Humoral immune response anti K99 pilus from enterotoxigenic *Escherichia coli* in experimentally inoculated calves

CAROLINA BUSTOS[1], LIVIO ZURITA[2], PEDRO SMITH[3], PATRICIA VALLEJOS[1], JUAN-CARLOS AGUILLON[1], MARCIA LOPEZ[1] and ARTURO FERREIRA[1]

Departmentos de:
[1] Medicina Preventiva Animal,
[2] Ciencias Clínicas y
[3] Patología Animal,
Facultad de Ciencias Veterinarias y Pecuarias,
Universidad de Chile, Santiago, Chile

The bovine model is extremely interesting to study several basic aspects of mucosal local immunity. Many reports have shown that, in young calves, the infectivity of enterotoxigenic ***Escherichia coli*** *may be inhibited by passively administered antibodies anti K99 pilus. We have measured, by immunoradiometric assays, the IgG response anti K99 pilus in the serum of calves, deprived of colostrum and orally inoculated with enteropathogenic K99+* ***E. coli****. Although variable levels of IgG anti K99 pilus were detected, their protective value could not be ascertained* ***in vivo*** *due to the acute development of the infection. In an effort to correlate the presence of serum antibodies anti K99 pilus with their protective capacity, an* ***ex-vivo*** *assay to monitor the interaction of radiolabeled K99 pilus with the bovine mucosa was standardized. Paradoxically, although K99 pilus, purified by standard procedures, was recognized by polyclonal rabbit and calf antisera, its interaction with the bovine intestinal mucosa, quantitated in the* ***ex-vivo*** *system, was not inhibited by these reagents, indicating that the antibodies did not effectively block those K99 pilus domains involved in the interaction with mucosal receptors.*

Key words*: bovines,* ***Escherichia coli****, humoral immune response, pili K99.*

INTRODUCTION

From the basic immunological point of view, it is important to understand the main aspects of the interaction between a pathogen and the target cell. Usually, this interaction involves few molecules from the host cells and the aggressor. Attempts to modulate this interaction with antibodies directed against one of these molecules may be informative with regard to the efficiency of humoral or local immunity. On the other hand, neonatal diarrhea in domestic species of economic relevance constitutes an important problem, with short and long term consequences, either because of mortality or decreased productivity. Since this type of pathology can be caused by multiple factors (nutritional, immunological, infectious, management), the term acute undifferentiated diarrhea has been proposed (Gouet, 1983; Hjerpe, 1990). However, it is frequently

Correspondence to: Dr Arturo Ferreira, Laboratorio de Inmunología, Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Casilla 2, Correo 15. Sta. Rosa 11735, La Pintana, Santiago, Chile. Fax: (56-2) 541-7357. E-mail: aferreir@abello.seci.uchile.cl.

TABLE I

Experimental design

	VARIABLES		
Group	Inoculation	Colostrum	Treatment
A	–	+	–
B	+	–	+*
C	+	–	+**
D	+	–	–

Treatment (*per os* hydration with glucose / saline and lactated Ringer plus gentamicin) 1 h* and 6 h** post onset of diarrhea.

possible to identify an agent responsible *per se* for the diarrheic problem. Thus, in 50 to 60% of 5 day old calves, diarrhea is produced by enterotoxigenic *Escherichia coli* (ETEC) (Acres, 1985). The pathogenicity of this bacteria seems to be determined by an adherence factor or pilus and by an enterotoxin (Acres *et al*, 1982; Gaastra and de Graaf, 1982).

The pilus, also denominated fimbria, permits bacterial adherence to intestinal epithelium receptors, preventing expulsion of the agent by peristaltic movements, thus focusing the action of the secreted enterotoxin (Gaastra and de Graaf, 1982; Desmettre, 1983). Bovine ETEC display several types of pili: Att 25, K88, F17, and K99. The latter is the most frequently found in ETEC isolated from diarrheic calves (Hjerpe, 1990; Acres, 1985).

Immunity in the neonate bovine is passively acquired from the mother by serum derived antibodies, present in colostral and milk secretions (Desmettre, 1983; Kruse, 1983; Tizard, 1992). We postulate here that oral inoculation of newborn calves with *E. coli* can generate serum antibodies capable of modulating the interaction pilus K99/ mucosa. Within this context, we have evaluated the serum IgG immune response in colostrum deprived calves, orally inoculated with ETEC bacteria, since IgG, mostly derived from serum, constitutes a high proportion of protective immunoglobulins detected in ruminant gastrointestinal secretions (Butler, 1984). In addition, the K99 pilus capacity to bind to the intestinal mucosa and the effect of specific anti pilus antibodies on this binding were assessed.

MATERIALS AND METHODS

Experimental animals

Twenty Dutch Freisan calves were used in these experiments. The animals were kept confined, under standard management conditions and permanent veterinary surveillance. A single dose of 6 x 10^8 ETEC K99 + CFU, in 100 ml of milk substitute (Spray Fo Blue^{MR}), were administered *per os* to 15 colostrum deprived animals, between the first and second day of age. These animals were randomly divided into 3 groups of five calves each, subjected to different therapeutic protocols, as indicated in Table I. An antibiogram determined that gentamicin was the best choice for *E.coli*. Thus, gentamicin 5 mg/kg was injected i.m., twice a day. Additionally, a control group (5 calves), colostrum-fed and not experimentally inoculated was included (Table I).

Escherichia coli

The strain used in our experiments was isolated from a field neonatal diarrheic case. Enteropathogenicity was assessed by the calf ligated-gut assay (Myers *et al*, 1975) to determine the presence of thermostable enterotoxin type a and by specific antisera to

show the adherence factor K99 pilus (Smith *et al*, 1988).

Antigen K99 pilus

It was obtained as previously described (Karkhanis and Bhogal, 1986). Its purity was determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and compared with standard K99 pilus from the *E. coli* obtained from the Reference Center of the University of Pennsylvania , Pennsylvania, USA.

Immunologic assays

An immunoradiometric assay (IRMA) (Catt and Treager, 1967) was standardized to measure bovine anti K99 pilus IgG . Briefly, microtitration plates were coated with 10 µg/ml pilus K99. After overnight incubation, the wells were washed, saturated with bovine serum albumin, washed again, and sera from experimental animals were added. After incubation and washing, a rabbit IgG, ^{125}I-labelled and affinity purified, anti-bovine IgG, was added. After incubation and washing, radioactivity bound to the solid phase was measured. In some assays, the results obtained with the IRMA were correlated with an immunoenzymatic assay (ELISA) (Voller and Bidwell, 1986; Smith *et al*, 1994). To determine that the response was indeed directed against K99 pilus, Western immunoblotting (Towbin *et al*, 1979) and immunodot (Hawkes *et al*, 1982) assays were also used.

Antisera

Antisera from adult bovine was obtained from one animal immunized with the whole ETEC, inactivated with formol, by standard

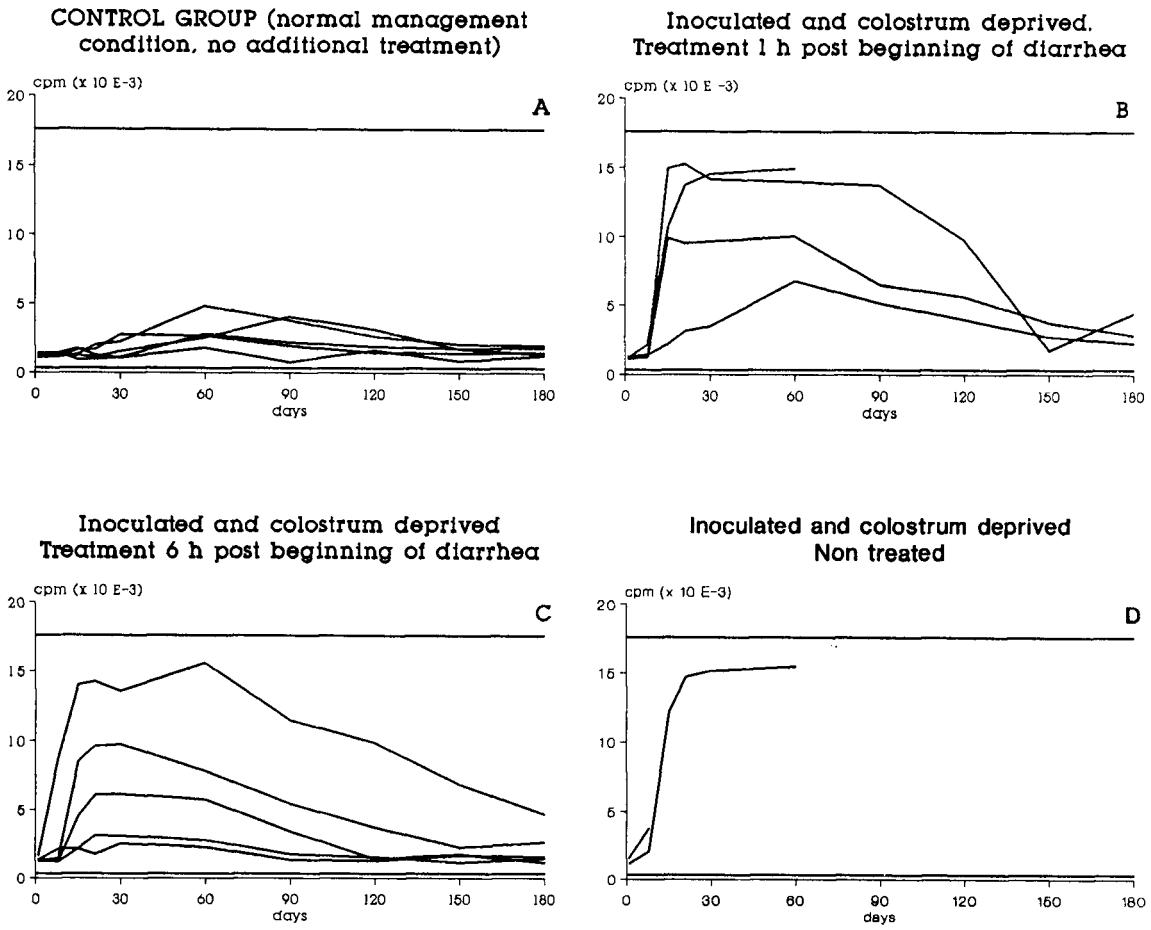

Fig 1. Kinetics of anti K99 pili seric IgG production in calves orally inoculated with ETEC. Top and bottom lines correspond to positive (immune adult bovine serum) and negative (fetal calf serum) controls, respectively.

procedures (Acres *et al*, 1979). Fetal calf serum (Sigma Chemical Co.) was used as negative control. The rabbit anti pili K99 antiserum was generated by serial inoculations, 100 μg each, of the antigen pili K99, purified from an autochthonous field *E. coli* strain, in a 3-month old New Zealand white female, following standard protocols (Coligan *et al*, 1992).

Ex-vivo assay

Ileal intestinal mucosae from adults and fetuses were obtained immediately after the animals were slaughtered in an authorized, veterinary inspected facility. Specimens were placed in ice-cold Tyrode solution. Upon arrival at the laboratory, intestinal samples were fixed between two polyvinylchloride (PVC) tube segments, in a system similar to a mini embroidery frame. The ligands (radiolabeled pili K99 and polyclonal antisera) were assayed on the mucosal epithelium facing the inside of the inner tube. Prior to the assay, the mucosa was gently washed with Tyrode solution. Purified pilus was radiolabeled with ^{125}I by the iodogen method (Fraker and Speck, 1978). The specific activity obtained was 8 x 10^6 cpm/μg. The pilus was incubated with the mucosa in the presence or absence of specific polyclonal antibodies. After several washes, bound radioactivity was measured.

RESULTS AND DISCUSSION

The IgG response of animals orally inoculated with ETEC was monitored by IRMA, during 180 days (Figure 1). Group A corresponds to non inoculated animals, in which only marginal signals were detected. Groups B, C and D correspond to surviving inoculated calves and they showed variable responses against K99 pilus. Their responses were higher than those of Group A. The responses reached their peaks 7 days post inoculation and were still detectable after 3 months.

A great variability (from high to low responders), among groups and among animals of the same group, was observed. Comparison of groups B and C against group D illustrates the importance of the treatment, even when established late in the course of infection. The unreported fact that oral inoculation with ETEC is able to induce the presence of serum antibodies is demonstrated here, when groups B and C are compared with group A. In group D, most animals died from the infection at two days post inoculation. In this group, one animal survived with persistent diarrhea, in spite of displaying high anti K99 pilus antibodies.

IRMA was used in the experiments reported here since it is frequently more sensitive than immunoenzymatic assays (ELISA). Subsequent comparison of both assays showed that the regression curves obtained for the variables, absorbance at 490 nm (ELISA) *versus* cpm (IRMA), when sera from an immune calf and adult bovine were titrated, provided a highly significant correlation ($r = 0.98$, $p < 0.01$, $df = 4$), indicating that ELISA may be used in the future for these purposes (results not shown).

When reactivity with K99 pilus of sera from immune and non immune bovines was assessed by Western immnoblotting, both inoculated and control animals showed weak reactivity with the antigen, as compared with the reference polyclonal rabbit antiserum (results not shown). Since sera from inoculated bovines were indeed reactive with the antigen by IRMA (Figure 1, Groups B and C), it is possible that the interactions detected in Western immunoblotting were not specific and that most, if not all, the relevant epitopes were lost, due to the stringent conditions of that assay (presence of methanol, SDS, and denaturing conditions). For these reasons, the results were further assayed by immunodot, where the conditions are not denaturing. As shown in Figure 2, both the rabbit polyclonal antiserum (tracks 3a and 3b) and an immune calf serum (tracks 2a and 2b) showed strong specific reactivity against the purified antigen, as compared with fetal calf serum (tracks 1a and 1b). The reference pilus did not react with the radioactive immunoprobe, as shown in track 4.

Fetal calf serum was used as a negative control in the experiments reported above, because sera from colostrum-deprived neonates consistently showed weak signals in

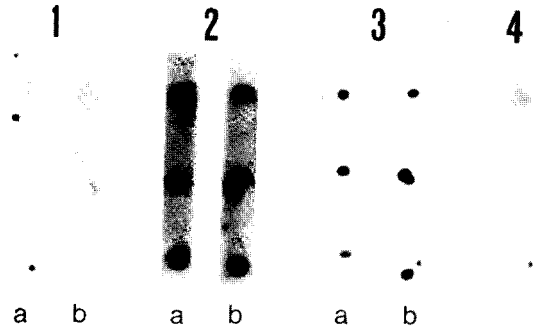

Fig 2. Radioautography of immunodot performed with reference (tracks 1a, 2a, 3a and 4) or experimental (tracks 1b, 2b and 3b) pili K99: Fetal bovine serum (tracks 1a and 1b); serum from calf orally inoculated with ETEC K99+, 6th bleeding (tracks 2a and 2b); polyclonal reference anti pili K99 serum (tracks 3a and 3b) were used as source of bovine antibodies. Bovine antibodies, but not the radioactive immunoprobe, were omitted in track 4.

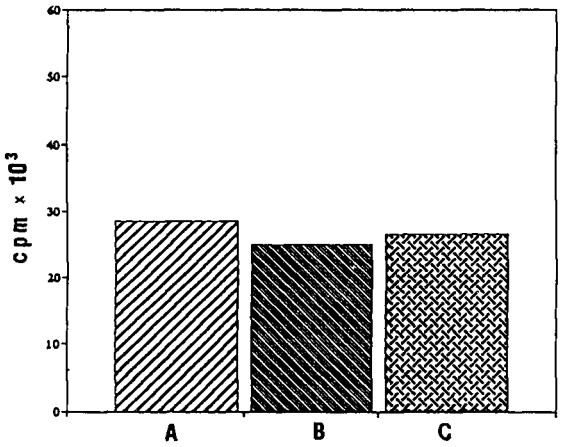

Fig 3. Lack of effect of anti pilus K99 rabbit polyclonal antibodies on the interaction pilus-intestinal mucosa. **A**: Without serum; **B**: Immune serum; **C**: Preimmune serum. Sera were used in a 1:100 dilution.

immunodot and Western immnunoblotting, suggesting that marginal levels of anti K99 pilus antibody may be present in these animals. This idea is consistent with recent observations (Landero, Ferreira, Vallejos, Bustos, Zurita, manuscript submitted), showing that small radioactive signals are detected in the sera of colostrum-deprived calves, born from mothers inoculated with radioactive bovine IgG.

Paradoxically, although K99 pilus, purified by standard procedures, was recognized by polyclonal rabbit antisera in IRMA, ELISA and immunodot, its interaction with the bovine intestinal mucosa, quantitated in the *ex-vivo* system, was not inhibited when this reagent was used in high concentration (Figure 3). Although the intestinal receptor for K99 pilus has been identified (Ono *et al*, 1989), previous observations proposed that the union of ETEC to the intestinal epithelium was mediated, at least partially, by mucus (Mouricout and Julien, 1987). If this kind of interaction is operative, it may not be necessarily inhibited by antibodies to pilus. It could be argued that the antibodies did not effectively block those K99 pilus domains involved in the interaction with mucosal receptors. Alternatively, we can not rule out the possibility that the affinity of the receptor for the ligand is much higher than that of the antibodies for the pilus.

In conclusion, although pilus K99, administered orally in the context of whole bacteria, is able to induce an IgG humoral immune response, the resulting antibodies, in spite of recognizing the antigen by several criteria, are unable to modulate its interaction with the mucosa.

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