Journal of Medical Microbiology (2004), **53**, 267–272 DOI 10.1099/jmm.0.05359-0

Differences in lymphocyte subpopulations and cell counts before and after experimentally induced swine dysentery

Robert Jonasson,[1] Anders Johannisson,[2] Magdalena Jacobson,[1] Claes Fellström[1] and Marianne Jensen-Waern[1]

Correspondence
Robert Jonasson
Robert.Jonasson@kirmed.slu.se

[1]Department of Large Animal Clinical Sciences, Unit of Comparative Physiology and Medicine, Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences, PO Box 7018, S-750 07 Uppsala, Sweden

[2]Department of Anatomy and Physiology, Swedish University of Agricultural Sciences, Uppsala, Sweden

The aim of this study was to examine the levels of circulating leukocytes and lymphocyte subpopulations before and immediately after experimentally induced swine dysentery. Twenty-one healthy crossbred pigs (~22 kg) were orally inoculated with *Brachyspira hyodysenteriae*. Blood was sampled before inoculation and when clinical signs of swine dysentery occurred. Pigs that remained healthy were sampled when killed. Total and differential white blood cell counts were performed, and lymphocyte subpopulations were analysed using flow cytometry. Following a mean incubation period of 13 days, 12 pigs developed swine dysentery, whereas nine remained healthy throughout the study. Before inoculation, pigs that subsequently developed swine dysentery displayed higher levels of circulating $\gamma\delta$ T cells (mean ± SE; 30·7 ± 3·5 %) compared with pigs that remained healthy (14·9 ± 1·4 %). Sick animals also displayed lower levels of $CD8^+$ cells (24·6 ± 1·5 %), cytotoxic/suppressor T cells (10·9 ± 1·3 %) and $CD4^+$ $CD8^-$ T cells (8·1 ± 1·0 %) than the pigs that remained healthy (34·9 ± 3·1 %; 17·6 ± 2·0 %; 13·6 ± 2·3 %). No difference was observed in leukocyte counts before inoculation. At onset of swine dysentery, there was an increase in monocytes (from 1·5 ± 0·2 × 10^9 to 3·8 ± 0·5 × 10^9 l^{-1}) and $CD4^+$ $CD8^+$ T cells (from 5·8 ± 0·9 to 8·9 ± 0·7 %). In conclusion, $\gamma\delta$ T cells and $CD8^+$ cells may be associated with susceptibility to experimentally induced swine dysentery, whereas monocytes and $CD4^+$ $CD8^+$ T cells appear to be the major responding leukocytes during the disease.

Received 23 June 2003
Accepted 28 October 2003

INTRODUCTION

Swine dysentery is caused by the Gram-negative spirochaete *Brachyspira hyodysenteriae*, which colonizes the large intestine and gives rise to excessive mucus production, haemorrhage and tissue necrosis. The disease is controlled by the use of antimicrobial treatments and eradication programmes. Emerging antimicrobial resistance is an increasing problem (Buller & Hampson, 1994; Molnar, 1996), and further understanding of the humoral and cellular immune response against *B. hyodysenteriae* is imperative for the development of prophylactic measures. However, few studies have focused on the immune response during swine dysentery.

There is evidence of a B-cell-mediated humoral immune response, with circulating IgG, IgA and IgM antibodies and locally produced mucosa-associated sIgA, against outer-membrane proteins and lipopolysaccharides of *B. hyodysenteriae* (Joens *et al.*, 1984; Rees *et al.*, 1989a, b). These antibodies have been detected after challenge in pigs previously vaccinated with formalin-inactivated *B. hyodysenteriae* (Fernie *et al.*, 1983) and in pigs that survived the disease (Rees *et al.*, 1989a). The appearance of these antibodies has not been related to recovery from the disease (Rees *et al.*, 1989a), but may be involved in protection against reinfection (Joens *et al.*, 1979). Recovery from experimentally induced swine dysentery has been associated with an increased percentage of circulating $CD8^+$ $CD4^-$ cells and an *in vitro* proliferate response of these cells against *B. hyodysenteriae* antigens (Waters *et al.*, 2000). Immunization studies in pigs with pepsin-digested *B. hyodysenteriae* bacterin indicate an increase in both mucosal and peripheral blood $CD8^+$ cells (Waters *et al.*, 1999b).

The porcine T cell repertoire is distinguished by large

This paper was presented at the Second International Conference on Colonic Spirochaetal Infections in Animals and Humans, Edinburgh, UK, 2–4 April 2003.

numbers of circulating $CD4^+$ $CD8^+$ and $\gamma\delta$ T cells, but their roles in the immune response are not fully understood. Extrathymic $CD4^+$ $CD8^+$ T cells in pigs increase with age and are considered to be mature antigen-experienced memory/effector cells (Pescovitz *et al.*, 1994; Zuckermann & Husmann, 1996). The levels of $\gamma\delta$ T cells increase in response to a variety of infections in different species such as mice (Hiromatsu *et al.*, 1992), young cattle (Koets *et al.*, 2002), chickens (Rothwell *et al.*, 1995) and humans (Munk *et al.*, 1990). These cells are considered important in the early response against infections at epithelial surfaces (Skeen & Ziegler, 1993; Boismenu & Havran, 1994; Ishigami *et al.*, 1999) and in the immune response of young pigs before maturation of other lymphocyte subsets (Yang & Parkhouse, 1996).

The aim of the present study was to examine the levels of different circulating leukocytes and lymphocyte subpopulations before and during experimentally induced swine dysentery.

METHODS

Animals. The ethical committee for animal experiments (Uppsala, Sweden) approved the experimental design.

Twenty-one clinically healthy crossbred pigs (Yorkshire × Swedish Landrace) of mixed sexes, with a mean weight of 21·5 kg (range 17–25 kg), were obtained from two commercial piglet-producing herds known to be free from swine dysentery. All pigs were acclimatized in pens with concrete floors and straw as bedding for at least 1 week (Department of Large Animal Clinical Sciences, Swedish University of Agricultural Sciences). Twelve pigs were group-housed with three animals per pen, and nine were housed individually. They were fed twice daily with the same batch of a commercial finisher diet without growth promoters (Singel Veg SPK; Fori HB, Lidköping, Sweden) and had *ad libitum* access to water. Faecal samples from all pigs were cultured and found to be negative for *B. hyodysenteriae.*

Experimental design. In order to increase susceptibility to *B. hyodysenteriae* infection, the pigs were fed a diet for 7 consecutive days in which every second meal was substituted with pure soybean meal. The straw bedding was replaced with a synthetic fur blanket to minimize fibre ingestion. All pigs were orally inoculated for three consecutive days with 30 ml brain heart infusion broth containing approximately 10^7–10^9 *B. hyodysenteriae* strain B204 ml^{-1}. The bacteria were propagated as described previously by Fellström & Gunnarsson (1995) and the growth and purity were evaluated by phase-contrast microscopy.

After the challenge, clinical health examinations and collection of rectal swabs for examination of *Brachyspira* spp. shedding were performed daily. The consistency and presence of blood and/or mucus in the faeces were recorded. Depending on the severity of disease, the pigs were euthanized by captive bolt and exsanguination 2–6 days after clinical symptoms, which occurred 8–17 days (a mean of 13) after inoculation. Immediately after euthanasia, a complete necropsy was performed on all diseased animals. No clinical signs of illness, other than those associated with swine dysentery, were observed throughout the study, and the pigs that remained clinically healthy were euthanized between 21 and 27 days (a mean of 25 days) after inoculation.

Blood sampling. Blood samples were collected on two occasions. All animals were sampled before the provocative diet was given. Pigs developing swine dysentery were sampled at onset of first clinical signs, i.e. diarrhoea, and healthy pigs that did not develop any clinical signs of disease were sampled when killed. All blood samples were obtained from the jugular vein into EDTA vacutainer tubes. The EDTA-preserved blood was analysed with an electronic cell counter (Cell-Dyn 3500; Abbott) for total and differential white blood cell counts.

Staining of peripheral blood leukocytes. EDTA blood samples used in the flow cytometric analyses were rotated at room temperature until immunostaining, which was performed within 24 h. Erythrocytes were lysed using a buffer containing 0·155 M NH_4Cl followed by centrifugation at 1500 r.p.m. (∼380 ***g***) for 10 min at 4 °C. The leukocyte pellet was washed twice with PBS before resuspension in PBS with 5 % fetal calf serum. During a 30-min incubation with commercially available monoclonal primary antibodies (VMRD; Pullman, WA, USA) and appropriate isotype controls (Dako), half a million cells ml^{-1} were double-stained according to the procedure in Table 1. The cells were then washed and incubated with phycoerythrin (PE)-conjugated (Becton Dickinson) and FITC-conjugated (Caltag) secondary antibodies. Following incubation, the cells were washed, resuspended and finally fixed in PBS with 1 % paraformaldehyde until flow cytometric analyses.

Flow cytometric analyses. Stained cells were washed twice with PBS and quantified in a FACStar Plus or a BD LSR flow cytometer (Becton

Table 1. Monoclonal primary antibodies used during the double-staining procedure

Double staining no.	Anti-IgG2a PE conjugate		Anti-IgG1 FITC conjugate	
	IgG2a isotype	Clone no.	IgG1 isotype	Clone no.
1*	–	–	–	–
2	Isotype control	X943	Isotype control	X931
3	CD4	PT90A	CD3	8E6
4	CD4	PT90A	CD8	PT36B
5	CD8β	PG164A	CD8	PT36B
6	CD4	PT90A	CD25	PGBL25A
7	CD4	PT90A	$\gamma\delta$ (Po-TcR1-N4)	PGBL22A
8	CD8β	PG164A	CD 21	BB6-11C9

*Auto-fluorescence control with no antibodies added.

Dickinson) by the collection of forward and orthogonal light scatter and FITC and PE fluorescence. In each sample, 30 000 cells were recorded. Lymphocytes were gated by size and granularity with forward and orthogonal light scatter and further analysed for FITC and/or PE staining. The results were evaluated with region analysis using Cellquest software (Becton Dickinson), except in the case of the CD4/CD8 double staining, where quadrant analysis was used.

Statistical analyses. All data were normally distributed and presented as means ± SE. An unpaired *t*-test was used to compare differences in cell population before inoculation between the group that remained healthy and the group that developed swine dysentery; the differences within each group before and after inoculation were analysed with a paired *t*-test. Data were regarded as significantly different at $P < 0{\cdot}05$. Statistical calculations were performed with SigmaStat software (SPSS Science).

RESULTS

After a mean incubation period of 13 days (range 8–17 days), 12 of the 21 challenged pigs developed swine dysentery (six of the group-housed and six of the individually housed). Seven of these 12 developed haemorrhagic diarrhoea and five animals showed milder symptoms, with non-haemorrhagic diarrhoea. All pigs with clinical signs shed *B. hyodysenteriae*, and post-mortem examinations confirmed the disease. Nine animals remained clinically healthy throughout the study. Of these, three occasionally shed *B. hyodysenteriae*, but without any clinical symptoms or differences in leukocyte populations or lymphocyte subpopulations.

Leukocyte counts and lymphocyte subpopulations before inoculation

The pigs that subsequently developed swine dysentery displayed a higher percentage of $\gamma\delta$ T cells and lower percentages of $CD8^+$, $CD8^+$ $CD8\beta^+$ and $CD4^+$ $CD8^-$ T cells ($30{\cdot}7 \pm 3{\cdot}5$, $24{\cdot}6 \pm 1{\cdot}5$, $10{\cdot}9 \pm 1{\cdot}3$ and $8{\cdot}1 \pm 1{\cdot}0$ %, respectively) before inoculation than the pigs that remained healthy ($14{\cdot}9 \pm 1{\cdot}4$, $34{\cdot}9 \pm 3{\cdot}1$, $17{\cdot}6 \pm 2{\cdot}0$ and $13{\cdot}6 \pm 2{\cdot}3$ %, respectively) (Fig. 1). The higher proportion of $CD8^+$ cells present in the healthy group was mainly accounted for by cells that co-expressed the β-subunit of CD8 ($CD8^+$ $CD8\beta$+) and therefore had the phenotype of cytotoxic T cells. There were no differences in the total numbers of neutrophils, monocytes or lymphocytes between the two groups of animals before inoculation (Table 2).

Leukocyte counts and lymphocyte subpopulations after inoculation

At onset of the first clinical signs of disease, the number of monocytes increased (from $1{\cdot}5 \pm 0{\cdot}2 \times 10^9$ to $3{\cdot}8 \pm 0{\cdot}5 \times 10^9\ l^{-1}$) in pigs that developed swine dysentery (Table 2). An increase in neutrophils was observed in both groups. The total number of circulating lymphocytes remained unchanged in both groups, but a shift within the lymphocyte subpopulations occurred at onset of disease; the total numbers of T cells ($CD3^+$) increased in both the swine dysentery group and the healthy group. This increase was mainly due to $CD4^+$ $CD8^+$ T cells (from $5{\cdot}8 \pm 0{\cdot}9$ to $8{\cdot}9 \pm 0{\cdot}7$ %) in the swine dysentery group and to $\gamma\delta$ T cells in the healthy group. Although the level of $\gamma\delta$ T cells increased from 14·9 to 20·6 % in the healthy group, it was lower than that detected in the swine dysentery group (29·0 %). The increase in monocytes was larger in pigs with haemorrhagic diarrhoea ($n = 7$; $1{\cdot}5 \pm 0{\cdot}2 \times 10^9$ to $4{\cdot}7 \pm 0{\cdot}6 \times 10^9\ l^{-1}$) than in pigs with milder non-haemorrhagic diarrhoea ($n = 5$; $1{\cdot}5 \pm 0{\cdot}4 \times 10^9$ to $2{\cdot}5 \pm 0{\cdot}7 \times 10^9\ l^{-1}$) ($P < 0{\cdot}001$). There were no changes in $CD21^+$ cells and no detectable levels of $CD25^+$ cells in either group.

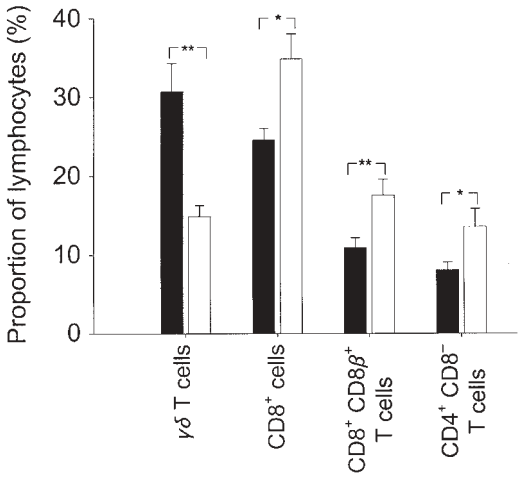

Fig. 1. Percentages of $\gamma\delta$ T cells, $CD8^+$ cells, $CD8^+$ $CD8\beta^+$ and $CD4^+$ $CD8^-$ T cells in the blood before inoculation. Filled bars denote pigs that subsequently developed swine dysentery ($n = 12$) and open bars represent pigs that remained healthy ($n = 9$). *, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$.

DISCUSSION

The pigs were from herds known to be free of swine dysentery and displayed no signs of clinical disease before *B. hyodysenteriae* inoculation. There were marked inter-individual differences in the lymphocyte subpopulation levels before inoculation: pigs with high levels of $\gamma\delta$ T cells and low levels of $CD8^+$ cells (mainly $CD8^+$ $CD8\beta$+ T cells) and $CD4^+CD8^-$ T cells were more susceptible to swine dysentery. The reason for these differences between healthy animals may be genetic variation or early exposure of young pigs to certain antigens that trigger shifts within lymphocyte populations. The infection model used in the present study was considered satisfactory, as approximately 60 % of the inoculated animals developed swine dysentery after a mean incubation period of 13 days.

An *in vitro* study (Waters *et al.*, 1999a) with *B. hyodysenteriae* whole-cell sonicates has shown a proliferate response of porcine $CD3^+$ cells, of which $CD8^+$, $CD4^+$ $CD8^+$ and $\gamma\delta$ T cells were particularly responsive. The $\gamma\delta$ T cells have been suggested as especially important for young pigs, and the percentage generally decreases with age as $\alpha\beta$ T cells become

Table 2. Leukocyte counts and lymphocyte subpopulations in the swine dysentery and healthy groups

Cell type	Swine dysentery group ($n = 12$)		Healthy group ($n = 9$)	
	Before inoculation	Euthanasia	Before inoculation	Euthanasia
Cell count ($\times 10^{-9}$ l^{-1})				
Leukocytes	18·5 ± 2·1	23·9 ± 1·7	19·3 ± 1·5	21·2 ± 1·6
Neutrophils	7·3 ± 1·2	11·6 ± 1·2^{a}*	7·6 ± 0·7	10·7 ± 1·5^{a}
Monocytes	1·5 ± 0·2	3·8 ± 0·5^{c}	2·1 ± 0·3	2·3 ± 0·4
Lymphocytes	9·1 ± 1·3	8·3 ± 1·0	9·5 ± 1·5	8·1 ± 0·9
Lymphocyte subpopulation (%)				
T cells (CD3$^+$)	52·7 ± 3·7	61·7 ± 3·9^{b}	53·6 ± 4·1	60·4 ± 4·0^{a}
$\gamma\delta$ T cells	30·7 ± 3·5^{B}†	29·0 ± 3·6	14·9 ± 1·4	20·6 ± 1·3^{b}
CD3$^+$ CD4$^+$ T cells	14·4 ± 1·5	17·4 ± 1·4^{b}	20·1 ± 2·9	22·3 ± 3·2
CD8$^+$ cells	24·6 ± 1·5^{A}	29·9 ± 2·7	34·9 ± 3·1	30·5 ± 2·0
CD8$^+$ CD8β^+ T cells	10·9 ± 1·3^{B}	11·6 ± 0·7	17·6 ± 2·0	15·2 ± 1·7
CD4$^+$ CD8$^-$ T cells	8·1 ± 1·0^{A}	8·8 ± 0·8	13·6 ± 2·3	14·4 ± 2·8
CD4$^+$ CD8$^+$ T cells	5·8 ± 0·9	8·9 ± 0·7^{c}	9·0 ± 2·0	8·7 ± 1·2
B cells (CD21$^+$)	20·4 ± 1·9	20·9 ± 3·0	24·4 ± 1·5	22·1 ± 1·9

*Values that are significantly different from the value before inoculation within a group are indicated by *a* ($P < 0·05$), *b* ($P < 0·01$) and *c* ($P < 0·001$).

†Values that are significantly different before inoculation compared with the healthy group are indicated by *A* ($P < 0·05$) and *B* ($P < 0·01$).

more numerous and more important for the immune system (Yang & Parkhouse, 1996). In mice, intestinal intra-epithelial $\gamma\delta$ T cells stimulate both *in vitro* proliferation and differentiation of epithelial cells (Boismenu & Havran, 1994). The absence of $\gamma\delta$ T cells in $\gamma\delta$ T-cell-receptor mutant mice is associated with decreased epithelial proliferation and exaggerated intestinal damage due to subsequent failure in regulating the consequences of T-cell responses (Komano *et al.*, 1995). This is consistent with findings that $\gamma\delta$ T cells can modulate the proliferative response of antigen-stimulated bovine CD4$^+$ T cells (Chiodini & Davis, 1992), suggesting a role for $\gamma\delta$ T cells as immunoregulatory cells. Taken together, these findings suggest that pigs with high levels of $\gamma\delta$ T cells may be less susceptible to infections. However, in the present study, pigs with large numbers of $\gamma\delta$ T cells were more susceptible to swine dysentery. This is consistent with a previous study on mice, in which a higher susceptibility to infections with *Salmonella choleraesuis* was found in normal mice compared with $\gamma\delta$ T-cell-deficient mice (Emoto *et al.* 1995). Therefore, for protection against some infections, the presence of $\gamma\delta$ T cells may not be advantageous for the animal. Furthermore, in studies with other microbes, $\gamma\delta$ T-cell-deficient mice show a lower morbidity and mortality after *Trypanosoma cruzi* infections (Santos Lima & Minoprio, 1996) and a faster and more complete recovery from *Pneumocystis carinii* infections, compared with normal mice (Steele *et al.*, 2002). These results may be explained by the ability of $\gamma\delta$ T cells to down-regulate both the recruitment and the functions of CD8$^+$ T cells during infections, as CD8$^+$ T cells are major producers of the cytokine IFN-γ (Steele *et al.*, 2002), which is important for the activation and enhancement of macrophage phagocytosis (Murray, 1990). Large numbers of CD8$^+$ T cells are present in the intra-epithelial region of the intestine (Fujihashi *et al.*, 1990; Guy-Grand & Vassalli, 1993) and these have been shown to secrete IFN-γ locally (Taguchi *et al.*, 1990). Even though $\gamma\delta$ T cells are efficient producers of IFN-γ (Tsukaguchi *et al.*, 1995), $\gamma\delta$ T-cell-deficient mice produce larger quantities of IFN-γ than normal ($\gamma\delta^+$) mice (Williams *et al.*, 1996; Santos Lima & Minoprio, 1996; Steele *et al.*, 2002). Thus, it could be speculated that animals with high levels of $\gamma\delta$ T cells are more susceptible to infections in which CD8$^+$ cells and the production of IFN-γ are important for host immune defence. CD8$^+$ cells are a major responding subset in swine dysentery (Waters *et al.*, 2000), and *B. hyodysenteriae* bacterin triggers production of IFN-γ *in vitro* by peripheral blood mononuclear cells (Waters *et al.*, 1999a). Therefore, animals with large numbers of $\gamma\delta$ T cells, and thus a down-regulated CD8$^+$ T-cell response, may produce lower levels of IFN-γ. Furthermore, it is possible that reduced levels of IFN-γ subsequently reduce the activation of macrophages in these animals prior to inoculation, thus increasing susceptibility to the infection in these animals if the clearance is dependent on macrophage phagocytosis. In the present study, the number of monocytes increased in pigs with swine dysentery, which indicates that the possible main clearance of *B. hyodysenteriae*

is through macrophages. Interestingly, the increase in monocytes was larger in pigs with haemorrhagic diarrhoea compared with pigs with milder non-haemorrhagic diarrhoea.

In the present study, there was no increase in CD8β^+ T cells in the swine dysentery group, which is in agreement with the findings of Waters *et al.* (2000). The proliferation of CD8$^+$ cells in pigs with swine dysentery appears to be related to CD4$^+$ CD8$^+$ T cells and not to cytotoxic/suppressor T cells or $\gamma\delta$ T cells, which also express the $\alpha\alpha$-homodimer of the CD8 receptor. In pigs, CD4$^+$ CD8$^+$ T cells have been reported to increase substantially with age (Yang & Parkhouse, 1996), from a few per cent in young animals up to 60 % in adults. However, in the present study, the increase in CD4$^+$ CD8$^+$ T cells in the swine dysentery group was not detected in the healthy group and was therefore unlikely to be related to age. In a study by Waters *et al.* (2000), there was no increase in CD4$^+$ CD8$^+$ T cells *in vivo* during swine dysentery, but a proliferation of these cells *in vitro* was seen in response to recall antigens in cultures from vaccinated pigs. The increase in CD4$^+$ CD8$^+$ T cells in this study could be attributed to their role as memory/effector cells of an antigen-experienced lymphocyte population (Pescovitz *et al.*, 1994; Zuckermann & Husmann, 1996). These lymphocytes produce IL10 and may therefore participate in the antibody production through the activation, growth and differentiation enhancement of B cells (Levy & Brouet, 1994; Ober *et al.*, 1998). Pigs are unique in their unusually high levels of CD4$^+$ CD8$^+$ T cells; therefore, the role of these cells during swine dysentery may be important.

In blood samples taken at onset of dysentery, no changes in B cell (CD21$^+$) levels were detected, but it cannot be excluded that an increase may have been found later. *B. hyodysenteriae*-specific antibodies have been detected in both colon washings and sera 4 and 7 days after first clinical signs of swine dysentery (Joens *et al.*, 1984). However, this antibody response has mainly been an indication of a prolonged or recent exposure to *B. hyodysenteriae* and unrelated to recovery or protection (Rees *et al.*, 1989a). T cells but not B cells from pigs vaccinated with *B. hyodysenteriae* antigens proliferate in response to *in vitro* stimulation with *B. hyodysenteriae* whole-cell sonicate (Waters *et al.*, 1999a). This raises the possibility that recovery from swine dysentery is mainly dependent on non-humoral defences through the actions of CD8$^+$ T cells and macrophages. In the absence of B-cell proliferation and the production of protective antibodies, there could be other important pathways for enhancement or opsonization of macrophage response against *B. hyodysenteriae*. Inoculation of colonic loops with *B. hyodysenteriae* and immune sera from pigs that have recovered from swine dysentery passively protects the colon from infection, whereas heat-inactivated immune sera give little protection (Joens *et al.*, 1985). This may indicate that involvement of a complement is important and could be the main route of opsonization.

The increase in neutrophils that was observed in both groups may be related to the stress caused by restraint during blood sampling, but the short duration of this stress (a few minutes) has previously been shown to have no influence on neutrophil counts (Magnusson *et al.*, 1998).

In conclusion, susceptibility to experimentally induced swine dysentery may be related to differences in lymphocyte subpopulations before inoculation. High levels of circulating $\gamma\delta$ T cells, and low levels of CD8$^+$ cells (mainly CD8$^+$ CD8β^+ T cells) and CD4$^+$ CD8$^-$ T cells were observed in pigs that subsequently developed swine dysentery. Further, increases in monocytes and CD4$^+$ CD8$^+$ T cells were noted during the disease. However, more studies are required to confirm these results and, in future studies, blood samples should be obtained more frequently from both challenged and non-challenged animals.

ACKNOWLEDGEMENTS

The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning financially supported this study. The BD LSR flow cytometer was financed with a grant from the Swedish Research Council. Thanks are due to Professor Caroline Fossum and Associate Professor Birgitta Essén-Gustavsson for critical reading of the manuscript.

REFERENCES

Boismenu, R. & Havran, W. L. (1994). Modulation of epithelial cell growth by intraepithelial $\gamma\delta$ T cells. *Science* **266**, 1253–1255.

Buller, N. B. & Hampson, D. J. (1994). Antimicrobial susceptibility testing of *Serpulina hyodysenteriae*. *Aust Vet J* **71**, 211–214.

Chiodini, R. J. & Davis, W. C. (1992). The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic γ/δ T lymphocytes which prevent CD4+ activity. *Microb Pathog* **13**, 447–463.

Emoto, M., Nishimura, H., Sakai, T., Hiromatsu, K., Gomi, H., Itohara, S. & Yoshikai, Y. (1995). Mice deficient in $\gamma\delta$ T cells are resistant to lethal infection with *Salmonella choleraesuis*. *Infect Immun* **63**, 3736–3738.

Fellström, C. & Gunnarsson, A. (1995). Phenotypical characterisation of intestinal spirochaetes isolated from pigs. *Res Vet Sci* **59**, 1–4.

Fernie, D. S., Ripley, P. H. & Walker, P. D. (1983). Swine dysentery: protection against experimental challenge following single dose parenteral immunisation with inactivated *Treponema hyodysenteriae*. *Res Vet Sci* **35**, 217–221.

Fujihashi, K., Taguchi, T., McGhee, J. R., Eldridge, J. H., Bruce, M. G., Green, D. R., Singh, B. & Kiyono, H. (1990). Regulatory function for murine intraepithelial lymphocytes. Two subsets of CD3+, T cell receptor-1+ intraepithelial lymphocyte T cells abrogate oral tolerance. *J Immunol* **145**, 2010–2019.

Guy-Grand, D. & Vassalli, P. (1993). Gut intraepithelial T lymphocytes. *Curr Opin Immunol* **5**, 247–252.

Hiromatsu, K., Yoshikai, Y., Matsuzaki, G., Ohga, S., Muramori, K., Matsumoto, K., Bluestone, J. A. & Nomoto, K. (1992). A protective role of γ/δ T cells in primary infection with *Listeria monocytogenes* in mice. *J Exp Med* **175**, 49–56.

Ishigami, M., Nishimura, H., Yoshioka, K., Kakumu, S. & Yoshikai, Y. (1999). The role of intrahepatic $\gamma\delta$-T cells for liver injury induced by *Salmonella* infection in mouse. *Microbiol Immunol* **43**, 461–469.

Joens, L. A., Harris, D. L. & Baum, D. H. (1979). Immunity to swine dysentery in recovered pigs. *Am J Vet Res* **40**, 1352–1354.

Joens, L. A., DeYoung, D. W., Cramer, J. C. & Glock, R. D. (1984). The immune response of the porcine colon to swine dysentery. In *Proceedings of the International Pig Veterinary Society Congress*, Ghent, Belgium, p. 187.

Joens, L. A., DeYoung, D. W., Glock, R. D., Mapother, M. E., Cramer, J. D. & Wilcox, H. E., III (1985). Passive protection of segmented swine colonic loops against swine dysentery. *Am J Vet Res* **46**, 2369–2371.

Koets, A., Rutten, V., Hoek, A., van Mil, F., Muller, K., Bakker, D., Gruys, E. & van Eden, W. (2002). Progressive bovine paratuberculosis is associated with local loss of $CD4^+$ T cells, increased frequency of $\gamma\delta$ T cells, and related changes in T-cell function. *Infect Immun* **70**, 3856–3864.

Komano, H., Fujiura, Y., Kawaguchi, M. & 10 other authors (1995). Homeostatic regulation of intestinal epithelia by intraepithelial $\gamma\delta$ T cells. *Proc Natl Acad Sci U S A* **92**, 6147–6151.

Levy, Y. & Brouet, J. C. (1994). Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J Clin Invest* **93**, 424–428.

Magnusson, U., Wattrang, E., Tsuma, V. & Fossum, C. (1998). Effects of stress resulting from short-term restraint on *in vitro* functional capacity of leukocytes obtained from pigs. *Am J Vet Res* **59**, 421–425.

Molnar, L. (1996). Sensitivity of strains of *Serpulina hyodysenteriae* isolated in Hungary to chemotherapeutic drugs. *Vet Rec* **138**, 158–160.

Munk, M. E., Gatrill, A. J. & Kaufmann, S. H. (1990). Target cell lysis and IL-2 secretion by γ/δ T lymphocytes after activation with bacteria. *J Immunol* **145**, 2434–2439.

Murray, H. W. (1990). Gamma interferon, cytokine-induced macrophage activation, and antimicrobial host defense. *In vitro*, in animal models, and in humans. *Diagn Microbiol Infect Dis* **13**, 411–421.

Ober, B. T., Summerfield, A., Mattlinger, C., Wiesmuller, K. H., Jung, G., Pfaff, E., Saalmuller, A. & Rziha, H. J. (1998). Vaccine-induced, pseudorabies virus-specific, extrathymic CD4+ CD8+ memory T-helper cells in swine. *J Virol* **72**, 4866–4873.

Pescovitz, M. D., Sakopoulos, A. G., Gaddy, J. A., Husmann, R. J. & Zuckermann, F. A. (1994). Porcine peripheral blood CD4+/CD8+ dual expressing T-cells. *Vet Immunol Immunopathol* **43**, 53–62.

Rees, A. S., Lysons, R. J., Stokes, C. R. & Bourne, F. J. (1989a). Antibody production by the pig colon during infection with *Treponema hyodysenteriae*. *Res Vet Sci* **47**, 263–269.

Rees, A. S., Lysons, R. J., Stokes, C. R. & Bourne, F. J. (1989b). The effect of parenteral immunisation on antibody production in the pig colon. *Vet Immunol Immunopathol* **23**, 171–178.

Rothwell, L., Gramzinski, R. A., Rose, M. E. & Kaiser, P. (1995). Avian coccidiosis: changes in intestinal lymphocyte populations associated with the development of immunity to *Eimeria maxima*. *Parasite Immunol* **17**, 525–533.

Santos Lima, E. C. & Minoprio, P. (1996). Chagas' disease is attenuated in mice lacking $\gamma\delta$ T cells. *Infect Immun* **64**, 215–221.

Skeen, M. J. & Ziegler, H. K. (1993). Induction of murine peritoneal γ/δ T cells and their role in resistance to bacterial infection. *J Exp Med* **178**, 971–984.

Steele, C., Zheng, M., Young, E., Marrero, L., Shellito, J. E. & Kolls, J. K. (2002). Increased host resistance against *Pneumocystis carinii* pneumonia in $\gamma\delta$ T-cell-deficient mice: protective role of gamma interferon and $CD8^+$ T cells. *Infect Immun* **70**, 5208–5215.

Taguchi, T., McGhee, J. R., Coffman, R. L., Beagley, K. W., Eldridge, J. H., Takatsu, K. & Kiyono, H. (1990). Analysis of Th1 and Th2 cells in murine gut-associated tissues. Frequencies of CD4+ and CD8+ T cells that secrete IFN-γ and IL-5. *J Immunol* **145**, 68–77.

Tsukaguchi, K., Balaji, K. N. & Boom, W. H. (1995). CD4+ $\alpha\beta$ T cell and $\gamma\delta$ T cell responses to *Mycobacterium tuberculosis*. Similarities and differences in Ag recognition, cytotoxic effector function, and cytokine production. *J Immunol* **154**, 1786–1796.

Waters, W. R., Pesch, B. A., Hontecillas, R., Sacco, R. E., Zuckermann, F. A. & Wannemuehler, M. J. (1999a). Cellular immune responses of pigs induced by vaccination with either a whole cell sonicate or pepsin-digested *Brachyspira* (*Serpulina*) *hyodysenteriae* bacterin. *Vaccine* **18**, 711–719.

Waters, W. R., Sacco, R. E., Dorn, A. D., Hontecillas, R., Zuckermann, F. A. & Wannemuehler, M. J. (1999b). Systemic and mucosal immune responses of pigs to parenteral immunization with a pepsin-digested *Serpulina hyodysenteriae* bacterin. *Vet Immunol Immunopathol* **69**, 75–87.

Waters, W. R., Hontecillas, R., Sacco, R. E., Zuckermann, F. A., Harkins, K. R., Bassaganya-Riera, J. & Wannemuehler, M. J. (2000). Antigen-specific proliferation of porcine $CD8\alpha\alpha$ cells to an extracellular bacterial pathogen. *Immunology* **101**, 333–341.

Williams, D. M., Grubbs, B. G., Kelly, K., Pack, E. & Rank, R. G. (1996). Role of γ-δ T cells in murine *Chlamydia trachomatis* infection. *Infect Immun* **64**, 3916–3919.

Yang, H. & Parkhouse, R. M. (1996). Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. *Immunology* **89**, 76–83.

Zuckermann, F. A. & Husmann, R. J. (1996). Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology* **87**, 500–512.