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

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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 slgA, 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, b), 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− T 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
numbers of circulating CD4⁺ CD8⁺ and γδ 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 & Hausmann, 1996). The levels of γδ 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 ad libitum growth promoters (Singel Veg SPK; Fori HB, Lidköping, Sweden) and twice daily with the same batch of a commercial finisher diet without known to be free from swine dysentery. All pigs were orally inoculated for three consecutive days with 30 ml brain heart infusion broth containing approximately 10⁷–10⁹ B. hyodysenteriae strain B204 ml⁻¹. The bacteria were propagated as described previously by Fellstrom & 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 Brachy 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 NH₄Cl 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

<table>
<thead>
<tr>
<th>Double staining no.</th>
<th>Anti-IgG2a PE conjugate</th>
<th>Anti-IgG1 FITC conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG2a isotype</td>
<td>Clone no.</td>
</tr>
<tr>
<td>1*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Isotype control</td>
<td>X943</td>
</tr>
<tr>
<td>3</td>
<td>CD4</td>
<td>PT90A</td>
</tr>
<tr>
<td>4</td>
<td>CD4</td>
<td>PT90A</td>
</tr>
<tr>
<td>5</td>
<td>CD8γδ</td>
<td>PG164A</td>
</tr>
<tr>
<td>6</td>
<td>CD4</td>
<td>PT90A</td>
</tr>
<tr>
<td>7</td>
<td>CD4</td>
<td>PT90A</td>
</tr>
<tr>
<td>8</td>
<td>CD8γδ</td>
<td>PG164A</td>
</tr>
</tbody>
</table>

*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 $P < 0.05$. 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 γδ T cells and lower percentages of CD8$^+$, CD8$^+$CD8β$^+$ and CD4$^+$ CD8$^-$ T cells (30.7 ± 3.5, 24.6 ± 1.5, 10.9 ± 1.3 and 8.1 ± 1.0 %, respectively) before inoculation than the pigs that remained healthy (14.9 ± 1.4, 34.9 ± 3.1, 17.6 ± 2.0 and 13.6 ± 2.3 %, respectively) (Fig. 1). The higher proportion of CD8$^+$ cells present in the healthy group was mainly accounted for by γδ 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). *, $P < 0.05$; **, $P < 0.001$.

![Fig. 1. Percentages of γδ T cells, CD8$^+$ cells, CD8$^+$ CD8β$^+$ 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). *, $P < 0.05$; **, $P < 0.001$.](image)

**swine dysentery group and to γδ T cells in the healthy group.** Although the level of γδ 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; 1.5 ± 0.2 × 10$^9$ to 4.7 ± 0.6 × 10$^9$ l$^{-1}$) than in pigs with milder non-haemorrhagic diarrhoea (n = 5; 1.5 ± 0.4 × 10$^9$ to 2.5 ± 0.7 × 10$^9$ l$^{-1}$) ($P < 0.001$). There were no changes in CD21$^+$ cells and no detectable levels of CD25$^+$ cells in either group.

### 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 γδ T cells and low levels of CD8$^+$ cells (mainly CD8$^+$ CD8β$^+$ 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 γδ T cells were particularly responsive. The γδ T cells have been suggested as especially important for young pigs, and the percentage generally decreases with age as αβ T cells become
more numerous and more important for the immune system (Yang & Parkhouse, 1996). In mice, intestinal intra-epithelial γδ T cells stimulate both in vitro proliferation and differentiation of epithelial cells (Boismenu & Havran, 1994). The absence of γδ T cells in γδ 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 γδ T cells can modulate the proliferative response of antigen-stimulated bovine CD4+ T cells (Chiodini & Davis, 1992), suggesting a role for γδ T cells as immunoregulatory cells. Taken together, these findings suggest that pigs with high levels of γδ T cells may be less susceptible to infections. However, in the present study, pigs with large numbers of γδ 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 γδ T-cell-deficient mice (Emoto et al. 1995). Therefore, for protection against some infections, the presence of γδ T cells may not be advantageous for the animal. Furthermore, in studies with other microbes, γδ 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 γδ 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., 1995; Guy-Grand & Vassalli, 1993) and these have been shown to secrete IFN-γ locally (Taguchi et al., 1990). Even though γδ T cells are efficient producers of IFN-γ (Tsukaguchi et al., 1995), γδ T-cell-deficient mice produce larger quantities of IFN-γ than normal (γδ+ ) mice (Williams et al., 1996; Santos Lima & Minoprio, 1996; Steele et al., 2002). Thus, it could be speculated that animals with high levels of γδ 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 γδ 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

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

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Swine dysentery group (n = 12)</th>
<th>Healthy group (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before inoculation</td>
<td>Euthanasia</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>18±5 ± 2.1</td>
<td>23±9 ± 1.7</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7±3 ± 1.2</td>
<td>11±6 ± 1.2*</td>
</tr>
<tr>
<td>Monocytes</td>
<td>1±5 ± 0.2</td>
<td>3±8 ± 0.5*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>9±1 ± 1.3</td>
<td>8±3 ± 1.0</td>
</tr>
<tr>
<td><strong>Lymphocyte subpopulation (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T cells (CD3+)</td>
<td>52±7 ± 3.7</td>
<td>61±7 ± 3.9b</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>30±7 ± 3.5b†</td>
<td>29±0 ± 3.6</td>
</tr>
<tr>
<td>CD3+CD4+ T cells</td>
<td>14±4 ± 1.5</td>
<td>17±4 ± 1.4b</td>
</tr>
<tr>
<td>CD8+ cells</td>
<td>24±6 ± 1.5A</td>
<td>29±9 ± 2.7</td>
</tr>
<tr>
<td>CD8+ CD8β+ T cells</td>
<td>10±9 ± 1.3b†</td>
<td>11±6 ± 0.7</td>
</tr>
<tr>
<td>CD4+ CD8+ T cells</td>
<td>8±1 ± 1.0A</td>
<td>8±8 ± 0.8</td>
</tr>
<tr>
<td>CD4+ CD8− T cells</td>
<td>5±8 ± 0.9</td>
<td>8±9 ± 0.7c</td>
</tr>
<tr>
<td>B cells (CD21+)</td>
<td>20±4 ± 1.9</td>
<td>20±9 ± 3.0</td>
</tr>
</tbody>
</table>

*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).
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 CD8+$^+$ 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 γδ T cells, which also express the αβ-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 would 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 γδ 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

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