Immunological alterations during the clinical and recovery phases of experimental swine dysentery

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The aim of this study was to examine changes in the systemic immune response during the incubation period and following the onset of clinical swine dysentery, including the recovery period. Ten healthy conventional pigs were inoculated with *Brachyspira hyodysenteriae*. Blood was sampled at pre-inoculation, at days 4 and 14 post-inoculation, during the first 4 days with clinical signs of dysentery and at days 1, 3, 7, 11 and 15 of the recovery period. Eight pigs developed haemorrhagic diarrhoea. Flow-cytometric analyses of lymphocyte subpopulations showed that all animals, including the two that remained healthy, had an increase in CD8 α ⁺ CD4[−] cells and $\gamma\delta$ T cells at days 4 and 14 post-inoculation. In addition, an increase in CD4⁺ CD8 α ⁺ cells and CD8 α ⁺ CD8 β ⁺ cells was observed at days 4 and 14 post-inoculation in animals that developed dysentery. During clinical signs of dysentery, the acute-phase protein serum amyloid A was increased. There was a two- to threefold increase in both neutrophils and monocytes during signs of dysentery and at the beginning of the recovery period. The numbers of CD8 α ⁺ CD8 β [−] CD4[−], CD45RA[−] lymphocytes also increased during the dysentery period. Circulating CD21⁺ cells and CD21⁺ CD45RA[−] cells decreased at the end of the incubation period, during signs of dysentery and at the beginning of the recovery period. The dysentery-affected animals developed antibodies to *B. hyodysenteriae*-specific antigens (~16 kDa and ~30 kDa) from the first day of recovery, and $\gamma\delta$ T cells showed an increase during the recovery period. In comparison with pre-inoculation, increased numbers of monocytes, neutrophils, CD8 α ⁺ CD8 β [−] CD4[−] lymphocytes and CD45RA[−] lymphocytes were observed during clinical dysentery. Increased numbers of neutrophils, $\gamma\delta$ T cells and specific antibodies were seen during the recovery period.

Received 24 January 2006

Accepted 27 March 2006

INTRODUCTION

Taylor and Alexander (1971) showed that swine dysentery is caused by the Gram-negative spirochaete *Brachyspira hyodysenteriae*. The main clinical signs are severe muco-haemorrhagic diarrhoea and a reduced general appearance. The typical manifestations in the large intestine are excessive mucus production, haemorrhage and tissue necrosis. Due to the increased antimicrobial resistance of *B. hyodysenteriae* to the few antibiotic treatments available (Lobova *et al.*, 2004), a better knowledge of the host responses during infection is essential for development of prophylactic measures. In pigs, macrophages and neutrophils are found in colonic lesions at later stages of swine dysentery, but the role of these cells is still unclear (Albassam *et al.*, 1985). Experimental infections with *B. hyodysenteriae* in mice demonstrate that neutrophils are of great importance in mediating the severe oedema and structural lesions that occur in the intestine during the infection (Sacco *et al.*, 2000), and may thus be part of the

pathogenesis. However, the commensal intestinal flora has also been shown to be necessary for the development of the disease (Whipp *et al.*, 1979; Neef *et al.*, 1994). Among lymphocyte subsets, CD8⁺ cells appear to be of particular importance in the genesis of swine dysentery (Waters *et al.*, 1999a, b; Jonasson *et al.*, 2004), with low levels of circulating CD8 α ⁺ cells and high levels of circulating $\gamma\delta$ T cells before experimental inoculation shown to increase susceptibility to swine dysentery (Jonasson *et al.*, 2004). The mechanism of action of CD8⁺ cells against *B. hyodysenteriae* is largely unknown, but immune-mediated mucosal damage in mice has been shown to be prevented by CD8⁺ T cells (Jones *et al.*, 2002). CD8⁺ cells are important producers of IFN- γ (Steele *et al.*, 2002), which activates macrophages (Murray, 1990) and may also enhance their response against *B. hyodysenteriae*. In a study by Hontecillas *et al.* (2002), swine-dysentery-affected pigs had upregulated expression of IFN- γ in colonic lymph nodes.

The duration and characteristics of the immune response in the recovery period after the acute phase of dysentery have

Abbreviations: PE, phycoerythrin; SAA, serum amyloid A.

not been studied thoroughly. It has been shown that pigs that have recovered from swine dysentery are immune to reinfection (Joens *et al.*, 1979, 1983). This immunity appears to be antibody mediated, since colonic loops are protected against infection after passive transfer of convalescent serum antibodies (Joens *et al.*, 1979; Harris & Glock, 1981). While *B. hyodysenteriae*-specific antibodies have been detected during and after swine dysentery (Joens *et al.*, 1984, 1985; Rees *et al.*, 1989; Wright *et al.*, 1989), they have not been related to recovery from disease but instead regarded as an indication of prolonged or recent exposure to *B. hyodysenteriae* (Rees *et al.*, 1989). To date, the protection induced by vaccines has only been associated with reduced severity of lesions without decreasing bacterial shedding (Waters *et al.*, 1999a, b; Hontecillas *et al.*, 2002) or induction of *B. hyodysenteriae*-specific IgA secretion (La *et al.*, 2004). Thus, the relative importance of cell-mediated and antibody-mediated immunity for the recovery from infection with *B. hyodysenteriae* is not known.

The present study was therefore designed to allow examination of the proportional changes in some selected subpopulations of porcine blood lymphocytes and the development of specific antibodies during incubation, the acute phase and the recovery period of experimentally induced swine dysentery.

METHODS

Animals and housing. The Ethical Committee for Animal Experiments, Uppsala, Sweden, approved the experimental design.

Ten clinically healthy crossbred pigs (Yorkshire $\times$ Swedish Landrace) of mixed sexes were obtained at the age of 8–10 weeks from a conventional piglet-producing herd known to be free from swine dysentery. This herd is regularly health-monitored by swine specialists from the university. Following arrival at the experimental facilities at the Department of Clinical Sciences, the animals were acclimatized for at least 1 week. The pigs were housed individually, were fed twice daily with a commercial finisher diet without growth promoters (Singelveg SPK, Lantmännen) and had free access to water. The pens had concrete floors with straw as bedding material during acclimatization and synthetic fur blankets during the experimental period in order to minimize fibre ingestion. Faecal samples taken from all pigs at arrival were analysed by routine methods and found negative to parasite eggs, *Brachyspira* spp., *Salmonella* spp. and *Yersinia* spp. The pigs, which had a mean weight of 23 kg (range 20–29 kg) before inoculation, were weighed once a week for calculation of daily weight gain (DWG). The DWG was divided by the animals' live weight in order to avoid the effect of different growth rates during the fattening period.

Experimental design. The infection model included a provocative feeding regime that has previously been shown to facilitate infection with *B. hyodysenteriae* (Jacobson *et al.*, 2004). Briefly, 4 days prior to inoculation with *B. hyodysenteriae*, all pigs were fed a diet in which the commercial diet was substituted with pure soybean meal on every second feeding occasion for 7 consecutive days. During the last 3 days of this feeding regime, the pigs were inoculated orally once daily with 30 ml brain heart infusion (BHI) broth containing approximately 10^7 – 10^9 bacteria ml^{-1} of *B. hyodysenteriae* strain B204^T (ATCC 31212). The bacteria were propagated according to Fellström & Gunnarsson (1995), and bacterial growth, purity and motility were evaluated with phase-contrast microscopy.

Thorough clinical health examinations, including measurement of rectal temperature and evaluation of faeces with respect to consistency, and the presence of blood and mucus, were performed daily. The initial onset of signs of diarrhoea was defined as day 1 of the swine dysentery period. The time-point when the faecal consistency changed from diarrhoea to slightly loose or normal was defined as day 1 of the recovery period. The pigs that developed swine dysentery were euthanized approximately 20 days after recovery, and the pigs that remained clinically healthy throughout the study were euthanized 35 days after inoculation. After induction of anaesthesia with a mixture of 0.05 mg kg^{-1} medetomidine (Domitor vet) and 5 mg kg^{-1} Zoletil (tiletamine + zolazepam), euthanasia was performed with an overdose of pentobarbital sodium. A complete necropsy was performed on all animals.

Sampling of faeces and blood. Rectal swabs for culturing and detection of *Brachyspira* spp. were collected once a week until clinical signs of swine dysentery occurred, daily during the clinical signs of dysentery, and then once a week during the recovery period. Samples were cultured and evaluated according to Fellström & Gunnarsson (1995). Jugular venous blood was obtained in vacuum tubes from all pigs before the soybean diet and inoculum was given, and then 4 and 14 days after the last day of inoculation. During the acute phase with clinical signs of disease, blood was also sampled for 4 consecutive days, and then at days 1, 3, 7, 11 and 15 of the recovery period. Pigs that did not develop any clinical signs of disease were sampled once a week until the euthanasia that was performed after all of the dysenteric animals had recovered.

Analysis of peripheral blood leukocytes. Total and differential white blood cell counts were analysed with an electronic cell counter (Cell-Dyn 3500, Abbott). EDTA blood samples were rotated at room temperature until immunostaining, which was performed within 12 h, as previously described (Jonasson *et al.*, 2004). Briefly, erythrocytes were lysed, followed by centrifugation. The resulting leukocyte pellet was then washed twice and resuspended in PBS with fetal calf serum (FCS). Cells ($0.5 \times 10^6 \text{ ml}^{-1}$) were double-stained using monoclonal antibodies (VMRD) or isotype controls (DakoCytomation) as primary antibodies, as in Table 1. After washing, the cells were incubated with phycoerythrin (PE)- and FITC-conjugated secondary antibodies (CALTAG). The cells were washed, resuspended and finally fixed in PBS with paraformaldehyde until flow cytometric analyses were performed.

Flow cytometric analyses

A total of 30 000 cells per sample were analysed by forward light scatter and orthogonal light scatter in a BD LSR flow cytometer (BDBiosciences). Lymphocytes were gated using the light-scatter parameters and further evaluated for FITC and/or PE fluorescence. The results were evaluated by region analysis, except for the CD4/CD8 α double staining (no. 3 in Table 1), for which quadrant analysis was used. Evaluation was performed with the Cellquest software (BDBiosciences). The proportion of stained cells was multiplied by the number of lymphocytes determined in the total and differential counts in order to calculate the absolute number of lymphocytes in the various subpopulations.

Analysis of serum amyloid A (SAA). Blood samples without additives were centrifuged at 1500 g and serum was collected and stored at -80°C until analysed. The analysis of SAA was performed on serum samples diluted 1:500, using a commercially available ELISA (Tridelta Phase range SAA kit, Tridelta Development).

Analysis of serum antibodies

Sera from the following six sampling occasions were used: before the soybean diet and inoculum was given, at day 2 of clinical signs of

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

Double-staining no.	Anti-IgG1 FITC conjugate		Anti-IgG2a PE conjugate	
	IgG1 isotype	Clone no.	IgG2a isotype	Clone no.
1*	—	—	—	—
2	Isotype control	X0931	Isotype control	X0943
3	CD8 α	PT36B	CD4	PT90A
4	CD8 α	PT36B	CD8 β	PG164A
5	$\gamma\delta$ (Po-TcR1-N4)	PGBL22A	CD45 RA	PGB78A
6	CD 21	BB6-11C9	CD45 RA	PGB78A

*Auto-fluorescence control (no antibodies added).

dysentery and at days 1, 7, 11 and 15 of the recovery period. For the healthy pigs, the following three sampling times were used: before the soybean diet and inoculum was given, and 14 and 28 days after challenge. Sera from each pig at these separate sampling occasions were pooled, resulting in six pooled samples for the dysenteric pigs and three for the healthy pigs. In addition, sera from each of the swine-dysentery-affected pigs at recovery days 7, 11 and 15 were analysed separately for serum antibodies.

Analyses were performed with both unabsorbed and absorbed sera. The absorbed sera were absorbed with the type strains *Brachyspira intermedia* PWS/A^T (ATCC 51140), *Brachyspira innocens* B256^T (ATCC 29796), *Brachyspira murdochii* 56-150^T (ATCC 51284), *Brachyspira pilosicoli* P43/6/78^T (ATCC 51139) and *Brachyspira aalborgi* 513A^T (ATCC 43994). This procedure was performed in order to remove general antibodies to *Brachyspira* spp., thus leaving only those with specificity for *B. hyodysenteriae* strain B204^T (ATCC 31212). In brief, 100 μ l each of bacterial pellets of *B. innocens* and *B. pilosicoli*, and 10 μ l each of *B. intermedia*, *B. murdochii* and *B. aalborgi* were diluted in 1 ml resuspension buffer (50 mM Tris/HCl, 10 mM EDTA, pH 8.0) and sonicated on ice for 10 s 10 times with 20 s intervals. Five hundred microlitres of this mixture was added to every millilitre of 1:10-diluted sera and incubated for 90 min at 37 °C before use.

Western blot of unabsorbed or absorbed sera. Whole-cell proteins from the type strain of *B. hyodysenteriae* (B78^T) were separated by standard SDS-PAGE, according to Laemmli (1970). Bacterial samples corresponding to 2 μ g protein were mixed with sample buffer containing DTT before heat treatment for 5 min. The sample was applied to an SDS-polyacrylamide gel (12%) with a stacking gel on top and subjected to electrophoresis in a Mini-Protein II gel system (Bio-Rad). A pre-stained, broad-range protein marker from New England Biolabs was used as size ladder. The proteins were transferred onto a PVDF membrane (Towbin *et al.*, 1979) with a Mini Trans-Blot cell (Bio-Rad). The membrane was blocked in Tris-buffered saline (TBS) with dried non-fat milk powder (MP) and then incubated with sera (diluted 1:100) by using a multiscreen apparatus (Bio-Rad). Peroxidase-conjugated rabbit anti-swine IgG (DakoCytomation) was used as a secondary antibody. After a 30 min conjugate incubation on a shaker, the membranes were quickly rinsed and then washed. Bound antibodies were visualized with a chemiluminescent substrate using the ECL system and Hyperfilm ECL according to the manufacturer's instructions (Amersham Biosciences).

Statistical analyses. Data are presented as mean $\pm$ SEM. Data were calculated from eight animals with clinical signs and two healthy animals during pre-inoculation and 4 days post-inoculation. At 14 days post-inoculation, two animals in the group with clinical

signs had already developed dysentery ($n=6$). After these sampling points, one animal had to be euthanized and data are presented from seven dysenteric animals, with the exception of days 7, 11 and 15 of the recovery period, which are the means of six animals due to missing samples from different pigs on those days. The statistical calculations were performed with SigmaStat software (SPSS Science). Analysis of variance (ANOVA, Holm-Sidak method) for repeated measures was performed to compare differences in specific cell populations and SAA between measurements. Correlation analysis was performed with the Pearson product moment correlation. The data were regarded as significantly different at $P<0.05$.

RESULTS AND DISCUSSION

Clinical signs and SAA

The experimental protocol resulted in clinical signs of swine dysentery in eight out of 10 pigs. They developed swine dysentery with muco-haemorrhagic diarrhoea and shedding of *B. hyodysenteriae* after a mean incubation period of 17 days (range 7–31 days). Seven of the eight affected pigs recovered spontaneously without any need for treatment, but due to animal welfare considerations, one dysenteric animal had to be euthanized before termination of the experiment. Blood was collected both early and late during the incubation, the dysentery and the recovery period. Clinical signs of dysentery were evident for a mean of 7 days (range 3–17 days) before signs of recovery were observed. Five of the dysenteric pigs stopped shedding *B. hyodysenteriae* after a mean of 8 days of recovery, while two were still shedding at euthanasia. The two animals that remained clinically healthy did not shed *B. hyodysenteriae* on any occasion throughout the study. The mean daily weight gain per kilogram live weight for the swine-dysentery-affected pigs was 24 ± 1 g kg⁻¹ prior to disease, 0 ± 2 g kg⁻¹ during the disease, and 20 ± 0 g kg⁻¹ during the recovery period. The two animals that remained healthy had a mean daily weight gain of 25 ± 2 g kg⁻¹. Three of the swine-dysentery-affected pigs showed decreased appetite during the haemorrhagic phase of the diarrhoea, but none of the animals had fever. No clinical signs of illness other than those associated with swine dysentery were observed throughout the study. SAA is considered to be a clinically relevant acute-phase protein in pigs (Heegaard *et al.*, 1998). In the present study,

an SAA response was observed during the first 3 days of clinical signs, which is in agreement with Jacobson *et al.* (2004). The SAA levels were 21 ± 13 mg l⁻¹ before inoculation and 231 ± 187 , 154 ± 90 , 137 ± 82 and 25 ± 10 mg l⁻¹, respectively, during the first 4 days of swine dysentery. At day 1 of the recovery period, the SAA level was 15 ± 7 mg l⁻¹, and at day 7 of the recovery period, it was 4 ± 3 mg l⁻¹. Even though there were large inter-individual differences in the SAA response, the majority of the animals had a 30- to 60-fold increase. However, no relationship between SAA levels and severity of clinical signs was observed. The production of SAA is induced by the presence of LPS and the pro-inflammatory cytokines IL-1 and TNF- α (van Snick, 1990; for review see Jensen & Whitehead, 1998). *B. hyodysenteriae* has been shown to possess biologically active LPS (Nuessen *et al.*, 1982) that stimulates production of the pro-inflammatory cytokines IL-1 and TNF (Greer & Wannemuehler, 1989) and the neutrophil chemokine IL-8 (Sacco *et al.*, 1996). The production of SAA and its induction of tissue infiltration of monocytes and polymorphonuclear cells (Badolato *et al.*, 1994; Xu *et al.*, 1995; Hershkoviz *et al.*, 1997) likely influence the cellular response of the animals affected by swine dysentery.

Neutrophils, monocytes and lymphocyte subpopulations

In accordance with a previous study (Jonasson *et al.*, 2004), elevated levels of circulating monocytes, neutrophils and CD8 α ⁺ cells were seen during clinical signs of swine dysentery. The increase of monocytes/macrophages and neutrophils has also been shown in other studies (Wilcock & Olander, 1979; Albassam *et al.*, 1985). There was a two- to threefold increase of neutrophils during dysentery with a peak at the fourth day of clinical signs and at the beginning of the recovery period (Fig. 1). The migration of these neutrophils to tissues and their release of active inflammatory mediators, such as prostaglandins, NO, reactive oxygen species and hydrolytic enzymes, could be important contributors to the oedema and lesions associated with swine dysentery. This is supported by a study in which the severity of intestinal macro- and microscopic lesions, oedema, and epithelial erosions in *B. hyodysenteriae*-induced colitis in mice were found to be moderated when the levels of intestinal neutrophils were reduced or were zero (Sacco *et al.*, 2000).

A twofold increase in the number of monocytes was observed during clinical signs of dysentery (Fig. 1). *B. hyodysenteriae*-derived LPS has been reported to induce IL-1 secretion by murine macrophages (Greer & Wannemuehler, 1989), and increased serum concentrations of TNF- α have been shown following onset of swine dysentery (Nibbelink *et al.*, 1997). Macrophages are the major producers of TNF- α , which can facilitate lesion development, since the gastrointestinal tract is known to be sensitive to TNF- α (Beutler *et al.*, 1985). Potentially, local IL-1 and TNF- α production by intestinal *B. hyodysenteriae*-stimulated macrophages can contribute to the pathology of swine

dysentery. However, there was a positive correlation between the length of the incubation period and the pre-inoculation levels of peripheral monocytes (correlation coefficient 0.76; $P=0.03$; $n=8$). This relationship was also seen in pigs in a previous study (Jonasson *et al.*, 2004) in which the pigs with the highest levels of monocytes before inoculation had the longest incubation period (correlation coefficient 0.73; $P=0.003$; $n=14$). In the present study, two pigs had longer incubation periods than the other animals (29 and 31 days versus the mean of 15 ± 3 days for the other animals), and these two also had markedly higher levels of monocytes pre-inoculation (4.0 and 3.3×10^9 l⁻¹ versus the mean of $1.7 \pm 0.3 \times 10^9$ l⁻¹ for the other animals). The higher numbers of monocytes do not appear to influence the clinical development of dysentery, since in the previous study there was no difference in the monocyte levels before inoculation between animals that developed swine dysentery and those that remained healthy after inoculation (Jonasson *et al.*, 2004). Even though only two animals remained healthy after inoculation in the present study, they did not differ notably in monocyte levels before inoculation from those that developed dysentery.

Overall, the total numbers of lymphocytes increased 4 days after inoculation and at day 1 with clinical signs of dysentery. However, large inter-individual variations in the total numbers of lymphocytes were observed during the dysentery period and the beginning of the recovery period. Therefore, it is important to examine the alterations in the different lymphocyte subpopulations. While the results of lymphocyte subpopulations in this study are presented in absolute numbers, the relative numbers, expressed as a percentage, show similar results. The CD8 α ⁺ lymphocyte subpopulations increased after inoculation. Irrespective of the presence or absence of clinical signs of dysentery, all animals, including the pigs that remained healthy (Figs 2 and 3), showed a similar increase in circulating CD8 α ⁺ cells, CD8 α ⁺ CD4⁻ cells and $\gamma\delta$ T cells on days 4 and 14 post-inoculation (Figs 2 and 3). A similar rise was seen in CD8 α ⁺ CD8 β ⁺ cells 14 days post-inoculation (Fig. 2). These increases may be a response to the introduction of novel antigens, since vaccination studies with *B. hyodysenteriae* antigens have shown increased peripheral levels of CD8 α ⁺ $\gamma\delta$ T cells and CD8 α ⁺ natural killer cells (Bassaganya-Riera *et al.*, 2001; Waters *et al.*, 1999a). The response could also be directed against antigens present in the soybean meal included in the provocative diet, but since these alterations were still observed 14 days after the last soybean meal, this is less likely. Soybean is a rich source of linolenic acid, which can be converted to conjugated linolenic acid (CLA) by intestinal micro-organisms in cows (Dhiman *et al.*, 2000). We do not know whether this conversion also occurs in pigs. However, Bassaganya-Riera *et al.* (2001) have shown that dietary CLA increases circulating levels of CD8 α ⁺ $\gamma\delta$ T cells, cytotoxic T cells and CD8 α ⁺ natural killer cells in pigs. On the other hand, these changes were observed after several weeks on a CLA-rich diet, and are thus probably not applicable to the present study.

Fig. 1. Blood leukocyte populations of the dysentery-affected pigs: before inoculation, 4 and 14 days after inoculation, and during the swine dysentery and recovery periods. The mean incubation period was 17 days and the mean disease period was 7 days. The boundary of the box indicates the 25th percentile, the median and the 75th percentile. Whiskers indicate the 95th and 5th percentiles. The dotted lines behind each box plot show the average for the two animals that remained healthy. Pre-, 4 days post- and 14 days post-inoculation were the same in all animals, but the remaining sampling points for the healthy animals were from 21, 28 and 35 days post-inoculation. *Significant change from pre-inoculation.

The pigs that subsequently developed dysentery showed an increase in $CD4^+ CD8\alpha^+$ cells at 4 days post-inoculation. Even though the numbers of animals are small, this change was not observed in the two healthy animals. High levels of extrathymic $CD4^+ CD8^+$ T cells are typical for pigs, and they are considered to be mature antigen-experienced memory/effector cells (Pescovitz *et al.*, 1994; Zuckermann & Husmann, 1996). In a previous study, it was observed that the numbers of these double-positive cells increased in the blood during clinical signs of dysentery (Jonasson *et al.*, 2004). However, this was not observed in the present study, and their role remains unclear.

There was an increase in total $CD8\alpha^+$ cells during the dysentery period. The majority of these $CD8\alpha^+$ cells were $CD4^-$ and also, when comparing the curve characteristics of total $CD8\alpha^+$ cells with those of $CD8\alpha^+ CD8\beta^+$ T cells, they

appeared to be $CD8\beta^-$. The increase of $CD8\alpha^+$ cell populations during the clinical signs of disease appears to be of importance in swine dysentery, and the involvement of these populations is in accordance with findings by Waters *et al.* (2000) and Jonasson *et al.* (2004). In the present study, the proliferation of $CD8\alpha^+$ cells appears to consist of $CD8\alpha^+$ cells that are not cytotoxic/suppressor T cells ($CD8\beta^+$) or double-positive T cells ($CD4^+ CD8\alpha^+$). In a previous study we found that experimentally inoculated pigs with low levels of $CD8\alpha^+$ cells were more susceptible to swine dysentery than pigs with high levels of $CD8\alpha^+$ cells (Jonasson *et al.*, 2004). It was not possible to verify this in the present study, since only two animals remained healthy after inoculation. *B. hyodysenteriae* whole-cell sonicate has been shown to induce an *in vitro* proliferation response of porcine $CD8^+$ T cells, $CD4^+ CD8^+$ T cells and $\gamma\delta$ T cells (Waters *et al.*, 1999a). Thus, it appears that $CD8^+$ cell populations are an

Fig. 2. CD8⁺ lymphocyte subpopulations obtained by flow cytometry: before inoculation, 4 and 14 days after inoculation, and during the swine dysentery and recovery periods. The mean incubation period was 17 days and the mean disease period was 7 days. The boundary of the box indicates the 25th percentile, the median and the 75th percentile. Whiskers indicate the 95th and 5th percentiles. The dotted line behind each box plot shows the average for the two animals that remained healthy. Pre-, 4 days post- and 14 days post-inoculation were the same in all animals, but the remaining sampling points for the healthy animals were from 21, 28 and 35 days post-inoculation. *Significant change from pre-inoculation.

important subset in swine dysentery. In order to understand their role it may be necessary to study their cytokine production. It has been shown that CD8⁺ cells are major producers of the cytokine IFN- γ (Steele *et al.*, 2002), which is important for macrophage activation (Murray, 1990). The intraepithelial region of the intestine contains large numbers of CD8⁺ cells (Fujihashi *et al.*, 1990; Guy-Grand & Vassalli, 1993). These have been shown to secrete IFN- γ locally (Taguchi *et al.*, 1990), and there is *in vitro* production of IFN- γ by peripheral blood mononuclear cells in response to

B. hyodysenteriae bacterin (Waters *et al.*, 1999a). Therefore, the increase of CD8⁺ cells in swine dysentery could influence the pathology and should be further investigated.

No increase in CD4⁺ CD8 α^+ T cells or CD8 α^+ CD8 β^+ T cells was seen during dysentery or recovery. The numbers of $\gamma\delta$ T cells increased throughout the recovery period (Fig. 3). These cells showed a similar pattern of increase in the animals that remained healthy (Fig. 3). This increase of $\gamma\delta$ T cells has also been seen in immunization studies with

Fig. 3. Lymphocyte subpopulations obtained by flow cytometry (mean $\pm$ SEM): before inoculation, 4 and 14 days after inoculation, and during the swine dysentery and recovery periods. The mean incubation period was 17 days and the mean disease period was 7 days. The boundary of the box indicates the 25th percentile, the median and the 75th percentile. Whiskers indicate the 95th and 5th percentiles. The dotted line behind each box plot shows the average for the two animals that remained healthy. Pre-, 4 days post- and 14 days post-inoculation were the same in all animals, but the remaining sampling points for the healthy animals were from 21, 28 and 35 days post-inoculation. *Significant change from pre-inoculation.

B. hyodysenteriae in pigs (Waters *et al.*, 1999a, 2000; Bassaganya-Riera *et al.*, 2001). Since the percentage of $\gamma\delta$ T cells in pigs generally decreases with age, the observed change in the present study is unlikely to be age related (Yang & Parkhouse, 1996). The $\gamma\delta$ T-cell receptor has a broader specificity than the $\alpha\beta$ T-cell receptor (Constant *et al.*, 1994; Schoel *et al.*, 1994; Tanaka *et al.*, 1995), and the $\gamma\delta$ T-cell-antigen recognition and subsequent activation is not dependent upon class I or class II antigen processing followed by major histocompatibility complex (MHC) presentation (Schild *et al.*, 1994) or recirculatory migration through colonic lymph nodes (Hayday, 2000). Porcine $\gamma\delta$ T cells have also been shown to exhibit non-specific *in vitro* cytotoxicity (de Bruin *et al.*, 1997, 2000). Thus, $\gamma\delta$ T cells could be an important part of the early defence against *B.*

hyodysenteriae. Interestingly, in a previous study, pigs with high circulating levels of $\gamma\delta$ T cells before inoculation were more susceptible to the development of swine dysentery (Jonasson *et al.*, 2004). The absence of $\gamma\delta$ T cells in $\gamma\delta$ T-cell-receptor deficient mice is associated with exaggerated intestinal damage and decreased epithelial proliferation (Komano *et al.*, 1995). In another study, Hontecillas *et al.* (2005) have reported that swine dysentery induces a loss of intraepithelial colonic $\gamma\delta$ T cells, and that this is probably caused by the epithelial erosions that occur during the disease (Hutto & Wannemuehler, 1999; Hontecillas *et al.*, 2005). It is possible that this epithelial erosion and the subsequent loss of $\gamma\delta$ T cells may cause some of the immune responses observed during swine dysentery, through exposure to luminal antigens and bacteria other than *B.*

hyodysenteriae. Whether this loss of local $\gamma\delta$ T cells influences the levels of peripheral $\gamma\delta$ T cells is unknown. In the present study, there was no general decrease of peripheral $\gamma\delta$ T cells during clinical signs, but the two pigs with the longest episode of clinical signs (17 and 12 days versus the mean of 5 ± 1 days for the other animals) had the lowest levels of $\gamma\delta$ T cells during disease and the recovery period.

The CD45RA⁺ cells decreased during dysentery (data not shown) and correspondingly CD45RA⁺ cells increased during clinical signs of swine dysentery and then returned to pre-inoculation levels during recovery (Fig. 3). These alterations were not seen in the two animals that remained healthy. CD45 gene expression is regulated so that naïve T cells are predominantly CD45RA⁺, and then their expression switches to CD45RO⁺ through alternative splicing when activated (Janeway, 1992). The increase of CD45RA⁺ cells, i.e. decrease of CD45RA⁺ cells, could be interpreted as a decrease in naïve lymphocytes and thereby an increase in the levels of activated cells during clinical signs of dysentery.

The CD21⁺ cells decreased 14 days after inoculation and then remained at a lower level during the dysentery and recovery periods. There was no evidence of a B cell proliferation/increase in the peripheral blood. This does not necessarily mean that there was no activation of B cells, since local proliferation could occur that was not evident in the circulation. However, in the present study there was no decrease in CD45RA⁺ B cells (data not shown), and in a study performed with biopsy specimens from the colon of swine-dysentery-affected pigs, the numbers of colonic B cells showed no tendency to increase during the disease (M. Jacobson, personal communication). Pigs vaccinated with *B. hyodysenteriae* antigens show proliferation of T cells, but

not B cells, in response to *in vitro* stimulation with *B. hyodysenteriae* whole-cell sonicate antigens (Waters *et al.*, 1999a). The decrease seen in the present study could be related to the decrease of CD21⁺ cells that has been found to occur in pigs post-weaning (Solano-Aguilar *et al.*, 2001).

Other subpopulations that were stained for, such as CD4⁺ CD8 α ⁺ T cells (data not shown), did not change during the experimental period.

Antibody response

As shown in Fig. 4, antibodies to *B. hyodysenteriae* were only present in sera collected during the recovery period. As is shown with the absorbed sera in Fig. 4(b), some antibodies were specific for *B. hyodysenteriae* antigens. The bands at ~ 16 kDa are in agreement with the membrane-associated lipoprotein SmpA of *B. hyodysenteriae* (Thomas *et al.*, 1992; Thomas & Sellwood, 1993), and the weaker bands at ~ 30 kDa are in agreement with the membrane lipoprotein BmpB (Lee *et al.*, 2000). All except one of the swine-dysentery-affected pigs developed these specific serum antibodies (data not shown). The animal without *B. hyodysenteriae*-specific antibody production did not show any differences in pathology or recovery that could be correlated with the lack of specific antibodies, but this animal showed the highest levels of cytotoxic (CD8 β ⁺) T cells and double-positive (CD4⁺ CD8 α ⁺) T cells during incubation, and the highest level of CD8 α ⁺ CD4⁺ cells during clinical signs of dysentery. The production of *B. hyodysenteriae*-specific antibodies has been detected in sera during the acute phase of swine dysentery (Joens *et al.*, 1985), and in colonic washings and sera 4–7 days after onset of clinical signs (Joens *et al.*, 1984). However, their presence has not been related to recovery, but rather these antibodies

Fig. 4. Immunoblot analysis of whole-cell proteins separated by SDS-PAGE and incubated with serum from pigs before and after inoculation with *B. hyodysenteriae*. (a) Serum antibodies bound to whole-cell proteins from *B. hyodysenteriae* (B78^T). (b) *B. hyodysenteriae* specific antigens in sera after absorption with *B. intermedia*, *B. innocens*, *B. murdochii*, *B. pilosicoli* and *B. aalborgi*. Molecular size (kDa) markers are shown to the left of each panel. SD, swine dysentery; inoc., inoculation.

have mainly been regarded as an indication of a prolonged or recent exposure to *B. hyodysenteriae* (Rees *et al.*, 1989). In the present study, the recovery period coincided with the appearance of *B. hyodysenteriae*-specific serum antibodies. The presence of *B. hyodysenteriae*-specific serum antibodies in the swine-dysentery-affected pigs indicated some activation of B cells during recovery. Sera collected from the two inoculated but clinically healthy animals remained negative for antibodies to *B. hyodysenteriae* throughout the experimental period.

In conclusion, increased levels of circulating monocytes, neutrophils and CD8 α ⁺ cells correlate with the clinical signs of swine dysentery. These CD8 α ⁺ cells were not $\gamma\delta$ T cells, CD8 α ⁺ CD8 β ⁺ T cells or CD4⁺ CD8 α ⁺ T cells. During the recovery period, there were high levels of circulating neutrophils. Additionally, $\gamma\delta$ T cells were noted to increase during the recovery period. The dysentery-affected pigs showed decreased levels of CD21⁺ cells in blood during disease and recovery, but developed *B. hyodysenteriae*-specific antibodies from the first day of recovery. In order to understand this disease it is of importance to clarify the roles that cells play during clinical signs of swine dysentery, and the roles of cells and specific antibodies during recovery.

ACKNOWLEDGEMENTS

This study was financially supported by The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, and the Swedish Farmers Foundation for Agricultural Research (SLF). The Swedish Research Council financed the BDLSR flow cytometer. Thanks are due to Professor Caroline Fossum, Associate Professor Birgitta Essén-Gustavsson and Dr Magdalena Jacobson for critical reading of the manuscript.

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