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Differences in Development of Lymphocyte Subpopulations from Gut-Associated Lymphatic Tissue (GALT) of Germfree and Conventional Rats: Effect of Aging

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ABSTRACT. The aim of the study was to compare the phenotype of lymphocyte subpopulations of the GALT (gut-associated lymphatic tissue) in germfree (GF) and conventionally (CV) reared rats, *i.e.* to analyze the effect of microbial colonization on the development of intestinal lymphocyte subsets. Surface marker characteristics were studied in cell suspensions isolated from Peyer's patches, mesenteric lymph nodes, spleen and the intraepithelial lymphocyte compartment of 2- and 12-month old inbred AVN rats. The pattern of T lymphocyte phenotypes in Peyer's patches, mesenteric lymph nodes and spleen determined by FACS analysis did not reveal differences between GF and CV rats. In contrast, a 2-month conventionalization of GF rats led to substantial changes in the composition of intestinal intraepithelial lymphocyte subsets (IELs): increase of $CD4^+$, $CD8\alpha^+$, $CD8\beta^+$, TcR α/β^+ bearing lymphocytes was observed after colonization of rats with normal microflora. Surprisingly, the relative numbers of lymphocytes bearing TcR γ/δ^+ did not change during conventionalization. The effect of aging was also studied and differences in IELs composition of aged (GF) and (CV) rats were found to be more pronounced: 6,6 % and 30 % of lymphocytes bearing TcR α/β were present among IELs in two-month old GF and CV rats, respectively. 30 % of IELs in 2-month old GF rats, 80 % of IEL from 12-month old CV rats were found to bear TcR α/β. This finding demonstrates that during conventionalization and aging the TcR α/β bearing population of IELs substantially expands It suggests that mainly this lymphocyte subset responds to microflora stimuli and is probably involved in the protection of the epithelial cell layer of intestinal mucosa.

Gnotobiotic animals, living either in the absence of, or in association with, known viable heterologous agents, represent a suitable model for the microbiologists and immunologists. The immunological capacity of the newborn organism is not fully mature: the insufficiently adapted organism is delivered into an environment full of microorganisms. The lymphatic tissue of the intestine responds to antigenic stimuli by rapid development that is particularly apparent during the first days after birth. In our previous studies we compared the immunological capacities of GF rabbits with CV reared partners and showed that the antigenic stimulation by microflora fundamentally affects not only the development of GALT but also the capacity of the whole immune system (Tlaskalová-Hogenová and Štěpánková 1980; Štěpánková and Kovářů 1978). Nevertheless, in other species, *e.g.*, in mice, the development of the immunological capacity in the GF and CV individuals was reported to be comparable (Bosma *et al.* 1967). The rearing conditions for this two types of animals in the GF isolators were similar, except that GF rabbits were always delivered by Caesarean section, transferred into a GF environment and fed on an artificial diet (*i.e.* reared as a first generation). GF mice and rats were born in the isolator and for the first few weeks were suckled by mother. We have shown that the administration of these artificial diets during the early postnatal period in rats affected the immune status (Tlaskalová-Hogenová *et al.* 1983; Štěpánková *et al.* 1985).

The aim of the present work was to study T lymphocyte subpopulations present in the GALT of the small intestine in GF rats, which were breast-fed and reared in GF conditions for many generations and compared with CV-reared rats.

MATERIALS AND METHODS

Animals. Germfree (GF) inbred rats (AVN, F89, Prague) were reared in plastic isolators for 10 generations under germfree conditions. The conventionalized (CV) group was obtained from GF rats by removing the animals from isolators and conventionalizing them by feces of healthy conventional rats with normal microflora. In our experiments, F1 generation of the CV rats was used. Rats were fed with granulated gluten-free diet, consisting of dried cow milk, soya and corn (Štěpánková 1979, 1997).

Cell isolation. Lymphocytes were isolated from mesenteric lymph nodes, Peyer's patches, spleen and intraepithelial compartment. Lymph nodes, spleen and Peyer's patches were removed, cut with scissors and minced with a forceps in the RPMI 1640 medium (*Gibco Biocult*) supplemented with 10 % of heat-inactivated fetal calf serum (FCS), penicillin, gentamicin and streptomycin. Cell suspensions were filtered through gauze and nylon mesh (Nybolt 40, *Nybolt*, Switzerland). IELs were isolated according to the method

of Tagliabue *et al.* (1981) with modifications (Štěpánková *et al.* 1996). The lumen of the small intestine was washed with saline solution until free of detritus. Segments of the small intestine were clamped at both ends and rubbed gently by hand with RPMI medium (pH 6.05) and 1 mmol/L DTT (1,4-dithiothreitol; *Sigma*). The cell suspensions were passed through a nylon wool column: nylon wool was packed in a plastic syringe and incubated for 15 min at 37 °C in the RPMI medium with 10 % FCS prior to use. Cells were incubated in the column at 37 °C for 15 min, then flushed out with the warm RPMI medium. The cell suspension was then layered onto the Ficoll-Hypaque gradient, centrifuged (600 ***g***, 20 min), washed 3-times and the cell viability was determined (always higher than 90 %).

Flow cytometry. Cells were suspended in phosphate buffered saline containing 2.5 % heat inactivated normal pig serum, 0.1 % sodium azide (PBS-NPS) and single stained for flow cytometry. W3/25 (anti rat CD4) and OX8 (anti rat CD8α) moAbs (*Serotec*) labeled with FITC in our laboratory were used for determining T cells subsets. FITC-labeled swine anti-rat Ig polyclonal antibodies (SwARa-FITC, *ÚSOL*, Prague) were used for B cell detection. Neat supernatants of V65 (anti rat TcR γ/δ) – a gift from Dr. Hünig, Würzburg), R 73 (anti rat TcR α/β), 341 (anti rat CD 8β) (*Serotec*) and RGL-1 (CD103, gift from Dr. Cerf-Bensussan, Paris) were used for indirect staining. The RGL-1 mAb target is related to the homing of lymphocytes into the gut mucosa (Cerf-Bensussan *et al.* 1986). FITC labeled, rat splenocyte adsorbed swine anti-mouse Ig polyclonal antibodies (SwAM-FITC, *Sevac*, Prague) were used as the secondary reagent. No reactivity of the secondary reagent with rat cells was observed without the presence of primary antibodies. Flow cytometry was performed on the FACSort (*Becton-Dickinson*) cytometer. Dead cells were excluded from analysis by propidium iodide staining. The data in the list mode were analyzed using the PC LYSYS II software (*Becton-Dickinson*).

RESULTS AND DISCUSSION

Microbial colonization of GF rats with specific pathogen-free (SPF) microflora significantly increases the amount of IELs with the T phenotype in the small intestine in comparison with GF rats (Table I). These results confirm the speculation that the introduction of microbial antigens into a GF organism first stimulates homing of IELs into the small intestine.

Table I. Phenotype of lymphocytes from mesenteric lymph node, Peyer's patches and spleen of 2-month-old GF and CV rats (% of cells)[a]

Lymphocyte marker	MLNC		Peyer's patches		Spleen	
	GF	CV	GF	CV	GF	CV
$CD4^+$	42 (36–46)	46 (46–47)	20 (12.7–25)	22 (20–24)	37 (30–43)	34 (34–35)
$CD8\alpha^+$	21 (16–25)	19 (18–19.4)	8 (5–10)	5 (4–6)	23 (22–24)	24 22–26)
$CD8\beta^+$	17 (16–22)	19 (16–19)	6 (5–7)	5 (3.6–6.6)	18 (15–21)	18 (18–19)
TcR α/β^+	56 (50–60)	60 (59–61)	22 (19–24)	23 (22–24)	43 (42–43)	45 (44–46)
TcR γ/δ^+	1.2 (1.0–1.5)	1 6 (1.2–1.8)	0.8 (0.5–0.9)	0.6 (0.4–0.8)	3.1 (3.0–3.7)	2.8 (2.6–3.0)
sIg^+	40 (37–42)	31 (30–31)	77 (73–80)	72 (72–73)	51 (50–54)	49 (48–50)

[a] $n = 4–5$.

In order to characterize subpopulations of T lymphocytes in our cell preparations the expression of the following surface markers was determined: CD4, CD8α, CD8β, TcR α/β and TcR γ/δ. The results are summarized in Table II. The pattern of T-lymphocyte phenotypes in mesenteric lymph nodes, spleen and Peyer's patches was not different in CV and GF animals. It suggests that microbial antigens do not cause phenotypic alterations of T-lymphocytes in these organs. In mice, Umesaki *et al.* (1993) showed that there is

Table II. Phenotype of IEL from small intestine of rats[a]

Type of cell subsets		Positive cells[b], %
CD4	GF	1.2 ± 0.3
	CV	8.7 ± 1.2
CD8α	GF	12 7 ± 2 3
	CV	29 9 ± 2.0
TcR α/β	GF	6.6 ± 1 5
	CV	30.0 ± 5.0
TcR γ/δ	GF	6 1 ± 1.1
	CV	10.6 ± 6.1
CD8β	GF	6.3 ± 0.2
	CV	19.4 ± 1.2
CD8 α/α	GF	5.0 ± 2.8
	CV	6.2 ± 3.8

[a]2-month old germ-free (GF) and conventional (CV) animals.

[b]Error ranges indicate the minimum and maximum value in the group; CD103 (RGL) is 81 % in GF and 79 % in CV; CD8 α/α was calculated as CD8α – CD8β; $n = 4$.

a great difference in the origin and mechanism of the response to microbial association between IELs and T cells in GALT. Recently, Helgeland *et al.* (1996) have published an interesting paper about the relative proportions of T-lymphocytes ($CD8\alpha^+$, $CD8\beta^+$ and TcR α/β^+ subsets) in mesenteric lymph nodes and IELs in 10-week old GF and conventionalized rats. Their data principally correspond with our results. In mesenteric lymph nodes, however, we found slightly higher levels of T cells. We speculate that this small discrepancy can be caused by a difference in the genetic background of animals similarly as described by Takimoto *et al.* (1992) in the mouse model.

The major function of IELs is probably the destruction of altered or impaired enterocytes. IELs, independently of their surface phenotypes, contain intracytoplasmic granules identical to those observed in clones of cytotoxic T cells or in NK cells (Guy-Grand *et al.* 1991). These granules contain serine esterases of the granzyme family and perforin. The subpopulations of T-lymphocytes in the small intestine were markedly changed after microbial colonization. Conventionalization of GF rats significantly increased the proportion of IELs $CD4^+$, $CD8\alpha^+$, $CD8\beta^+$, and TcR α/β^+ phenotype (Table II). Moreover, long-lasting conventionalization of rats caused an increase in the percentage of TcR positive IELs, especially TcR α/β^+ cells. Whereas in 2-month old CV rats TcR α/β^+ subpopulation represented about 30 % of T lymphocytes, in 12-month old CV rats it was almost 80 % (Table III). These data correspond with the results previously published by Takimoto *et al.* (1992) in mice and further support the hypothesis that with aging a unique subpopulation of TcR α/β IELs expands to the small intestine and significantly contributes to the first line of defense in the intestinal epithelium against potential microbial invasion.

Table III. Comparison of IELs from 2- and 12-month old rats[a]

Type of cell subsets		Age, months	Positive cells[b], %
CD4	GF	2	1.1 ± 0.1
		12	11.9 ± 3.9
	CV	2	8.7 ±1.1
		12	39.6 ± 4.3
CD8	GF	2	10.9 ± 3.0
		12	28.2 ± 4.5
	CV	2	29.9 ± 1.1
		12	51.0 ± 7.7
TcR α/β	GF	2	6.5 ± 1.6
		12	28.2 ± 3.7
	CV	2	29.9 ± 5.0
		12	79.8 ± 5.0

[a]Germ-free (GF) and conventional (CV) animals.

[b]Error ranges indicate the minimum and maximum value in the group; $n = 3$–4.

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