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Number and distribution of T lymphocytes in the small intestinal mucosa of calves inoculated with rotavirus

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Abstract

An understanding of the immune response to rotavirus is needed to develop effective prophylaxis. There is evidence that cell-mediated responses may be involved and to extend these observations, rotavirus antigen and the three major T cell subsets, BoCD4$^+$, BoCD8$^+$, and BoWC1$^+$ γ/δ lymphocytes were immunostained in tissue sections from calves killed at 2, 4, 6, 8 and 10 days post inoculation and quantified by image analysis. It was established that in control calves, BoCD4$^+$ lymphocytes were predominantly in the lamina propria, while the majority of BoCD8$^+$ and BoWC1$^+$$\gamma/\delta$ lymphocytes were in the epithelium. Rotavirus infection was seen throughout the small intestine with the greatest amount of viral antigen detected at 4 days post inoculation in the mid and distal small intestine. Increased numbers of all subsets were detected; small increases in intraepithelial BoCD4$^+$ and BoWC1$^+$ γ/δ T lymphocytes were observed especially in the distal small intestine, while larger increases in BoCD8$^+$ cells were detected in the epithelium and lamina propria of the proximal, mid and distal small intestine. The timing and location of these increases in T lymphocyte subsets is indicative of a specific immune response involving BoCD8$^+$ and BoWC1$^+$ γ/δ T lymphocytes.

Abbreviations

mAb, monoclonal antibodies; NK, natural killer.

Introduction

Rotaviruses have been identified as the most important viral enteropathogen of calves in the second week of life in the UK (Reynolds et al., 1986; Snodgrass et al., 1986) and as the major cause of acute viral diarrhoea in infants and young children, causing $5–10\times10^6$ deaths every year in developing countries (Kapikian and Chanock, 1990). An understanding of immune responses to rotavirus is needed to develop effective prophylaxis which would benefit the health and welfare of both species.

Serotype-specific neutralising antibody is one of the immune mechanisms against rotavirus infection (Theil, 1990), but heterotypic protection was observed in the absence of rotavirus-specific neutralising antibody in infants (Clark et al., 1988) and calves (Bridger and Oldham, 1987; Woode et al.,

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1987), indicating other mechanisms in rotavirus immunity. Cytotoxic T cells contributed to the immune response of mice to rotavirus; after oral or parenteral inoculation with rotavirus, virus-specific cytotoxic activity was detected among splenic and intraepithelial lymphocytes (Offit and Dudzik, 1989). Subsequently, in mice with severe combined immunodeficiency, a chronic rotavirus infection was cleared by adoptive transfer of immune $CD8^+$ T lymphocytes (Dharakul et al., 1990) and passive protection against rotavirus-induced diarrhoea was induced in the absence of virus-specific antibodies using the same technique (Offit and Dudzik, 1990).

If lymphocytes in the calf small intestinal mucosa are involved in immune mechanisms against rotavirus, a change in the number and distribution of lymphocyte subsets might be expected in response to infection and a study of these changes might indicate the cell types involved in immune responses. Three major T cell populations are found in cattle. The present study establishes the number and distribution of these three lymphocyte subsets in the epithelium and lamina propria of villi in control calves and investigates changes in these subsets in calves inoculated with bovine rotavirus.

Materials and methods

Calves

Ten gnotobiotic calves (Dennis et al., 1976; Hoare et al., 1976) aged 10 days were inoculated orally with 10^6 $TCID_{50}$ of rotavirus C3-160 and four controls were given saline. Rotavirus C3-160 is a cloned virus of low virulence, which infects 10-day-old calves without causing diarrhoea (Bridger and Pocock, 1986; Bridger and Oldham, 1987). Two inoculated and one control calf were killed at 2, 4, 6 and 8 days after inoculation, and two inoculated calves at 10 days after inoculation.

Necropsy procedures

Calves were anaesthetised with pentobarbitone sodium (Sagatal, May and Baker, Dagenham, UK). Lengths of small intestine (10 cm) were ligated at nine sites and the sacs filled in situ with formol sublimate (Hall et al., 1985). Samples of tissue were removed from the proximal small intestine at the ligament of Treitz, from the distal small intestine adjacent to the ileocaecal junction and from the mid small intestine. They were immersed in OCT compound (Miles Scientific, Elkhart, USA), frozen in isopentane in liquid nitrogen and stored at $-20°C$. The sacs infused with fixative were removed, immersed in fixative and the animal killed by anaesthetic overdose.

Location of rotavirus antigen

Fixed tissue from nine small intestinal sites was processed into paraffin wax. Sections were cut at 5 μm, dewaxed, hydrated and stained for rotavirus antigen using an antiserum to rotavirus in an indirect peroxidase technique (Hall et al., 1993).

To assess the distribution of infection in the small intestine, the number of villi on which immunostaining to rotavirus antigen was or was not detected was counted and the percentage of infected villi calculated. Villi were counted in one section from each of the nine small intestinal sites from each of the two calves killed at 2, 4 and 6 days post inoculation.

Location of lymphocyte subsets

To distinguish between lymphocytes expressing different surface marker molecules, frozen tissues from proximal, mid and distal small intestine were cut on a cryostat at 8 μm and stored with silica gel at −20°C until they were immunostained using monoclonal antibodies (mAb) (Table 1). The specificities of these antibodies were defined at the First International Workshop on Leukocyte Antigens in Cattle, Sheep and Goats (Howard and Morrison, 1991). Samples were not available from one calf killed at 4 days post inoculation. Cell culture fluid, diluted 1/5 in phosphate buffered saline containing 0.01% sodium azide (Sigma, Pool, UK) or ascitic fluid diluted 1/1000 was applied to each section and an avidin-biotin immunoperoxidase technique (Vectorstain ABC, Peterborough, UK) was used with 3, 3′-diaminobenzidene tetrahydrochloride as substrate (Sigma) (Howard et al., 1988).

The areas of villus epithelium and villus lamina propria that were immunostained using each mAb were measured from five villi from the proximal,

Table 1
Monoclonal antibodies to bovine lymphocyte antigens used in immunohistology

mAb	Bovine antigen recognised	Human homologue	Distribution	Reference
IL-A11	BoCD4	CD4	T helper/inducer	Baldwin et al., 1986
CC63	BoCD8	CD8	T suppressor/ cytotoxic	Ellis et al., 1986
			α chain specific	MacHugh et al., 1991
CC15	BoWC1	–	BoCD4⁻, BoCD8⁻, BoCD2⁻, expressing gamma/delta T cell receptor	Clevers et al., 1990

mid and distal small intestine. Villi sectioned longitudinally were chosen at random and measurements made with an Optomax V image analyser (Synoptics, Cambridge, UK). Stained areas of epithelium and lamina propria were expressed as the mean percentage area of epithelium and lamina propria per villus. It has been assumed that the area stained by each mAb was proportional to the number of cells present in the tissue which expressed the antigen recognised by the particular mAb as previously reported (Parsons et al., 1989). Statistical analysis of the data was not possible because small groups of gnotobiotic calves were studied.

Results

Control calves—location of lymphocyte subsets

The majority of BoCD4$^+$ lymhocytes occurred in the lamina propria, while most BoCD8$^+$ and BoWC1$^+$ lymphocytes were in the epithelium. In the proximal, mid and distal small intestine, the ratio of lamina propria to intraepithelial lymphocytes was 4.6:1, 3.4:1 and 7.7:1 for BoCD4 cells, 1:4.1, 1:6.4 and 1:4.6 for BoCD8$^+$ cells and 1:2.3, 1:3.1 and 1:4.0 for BoWC1$^+$cells.

The majority of cells in the lamina propria were BoCD4$^+$, while most intraepithelial lymphocytes were BoCD8$^+$ except in the distal small intestine where there were eqivalent numbers of BoCD8$^+$ and BoWC1$^+$. In the proximal, mid and distal small intestine, the ratio of BoCD4$^+$:BoCD8$^+$:BoWC1$^+$ cells in the epithelium was 1:4.3:1.8, 1.1:2.2:1 and 1:6.9:7.1, and in the lamina propria 5.9:1.3:1, 15.0:1.3:1 and 4.8:1:1.5.

Inoculated calves—location of virus antigen

Infected villi were present throughout the small intestine in calves killed on 2, 4 and 6 days post inoculation. Infected villi were not detected in sections from control calves or from calves killed 8 and 10 days after inoculation. In calves killed 2 days after inoculation, the percentage of infected villi did not exceed 4%. The greatest amount of viral antigen was found in the mid and distal small intestine 4 days after inoculation, where up to 85% of villi were infected (Fig. 1). The percentage of infected villi in calves killed 6 days after inoculation was less than 12% in all small intestinal samples, except for Sites 6 and 7 in one calf where 45% and 42% villi were infected. The number of infected enterocytes per villus were greatest at those intestinal sites where the largest percentage of infected villi were detected.

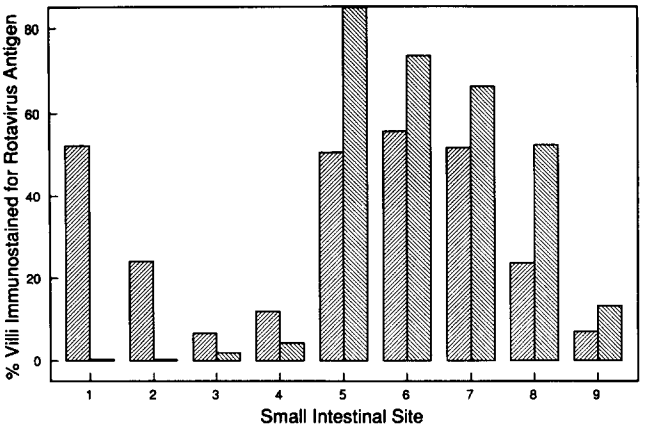

Fig. 1. Percentage of villi containing infected enterocytes detected by immunostaining of rotavirus antigen in sections from fixed tissue embedded in paraffin-wax from two calves killed at 4 days after inoculation.

Inoculated calves—location of lymphocyte subsets

BoCD4$^+$ lymphocytes

The number of intraepithelial BoCD4$^+$ cells in animals inoculated with rotavirus was increased above the upper limit of control animals in three of nine calves (33%) in the proximal and mid small intestine, and in five of nine calves (56%) in the distal small intestine (Figs. 2(a)–2(c)). These increases ranged from 1.68 to 3.77 times the mean of control animals with the levels increasing distally along the intestine and with the majority of increases occurring 6–10 days post inoculation.

Lamina propria BoCD4$^+$ lymphocytes were increased in number in inoculated calves above the upper limit of control values in four of 27 (15%) measurements (Figs. 3(a)–3(c)) with increases ranging between 2.07 and 3.17 times the mean of control animals and occurring 2–6 days post inoculation.

BoCD8$^+$ lymphocytes

Intraepithelial BoCD8$^+$ cells were increased in number in the proximal small intestine in three of nine (33%) calves, in the mid in six of nine (67%) calves and in the distal small intestine in seven of nine (78%) inoculated calves (Figs. 2(d)–2(f)). These increases ranged from 1.35 to 5.34 times the mean of control animals with the greatest increases in the mid and distal small intestine and with the majority of increases occurring 6–10 days post inoculation.

The number of lamina propria BoCD8$^+$ lymphocytes were increased above the upper limit of control animals in the proximal small intestine in six of

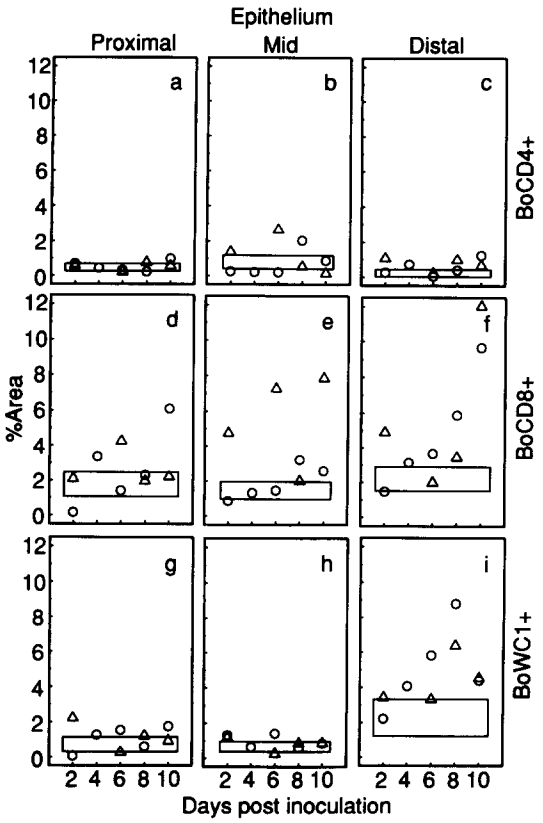

Fig. 2. The percentage of the area of epithelium immunostained using mAb against BoCD4$^+$ (a–c), BoCD8$^+$ (d–f) and BoWC1$^+$ (g–i) lymphocytes in the proximal (a, d and g), mid (b, e and h) and distal (c, f and i) small intestine in pairs of calves (0, △) killed at 2, 4 (one calf), 6, 8 and 10 days after inoculation. The boxes indicate the range of values in control calves for each mAb and each site.

nine (67%) calves, in the mid in eight of nine (89%) calves and in the distal small intestine in seven of nine (78%) calves (Figs. 3(d)–3(f)). These increases were greater than seen elsewhere and ranged from 1.03 to 5.92, 2.20 to 31.84 and 3.30 to 9.27 times the mean of control animals in the proximal, mid and distal small intestine respectively. Three-quarters of these increases occurred 6–10 days post inoculation.

BoWC1$^+$ lymphocytes

The most striking increase in the number of BoWC1$^+$ lymphocytes was observed in the epithelium of the distal small intestine where the numbers in seven of nine calves (78%) exceeded the upper limit of control animals (Fig. 2(i)). Increases were observed in the proximal small intestine in five of nine

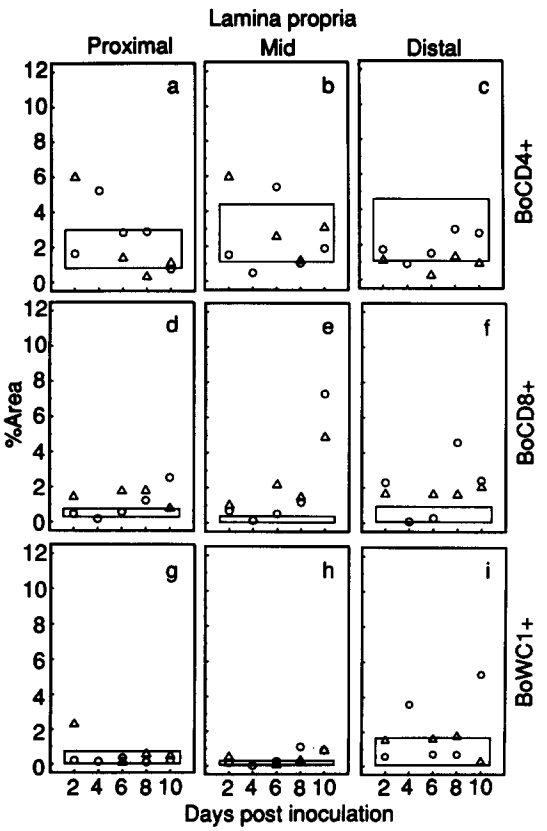

Fig. 3. The percentage of the area of lamina propria immunostained using mAb against BoCD4$^+$ (a–c), BoCD8$^+$ (d–f) and BoWC1$^+$ (g–i) lymphocytes in the proximal (a, d and g), mid (b, e and h) and distal (c, f and i) small intestine in pairs of calves (0, △) killed at 2, 4 (one calf), 6, 8 and 10 days after inoculation. The boxes indicate the range of values in control calves for each mAb and each site.

(56%) calves and in the mid in three of nine (33%) calves (Figs. 2(g) and 2(h)). Numbers ranged from 1.70 to 3.80 times the mean of control animals and were observed from 2 days post inoculation onwards.

Lamina propria BoWC1$^+$ lymphocytes were increased in number in the inoculated calves above the upper limit of control animals in eight of 27 (30%) measurements (Figs. 3(g)–3(i)). Numbers ranged from 1.63 to 7.15 times the mean of control calves with the majority of increases occurring 8–10 days post inoculation.

Discussion

Quantitation of lymphocytes in the mucosa of control calves demonstrated that, as in man, most CD4$^+$ cells are found in the lamina propria and the

majority of $CD8^+$ lymphocytes are located in the epithelium (Brandtzaeg et al., 1988; Ernst et al., 1985). Bovine $BoWC1^+$ γ/δ T cells were more numerous in the epithelium than lamina propria, providing further evidence that γ/δ T lymphocytes associate with mucosal surfaces (Lefrancois, 1991) . In addition, this study has shown that while most intraepithelial lymphocytes in the proximal and mid small intestine were $CD8^+$, equivalent numbers of intraepithelial $BoCD8^+$ and $BoWC1^+$ cells were found in the distal small intestine of calves.

MAb CC63, used to detect $BoCD8^+$ cells, identifies the α chain of the CD8 molecule (MacHugh et al., 1991) and recognises both $CD8\alpha/\alpha$ and $CD8\alpha/\beta$ T cells. The $CD8\alpha$ chain has also been detected on a cloned cell line with non-specific killer activity (Goddeeris et al., 1991) and on $BoCD2^+BoWC1^-\gamma/\delta TcR^+$ T cells (Sopp et al., 1991). Natural killer (NK) cells capable of non-specific cytotoxicity are $CD8^+$ in some species (Trinchieri, 1989) and non-specific cytotoxicity has been detected in cattle (Cook and Splitter, 1989; Godson et al., 1991), but expression of CD8 was not determined. The $CD8^+$ cells which were increased in number in the epithelium and lamina propria in the mid and lower small intestine, the site of most rotavirus infection, were probably cytotoxic T cells because these lymphocytes have been associated with rotavirus infection in mice. It is possible that some or all were other cell types expressing CD8; $CD8^+$ γ/δ TCR^+ T cells and homodimeric $CD8^+$ T cells have been detected by flow cytometry amongst bovine intraepithelial lymphocytes (Parsons et al., 1993a) . Cytotoxic T lymphocytes cleared rotavirus infection in SCID mice (Dharakul et al., 1990) and mice were passively protected from rotavirus-induced gastroenteritis by adoptive transfer of immune $CD8^+$ lymphocytes (Offit and Dudzik, 1990). The influx of $BoCD8^+$ cells was not immediate and the increase in numbers with time after inoculation was more typical of a specific immune response by cytotoxic lymphocytes than a non-specific response by NK cells (Welsh, 1978).

MAb CC15 recognises a 215/300 kDa antigen (WC1) present on about 90% of peripheral blood γ/δ TCR^+ T lymphocytes (Parsons et al., 1993b). Three mAbs that appear to identify all bovine γ/δ T cells in peripheral blood by flow cytometry unfortunately do not immunostain cells in cryostat sections (Parsons et al., 1993). In peripheral blood the small population of γ/δ TCR^+ cells that are $WC1^-$ are mostly $BoCD2^+BoCD6^+BoCD8^+$ (Sopp et al., 1991). The function of T lymphocytes expressing the γ/δ T cell receptor (TcR) is unknown. In man and mice, γ/δ T cells are associated with epithelial surfaces and are considered to be MHC-unrestricted, but may recognise antigen presented by other molecules found on epithelial cells (Balk et al., 1991; Wu et al., 1991; Kronenberg et al., 1992).

The timing and location of these increases in T lymphocyte subsets is indicative of a specific immune response involving $BoCD8^+$ and $BoWC1^+$ lym-

phocytes and this response may have been involved in the clearance of primary rotavirus infection.

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