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The effect of serum and colostrum immunoglobulins from buffaloes infected with *Toxocara vitulorum* on *T. vitulorum* larvae in vitro and in vivo in mice

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Abstract Serum and colostrum were collected from adult buffalo cows naturally infected with *Toxocara vitulorum*. When injected into mice, the colostrum reduced the number of larvae of *T. vitulorum* that migrated in the mice. Injection of particularly the IgG-containing fraction but also the IgM-containing fraction of Sephadex G200-chromatographed colostrum also passively protected mice. When incubated for 6 h in buffalo serum or colostrum or fractions of these from Sephadex G200 and diethylaminoethanol Sephadex, *T. vitulorum* larvae had their activity in vitro curtailed. When the larvae were then fed to mice, their ability to migrate was markedly inhibited as compared with that of larvae that had been incubated in fetal calf serum or in helminth-free sheep colostrum. Fractions of serum and colostrum containing IgG_1 had greater inhibitory effects both on the larvae in vitro and on their subsequent migration in mice than did IgG_2-containing fractions. IgM also inhibited the larvae as 2-mercaptoethanol reduction of IgM in the IgM-containing peak eluted from Sephadex G200 reduced the inhibitory activity of this peak, although the activity was not completely abrogated.

Introduction

The life cycle of *Toxocara vitulorum* in *Bos* and *Bubalis* species involves vertical transmission of infection from mother to young. Infective larvae ingested in eggs by adult animals are presumed to become dormant in the tissues (Warren 1971). Activation in the cow of these larvae, their growth and migration to the mammary gland and subsequent ingestion of the larvae in colostrum and milk over the first 5–6 days of lactation by the calf lead to adult *T. vitulorum* infection in the calf's small intestine (Warren 1971; Roberts 1990; Roberts et al. 1990). This infection can cause considerable morbidity and mortality among calves (Enyenihi 1969).

The pivotal role played in this life cycle by larvae within reproductive female cows has led to studies on antigens of the larvae and antibodies produced against them. In particular, Rajapakse et al. (1994) demonstrated antibodies against the excretion/secretion antigens of *T. vitulorum* larvae (larval ES) in serum and colostrum of buffalo cows by enzyme-linked immunosorbent (ELISA) and gel precipitation assays. High titres of anti-larval ES antibodies in buffalo cows' colostrum, and in the serum of their suckled calves, correlated with low *T. vitulorum* faecal egg counts in the calves to suggest that antibody might have a protective role against activating or migrating larvae. Also, *T. vitulorum* larvae migrate in mice and immunization of mice with larval ES antigens has induced protection against larval migration (Amerasinghe et al. 1992). Therefore, in the present study, antibody-mediated protection against *T. vitulorum* larvae was confirmed by the ability of buffalo serum or colostrum to inhibit migration of *T. vitulorum* larvae in mice.

Materials and methods

Mice

Albino mice of an outbred strain from the Faculty of Medicine, University of Peradeniya, were used at 5–6 weeks of age.

Serum and colostrum samples

Blood for serum was collected 1 month before and colostrum was collected immediately after parturition from four buffalo cows that had shown a high titre (1:6400) in their colostrum to larval ES by ELISA (Rajapakse et al. 1994). Low-titre colostrum was

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collected from three cows that had shown only a 1:50 ELISA titre to larval ES. Negative samples were fetal calf serum (FCS; Flow Laboratories, Scotland) and colostrum pooled from helminth-free sheep reared at the Department of Clinical Veterinary Medicine, University of Cambridge. Colostrum was centrifuged at 3000 *g* and 4° C for 30 min. The surface fat layer was removed, casein was precipitated with one drop of 1% $CaCl_2$ and 1% rennin (Sigma Chemical Co., USA) at 37° C for 1 h and the whey was recovered by centrifugation.

Chromatography

Serum and colostrum samples were fractionated using methods described by the manufacturer (Pharmacia, Sweden) and Fernando and Soulsby (1974). Samples were fractionated through Sephadex G200 in phosphate-buffered saline (PBS) by upward flow through 3 × 100-/2.6-cm columns at an hydrostatic pressure of 25 cm. The elution pattern gave the expected three peaks that occur on the chromatography of ruminant sera. The presence of IgM, the IgGs and albumin in the first, second and third peaks, respectively, from the buffalo serum and colostrum was confirmed by immunoelectrophoresis (IEP; Fernando and Soulsby 1974) using rabbit anti-bovine globulin (Cappel Laboratories, USA). The fractions comprising the three protein peaks were pooled and concentrated to 1 mg/ml in dialysis tubing by polyethylene glycol (Aquacide III, Calbiochem, USA). In some instances, the second IgG-containing peak from Sephadex G200 was refractionated on diethylaminoethanol (DEAE) Sephadex A25. The first peak was eluted with 0.056 *M* TRIS buffer (pH 8.6). A second protein peak was eluted with this buffer containing added 0.1 *M* NaCl. The two peaks were concentrated as described above and were shown by IEP to contain the expected IgG_2 (first peak) and IgG_1 (second peak).

2-Mercaptoethanol treatment

FCS and fraction 1 from Sephadex G200-filtered colostrum were treated with 2-mercaptoethanol (2-ME) and iodoacetamide as described by Deutsch (1963) and Fernando and Soulsby (1974). The IgM band seen on IEP of fraction 1 was no longer visible after 2-ME treatment.

Toxocara vitulorum

Eggs were collected from the faeces of young calves and embryonated in 0.1 *N* H_2SO_4. Embryonated eggs were washed, decorticated in saturated $Ca(OCl)_2$ for 20 min and washed extensively in PBS before infection of mice or larvae were hatched in vitro as described by Rajapakse et al. (1992).

In vitro treatment of larvae

Larvae freshly hatched from eggs were washed with PBS by centrifugation at 500 *g* for 5 min. In all, 40000 larvae were added to each millilitre of the appropriate serum or colostrum samples or fractions of these and were incubated at 37° C in 5% CO_2 for 6 h. At the end of this time the larvae were counted and a minimum of 300 larvae were examined at 25 × magnification under an inverted microscope. The viability of the larvae was judged subjectively; larvae were noted as moving actively as compared with being lethargic and coiled or immobile.

Infection of mice

Groups of mice were injected with different volumes of colostrum or FCS, all undiluted, or with fractions of colostrum at 1 mg/ml. Mice were restrained in a plastic tube and the sample was injected very slowly via a 26-gauge needle into the tail vein. The mice were infected 5 h later with 500 eggs in 0.2 ml PBS via a stomach tube. Other mice were given by stomach tube 500 larvae that had been pretreated with various serum or colostrum preparations.

Larval counts

At 5 days after infection, mice were euthanized by deep chloroform anaesthesia and cervical vertebral dislocation. The liver, lungs and kidneys were minced finely with scissors, digested in 1% pepsin/HCl at 37° C and then fixed with 6% formal saline. Larvae in all or in 20% (when present in large numbers) of a digest were counted at a 40–100 × magnification. The percentage of reduction in the number of larvae was calculated as:

$$\frac{\text{Larvae in control mice} - \text{Larvae in treated mice}}{\text{Larvae in control mice}} \times 100.$$

Group means were compared by Student's *t*-test or by Cochran's *t'*-test if inequality of variances occurred (Snedecor and Cochran 1980).

Results

Passive transfer of colostrum immunoglobulin

Mice in two experiments were injected either with FCS or with varying volumes of high-titre (1:6400, ELISA) colostrum whey or with its fractions eluted from Se-

Table 1 Recovery of *Toxocara vitulorum* larvae from mice passively immunized with colostrum whey and various Sephadex G200-chromatographed fractions of colostrum whey from cows infected with *T. vitulorum*

Volume of colostrum or FCS injected	Number of mice	Mean number of larvae recovered ±SE	% Reduction in larvae	*P* value
200 µl FCS	7	274.3±17.6		
200 µl colostrum	7	213.9±9.1	22.0	<0.01
500 µl colostrum	7	87.5±5.4	68.1	<0.001
2000 µl colostrum	7	17.0±2.3	93.8	<0.001
1000 µl FCS	6	159.8±8.6		
1000 µl colostrum unfractionated	6	15.3±1.5	90.4	<0.001
1000 µl fraction 1 (containing IgM)	6	48.6±8.0	64.6	<0.001
1000 µl fraction 2 (containing IgG)	6	26.7±4.8	83.4	<0.001
1000 µl fraction 3	6	151.2±8.1	5.4	>0.05

Table 2 Ability to migrate in mice of *T. vitulorum* larvae pretreated in vitro with serum and colostrum whey from cows infected with *T. vitulorum*

Pretreatment of larvae with	Number of mice	Mean number of larvae recovered ±SE	% Reduction in larvae	*P* value
1000 µl FCS	8	98.2±8.0		
1000 µl serum (1:6400 titre)	8	0.6±0.3	99.4	<0.001
1000 µl colostrum (1:6400 titre)	8	0.9±0.5	99.0	<0.001
1000 µl colostrum (1:50 titre)	8	72.3±3.5	26.4	<0.001

Table 3 Ability to migrate in mice of *T. vitulorum* larvae pretreated in vitro with Sephadex G200-chromatographed fractions of colostrum whey from cows infected with *T. vitulorum*

Pretreatment of larvae with	Number of mice	Mean number of larvae recovered ±SE	% Reduction in larvae	*P* value
1000 µl FCS	8	105.0±3.2		
1000 µl sheep colostrum	8	102.3±7.9	2.6	>0.05
1000 µl colostrum unfractionated	8	0.7±0.3	99.3	<0.001
1000 µl fraction 1 (containing IgM)	8	9.0±2.2	91.4	<0.001
1000 µl fraction 2 (containing IgG)	8	3.5±1.1	96.7	<0.001
1000 µl fraction 3	8	83.2±6.9	20.9	<0.05

phadex G200 and were then infected with *Toxocara vitulorum* eggs. The results are recorded in Table 1. All the different volumes of injected colostrum whey, as compared with FCS, induced a significant reduction ($P<0.01-<0.001$) in the numbers of larvae recovered from the mice. In addition, the level of protection increased with increasing doses of colostrum. Mice injected with 500 µl colostrum contained fewer larvae ($P<0.001$) than did those injected with 200 µl, and mice injected with 2000 µl harboured fewer larvae ($P<0.001$) than did those receiving 500 µl colostrum.

Fractions 1 and 2 from Sephadex G200-fractionated colostrum whey, containing IgM and IgG, respectively, but not fraction 3 (containing albumin), induced a significant reduction in the numbers of larvae recovered ($P<0.001$ as compared with mice receiving FCS; Table 1). The IgG-containing fraction 2 induced significantly greater ($P<0.005$) protection than did the IgM-containing fraction 1.

Effect of in vitro treatment of larvae with serum and colostrum whey

After 6 h of treatment in vitro with serum or colostrum of 1:6400 titre, 50%–60% of the larvae were lethargic and coiled. Also, there was a marked (>99%) reduction in the numbers of larvae treated with high-titred colostrum recovered from mice as compared with larvae treated with FCS ($P>0.001$; Table 2). Larvae treated with either FCS or colostrum of low titre (1:50) remained very active. Nevertheless, fewer larvae pretreated with low-titre colostrum were recovered from the mice as compared with larvae pretreated with FCS ($P<0.001$; Table 2). The low-titre colostrum was not as effective at reducing the numbers of larvae as were the high-titre serum and colostrum ($P<0.001$ for both).

Effect of pretreatment of larvae with Sephadex G200 fractions of colostrum whey

Incubation of larvae in vitro in sheep colostrum did not affect the motility of the larvae in vitro and did not reduce the migration of the larvae in mice as compared with larvae pretreated with FCS ($P<0.05$; Table 3). Again, 50%–60% of larvae became lethargic and coiled after incubation with immune colostrum whey (1:6400) or with its IgG- or IgM-containing fractions from Sephadex G200. Also, very few ($P<0.001$) of the larvae thus treated were recovered from the mice to which they had been fed (Table 3). Although no immunoglobulin was detected by IEP in fraction 3, 22% of the larvae treated with fraction 3 were lethargic and coiled. As compared with pretreatment with FCS, fraction 3 also reduced the infectivity of the larvae for mice ($P<0.05$; Table 3), although this reduction was not marked and was significantly less pronounced ($P<0.001$) than the reduction induced by the IgG- and IgM-containing fractions.

Effect of pretreatment of larvae with DEAE fractions of serum and colostrum whey

Of the larvae treated with fraction B of high-titre (1:6400) colostrum whey containing IgG_1, 60% were lethargic and coiled and another 23% showed only little movement. Of the larvae treated with fraction A, the

Table 4 Ability to migrate in mice of *T. vitulorum* larvae pretreated in vitro with DEAE Sephadex A25 fractions of serum and colostrum whey from cows infected with *T. vitulorum*

Pretreatment of larvae with	Number of mice	Mean number of larvae recovered ±SE	% Reduction in larvae	*P* value
1000 µl FCS	7	198.2±4.9		
1000 µl colostrum unfractionated	7	6.2±1.8	96.9	<0.001
1000 µl colostrum fraction A (IgG_2)	7	162.7±7.6	17.9	<0.005
1000 µl colostrum fraction B (IgG_1)	7	56.0±4.9	71.7	<0.001
1000 µl FCS	8	211.8±7.5		
1000 µl serum unfractionated	8	1.0±0.5	99.5	<0.001
1000 µl serum fraction A (IgG_2)	8	94.7±10.0	55.3	<0.001
1000 µl serum fraction B (IgG_1)	8	45.7±9.8	78.4	<0.001

Table 5 Ability to migrate in mice of *T. vitulorum* larvae pretreated in vitro with the IgM-containing fraction of colostrum whey eluted from Sephadex G200 in reduced with 2-ME

Pretreatment of larvae with	Number of mice	Mean number of larvae recovered ±SE	% Reduction in larvae	*P* value
1000 µl FCS	8	209.3±8.4		
2-ME-treated FCS	8	205.2±10.5	2.0	>0.05
1000 µl colostrum unfractionated	8	5.7±1.6	97.3	<0.001
Colostrum fraction 1 (IgM)	8	46.7±8.8	77.7	<0.001
2-ME-treated colostrum fraction 1 (IgM)	8	135.3±10.6	35.4	<0.001

IgG_2 fraction, 31% were coiled and inactive. The infectivity of the pretreated larvae for mice followed the same pattern in that whereas both fractions significantly reduced the numbers of larvae migrating in mice as compared with FCS-treated larvae ($P<0.005-<0.001$; Table 4), the IgG_1-containing fraction had a much greater inhibitory effect ($P<0.001$) than did that containing IgG_2.

The comparable IgG_1- and IgG_2-containing fractions from immune serum caused 73% and 68% of the larvae, respectively, to become lethargic and coiled, and 6% of the larvae in fraction B also showed small precipitates at their oral orifice. As compared with FCS, both fractions significantly reduced the numbers of larvae migrating in the mice ($P<0.001$; Table 4). As with the colostrum IgG isotypes, significantly fewer ($P<0.001$) larvae were recovered after pretreatment with the IgG_1-containing fraction B as compared with the IgG_2-containing fraction A.

Effect of 2-ME treatment of immunoglobulin from colostrum whey

Reduction with 2-ME and iodoacetamide did not itself affect the viability of the larvae. Thus, larvae incubated in FCS that either had or had not been treated with 2-ME remained active and the larvae were equally infective for mice ($P>0.05$; Table 5). After incubation in untreated fraction 1 (IgM-containing) or in 2-ME-treated fraction 1 from Sephadex G200, only a few of the larvae, namely 12% and 8%, respectively, were sluggish. Nevertheless, as compared with FCS, both the untreated and the 2ME-treated fractions 1 significantly reduced the infectivity of the larvae for mice ($P<0.001$; Table 5), but 2-ME treatment did significantly reduce ($P<0.001$) the efficacy of fraction 1.

Discussion

This study demonstrated that colostrum and serum, and various fractions thereof, from buffalo cows naturally infected with *Toxocara vitulorum* (a) passively transferred protection against *T. vitulorum* larvae to mice, (b) adversely affected the in vitro activity of the larvae and (c) reduced the infectivity for mice of the larvae thus treated.

Passive transfer of immunity by means of serum and colostrum and in vitro lethal effects induced by these have been reported for other helminth infections. In both serum and colostrum, the highest levels of activity against *T. vitulorum* were present in the IgG fraction, particularly the IgG_1 fraction. IgG_2 activity did, however, seem higher in colostrum than in serum, although these were not directly compared within the same experiment. Fernando et al. (1987, 1989) had also demonstrated IgG in serum precipitates on larvae of *T. vitulorum* and serum IgG_1 was the main immunoglobulin isotype reactive in ELISA against antigens extracted from *T.*

vitulorum larvae. IgG antibodies are clearly important in other parasite infections as well. IgG antibodies were 'lethal' for schistosomula of *Schistosoma mansoni* in vitro and passively transferred immunity against *S. mansoni* to mice and rats (Clegg and Smithers 1972; Grzych et al. 1982; Capron and Dessaint 1987). Serum IgG_1 and IgG_{2a} in mice and rats, respectively, were responsible for passive transfer of immunity against *Taenia taeniaeformis* (Leid and Williams 1974; Musoke and Williams 1975). Similarly, transfer of immunity against *Ascaris suum* in guinea pigs was associated with serum IgG_2 as well as a fraction containing both IgG_1 and IgE (Khoury et al. 1977).

IgM is inactivated by 2-ME reduction (Deutsch 1963; Fernando and Soulsby 1974). Therefore, colostrum IgM seems responsible at least in part for the activity against *T. vitulorum* larvae observed in the first macroglobulin peak eluted from Sephadex G200. Activity against *A. suum* and *T. taeniaeformis* in an IgM-containing fraction of serum, although at low levels as compared with that associated with the IgG isotypes, has also been identified (Khoury et al. 1977; Lloyd and Soulsby 1978). 2-ME reduction of the first peak of colostrum did not eliminate all its activity against *T. vitulorum*, however. It might be possible to suggest that the remaining activity is associated with IgA immunoglobulins in colostrum. Thus, IgA is common in colostrum, secretory IgA elutes in the macroglobulin peak and IgA antibodies are important in protection against parasites such as *T. taeniaeformis* in mice and *Haemonchus contortus* in sheep (Lloyd and Soulsby 1978; Gill et al. 1993).

The third colostrum peak was shown by IEP to contain albumin and no immunoglobulin. This peak did not passively transfer protection to mice, but when used to treat larvae in vitro, it did have some deleterious effects on the ability of the larvae to migrate in mice. It seems probable that IgG immunoglobulins, at a level below that detected by IEP and the passive transfer studies, did indeed trail into the third peak and that in vitro incubation studies were more sensitive at detecting these immunoglobulins.

The mechanism(s) of action of these immunoglobulins against *T. vitulorum* larvae was not determined. Fernando et al. (1987) had observed precipitates on and sluggish, feeble movements of *T. vitulorum* larvae incubated in immune buffalo serum or colostrum for 24–96 h. This study demonstrates that these effects may occur in vitro within 6 h of incubation. The antibodies, therefore, could adversely affect metabolism of the larvae. Also, the in vitro-attached antibody could react as a receptor(s) for antibody-dependent, cell-mediated cytotoxocity as the larvae begin their migration in mice.

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