Preliminary Characterization of Cell-free K99 Antigen Isolated from *Escherichia coli* B41

By J. A. MORRIS, A. E. STEVENS AND W. J. SOJKA

Central Veterinary Laboratory, Weybridge, Surrey

(Received 11 October 1976)

SUMMARY

The K99 antigen of *Escherichia coli* B41 was isolated by isoelectric precipitation from heated bacterial suspensions. Chromatography and immunoabsorption experiments suggested that the mannose-resistant haemagglutinating activity of partially purified preparations of antigen was K99. The antigen was partially susceptible to bacterial proteases and was inactivated by periodate oxidation. Haemagglutination inhibition experiments with sugars and absorption of K99 with antisera to human blood groups A and B substances suggested that K99 contains a terminal α -linked *N*-acetylgalactosamine moiety, which is involved in the haemagglutination reaction, and an adjacent terminal α -linked galactose moiety, which plays no part in the reaction.

INTRODUCTION

Most *Escherichia coli* strains enteropathogenic for calves and lambs contain a common surface antigen which was originally referred to as Kco (Sojka, unpublished; Smith & Linggood, 1972) but has now been established as the *E. coli* K99 antigen (Ørskov *et al.*, 1975). Smith & Linggood (1972) demonstrated that the production of K99 was controlled by a transmissible plasmid and that strains which possessed the K99 plasmid proliferated in the anterior small intestine of calves and lambs while those pretreated with acridine orange did not. They therefore suggested that the K99 antigen may facilitate adhesion to the intestinal epithelium of lambs and calves in a similar manner to the K88 antigen found in some of the pig enteropathogenic strains (Smith & Linggood, 1971; Jones & Rutter, 1972).

To study the pathogenesis of enteric colibacillosis in calves and lambs and hence develop an effective vaccine, cell-free preparations of the K99 antigen are needed. This paper reports the isolation and chemical properties of partially purified K99 from *E. coli* B41, the recommended reference strain (Ørskov *et al.*, 1975).

METHODS

Preparation of K99 antigen. The K99 antigen was isolated by a method similar to that used to isolate the K88 antigen (Stirm *et al.*, 1967). *Escherichia coli* B41 (O101:K99:H-) was grown on fresh blood agar in Roux flasks for 18 h at 37 °C and harvested in saline. The K99 antigen was extracted by gently shaking the bacterial suspension at 60 °C for 30 min, removing the bacteria by centrifuging and adjusting the pH of the supernatant fluid to 4.0 with dilute acetic acid. Crude antigen precipitated overnight at 4 °C; it was washed in 10 mM-acetate buffer pH 4.0, and then redissolved in 100 mM-phosphate buffer pH 7.5. The K99 was partially purified by five cycles of precipitation and redissolved to give a protein concentration of 8 mg ml⁻¹.

Antisera. Antiserum to K99 was prepared in rabbits using *E. coli* strain 'B117' (O8:K85ab,K99-H-). It was rendered monospecific by absorption with *E. coli* strains G7 (O8:K87,K88ab) and E6811 (O141:K85ab) to remove agglutinins to O8 and K85ab antigens using the techniques described by Sojka (1965).

Antisera to human blood groups A and B substances were a gift from the South London Regional Blood Transfusion Centre.

Serological techniques. Ouchterlony double diffusion tests were performed in agar gels (Stirm *et al.*, 1967) using 20 μ l samples. Plates were incubated in a humidity cabinet at 37 °C for 3 days. Specific precipitin tests were used to identify K99 during the isolation and purification processes but these were unnecessary once it was established that the antigen could be assayed by haemagglutination.

Haemagglutination tests. Direct haemagglutination tests were performed in microtitre trays using 50 μ l portions of 3 % red blood cells and K99 preparations. Trays were incubated at 4 °C and examined after 2 h. Sheep red blood cells were used in all of the studies and human blood group O red cells were used as indicated. The highest dilution of antigen which gave complete agglutination was defined as one haemagglutinating unit (h.u.). Standard preparations of K99 usually contained 128 h.u.

Column chromatography. Gel filtration was done in columns (26 $\times$ 700 mm) of Sepharose 4B (Pharmacia) using 100 mM-Tris/HCl buffer, pH 8.0. Fractions (2.0 ml) were eluted by upward development of eluant at a flow rate of 20 ml h⁻¹ at room temp. Ion exchange chromatography was done on columns (9 $\times$ 300 mm) of QAE Sephadex A50 (Pharmacia) equilibrated with 100 mM-phosphate buffer pH 7.0. Antigen from the void volume of the Sepharose 4B column was dialysed against starting buffer and a sample (2 mg protein) was applied to the Sephadex column which was developed using an increasing stepwise NaCl gradient in 10 mM-phosphate buffer pH 7.0 at room temp.

Chemical analysis. The protein content of K99 preparations was determined by the method of Lowry *et al.* (1951) using Wellcontrol sera as standard (Wellcome Reagents). Carbohydrate was estimated by the method of Dubois *et al.* (1956) using D-glucose as standard, and lipid was determined using a commercially available test kit for total lipid (Boehringer).

Degradation studies. The susceptibility of K99 to the following enzymes was examined: trypsin, carboxypeptidase and leucine aminopeptidase (each at 1 mg ml⁻¹), chymotrypsin (1.5 mg ml⁻¹), papain (2.5 mg ml⁻¹), bromelain, *Bacillus subtilis* protease, *Streptomyces griseus* pronase, α -amylase, β -amylase, α -glucosidase, β -galactosidase, muramidase and neuraminidase (each at 5 mg ml⁻¹), DNAase and lipase (each at 1 mg ml⁻¹). Enzymes were obtained from Sigma, except neuraminidase which was from Boehringer. All enzymes, except β -galactosidase, were incubated with K99 in 100 mM-phosphate buffer pH 7.5 for 18 h at 37 °C. The action of β -galactosidase was tested in 10 mM-acetate buffer pH 5.0; after incubation, the reaction mixture was adjusted to pH 7.5 by adding 100 mM-Na₂HPO₄. Controls without any enzyme were treated similarly. At first, the low molecular weight enzymes were removed by selective dialysis through Minicon S-125 concentrators (Amicon, High Wycombe, Buckinghamshire) before testing for K99 activity. It was subsequently found that at 4 °C the enzymes did not affect the haemagglutination tests, and in later experiments these were done directly on the reaction mixtures.

Susceptibility to periodate was determined by incubation at 4 °C for 30 min, and to dithiothreitol and formaldehyde by incubation at room temp. for 2 h and 30 min respectively. Each agent was removed from the reaction mixture using Minicon S-125 concentrators.

Table 1. *Chemical analysis of a standard preparation of K99 antigen*

Component	Concn (mg ml ⁻¹) in antigen:	
	After 1st precipitation	After 5th precipitation
Protein	13.90	8.20
Carbohydrate	0.96	0.55
Lipid	< 0.1	< 0.1
Protein:carbohydrate	14.5:1	14.9:1

Sugar blocking studies. Competitive inhibition of K99 haemagglutination was studied by titrating the antigen in the presence of an equal volume of 2 % (w/v) sugar solution. Sheep and human blood group O red cells were examined. Galactose, glucose and mannose, together with their amino and acylamino derivatives, *N*-acetylneuraminic acid, fucose, rhamnose and muramic acid were examined. Galactose, galactosamine, *N*-acetylgalactosamine, glucose, glucosamine and fucose were also examined at concentrations up to 15 % (w/v).

In some experiments sheep or human blood group O red cells were incubated with an equal volume of *N*-acetylgalactosamine for 3 h at 4 °C or at 37 °C, and then centrifuged and resuspended in buffer for use in the haemagglutination test with untreated K99. Preparations of K99 were treated similarly and the sugar was removed by selective dialysis in Minicon S-125 concentrators. The treated antigen was then titrated using untreated sheep red cells.

Immunoabsorption studies. Monospecific antiserum to K99 or human blood groups A or B substances was used to absorb preparations of K99. Equal volumes of antiserum and antigen were incubated at 37 °C for 18 h and any sediment was removed by centrifuging.

Suspensions of 3 % sheep red cells and human blood group O red cells were absorbed in a similar manner with antisera to human blood groups A or B substances, washed once in buffer and resuspended to their original concentration.

RESULTS

Column chromatography. Haemagglutinating activity and gel precipitin activity were detected in the material eluting in the void volume of Sepharose 4B columns. The precipitin and haemagglutinin eluted together as a single peak on QAE Sephadex A50 columns with 300 mM-NaCl. No other peaks were observed.

Chemical analysis. Only protein and carbohydrate were detected on analysis, and the ratio of one to the other did not alter significantly on repeated precipitation (Table 1).

Degradation studies. *Bacillus subtilis* protease and *Streptomyces griseus* pronase both reduced the activity of K99 preparations by 4 h.u. None of the other enzymes examined affected the activity of the antigen. Sodium periodate oxidation reduced the antigen activity by 32 h.u. whilst formaldehyde caused a reduction of 128 h.u.

Sugar blocking studies. Of the sugars examined, only *N*-acetylgalactosamine inhibited haemagglutination of sheep red cells by K99: activity decreased by 32 h.u. with a 2 % solution but by only 2 h.u. with a 1 % solution. When either K99 or sheep red cells were pre-treated with *N*-acetylgalactosamine, activity was unaffected.

Human blood group O red cells agglutinated when treated with 2 % *N*-acetylgalactosamine. Again none of the other sugars examined affected the haemagglutination of these human red cells by K99.

Immunoabsorption. Absorption of K99 with antisera to K99 reduced the activity of the antigen by only 32 h.u. while antisera to human blood groups A or B substances each reduced the K99 activity by 128 h.u. These results showed no dependence on the species of red cell used to assay the absorbed antigen.

Sheep red cells absorbed with antisera to either human blood groups A or B substances gave the same haemagglutination titre with K99 as untreated red cells although some haemolysis occurred during absorption. There was no haemolysis when human blood group O red cells were absorbed and the haemagglutination titre using these cells was similarly unaffected.

DISCUSSION

The technique of extracting *E. coli* K99 antigen by isoelectric precipitation from heated bacterial suspensions differs from that described for the isolation of the K88 antigen only in the pH required (Stirm *et al.*, 1967). Throughout the chromatographic studies the gel precipitin activity of a K99 preparation could not be dissociated from the eluting mannose-resistant haemagglutinating activity. Similarly, absorption of antigen with monospecific antiserum to K99 abolished haemagglutination suggesting that the eluting mannose-resistant haemagglutinin in the extracts was probably K99. Chemical analysis showed that the major component of K99 was polypeptide, although traces of carbohydrate were also detected. The K88 antigen is also an eluting mannose-resistant haemagglutinin (Jones & Rutter, 1974) and is thought to be a pure protein usually contaminated with carbohydrate (Stirm *et al.*, 1967). Enzyme studies confirmed, in part, the chemical nature of K99, although the polypeptide was only susceptible to bacterial proteases. The resistance of K99 to mammalian proteases was not unexpected (see also Stirm, Ørskov & Ørskov, 1966). Trypsin and chymotrypsin are pancreatic endopeptidases active in the anterior small intestine. Carboxypeptidase is also a pancreatic enzyme while leucine aminopeptidase is in the intestinal mucosa (Mahler & Cordes, 1971).

The results of the periodate oxidation and chemical analysis are consistent with K99 being a glycoprotein, although the action of glycoside hydrolases could not confirm this. When a glycoprotein has an affinity for a cell membrane, the carbohydrate moiety is often responsible for its binding to a receptor on that membrane. Therefore treatment of K99 to remove or destroy carbohydrate might reduce the haemagglutination activity and, indeed, periodate oxidation did destroy the activity, although this could have resulted from the oxidation of any hydroxyamino acids which might be present in K99. Competitive inhibition of K99 haemagglutination by the sugars commonly associated with glycoproteins was only shown by *N*-acetylgalactosamine which suggested that the antigen contains a terminal *N*-acetylgalactosamine residue or that this sugar acts as the K99 receptor on the red cell membrane. This could not be resolved by haemagglutination experiments using K99 or red cells absorbed with *N*-acetylgalactosamine.

N-Acetylgalactosamine and galactose are the immunodominant sugars in human blood groups A and B substances respectively (Watkins, 1972). Consequently antisera used in the typing of human blood groups are monospecific for the relevant α -linked sugar. The decrease in haemagglutinating activity when K99 was absorbed with antiserum to human blood group A substance, and the unaffected activity of untreated antigen titrated with absorbed red cells, indicated that terminal *N*-acetylgalactosamine is part of the K99 antigen. Absorption experiments using antiserum to human blood group B substance implied that K99 also contains terminal α -linked galactose. However, as free galactose did not prevent haemagglutination the terminal galactose must be sufficiently close to terminal *N*-acetyl-

galactosamine for human blood group B antiserum to interfere with the binding of the latter to the red cell. Thus, the K99 antigen of *E. coli* B41 appears to be a glycoprotein containing a terminal α -linked *N*-acetylgalactosamine moiety, which is involved in the haemagglutination reaction with sheep and human blood group O red cells, and an adjacent terminal α -linked galactose moiety, which plays no part in the haemagglutination reaction.

Further studies on the adhesion of enteropathogenic *E. coli* should lead to a better understanding of the interactions between the pathogen and the host cell surface. This would be of value in controlling colibacillosis in farm animals either by using relevant vaccines or by seeking breeding stock which lack the K99 receptors on their intestinal mucosa.

We express our gratitude to Mrs M. Leak of the South London Regional Blood Transfusion Centre for gifts of antisera to human blood groups A and B substances. We also thank Miss P. Wiles for her excellent technical assistance.

REFERENCES

- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for the determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- JONES, G. W. & RUTTER, J. M. (1972). Role of the K99 antigen in the pathogenesis of neonatal diarrhoea caused by *Escherichia coli* in piglets. *Infection and Immunity* **6**, 918-927.
- JONES, G. W. & RUTTER, J. M. (1974). The association of K88 antigen with haemagglutinating activity of porcine strains of *Escherichia coli*. *Journal of General Microbiology* **84**, 135-144.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MAHLER, H. R. & CORDES, E. H. (1971). *Biological Chemistry*, 2nd edn. London: Harper & Row.
- ØRSKOV, I., ØRSKOV, F., SMITH, H. W. & SOJKA, W. J. (1975). The establishment of K99, a thermolabile, transmissible *Escherichia coli* antigen, previously called 'Kco', possessed by calf and lamb enteropathogenic strains. *Acta pathologica et microbiologica scandinavica* **B83**, 31-36.
- SMITH, H. W. & LINGGOOD, M. A. (1971). Observations on the pathogenic properties of K88, Hly and Ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. *Journal of Medical Microbiology* **4**, 467-486.
- SMITH, H. W. & LINGGOOD, M. A. (1972). Further observations on *Escherichia coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. *Journal of Medical Microbiology* **5**, 243-250.
- SOJKA, W. J. (1965). *Escherichia coli* in *Domestic Animals and Poultry*. Farnham Royal: Commonwealth Agricultural Bureaux.
- STIRM, S., ØRSKOV, I. & ØRSKOV, F. (1966). K88, an episome-determined protein antigen of *Escherichia coli*. *Nature, London* **209**, 507-508.
- STIRM, S., ØRSKOV, F., ØRSKOV, I. & MANSAB, B. (1967). Episome-carried surface antigen K88 of *Escherichia coli*. II. Isolation and chemical analysis. *Journal of Bacteriology* **93**, 731-739.
- WATKINS, W. M. (1972). Blood group substances. In *Glycoproteins*, vol. B, 2nd edn. Edited by A. Gottschalk. Amsterdam: Elsevier.