Prague 4

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ABSTRACT. The effect of antibodies in the intestinal tract was studied in germ-free baby pigs whose intestinal barrier was closed to macromolecules by the peroral administration of modified cow's milk for the first 72 hours after birth. They were then all contaminated with the pathogenic strain *Escherichia coli* 055 in amounts of 10^9 bacterial cells per animal. The controls, which were not given any antibodies, all died within 24 hours. All the experimental animals given 12.5—50 ml immune colostrum or serum survived, while of those given 50 ml normal serum or colostrum containing natural antibodies reacting with the *Escherichia coli* test strain, 50% survived. No circulating antibodies were found in the serum of the experimental animals after the administration of serum or colostrum. The antibodies present in colostrum thus appear to protect the newborn organism directly in the intestinal tract, which is the first site of bacterial invasion, as well as after infiltration into the blood stream.

Comparative study of colostral and blood serum immunoglobulins (Rejnek, Kostka & Trávníček, 1966) showed that the main component of porcine colostrum, which is the only source of maternal antibodies for the newborn organism (in pigs, the epitheliochorial placenta is impermeable to all types of antibodies) was γ-G-type immunoglobulin. The haemochorial type of placenta, which occurs in man, is permeable to IgG, but not to IgA and IgM; these last two types of immunoglobulins (IgA and IgM), however, are the main components of human colostrum (Hansen & Johanson, 1962; Rejnek, Škvařil & Doležal, 1960). Colostrum thus appears to be a source from which the newborn infant obtains types of antibodies not received during intrauterine life.

This hypothesis is weakened by the fact that antibody transfer by absorption from the colostrum into the blood stream has never been demonstrated in man, as found by Kuttner and Ratner in 1923. We therefore felt that it was important to determine whether colostral antibodies would provide protection against gastrointestinal infection, i.e. whether they would take effect on the bacteria directly in the alimentary tract. We carried out experiments with precolostral baby pigs reared in a germ-free environment. This model was chosen because pigs do not receive any antibodies from the mother via the placenta (Šterzl, Rejnek & Trávníček, 1966) and die if reared under conventional conditions on an antibody-free diet (Trnka *et al.*, 1959) or if inoculated with a pathogenic strain under germ-free conditions. Another advantage of these animals is that their intestinal barrier, which is permeable to macromolecules during the postnatal period, can be closed by the peroral administration of any, even heterologans protein within 12—36 hours after birth (Payne & Marsh, 1962; Brambell, 1958; Lecce, Matrone & Morgan, 1961). We were thus able to study the effect of antibodies on a given bacterial strain in an animal with no antibodies of its own and whose capacity for antibody transfer across the intestinal tract could be regulated as required.

MATERIALS AND METHODS

Germ-free baby pigs obtained by hysterectomy were kept in special isolators enabling them to be reared in a germ-free environment. The rearing method was described in an earlier paper (Trávníček *et al.*, 1966).

Normal and immune colostrum was obtained at the time of birth from gravid sows (normal and immunized with the pathogenic strain *Escherichia coli* 055). Immunized sows were inoculated intravenously with seven doses of formalinized bacterial suspension at 5-day intervals, in amounts increasing from 10^8 to $3.5 \times \times 10^9$ bacterial cells.

Since the colostrum could not be sterilized by the usual methods, it was spun for one hour at 20,000 rpm (50,000 G) and only the middle layer, free from sediment and floating particles, was used. When controlling sterility, only a few of the samples were found to be contaminated (1—5 microorganisms per ml.). All the colostrum samples were stored in the frozen state until required.

Normal and immune serum was obtained from normal and immunized gravid sows by exsanguination after hysterectomy. The sera were sterilized by filtering through a G-5 bacterial filter.

Lipopolysacharide was isolated by extracting it from the bacteria by Westphal's phenol method (Westphal *et al.*, 1952) and by precipitating the extract with alcohol.

For immunoelectrophoresis, the technique of Škvařil and Rejnek (1958) was employed. Precipitation was carried out with rabbit antisera against porcine serum and colostrum, prepared by the method described in a previous paper (Rejnek, Kotýnek & Kostka, 1965).

Radioimmunoelectrophoretic analyses were carried out by the method of Rejnek and Bednařík (1960), using ^{51}Cr-labelled lipopolysaccharide. *Escherichia coli* 055 lipopolysaccharide (50 mg) was incubated in a thermostat for 20 hours at 37° C with 0.5 mC ^{51}Cr in the form of sodium chromate with a specific activity of 62.2 mC/ /mg; after incubation, the free chromate was removed by repeatedly precipitating the lipopolysaccharide with alcohol (Braude *et al.*, 1955). After eluting the excess protein, the immunoelectrophoretic analyses were incubated for 12 hours at 0° C in ^{51}Cr-lipopolysaccharide solution; free antigen was then removed by active washing and the analyses were dried and tested by autoradiography (24 hours' exposure), using Agfa Duro X-ray film as the sensitive material.

In passive haemagglutination (Kabat & Mayer, 1961), Takatsy's micromethod (Takatsy, 1955) was employed, using *Escherichia coli* 055 lipopolysaccharide adsorbed to 1 ml 2.5% sheep red cell suspension in a concentration of 50 μg/ml.

RESULTS

The control group of eight germ-free baby pigs was fed for 72 hours after hysterectomy on modified cow's milk and was then infected perorally with a living *Escherichia coli* 055 suspension in amounts of 10^9 bacterial cells. These animals all died within 24 hours after infection.

When testing the protective effect of the colostrum and serum of normal animals, sterile precolostral baby pigs were fed for the first 72 hours after birth on modified cow's milk. They were then contaminated with the same dose of *Escherichia coli* as the controls and two hours later were given colostrum or serum per os in amounts of 50 and 25 ml. After that they continued to be fed on modified cow's milk. The results of this experiment (Table 1) show that the administration of 50 ml normal colostrum or serum led to the survival of about 50% of the experimental animals, while the rest died later than the controls. The protective effect of 25 ml normal colostrum or serum was evidently very low, as the experimental animals died only slightly later than the controls; this is in conformity with the low titre of "natural" antibodies in normal

Table 1. Effect of normal porcine colostrum and serum on germ-free baby pigs contaminated with the pathogenic strain *Escherichia coli* 055

Group of animals	Animal No.	Dose in ml	Type of protein administered	Antibody titre	Antibody titre in baby pig's serum		Time of death in hours (S = survived)
					Before admin. protein	After admin. protein	
I	1	50	norm. colostr.	1 : 32	0	0	S
	2	50	norm. colostr.	1 : 32	0	0	48
	3	50	norm. colostr.	1 : 32	0	0	S
	4	50	norm. colostr.	1 : 32	0	0	48
	5	50	norm. colostr.	1 : 32	0	0	72
	6	50	norm. colostr.	1 : 32	0	0	S
	7	50	norm. colostr.	1 : 32	0	0	S
	8	50	norm. colostr.	1 : 32	0	0	S
II	1	25	norm. colostr.	1 : 32	0	0	24
	2	25	norm. colostr.	1 : 32	0	0	24
	3	25	norm. colostr.	1 : 32	0	0	72
	4	25	norm. colostr.	1 : 32	0	0	24
III	1	50	norm. serum	1 : 128	0	0	S
	2	50	norm. serum	1 : 128	0	0	72
	3	50	norm. serum	1 : 64	0	0	48
	4	50	norm. serum	1 : 64	0	0	S
IV	1	25	norm. serum	1 : 64	0	0	72
	2	25	norm. serum	1 : 64	0	0	24
controls	1	—		—	—	—	24
	2	—		—	—	—	24
	3			—	—	—	24
	4	—		—	—	—	24

colostrum and serum reacting with the test strain, *Escherichia coli* 055.

The question of whether the experimental animals were protected by the actual direct action of the antibodies in the intestinal tract, or whether some of the antibodies crossed the closed intestinal barrier into the blood stream was resolved by immunoelectrophoretic analysis and by antibody assay of serum samples collected from the experimental animals two hours after the peroral administration of colostrum or serum. The two-hour interval was chosen because Morris (1964), who studied the transmission of antibodies across young mouse gut, found the maximum serum concentration two hours after administration and because, by administering ^{131}I-labelled γ-globulin, we also found that significant amounts of administered foreign protein were absorbed in baby pigs during this period.

The results of immunoelectrophoretic analysis (Fig. 1) and of antibody assay (Tab. 1) failed to demonstrate the presence of the administered antibodies in the serum of any of the experimental animals. Immunoelectrophoresis indicated the presence of traces of γ-globulin in most of the samples, but comparison with serum samples collected before administration of the protein showed that they were only traces of the γ-globulin present in the serum of colostrum-free baby pigs (Šterzl *et al.*, 1960; Franěk & Říha, 1964).

Since the 50% survival rate in the first experiment was evidently due to the low

Table 2. Effect of hyperimmune porcine serum and colostrum on germ-free baby pigs contaminated with the pathogenic strain *Escherichia coli* 055

Group of animals	Animal No.	Dose in ml	Type of protein administered	Antibody titre	Antibody titre in baby pig's serum		Time of death in hours (S = survived)
					Before admin. protein	After admin. protein	
I	1	50	immune colostr.	1 : 25,600	0	0	S
	2	25	immune colostr.	1 : 25,600	0	0	S
	3	25	immune colostr.	1 : 25,600	0	0	S
	4	12.5	immune colostr.	1 : 25,600	0	0	S
	5	12.5	immune colostr.	1 : 25,600	0	0	S
II	1	50	immune serum	1 : 51,200	0	0	S
	2	25	immune serum	1 : 51,200	0	0	S
	3	12.5	immune serum	1 : 51,200	0	0	S
	4	12.5	immune serum	1 : 51,200	0	0	S
controls	1	—	—	—	—	—	24
	2	—	—	—	—	—	24
	3	—	—	—	—	—	24
	4	—	—	—	—	—	24

titre of normal natural serum and colostral antibodies reacting with *Escherichia coli* 055, in the next experiments we used the colostrum and serum of a gravid sow immunized with a formalinized suspension of the same strain. The technique was basically the same, i.e. the experimental animals were fed on modified cow's milk for 72 hours after birth and were contaminated with the same dose of *Escherichia coli* 055. In these experiments colostrum and serum were administered in amounts of 50, 25 and 12.5 ml per animal.

As seen from the results (Table 2), all the animals given immune colostrum or serum in any of the above amounts survived, while all the control animals, which were contaminated but received no protein, died within 24 hours. Antibody assay of serum samples taken from the experimental animals two hours after administration of the protein again showed that perorally administered antibodies did not infiltrate into the blood stream under the given experimental conditions.

As part of these experiments we also thought we ought to determine which of the immunoglobulin fractions was responsible for the antibody activity of the given immune serum and colostrum, i.e. whether the effective antibodies in the two experimental groups were of the same type. The results of radioimmunoelectrophoretic analysis of samples of immune serum and colostrum are given in Fig. 2. This shows that the antibody activity of the hyperimmune serum and colostrum used was also bound only to the γ-macroglobulin fraction, as γ-G-globulin, which was present in both materials in much larger amounts, displayed no traces of radioactivity.

DISCUSSION

The results of the above experiments show that germ-free baby pigs are a satisfactory model for study of the antibodies contained in maternal colostrum and milk. During the immediate postnatal period, young pigs have practically no immunoglobulins, except for a very small amount of young piglet γ-globulin, which does not possess antibody activity (Franěk & Říha 1964; Šterzl *et al.*, 1960). Antibodies are transmitted by colostrum and milk only for a relatively short time, however.

Brambell (1958) found that the postnatal period for the passive transfer of immunity in pigs was 36 hours. Other authors, such as Barrick, Matrone and Osborne (1954), Olsson (1959) and Lecce and Matrone (1960) showed that intestinal absorption stops completely within 48 hours. These results are also confirmed by the studies of Asplund, Grummer and Phillips (1962) and of Speer *et al.* (1959). Payne and Marsh (1962) made a detailed study of the permeation of γ-globulin and of closure of the intestinal barrier and found that if baby pigs were fed either on colostrum or on modified cow's milk, absorption stopped 12 hours after birth, when the epithelial cells of the intestinal mucosa filled with γ-globulin or other soluble protein. If the animals were left to starve, however, the barrier did not close and absorption of γ-globulin was still found 106 hours after birth. Similar results were obtained by Lecce and Morgan (1962) in a study of the absorption of polyvinylpyrrolidone.

These findings thus merely underline the question of the function of the antibodies contained in colostrum and milk, which, although they are the only source of maternal antibodies for the newborn young in this species, can infiltrate into the blood stream for only a few hours after birth. Owen *et al.* (1961) studied the effect of the administration of immune globulins on survival and on the serum protein spectrum in colostrum-free baby pigs reared under conventional conditions. They found that the animals survived much better if immune globulins were administered perorally, even at a time when antibodies were no longer absorbed from the intestinal tract. They assumed that the immune globulins acted as copro-antibodies. Similarly, Salajka (1966), who studied the aetiopathogenesis of colienterotoxaemia in weaned piglets, showed that the prevalence of haemolytic specific pathogenic serotypes of *Escherichia coli* in young pigs after weaning was associated with the disappearance of immunoglobulins transferred in the maternal milk. He assumed that the growth of specific pathogenic strains in the alimentary tract of suckling pigs was inhibited by antibodies contained in the milk.

As seen from the results of the present experiments, the antibodies contained in normal colostrum or serum protect the newborn organism to some extent against gastrointestinal infection, since 50% of the infected animals given normal colostrum or serum in amounts of 50 ml per animal survived, while all the control animals died. In no case, however, were the antibodies contained in the administered colostrum or serum found in the serum of the experimental animals, confirming that the intestinal barrier was closed at the time of their administration. In our opinion, this experiment provides adequate evidence that the antibodies contained in colostrum and serum act directly in the baby pig's alimentary tract. It might be objected, however, that the protective effect is due not to antibodies, but (for example) to nonspecific bactericidal factors present in the serum and colostrum. In further experiments we therefore used hyperimmune serum and colostrum. These experiments confirmed that the protective effect was definitely due to the action of antibodies, since all the experimental animals survived, even when given only one quarter of the dose which provided protection in the experiments with normal colostrum and serum. Again, no antibody activity was found in the serum of the experimental animals, even after a dose of 50 ml hyperimmune colostrum or serum. According to the results of Payne and Marsh (1962), the absorption of γ-globulin is proportionate to the amount administered, maximum infiltration occurring after a dose of 60 ml colostrum. This result can be regarded as sufficiently convincing, since even if the amount of antibody absorbed was very small, with an initial titre of 1 : 25,000 for colostrum

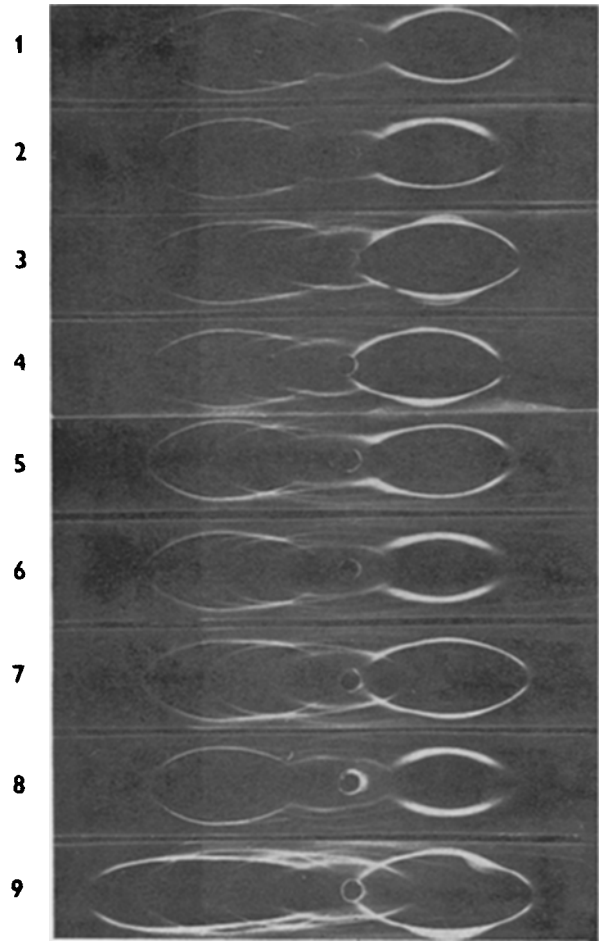

Fig. 1. Immunoelectrophoretic analysis of serum of experimental baby pigs before and two hours after administration of 50 ml colostrum. Strips 1—4: serum before administration of protein; 5—8: after administration of protein; 9: serum of normally suckled baby pig. Precipitated with antiserum against porcine serum proteins.

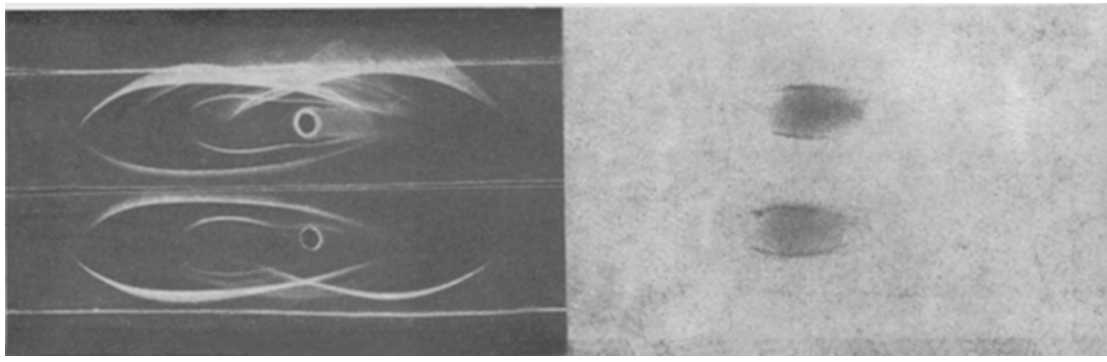

Fig. 2. Radioimmunoelectrophoretic analysis of immune porcine serum and colostrum. Strip 1 — serum; 2 — colostrum. Immunoelectrophoretogram on left, autoradiogram on right. Antiserum against porcine serum proteins was used in the marginal canals and antiserum against porcine IgM in the central canal.

and of 1 : 50,000 for serum it would still be possible to detect it in the baby pig's serum.

Another problem which needed to be resolved was the question of whether the antibodies against the test strain, *Escherichia coli* 055, in normal and hyperimmune serum and colostrum are carried by the same or different types of immunoglobulins. It is quite well known that "natural" antibodies in the sera of non-immunized individuals are carried by type M immunoglobulins (Landy & Weidanz, 1964). After active immunization with bacterial antigens, however, type γ-G antibodies are also usually present, in varying amounts (Weidanz, Jackson & Landy, 1964).

When investigating this problem, we used radioimmunoelectrophoresis with ^{51}Cr-labelled lipopolysaccharide isolated from the test strain of *Escherichia coli*, which, by being able to detect simple antigen-antibody complexes, makes it possible to determine all the antibodies present, irrespective of their serological heterogeneity, i.e. of their ability to participate in a reaction in which a different process from the adsorption of antigen to antibody is being studied (e.g. haemagglutination, haemolysis, etc). It is known that the haemagglutination reaction, for example, does not detect all antibodies (Benedict, 1965). The results of this experiment showed that the antibody activity of hyperimmune serum and colostrum was bound only to the γ-M immunoglobulins and that the results of the experiments with normal and hyperimmune serum and colostrum are therefore also capable of comparison from this aspect.

It can be concluded from the results submitted above that sterile colostrum-free baby pigs contaminated with a dose of the pathogenic strain *Escherichia coli* 055 causing death within 24 hours can be protected by the peroral administration of colostrum or serum (the dose depends on the antibody concentration) at a time when antibodies are no longer absorbed into the blood stream. The antibodies present in the colostrum and milk of mammals thus appear to be of significance not only from the aspect of the transfer of antibodies into the blood stream of the newborn, but also in providing protection against gastrointestinal infection directly in the alimentary tract.

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