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Lymphocyte subsets in jejunal and ileal Peyer's patches of normal and gnotobiotic minipigs

H. J. ROTHKÖTTER & R. PABST *Centre of Anatomy, Medical School of Hannover, Hannover, FRG*

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SUMMARY

The size and location of Peyer's patches (PP) in the jejunum and ileum and their composition of lymphocyte subsets (B, $CD2^+$, $CD4^+$, $CD8^+$) have been studied in conventional and gnotobiotic Göttingen minipigs. Each PP in the small intestine remained at the same site and was of comparable length between 2 and 12 months of age. In 1·5-month-old conventional minipigs the histology of the compartments differed between the continuous PP in the terminal ileum (ilPP) and the discrete PP in the jejunum (jejPP). No such difference was seen in gnotobiotic or in 12-month-old animals. The composition of lymphocyte subsets showed striking differences with significantly more B and less T, $CD4^+$ and $CD8^+$ cells in ilPP in 1·5-month-old minipigs in comparison with 12-month olds. Mesenteric lymph nodes and jejPP displayed a typical pattern of lymphocyte subsets. The size of the lymphocyte compartments in PP and their cellular composition depends largely on age and microbial influences from the gut lumen, which might be of major importance for studies on the function of the gut-associated immune system in the pig.

INTRODUCTION

The intestinal immune system is not only important in initiating tolerance and playing a role in the barrier function of the gut, but is of great clinical relevance, e.g. in the fields of food allergy and inflammatory diseases of the intestine (Newby & Stokes, 1984; Bienenstock & Befus, 1985; Brandtzaeg, 1985). The intra-epithelial lymphoid cells, those in the lamina propria and the Peyer's patches (PP), together form one of the largest accumulations of lymphocytes in the mammalian body.

In pigs, the development and distribution of Ig-bearing lymphocytes in the lamina propria (Brown & Bourne, 1976; Allen & Porter, 1977; Butler, Klobasa & Werhahn, 1981) and the functional characteristics of intra-epithelial lymphocytes (Wilson, Stokes & Bourne, 1986) have been described. Lymphocyte subsets in PP have not yet been characterized, although a panel of monoclonal antibodies against pig lymphocyte subsets now exists (Lunney & Pescovitz, 1988). In several species, such as sheep, pig and dog, there are two types of PP defined by their different localization, structure and function: several discrete patches in the jejunum and upper ileum (jejPP) and a long continuous patch in the terminal ileum (ilPP) (Binns & Licence, 1985; Reynolds, Pabst & Bordmann, 1985; Reynolds, 1987; Binns & Pabst, 1988; Pabst *et al.*, 1988). Peyer's patches play an essential role in antigen uptake through the specialized epithelial M cells, in initiating immune responses, in B-lymphocyte proliferation and in the production of IgA precursors (reviewed by Pabst, 1987).

In a recent study we described the post-natal development of jejPP and ilPP in pigs with respect to number, length and lymphocytopoiesis (Pabst *et al.*, 1988). One aspect of the present experiments was to examine whether PP are localized at the same place throughout life or whether some PP regress while others are newly formed. Therefore, the size and location of all PP were determined in 2-month-old minipigs and this was repeated when the animals were 10 months older. The other main aims of the present study were to look for differences in the lymphocyte subsets in the two types of PP, to find out whether there are differences between young and adult pigs and to compare the lymphocyte composition in conventionally reared pigs with that in gnotobiotic pigs.

MATERIALS AND METHODS

Twenty-six miniature pigs of the Göttingen breed were used for this study. There were four experimental groups. (A) Six minipigs were laparotomized at an age of 2 months under methitural anaesthesia (Thiogenal, Merck, Darmstadt). The number and length of all PP in the small intestine and the distance between individual PP were measured. At 12 months of age the same measurements were repeated immediately after killing the pigs by an overdose of methitural. (B) Five conventionally reared female minipigs (mean age 1·5 months). (C) Five conventionally reared female minipigs (mean age 12 months). (D) Ten germ-free pigs (~1·5 months). These piglets were born by Caesarean section and kept under sterile conditions (Dziaba,

Correspondence: Dr H. J. Rothkötter, Zentrum Anatomie 4150, Medizinische Hochschule Hannover, Postfach 610180, D-3000 Hannover 61, FRG.

Lambrecht & Petzoldt, 1985) to be used in a different study devised by Dr K. Petzoldt, Veterinary School of Hannover. These animals were fed throughout the whole period with a diet containing milk, vitamins and necessary minerals, which was sterilized by cobalt irradiation. In Groups B, C and D the length of the gut and PP was measured and lymphocyte subsets determined in cell suspensions.

Cell separation and surface staining

The jejPP and ilPP and mesenteric lymph nodes were excised, gently minced, and then washed with RPMI-1640 medium. The cells were centrifuged (400 *g*, 10 min) through 2 ml fetal calf serum (FCS) and resuspended in RPMI−1640 with 0·5% bovine serum albumin (BSA). The lymphocyte cell suspensions were pipetted into microtitre plates ($1{\cdot}5 \times 10^6$ per well) and after two washing procedures (phosphate-buffered saline (PBS), 0·5% BSA, 0·1% NaN_3, at 200 *g* for 1 min) 20 μl of monoclonal antibodies (mAb) (1:50) were added for 30 min at 4°. The following mAb were used: anti-CD2 (Mac 80, a rat anti-pig CD2, personal gift of Dr R. M. Binns, AFRC Cambridge, U.K.; anti-CD4 (Pescovitz, Lunney & Sachs, 1984, 1985); anti-CD8 (mAb 295/33, Jonjić & Koszinowski, 1984, and mAb 76-2-11, Pescovitz *et al.*, 1984, 1985); and 8/1, which detects an antigen on resting T cells, monocytes and cells of the myeloid lineage (Saalmüller *et al.*, 1987). Ascites containing the mAb anti-CD4, anti-CD8 and 8/1 were kindly donated by Drs Koszinowski, Reddehase and Saalmüller (FRC for Virus Diseases of Animals, Tübingen, FRG). The suspensions were washed twice and the second antibody added for 30 min at 4° [RITC-coupled $F(ab')_2$ goat anti-mouse; Tago, Nordwald, Hamburg; or RITC-coupled $F(ab')_2$ goat anti-rat; Dianova, Hamburg]. B lymphocytes were determined on cell suspensions incubated with a TRITC-labelled rabbit anti-pig Ig polyclonal antiserum (Sigma, Munich). The washed lymphocyte cell suspensions were resuspended in 200 μl PBS containing 0·5% BSA and 0·1% NaN_3. To prevent clumping the suspensions were pipetted into plastic tubes containing 300 μl of a special medium (0·9% NaCl, 5% BSA, 2·7 mM NaEDTA, pH 7·2).

Cytocentrifuge preparations (200 *g*, 5 min) of these labelled cell suspensions on glass slides were dried (1 hr) and fixed (12 min) in ice-cold absolute ethanol with 5% acetic acid. The slides were flushed three times with PBS and the spots covered with a drop of a solution containing 9 ml 87% glycerol, 1 ml PBS and 250 mg DABCO (1.4 diazobicyclo-2.2.2.-octane; Sigma). This medium kept the cells moist and increased the fluorescence (Opstelten *et al.*, 1986). The coverslips were sealed with nail varnish. These slides could be used for evaluation for at least 3 months when kept at 4°. A Zeiss fluorescence microscope was used and at least 500 cells were counted to determine the labelling index. The mean and standard error was calculated and differences were taken as significant when they reached $P < 0{\cdot}05$ in Student's *t*-test.

RESULTS

Number, size and position of PP

The total length of the jejunum and ileum did not differ significantly between 2 months (911 ± 41·6 cm) and 12 months (880 ± 42 cm). The relative part of the gut containing PP was also comparable: 18·4 ± 0·7% and 18·7 ± 0·7%, respectively. The location of each individual PP was surprisingly constant. By comparing the distance between two PP and their size nearly all PP at 2 months of age could be re-identified at 12 months of age. The number of PP was not completely identical because a few small ones had been overlooked during operation or at autopsy, but the overall pattern was comparable (Fig. 1).

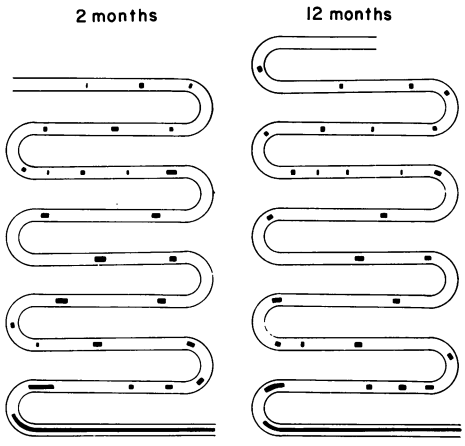

Figure 1. Schematic drawing of the position of the PP in the gut in a representative animal at 2 and 12 months of age. The length of the jejPP and the distances between them are shown on the same scale.

The comparison of age-matched conventional and gnotobiotic pigs revealed significantly different lengths in the small intestine (conventional 594 ± 45 cm; gnotobiotic 408 ± 10 cm) and in the total length of PP (conventional 125·7 ± 18·4 cm; gnotobiotic 94·7 ± 5·3 cm). However, relative length covered by PP was comparable in conventional (21·3 ± 0·4%) and gnotobiotic pigs (23·3 ± 0·7%). Thus, the size of the gut and PP seem to be influenced by microbial contents to the same extent.

Histology of jejPP and ilPP

Jejunal PP showed the typical compartmentalization of PP with round follicles, interfollicular areas and dome. In older minipigs ilPP had a comparable structure to jejPP. In the 1·5-month-old animals, however, the follicles were oval-shaped and the interfollicular area was much smaller than in older animals or jejPP (Fig. 2.) In gnotobiotic pigs the compartments of the PP were less well developed. The ilPP showed only small developing follicles, but in jejPP the different compartments could be differentiated.

Lymphocyte subsets in PP and mesenteric lymph nodes

The two anti-CD8 monoclonal antibodies produced similar results with a tendency for a small percentage more cells to be stained with the mAb 295/33. Only the CD8 values obtained by staining with this antibody are shown in the figures.

The continuous ilPP in the terminal ileum of pigs showed differences in lymphocyte migration, with a low entry in most parts but an increasing entry in the very last 10–15 cm before the ileocaecal junction, reaching the value of the jejPP (Binns & Licence, 1985). Lymphocyte cell suspensions prepared from the mid- and end portion of ilPP (the last 10 cm) were classified by monoclonal antibodies. As in all groups studied, no differences were found in the proportion of B cells, T cells or T-cell subsets at these two sites (Fig. 3); in the following only the data from the

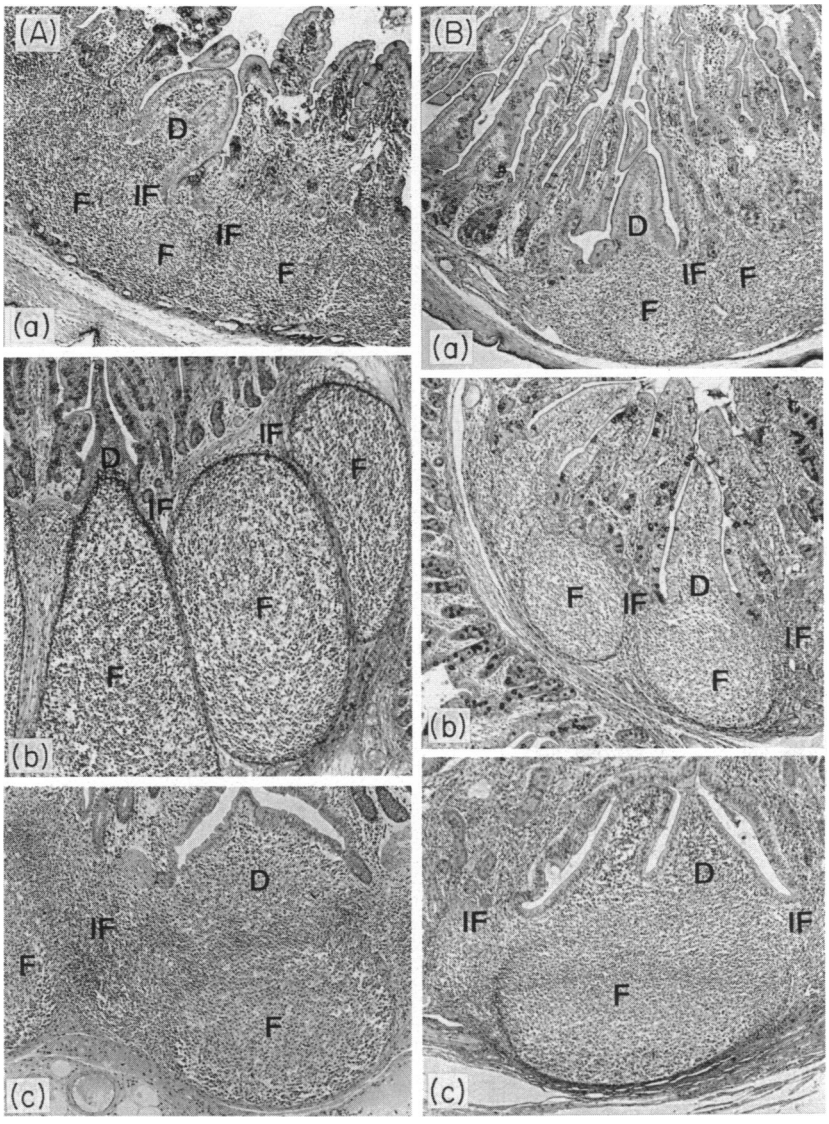

Figure 2. Histological picture of the ilPP (A) and jejPP (B) of gnotobiotic (a), 1·5-month-old (b) and 12-month-old (c) minipigs (4 μm, glycol methacrylate, H & E). The follicle (F), interfollicular area (IF) and the dome area (D) are indicated. All figures are at the same magnification ($\times$ 42).

mid-portion of ilPP were plotted for comparison with jejPP and mesenteric lymph nodes. Comparison of the lymphocyte subsets of different age groups, conventional and gnotobiotic animals showed interesting differences (Fig. 4): in 1·5-month-old minipigs the ilPP showed a strikingly different lymphocyte composition, with very high numbers of B cells and minute numbers of T cells. The differences compared with 12-month-old pigs were highly significant for all subsets. Comparison of the proportion of B and T lymphocytes in gnotobiotic and conventional animals of the same age showed significant differences. In jejPP the proportion of B cells decreased significantly from 1·5 to 12 months of age. In gnotobiotic pigs $CD4^+$ and $CD8^+$ were found significantly less often than in conventional animals of comparable age. In the mesenteric lymph nodes of conventional animals the number of $CD8^+$ cells increased and the B cells decreased significantly with age. In all three organs the proportion of $8/1^+$ lymphocytes was significantly smaller than that of the $CD2^+$ lymphocytes in gnotobiotic and 1·5-month-old minipigs. In older animals this difference reached significance only in the mesenteric lymph nodes.

When the number of lymphoid cells that had neither the CD2 nor B-cell surface marker was calculated, it was obvious that in ilPP about 35% of the cells were Null cells, with no significant differences between the groups of animals, In mesenteric lymph nodes and jejPP the number of Null cells increased significantly with age. In this respect, the gnotobiotic minipigs were comparable to age-matched conventional animals.

More interesting aspects became apparent when $CD4^+$ and $CD8^+$ lymphocytes were added and compared to the number of $CD2^+$ cells. Only in gnotobiotic animals did many $CD2^+$ cells have neither the CD4 nor CD8 marker, e.g. about 20% in ilPP. In conventional 1·5-month-old animals the sum of the T-cell subsets reached the $CD2^+$ values. In the older pigs, however, the

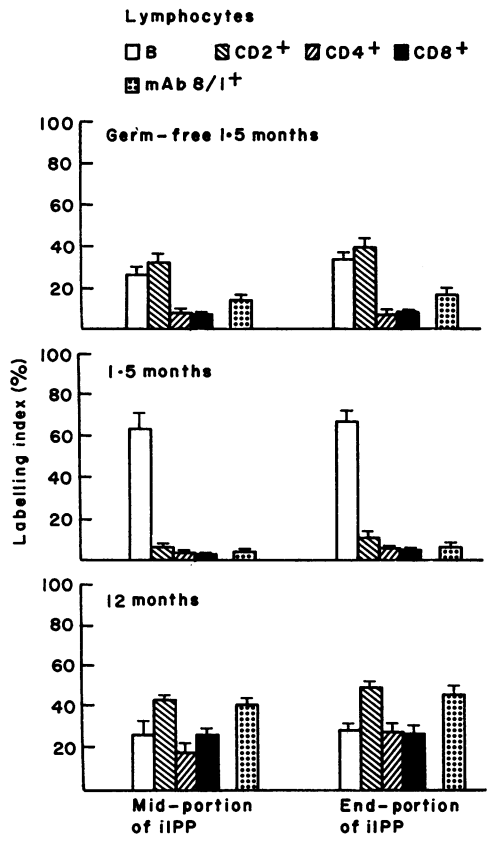

Figure 3. Subsets of pig lymphocytes in the mid-portion and the end portion of the continuous ilPP (mean ± SE).

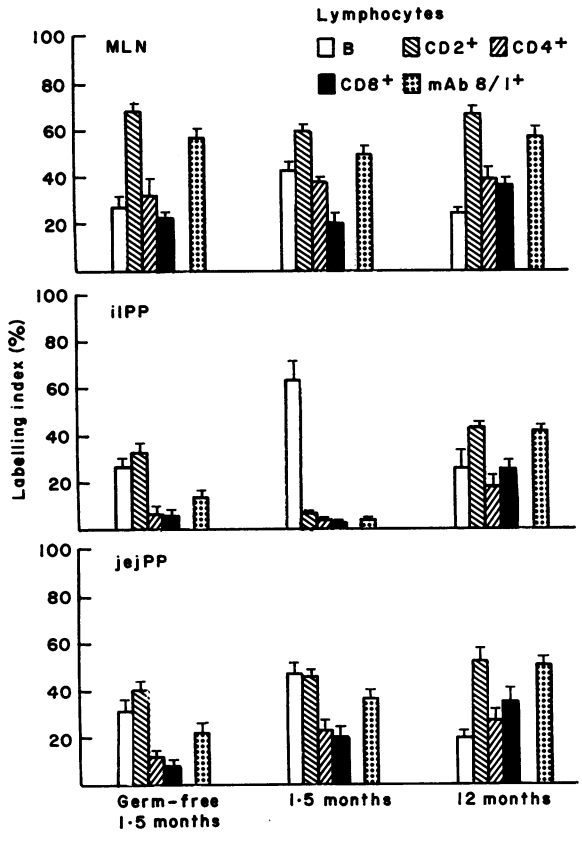

Figure 4. Lymphocyte subsets in the mesenteric lymph nodes (MLN), ilPP and jejPP from gnotobiotic, 1·5-month-old and 12-month-old minipigs.

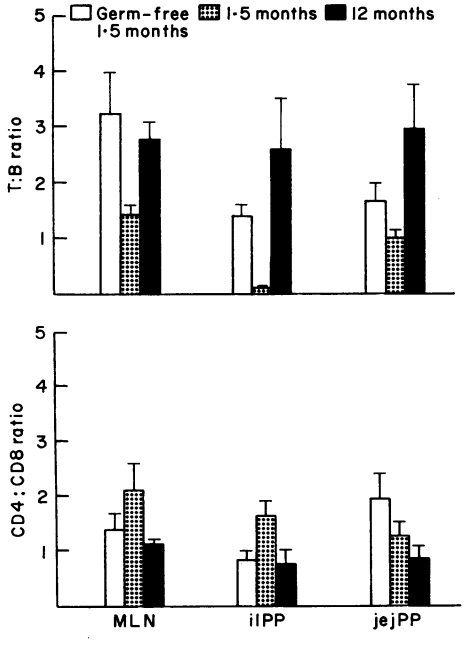

Figure 5. T/B and $CD4^+/CD8^+$ ratios in the mesenteric lymph nodes, ilPP and jejPP in minipigs of different ages and microbial status.

Table 1. Percentages of B cells, T cells ($CD2^+$) and T-cell subsets ($CD4^+$ or $CD8^+$) in PP of different species

	Pigs (1·5 months)		Lambs* (2–3 months)		Rats† (adult)	Rats‡ (adult)
B	ilPP	63·1 ± 7·8	ilPP	77·8 ± 8·6	64·4 ± 1·7	55·0 ± 9·5
	jejPP	46·4 ± 4·8	jejPP	61·7 ± 11·4		
$CD2^+$	ilPP	6·3 ± 1·3	ilPP	3·9 ± 4·4	15·5 ± 1·1	18·2 ± 4·2
	jejPP	45·4 ± 2·9	jejPP	30·3 ± 7·8		
$CD4^+$	ilPP	3·4 ± 0·8		ND	14·3 ± 1·8	10·0 ± 3·2
	jejPP	22·9 ± 4·1				
$CD8^+$	ilPP	2·5 ± 0·8		ND	4·5 ± 0·7	6·4 ± 4·4
	jejPP	20·2 ± 4·0				

* J. D. Reynolds and R. Pabst, unpublished observations.

† J. Westermann, S. Ronneberg and R. Pabst, unpublished observations.

‡ Lyscom & Bruton (1982).

number of $CD4^+$ plus $CD8^+$ lymphocytes outnumbered the $CD2^+$ cells, indicating a considerable number of double-positive lymphocytes. When the ratios of T/B cells were plotted (Fig. 5) the differences were obvious between jejPP and ilPP in young and adult animals and in age-matched conventional pigs compared with gnotobiotic ones. For the $CD4^+/CD8^+$ ratio the differences were far less pronounced (Fig. 5).

DISCUSSION

In a recent study we described the post-natal development of jejPP and ilPP in normal pigs (Pabst *et al.*, 1988). The present data in minipigs support and extend these observations. Age and microbial contents of the gut increase the size of PP but the number and the position of the individual patches remain constant. There is no continuous development of new jejPP or regression of old jejPP in pigs. In Landrace pigs up to an age of 42 days no histological differences were found between jejPP and ilPP (Pabst *et al.*, 1988). The elongated, prominent follicles and small interfollicular areas in young conventional minipigs are comparable to those in lambs (Reynolds *et al.*, 1985), and also in pigs of 1 month of age (Binns & Licence, 1985). In lambs, the ilPP is partly comparable to a primary organ for B lymphocytes (Gerber, Morris & Trevella, 1986; Reynolds, 1987). The ilPP of pigs might play a similar role as indicated by the major differences in lymphocyte subset composition related to age and its regression with age in contrast to the permanent structure of jejPP (Binns & Licence, 1985; Pabst *et al.*, 1988).

Despite the panel of monoclonal antibodies available now for pig lymphocytes (summarized by Lunney & Pescovitz, 1988) no data have been published on the composition of PP in pigs. Jonjić *et al.* (1987) showed that lymphocyte subsets are localized in pig PP as in other species by immunohistology. In a preliminary study using rosetting techniques more T cells were identified in jejPP than in ilPP (Binns & Licence, 1985), which is in agreement with the present data. It remains to be seen what functional role the cells without markers for T or B cells play, these cells being more numerous in gnotobiotic piglets. Comparing the present data with lymphocyte subsets in lambs and rats (Table 1), the young pigs and lambs have a comparable number of T and B lymphocytes in jejPP and ilPP, while rat PP are intermediate between these two PP types. It would be of interest to study whether PP in younger rats consist of more B and less T cells and whether there is perhaps a difference between PP of different localizations.

The antibody 8/1 identifies resting T cells as the antigen is lost after stimulation *in vitro* (Saalmüller *et al.*, 1987). Based on these *in vitro* data one would expect that in gnotobiotic piglets there would be less activated lymphocytes than in conventional pigs. Just the opposite was found, i.e. in the gnotobiotic animals significantly less 8/1-positive cells were identified than by the pan T-cell marker. Further immunohistological studies might help to interpret these data. The double-positive population ($CD4^+$, $CD8^+$), as identified in pigs by the FACS (Saalmüller *et al.*, 1988), was also found in the two types of pig PP showing an increased frequency with age.

Pigs have proven to be excellent animal models for studying the small intestine enzyme development (Shulman, Henning & Nichols, 1988), food sensitivity (Stokes, Miller & Bourne, 1987), enteropathogenic E. coli enteritis (Tzipori *et al.*, 1985) and transmissible gastroenteritis virus (Chu, Glock & Ross, 1982). In future, such studies as well as oral vaccination experiments, should include lymphocyte subsets in the two types of small intestinal PP.

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