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Studies of Bactericidal Activity to *Escherichia coli* of Porcine Serum and Colostral Immunoglobulins and the Role of Lysozyme with Secretory IgA

I. R. Hill and P. Porter

Unilever Research Laboratory, Colworth/Welwyn, Sharnbrook, Bedfordshire

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Summary. Gel filtration and immune inhibition techniques were used to study bactericidal activities of IgG, IgM and IgA against smooth strains of *Escherichia coli* 0141 and 08 in sow serum and colostrum and post-colostral piglet serum. Bactericidal activity in sow sera was primarily associated with IgM and a low molecular weight IgG component, 7S IgG activity was less frequently observed. In colostral whey fractions and post-colostral piglet sera, in the absence of lysozyme, bactericidal antibody activity was associated with IgM and 7S IgG. In post-colostral serum bactericidal antibody was also attributable to a low molecular weight form of IgG.

IgA in serum from the sow and neonate showed no bactericidal activity, even in the presence of lysozyme, whereas in colostrum secretory 11S IgA had bactericidal activity, but only in the presence of complement and lysozyme.

INTRODUCTION

Recent studies of the pig have described the transfer of specific immunoglobulins to the colostrum and milk (Porter, 1969; Bourne and Curtis, 1973) and their subsequent intestinal absorption by the neonate (Porter and Hill, 1970, Curtis and Bourne, 1971). The role of these immunoglobulins as antibodies to *E. coli* has been studied using antiglobulin haemagglutination techniques (Porter, Noakes and Allen, 1970) and these investigations suggest that IgA is an important local antibody in the passive defence of the young pig. The antibacterial role of IgA in external secretions has been questioned because of its apparent inability to fix complement (Ishizaka, Ishizaka, Borsoz and Rapp, 1966). However, human colostral IgA has been shown to lyse *E. coli* in the presence of lysozyme and complement (Adinolfi, Glynn, Lindsay and Milne, 1966) and the evidence for this bactericidal action has recently been re-examined and substantiated (Burdon, 1973).

It is questionable whether complement is present or can even survive in secretions in the gut, but studies of IgA in relation to alimentary tract defence in the young pig presents a special case since IgA is absorbed from the colostrum by the neonate. Furthermore IgA is present in sow colostrum in several different molecular classes and there is some selectivity against intestinal absorption of secretory 11S IgA by the piglet (Porter, 1973).

Studies of bactericidal antibodies have previously been carried out in the pig (Stertzl, Kostka and Lanc, 1962; Webb and Muschel, 1968) without examining the significance of

specific immunoglobulin classes. The present paper describes investigations of bactericidal activity against *E. coli* of immunoglobulins in sow serum, colostrum and the serum of suckling pigs and examines the role of lysozyme in the antibacterial function of IgA.

MATERIALS AND METHODS

Gel filtration and chromatography

Chromatography was carried out on Sephadex G-200 (45 × 2·5 cm) using 0·1 M Tris-buffered saline, pH 7·2.

Isolation of specific immunoglobulins

The preparation of porcine immunoglobulins IgG, IgM and IgA has been described by Porter (1969). The isolation of different molecular sizes of IgA from post-colostral piglet serum has been described by Porter (1973).

Affinity chromatography in the preparation of rabbit antiglobulins for porcine IgG, IgM and IgA

Emulsions of immunoglobulins in Freund's Bacto-Adjuvant, Incomplete, were injected subcutaneously at several sites into New Zealand White rabbits for antisera production. The antisera were rendered specific for porcine γ-, μ- or α-chains by absorption with purified non-specific immunoglobulins linked to cyanogen bromide-activated Sepharose 4B (Pharmacia) or the antiglobulins were isolated by affinity chromatography using these immunoabsorbents.

Quantitative estimation of immunoglobulins

The radial immunodiffusion technique of Mancini, Carbonara and Heremans (1965) was used to assay levels of immunoglobulins in colostral whey and sera.

Bactericidal antibody tests

Bactericidal tests were performed by a quantitative photometric assay (Muschel and Treffers, 1956). 0·05 ml volumes of serum or colostrum or Sephadex G-200 fractions of these, were supplemented with 0·05 ml rabbit complement (absorbed with the bacterium under test) and where necessary 0·05 ml lysozyme (to produce a final concentration of 30 μg/ml). Bacterial cultures in brain heart infusion (Oxoid) were prepared, and standardized to an optical density of $0{\cdot}15 \pm 0{\cdot}01$ at 650 nm in a Unicam SP500 Spectrophotometer. To whole sera and colostral whey 0·05 ml volumes of a 10-fold dilution of the culture were added, whilst to G-200 fractions of sera and colostral whey 0·05 ml volumes of a 100-fold dilution were added. Higher concentrations of bacteria were also used to ascertain whether bactericidal activity was being inhibited by antibody excess (Muschel and Treffers, 1956) but on no occasion was this so. Diluent 0·85 per cent saline containing 0·003 M $MgCl_2$ and 0·0004 M $CaCl_2$ and buffered at pH 7·2 with 0·05 M Tris (2-amino-2-(hydroxymethyl)-1,3-propandiol) was added to a total volume of 0·25 ml. Controls on bacterial growth included (a) serum or colostrum (complement omitted), (b) complement (serum or colostrum omitted) and (c) diluent alone. After a reaction period of 1 hour at 37° bactericidal activity was stopped by the addition of 1·0 ml Brain Heart Infusion (Oxoid). Incubation at 37° was allowed to continue until control preparations, without antibody, reached an optical density of 0·3–0·4. Bactericidal activity was then assayed photometrically.

Serum from piglets and sows

Sows were bled from an ear vein and post-colostral piglets (2 days old, maintained on the sow) from the anterior vena cava, and the blood allowed to clot. Serum was removed, cleared by centrifugation and stored at −20°.

Preparation of sow colostral whey

Sow colostrum collected at parturition, was centrifuged at 100,000 *g* for 30 minutes at 20° (Bohren and Wenner, 1961).

Bacteria

Bactericidal activity was assayed against two smooth strains of *E. coli* isolated from pigs. Haemolytic strain A499 (0141; K85a,c (B); H4) was isolated from a herd of pigs undergoing a severe outbreak of gastro-enteritis; non-haemolytic strain A517 (08; H^-) was isolated from healthy pigs.

Complement

Serum was obtained from rabbits by cardiac puncture. For use with A499 (0141) the natural antibodies were absorbed with the organism to be tested (Muschel and Treffers, 1956). Absorption of serum with A517 (08), however, removed complementary activity in addition to natural antibodies (possibly due to the inactivating action of endotoxin in the presence of antibody (Kostka and Stertzl, 1962)). Sera with very low levels of *E. coli* 08 antibodies were thus used, unabsorbed, as a source of complement in tests involving *E. coli* 08. The bacteria were removed by centrifugation and membrane filtration, and the sterile complement stored at −20° and used within 4 weeks. The activity of the sera was tested in a haemolytic system using sheep erythrocytes. Bacterial absorption was found to remove lysozyme completely from the source. Sera not absorbed by bacteria were absorbed with bentonite (Wardlaw, 1962) to remove lysozyme.

Immune inhibition of bactericidal antibody

Serum, colostral whey or Sephadex G-200 fractions of these were absorbed with specific rabbit anti-IgG, -IgM and -IgA globulins. One volume of the specific antiglobulin was added to three volumes of the test serum, colostral whey or fractions.

Assay of lysozyme

Lysozyme was assayed using the method of Osserman and Lawlor (1966). Lysozyme activity against *Micrococcus lysodeikticus* (Sigma, London) is expressed as the equivalent weight in μg/ml of three times crystallized egg white lysozyme (Sigma, London).

RESULTS

GEL FILTRATION AND IMMUNE INHIBITION STUDIES OF BACTERICIDAL ANTIBODIES IN SOW SERUM, COLOSTRAL WHEY AND POST-COLOSTRAL PIGLET SERUM

Gel filtration studies of *E. coli* 0141 and *E. coli* 08 bactericidal antibodies are shown for sow serum, colostral whey and piglet serum in Figs 1, 2 and 3 respectively. These figures give elution characteristics of immunoglobulins and also the effects on bactericidal antibody of absorbing selected fractions with rabbit antiglobulin specific for porcine IgG, IgA and IgM. Thus the antibodies are characterized by elution characteristics in the

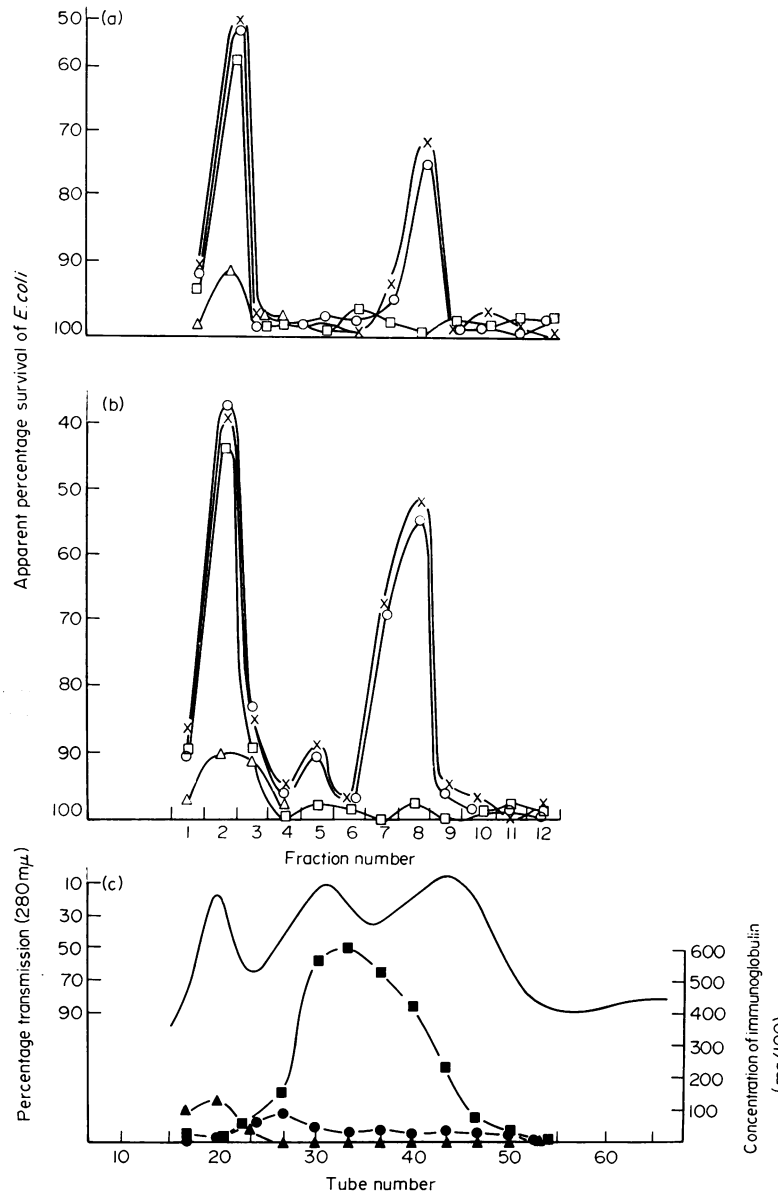

FIG. 1. Assays of sow serum fractions for bactericidal antibody activity to (a) haemolytic *E. coli* 0141 and (b) non-haemoltic *E. coli* 08; demonstrating effects of absorption with specific rabbit anti-pig IgG, IgM and IgA globulins. (×) Unabsorbed; (□) IgG absorbed; (△) IgM absorbed; (○) IgA absorbed. All fractions were supplemented with complement. Fractions were prepared by gel filtration on Sephadex G-200. (c) Pooling data and immunoglobulin assays using radial immunodiffusion are given. (■) IgG; (▲) IgM; (●) IgA.

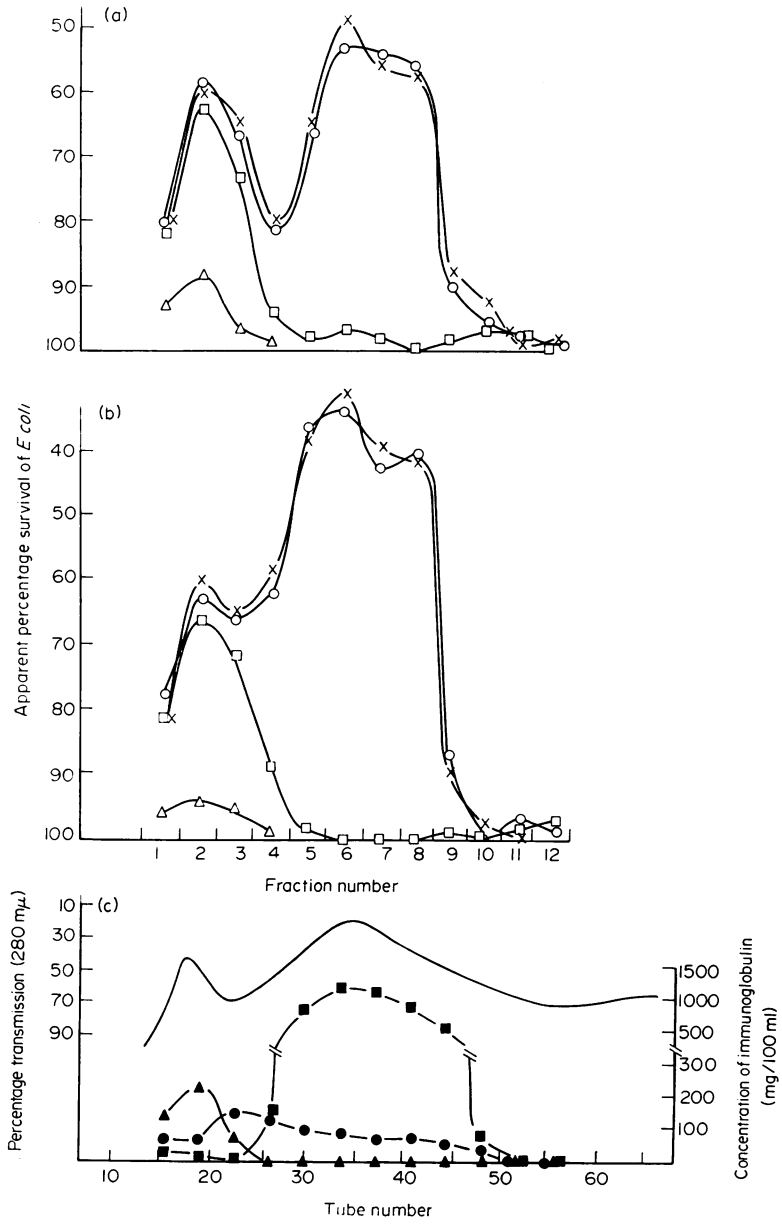

FIG. 2. Assays of colostral whey fractions for bactericidal antibody activity to (a) haemolytic *E. coli* 0141 and (b) non-haemolytic *E. coli* 08: demonstrating effects of absorption with specific rabbit anti-pig IgG, IgM and IgA globulins: (×) Unabsorbed; (□) IgG absorbed; (△) IgM absorbed; (○) IgA absorbed. All fractions were supplemented with complement. Fractions were prepared by gel filtration on Sephadex G-200. (c) Pooling data and immunoglobulin assays using radial immunodiffusion are given. (■) IgG; (▲) IgM; (●) IgA.

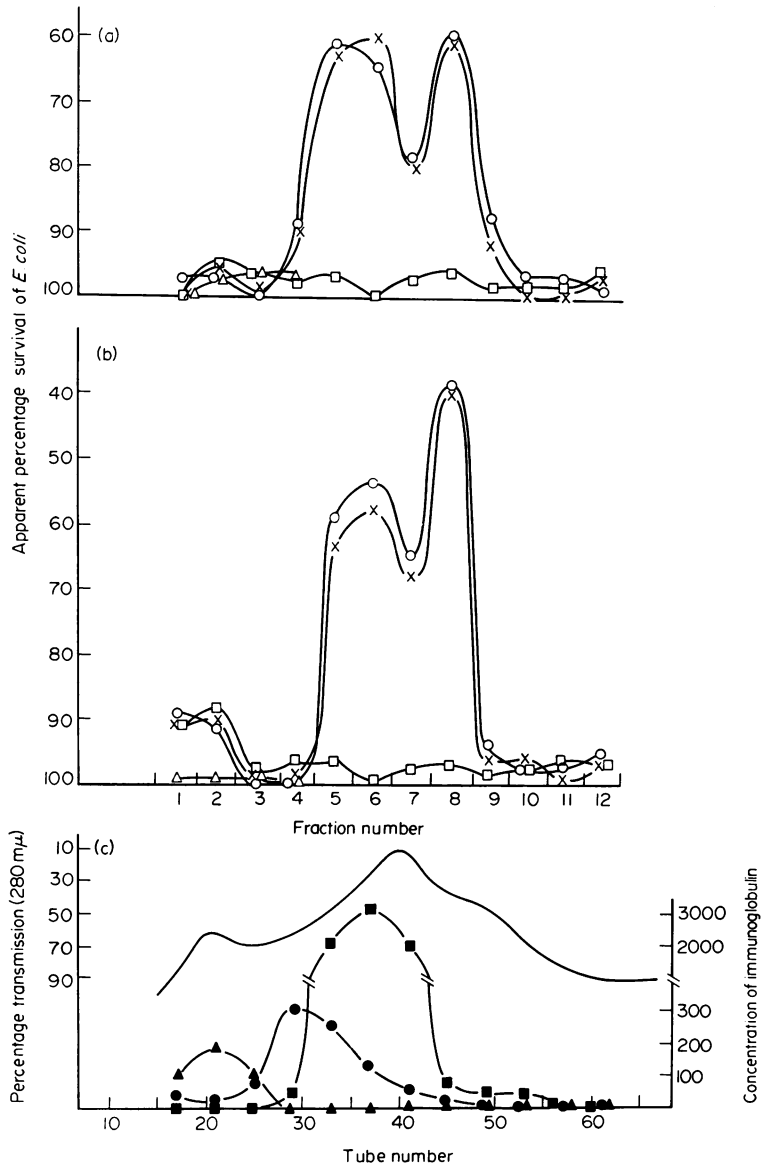

FIG. 3. Assays of post-colostral piglet (2-day-old) serum fractions for bactericidal antibody activity to (a) haemolytic *E. coli* 0141 and (b) non-haemolytic *E. coli* 08; demonstrating effects of absorption with specific rabbit anti-pig IgG, IgM and IgA globulins. (×) Unabsorbed; (□) IgG absorbed; (△) IgM absorbed; (○) IgA absorbed. All fractions were supplemented with complement. Fractions were prepared by gel filtration on Sephadex G-200. (c) Pooling data and immunoglobulin assays using radial immunodiffusion are given. (■) IgG; (▲) IgM; (●) IgA.

Sephadex G-200 protein profile and immune inhibition with specific rabbit anti-immunoglobulin.

In the Sephadex G-200 elution profile of sow serum (Fig. 1) bactericidal activity was identified in the exclusion peak and also peak 3 of the elution pattern. Little or no activity was detectable in peak 2 which would be attributable to 7S IgG. The anti-*E. coli* activity

in the exclusion peak was inhibited by rabbit anti-IgM globulin and the remainder of the activity in the chromatogram was inhibited by rabbit anti-IgG globulin. Thus the antibacterial activity in the low molecular weight peak of sow serum is similar to that previously defined in the precolostral serum of neonatal pigs (Porter and Hill, 1970). Rabbit anti-IgA globulin produced no inhibition in any fraction.

In similar studies with colostral whey (Fig. 2) fractions, 7S IgG played a predominant role as bactericidal antibody. Again no activity was attributable to IgA as indicated by lack of inhibition using the specific rabbit antiserum.

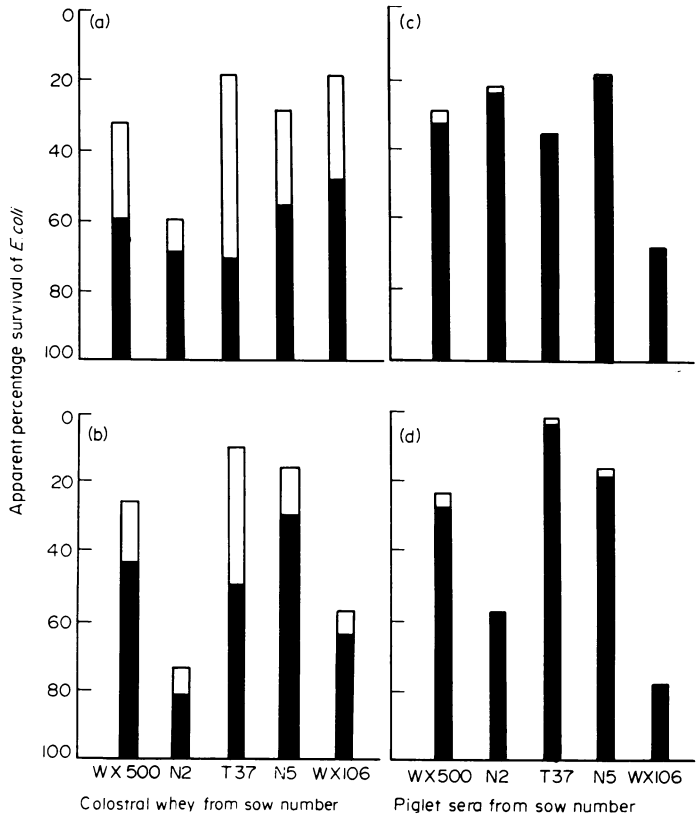

FIG. 4. Studies of the effects of immune inhibition of IgA in (a) and (b) colostral whey, and (c) and (d) post-colostral (2-day-old) piglet sera on their bactericidal antibody activity. (a) and (c), haemolytic *E. coli* 0141; (b) and (d), non-haemolytic *E. coli* 08. (□) Before absorption with rabbit anti-pig IgA. (■) After absorption.

Studies of post-colostral piglet sera (Fig. 3) showed that IgM played a minor role as a bactericidal antibody compared with activity attributable to IgG, which was associated with fractions in the 7S region and also peak 3. Immune inhibition of fractions with rabbit anti-IgA globulin did not reduce bactericidal activity in any fraction containing IgA. Thus in all gel filtration studies presented in Figs 1, 2 and 3 no bactericidal activity was attributable to IgA under the conditions of the test. Absorption of sow colostral whey with specific rabbit anti-IgA globulin resulted in a considerable reduction of bactericidal activity (Fig. 4), showing that activity in whole colostral whey was in part due to IgA,

whereas in Sephadex G-200 colostral whey fractions IgA activity was not demonstrated.

It thus appeared that in colostrum, a factor necessary for *E. coli* lysis by IgA was present, but was removed during Sephadex G-200 gel filtration, from the fractions tested. In view of the findings of Adinolfi *et al.* (1966) we examined the samples for the presence of lysozyme and also the effects of lysozyme on the bactericidal activity of IgA.

EFFECT OF LYSOZYME ON BACTERICIDAL ACTIVITY OF SOW SERUM, COLOSTRUM, POST-COLOSTRAL PIGLET SERUM AND IGA

Analyses of lysozyme concentrations in colostral whey and post-colostral piglet sera are given in Table 1. In colostral whey concentrations of from 5·0 to 46·8 μg/ml were recorded, whilst in piglet sera values were 4·7 to 39·5 μg/ml. Lysozyme could not be detected in Sephadex G-200 fractions 1 to 12, of colostral whey and piglet sera.

TABLE 1

LYSOZYME CONCENTRATIONS IN SOW COLOSTRAL WHEY AND POST-COLOSTRAL PIGLET SERA

Sow number	Sample	Lysozyme concentration (μg/ml)
WX 500	Colostral whey	10·0
	Piglet serum	11·7
N2	Colostral whey	24·3
	Piglet serum	20·5
T37	Colostral whey	8·7
	Piglet serum	6·0
N5	Colostral whey	46·8
	Piglet serum	4·7
WX 106	Colostral whey	39·8
	Piglet serum	39·5

TABLE 2

BACTERIOLYTIC ACTIVITY OF SECRETORY IGA AND SERUM IGA AGAINST *Escherichia coli* 08 AND 0141

IgA	Supplementation	Bacteriolytic activity (apparent percentage kill)	
		Escherichia coli 0141	*Escherichia coli* 08
Non-secretory 6·4S	Not supplemented	3	0
	Complement	1	4
	Lysozyme	0	1
	Lysozyme and complement	2	1
Non-secretory 9·3S	Not supplemented	1	2
	Complement	3	1
	Lysozyme	1	0
	Lysozyme and complement	1	1
Secretory 11S	Not supplemented	0	0
	Complement	1	0
	Lysozyme	2	1
	Lysozyme and complement	60	52

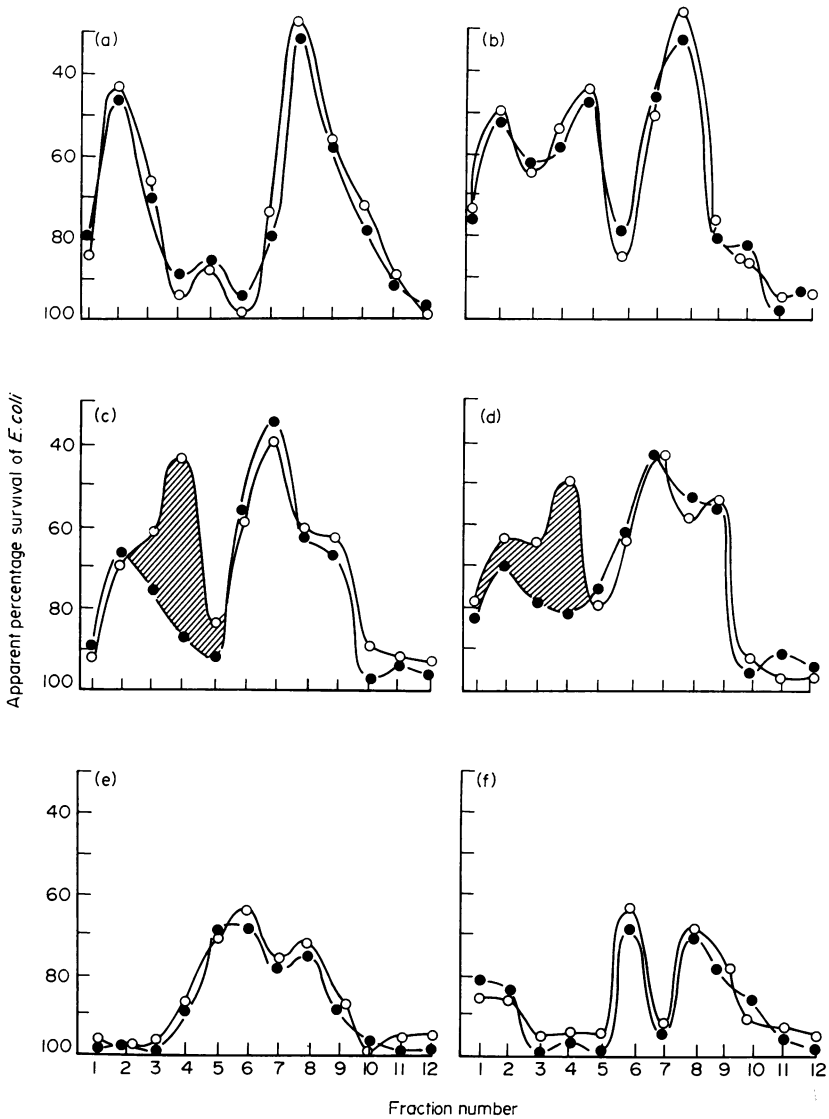

FIG. 5. Effects of lysozyme supplementation (30 μg/ml) on bactericidal antibody activity of Sephadex G-200 fractions of (a) and (b), sow serum, (c) and (d), colostral whey and (e) and (f), post-colostral piglet serum. (a), (c) and (d), haemolytic *E. coli* 0141; (b), (d) and (f), non-haemolytic *E. coli* 08. (●) Sephadex G-200 fraction with complement. (○) Sephadex G-200 fraction with complement and lysozyme.

Increasing the concentration of lysozyme in colostral whey and piglet sera (using egg white lysozyme) to 50 and 100 μg/ml failed to increase their bacteriolytic activity. Lysozyme alone at concentrations up to 100 μg/ml had no effect on *E. coli* 08 or 0141.

Sephadex G-200 fractions of sow serum and colostrum and post-colostral piglet serum were supplemented with lysozyme. Egg white lysozyme was supplied to a final concentration of 30 μg/ml. In sow and piglet sera no change in bactericidal activity was recorded. However, in colostral whey a considerable increase in activity against *E. coli* 08 and 0141

was apparent in fractions 3 and 4. (Fig. 5) The fractions showing increased bacteriolysis corresponded to the peak of colostral IgA in the region on the chromatogram which would relate to the secretory form of the immunoglobulin.

Purified preparations of secretory 11S IgA from milk and non-secretory 6·4S and 9·3S IgA from post-colostral piglet serum were similarly supplemented with 30 μg/ml of lysozyme (Table 2). Activity was observed with secretory IgA alone, and only in the presence of both complement and lysozyme.

DISCUSSION

Several workers (Stertzl *et al.*, 1962; Wardlaw, 1962; Tachibana, 1965) have reported that complement without the presence of antibody can kill rough strains of Gram-negative organisms. Others (Muschel and Jackson, 1963; Inoue, Tanigawa, Takubo, Satani and Amano, 1959; Goldman, Ruddy, Austen and Feingold, 1969) have questioned these observations and show an antibody requirement for killing rough organisms. The requirement for antibody and complement for killing smooth Gram-negative organisms has however been regularly shown and for the current studies smooth strains of *E. coli* were chosen.

The transfer of colostral immunoglobulins to the blood circulation of the young pig is important for survival since there is an almost total absence of transplacental transfer to the foetal circulation. In precolostral serum there is a low molecular weight 5S component in the IgG class (Stertzl, Kostka, Riha and Mandel, 1960; Franek, Riha and Stertzl, 1961) which has been demonstrated to have bactericidal activity against *E. coli* (Porter and Hill, 1970). However this component probably has a negligible role to play in protection of the neonate when one considers the large amounts of immunoglobulin acquired from the colostrum by intestinal absorption during the first few hours of life. In porcine colostrum IgG accounts for approximately 80 per cent of the total immunoglobulin and Brandenburg and Wilson (1973) have shown that this plays an important role in protection of the young pig against *E. coli* enteritis by neutralizing heat-labile enterotoxin and decreasing the rate of multiplication of *E. coli*.

The levels of bactericidal activity attributable to IgM relative to 7S IgG differ so markedly between the gel filtration profiles of sow serum and post-colostral piglet serum that there would appear to be some selectivity in the post-partum transfer of these immunoglobulins from mother to offspring. However, quantitative studies of serum immunoglobulins would suggest that there is no differentiation in the absorption of colostral IgM and IgG (Porter, 1969; Curtis and Bourne, 1971) and possibly these observations in the pig may be attributed to a preference for transfer of IgG_1 compared with IgG_2. This would not have been differentiated by antisera in our previous studies, but immunoelectrophoretic studies indicate that this is by no means as pronounced as that described in the bovine (Pierce and Feinstein, 1965). Selectivity of transfer of bactericidal antibodies has been described in the human, but this is due entirely to an absence of transplacental transfer of IgM (Gitlin, Rosen and Michael, 1963). Bactericidal antibody in human maternal serum is mainly associated with IgM and the low level of antibody which is transferred across the placenta to the foetal circulation is entirely associated with IgG.

Although IgA is a minor immunoglobulin in sow colostrum the pattern of lactation changes very rapidly during the first few days with a decline in total immunoglobulin, and IgA very soon predominates in the mammary secretions (Porter, Noakes and Allen, 1970).

Approximately 40 per cent of sow colostral IgA is derived by transudation with IgG from the serum (Bourne and Curtis, 1973); this has molecular characteristics of 6·4S and 9·3S and also lacks secretory component (Porter, 1973). It is of interest in the present studies that there was a differentiation between post-colostral serum IgA and colostral secretory IgA in relation to bactericidal activity in the presence of lysozyme. This again emphasises the selectivity against absorption of 11S IgA indicated previously in the neonatal pig (Porter, 1973) and demonstrates once again the lack of correlation between antibody activities attributable to IgA fractions of serum and secretions (Newcombe, Normansell and Stanworth, 1968).

Ishizaka *et al.* (1966) showed that IgA does not activate the first components of the complement sequence. However it is now known that IgA activates the alternative pathway which bypasses the early part of the complement system that gives rise to the conversion of C3 (Gotze and Muller-Eberhard, 1971); it is significant also that endotoxins of Gram-negative bacteria activate this pathway. These observations may account for the bacteriolytic activity of IgA which has been subject to question since the early observations of Adinolfi *et al.* (1966). However the role of lysozyme is also intriguing and deserves consideration in the context of local defence mechanisms. The participation of lysozyme in immune bacteriolysis was first described by Amano, Inai, Seki, Kashiba Fujikawa and Nishimura (1954) and the mechanism of action has since been investigated by Inoue *et al.* (1959), Wardlaw (1962) and Glynn and Milne (1967), mainly in relation to serum IgM. Lysozyme increased the rate of bactericidal activity of IgM with complement but bactericidal activity progresses without it, whereas secretory 11S IgA and complement are inactive in absence of lysozyme. A further interesting point emerges from recent studies of opsonizing activity in human duodenal fluid (Girard and Kalbermatten, 1970); lysozyme was observed to stimulate the phagocytic activity of IgA and IgM antibodies. Thus lysozyme must feature strongly in local defence mechanisms of the gut both in relation to passive and active immunity, since it is now known that active synthesis and secretion of IgM and IgA takes place in early life before the young animal is weaned (Allen and Porter, 1973).

Studies using the electron microscope have provided some information on the sequence of events in lysis of rough strains of *E. coli* by IgM. Feingold, Goldman and Kuritz (1968) suggest that the primary effect of serum antibody and complement is on the peripherally located lipopolysaccharide–phospholipid complex. This is generally, but not necessarily lethal. A lethal step is seen as the lysozyme degradation of the peptidoglycan polymer portion of the cell wall. In view of the lack of evidence of lytic activity of secretory IgA and complement in absence of lysozyme it may be rewarding to re-investigate this sequence of events in the secretory IgA bacteriolytic system.

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