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Characterization of the diffuse mucosal associated lymphoid tissue of feline small intestine

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Abstract

Characterization of the feline intestinal mucosal associated lymphoid tissue (MALT) will facilitate investigation of intestinal disease in the cat and promote the cat as an animal model for a range of human diseases which involve the intestinal lymphoid tissue. This includes inflammatory bowel disease, viral and non-viral associated intestinal lymphomas and immunodeficiency associated syndromes. Morphologic and phenotypic characterization of the normal small intestinal diffuse MALT in 22 SPF cats was performed using flow cytometry and cytology on isolated intestinal leukocytes from the intra-epithelial and lamina proprial compartments, as well as immunohistology on tissues from the feline duodenum, jejunum and ileum. The intra-epithelial compartment (IEC) was dominated by lymphocytes (>85%) which frequently contained intracytoplasmic granules. The most striking findings in the IEC were the elevated percentages of CD8α+ lymphocytes (40%), presumed to express CD8αα chains, and CD4−/CD8− (double negative) lymphocytes (44%), and the consistent presence of a minor subpopulation of CD3+/CD11d+ IELs (6%). Small percentages of CD4+ lymphocytes (10%) were observed such that the IEL CD4:CD8 ratio (0.25) was low. The LPC also contained a majority of T cells and few plasma cells. However, this compartment had reduced percentages of CD8α+ lymphocytes (28%) and increased percentages of CD4+ lymphocytes (27%) relative to the IEC. However, the LPL CD4:CD8 ratio (1.0) remained low compared with the ratio in peripheral blood. In feline MALT, MHC class II expression was lower than in other peripheral lymphoid compartments. The results of this study provide important reference values for future investigations involving feline intestinal lymphocytes and demonstrates that the leukocyte distribution and phenotypic characteristics of the feline diffuse MALT appear largely similar to the murine, rat and human counterparts.

Keywords: Feline; Small intestine; Mucosal; Lymphocyte; MALT; Phenotype

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1. Introduction

The mucosa of the small intestine constitutes one of the larger compartments of the lymphoid system and is composed of both diffuse and organized lymphoid tissue (Guy-Grand and Vassalli, 1993). The diffuse mucosal associated lymphoid tissue (MALT) is anatomically divided into the intra-epithelial compartment (IEC) and the lamina propria compartment (LPC). Leukocytes derived from each compartment have been differentiated by anatomical location as well as phenotype and function, thus defining a compartmentalization unique to the mucosal immune system (Lundqvist et al., 1995).

The lymphocyte subpopulation of the IEC, the intra-epithelial lymphocytes (IELs), have been extensively characterized in mouse (Lefrancois, 1991; Maloy et al., 1991; Guy-Grand and Vassalli, 1993), rat (Vaage et al., 1990; Fangmann et al., 1991; Takimoto et al., 1992; Santhi and Ramanadham, 1995; Kearsey and Stadnyk, 1996; Helgeland et al., 1997), and man (Spencer et al., 1989; Jarry et al., 1990; Guy-Grand and Vassalli, 1993; Machado et al., 1994; Lundqvist et al., 1995). Morphologically, a large percentage of IELs have intracytoplasmic azurophilic granules containing granzymes and perforin (Cerf-Bensussan and Guy-Grand, 1991; Mowat and Viney, 1997). Phenotypically, the majority of IELs are T cells with CD3/CD8 phenotype (Cerf-Bensussan and Guy-Grand, 1991; Guy-Grand and Vassalli, 1993; Mowat and Viney, 1997). A majority of IELs also co-express CD103, the αEβ7 integrin required for homing and retention of lymphocytes in the mucosal epithelium (Cepek et al., 1994). However, considerable phenotypic heterogeneity has been observed in IELs where CD4+/CD8+ (DP), CD4−/CD8− (DN) T cells and unusual subpopulations such as CD3−/CD8+ and CD4+/CD8αα+ lymphocytes are described (Lefrancois, 1991; Maloy et al., 1991; Guy-Grand and Vassalli, 1993; Mowat and Viney, 1997). Reasons for heterogeneity in this mucosal lymphoid pool are not well understood. However, this phenotypic diversity displayed by mucosal IELs is likely a reflection of the range of functions performed by IELs as the first line of defence against mucosal antigens, and is attributed to influences from both the intestinal local environment and the intestinal epithelium.

Although research has focused mainly on IEL biology, the lamina propria lymphocytes (LPLs) are also directly associated with inflammatory (Souza et al., 1996), hypersensitivity (Mowat and Viney, 1997) and HIV-1 associated intestinal disorders (Zeitz et al., 1994; Snijders et al., 1996). Although studied less extensively, LPLs have less phenotypic heterogeneity compared with IELs (Schieferdecker et al., 1992). However, a majority of LPLs in humans are reported to co-express molecules consistent with either activation, memory (James, 1991; Zeitz et al., 1991; Schieferdecker et al., 1992) or cytotoxic activity (Zeitz et al., 1991) and appear primed for cytokine production and response (Targan et al., 1995).

Interest in the mucosal lymphoid tissue of the domestic cat has grown as a comparative spontaneous and experimental animal model for various human diseases including inflammatory bowel disease, viral and non-viral associated intestinal lymphomas (Callanan et al., 1996; Jackson et al., 1996), and immunodeficiency associated syndromes (Beebe et al., 1994). The intestinal mucosal barrier and presumably, the MALT, also have a role in the pathogenesis of feline corona virus infection and the resultant development

of feline infectious peritonitis (Pedersen et al., 1981). However, characterization of feline normal intestinal MALT has not been reported. In this study, flow cytometry, immunohistology and cytology were used to characterize the diffuse MALT of the small intestine in normal cats. Additionally, we describe the distribution and expression of the novel leukointegrin CD11d (Danilenko et al., 1995) in the feline MALT. The most striking findings in this study were the elevated percentages of CD8α+ T cells, presumed to express CD8αα chains, and CD4−/CD8− (double negative) lymphocytes in the IEC, and the consistent presence of a minor subpopulation of CD3+/CD11d+ IELs. This contrasts with salient phenotypic characteristics of the feline LPL population which included increased percentages of CD4+ T cells, reduced numbers of CD8+ T cells, presumed to express both CD8α and β chains, and minimal expression of CD11d.

2. Materials and methods

2.1. Animals

Normal small intestine was obtained from 22 specific pathogen free cats (Feline Nutritional Laboratory, University of California at Davis, CA), comprised of nine juvenile (5–8 months age) and 13 adult (over 1 year age) animals. Intestinal samples were collected after sacrifice by intravenous injection of 10 ml saturated KCl which followed neuro-physiology or cardiology experimental protocols performed by other investigators. For this study, analysis of the IEC was performed on all 22 cats, while analysis of the LPC was performed on nine cats (three juvenile and four adults).

2.2. Isolation of IELs and LPLs

The IELs and LPLs were isolated using methods described for other species with minor modifications (Lundqvist et al., 1992; Santhi and Ramanadham, 1995; Kearsey and Stadnyk, 1996). The small intestine, extending from the duodenum to the ileo-cecocolic junction was excised. Fecal material and excess mucus were removed from the intestinal lumen with extensive flushing with RPMI 1640 medium pH 7.4 containing 2 mM dithiothreitol, 100 μl/ml penicillin and 100 μg/ml streptomycin (all from Fisher Scientific, Pittsburgh, PA). The intestine was opened longitudinally and cut into 2 cm fragments and the Peyer's patches were resected. The intestinal fragments were washed for 10 min with the solution described above prior to leukocyte extraction. Extraction of cells from the IEC consisted of incubation of washed intestinal fragments with stirring for an hour in $Ca^{2+}Mg^{2+}$ free Hanks balanced salt solution (HBSS, Fisher Scientific), containing 5 mM EDTA, 10% FetalClone I serum (Hyclone Inc., Oregon, UT) and 10 mM HEPES. Cell suspensions were collected after centrifugation of the supernatant at $200\times g$ for 5 min. This procedure was repeated twice for maximum leukocyte IEC yield. The intestinal segments were further washed with stirring for 1 h prior to extraction of leukocytes from the LPC. LPC leukocytes from nine animals were obtained after 6–8 h incubation of the IEL-extracted intestinal fragments on an orbital shaker (Bellco Glass Inc, Vineland, NJ) at $10\times g$ in RPMI 1640 pH 7.4 containing 0.01% collagenase type III

(Worthington Biochemical Corporation, Freehold, NJ), 10% FetalClone I serum, 10 mM HEPES. Collagenase type III was used for extraction of LPLs because of its low proteolytic activity and reduced likelihood of enzymatic degradation of cell surface antigens. The collagenase recognition sequence (PXGP) is rare in proteins other than collagen and is not present in the feline lymphocyte molecules for which sequences are known (CD4, CD8α and CD8β). The collagenase digested material was pressed through a stainless steel wire mesh (Fisher Scientific) to free the LPC leukocytes. The IEC and LPC leukocyte suspensions were filtered four times through columns packed with glass wool (Fisher Scientific). Following extraction and filtration, IEC and LPC leukocytes were washed with RPMI 1640 and resuspended in phosphate buffered saline pH 7.4 (PBS) with 10% v/v heat inactivated horse serum (Gibco BRL, Gaithersburg, MD) and 0.01% sodium azide (buffer A) for cytospin preparations and flow cytometry.

2.3. Preparation of cytology specimens

Morphologic evaluation, differential cell counts and immunocytology were performed on cytocentrifuged preparations of isolated IEC (n=22) and LPC (n=9). Approximately 5000 cells per slide were resuspended in 100 μl of buffer A and centrifuged at $200\times g$ for 5 min in a cytocentrifuge (Shandon, Pittsburgh, PA). Cytospins were air dried and stained with undiluted May-Grünwald for 8 min and Giemsa (both from VWR Scientific Products, San Francisco, CA), diluted 1:40 for 45 min or were fixed in acetone and stored at -70°C for immunocytology.

2.4. Monoclonal antibodies

A panel of monoclonal antibodies (mAbs), including feline specific and cross-reactive canine and human specific reagents, were used for flow cyometry and immunohistology (Table 1). CD79a, CD14 and CD3ε are raised to human specific antigens. CD11b, CD11d and CD18 are raised to canine specific antigens. Reagents that recognize CD4, CD8α, CD8β, CD21, CD22, CD49d and MHC class II are feline specific. All mAbs assessed cell surface molecule expression with the exception of CD3-12 and HM57, which were generated against synthetic peptides corresponding to conserved intra-cytoplasmic sequences of CD3ε and CD79a, respectively (Jones et al., 1993). CD3-12 was generated against the human CD3ε cytoplasmic peptide sequence which was conjugated to keyhole limpet hemocyanin (KLH) as follows: NH_2-ERPPPVPNPDYEP-OH. This reagent is cross-reactive to the cat because of the high degree of homology between the feline and human CD3ε amino acid sequence in this region (Woo and Moore, unpublished data).

Human CD3ε ERPPPVPNPDYEP

Feline CD3ε EKPPPVPNPDYEP

The specificity of CD8α and CD8β mAbs for each CD8 chain was confirmed by screening each mAb against the appropriately transfected feline recombinant CD8 constructs (Pecoraro et al., 1994; Pecoraro et al., 1996) (M. Shimojima, personal communication). The putative CD49d reagent is designated CD49d-like based on immunoprecipitation data and appropriate cell and tissue distribution (Woo and Moore, unpublished data).

Table 1
Monoclonal antibodies used for flow cytometry, immunohistology and immunocytology

Antigen	mAb[a]	Species	Reference/source
CD3ε	CD3–12[b]	Human	PF Moore (unpublished)
CD4	Fe1.7B12	Feline	Woo et al. (1997)
CD5	Fe1.1B11	Feline	Woo et al. (1997)
CD8α	Fe1.10E9	Feline	Woo et al. (1997)
CD8β	Fe5.4D2	Feline	PF Moore (unpublished)
CD21	Fe2.8F9	Feline	Woo et al. (1997)
CD22	Fe2.9F2	Feline	Woo et al. (1997)
CD79a	HM57	Human	Dako Inc., Carpinteria, CA
CD11b	Ca16.3E10	Canine	Danilenko et al. (1995)
CD11d	Ca16.3D3	Canine	Danilenko et al. (1995)
CD18	Ca1.4E9	Canine	Moore et al. (1990)
CD49d-like	Fe2.10E11	Feline	PF Moore (unpublished)
CD14	Tük 4	Human	Dako Inc., Carpinteria, CA
MHC class II	42.3	Feline	Rideout et al. (1990)

[a] All mAbs were generated in mouse with the exception of CD3–12 which was generated in rat.
[b] All mAbs were IgG1 isotype with the exceptions of Ca16.3D3 (CD11d) (IgG2b) and Tük 4 (CD14) (IgG2a).

For double label flow cytometry experiments, a combination of biotin (amino-hexanoyl-biotin *N*-hydroxysuccinimide, Zymed Laboratories Inc., San Francisco, CA), R-Phycoerythrin (R-PE, Molecular Probes Inc., Eugene, OR) or Fluorescein isothiocyanate (FITC, Molecular Probes Inc.) conjugated IgG mAbs were used. This included anti-CD4-biotin, anti-CD8α-R-PE, anti-CD11d-R-PE, anti-CD4-FITC, anti-CD8α-FITC, and anti-CD3ε-FITC.

2.5. Flow cytometry

For single label experiments, cells were resuspended in buffer A at a concentration of 2×10^7 cells per ml. Approximately, 1×10^6 cells were labeled with 25 μl of undiluted tissue culture supernatant (TCF) followed by FITC labeled horse anti-mouse IgG or anti-rat IgG (Vector Laboratories, Burlingame, CA) as described previously (Danilenko et al., 1992). An irrelevant mouse myeloma IgG1 hybridoma supernatant served as a negative control. For double label experiments, similar cell suspensions were incubated with titered amounts of directly conjugated mAbs as above. Biotin conjugated mAbs were detected using either neutralite-avidin-R-PE or neutralite-avidin-FITC (both from Southern Biotechnology, Birmingham, AL). Assessment of intracytoplasmic CD3ε was achieved using anti-CD3ε-FITC in conjunction with cell surface permeabilization using the Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA) as per manufacturer's instructions. Negative controls in both double label and permeabilization experiments consisted of resuspended cells stained with either no antibody or with an irrelevant unconjugated, R-PE or FITC- conjugated mouse IgG. All labeled cell suspensions were analyzed with a FACScan analytical cytometer (Becton Dickinson, San Jose, CA) equipped with Cell Quest software (Becton Dickinson). Phenotypic analysis

with single mAb labeling was performed on 22 cats for IELs, and nine cats for LPLs. Phenotypic analysis with double mAb labeling on IELs were performed on 12 of 22 cats.

2.6. Immunohistology and immunocytology

Tissue sampling of the duodenum, jejunum and ileum was also performed prior to IEL and LPL isolation techniques from four of nine juvenile and six of 13 adult cats. Tissues between 3 and 5 mm width were snap-frozen in OCT compound (Finetek Sakura USA, Torrance, CA) by immersion in 2-methylbutane (Fisher Scientific) cooled to freezing point in liquid nitrogen. Frozen tissue sections of 5 μm thickness and cytospin preparations from the IEC and LPC were stained by the avidin-biotin peroxidase method as previously described (Moore et al., 1990). Antibodies (Table 1) were applied to sections as tissue culture supernatants at a 1:10 dilution. An irrelevant isotype matched antibody or omission of the primary antibody was used as a negative control. Positive controls were performed using frozen sections of normal feline spleen. Additional blocking of non-specific sites was performed on cryosections of the intestine using an additional avidin-biotin blocking step (Avidin/Biotin Blocking Kit, Vector Laboratories) as per manufacturer's recommendations. Antibody staining was detected using the chromagen, 3-amino-9-ethyl-carbazole (AEC, Sigma, St. Louis, MO). Tissue sections and cytospin preparations were subsequently counterstained in Gill's hematoxylin (Fisher Scientific).

2.7. Statistical analysis

Values are given as percentage mean±S.D. and range (minimum–maximum). Statistical analysis of independent samples was done with the Kruskal–Wallis test (non parametric analysis of variance). Significant differences between groups were declared where p values less than 0.05 were present.

3. Results

3.1. Cell yield and differential cell counts

Consistent yields of leukocytes from the IEC were obtained from all samples from both juvenile and adult cats. Cytologic examination confirmed that both the IEC and LPC fractions had good purity with few epithelial cells in the IEC pool and minimal contamination of granular IELs in the LPC pool. Differential cell counts, performed on May–Grünwald Giemsa stained cytospin preparations, demonstrated that the IEC suspensions contained a majority of lymphocytes. However, significant differences in yield and lymphocyte morphology were observed between the two age groups. The cell yields from both the IEC and LPC were significantly reduced ($p<0.05$) in juvenile cats compared with adults (Table 2). Also, leukocytes from the IEC in juvenile cats had a significantly increased ($p<0.05$) subpopulation of granular lymphocytes compared with adults. These granular intra-epithelial lymphocytes (IELs), named for their morpholo-

Table 2
Cellular composition of leukocyte populations released from the IEC[a]

Cells	Juvenile cats (n=9)	Adults cats (n=13)
Total yield	$5.2\times10^8\pm2.6$	$2.6\times10^9\pm3.0$[b]
Total lymphocytes	89.5 ± 7.5[c] (45–75)	85.6±10.7 (40–73)
Granular lymphocytes	35.5±6.9 (25–47)	26.0±9.0[b] (11–45)
Globular leukocytes	3.9±2.9 (1–11)	2.1±2.7 (0–10)
Epithelial cells	2.0±1.3 (0–5)	3.1±3.7 (0–13)
Monocytes/macrophages	0.3±0.6 (0–2)	0.1±0.3 (0–1)
Neutrophils	3.4±3.8 (0–11)	5.3±2.3 (3–10)
Eosinophils	0.5±0.7 (0–1)	0.4±0.8 (0–2)
Mast cells	0.1±0.2 (0–1)	1.5±1.7 (1–4)

[a] Cell counts were performed on 300 cells for each sample.
[b] Significant differences (p<0.05) between juvenile and adult samples detected using the Kruskal–Wallis test.
[c] Values for differential cell counts are represented by the percentage mean±S.D. and the percentage range in parenthesis.

gically distinct intracytoplasmic azurophilic granules, were variable in size but rarely exceeded 12 μm in diameter. Their distinctive cytoplasmic granules also varied in number, size and shape and were either grouped in close proximity to the nucleus or diffusely distributed in the cytoplasm. With the exception of granular lymphocytes, similar percentages of leukocytes were present in the IEC of both juvenile and adult cats (Table 2). Globular leukocytes were the second major component of this compartment. These cells were approximately 15–20 μm in diameter, had oval nuclei with diffuse chromatin and contained large round intracytoplasmic granules. Differential cell counts on leukocytes from the LPC were not performed as elevated numbers of lysed cells were observed on cytospin preparations. However, LPC leukocytes were composed of a majority of small lymphocytes and few plasma cells. As in the IEC, mast cells, monocytes, macrophages and few polymorphonuclear cells were minor components which were also present in this compartment (Table 2).

3.2. IEL expression of T cell antigens

Despite a significant difference in cell yield and lymphocyte morphology between juvenile and adult cats, significant differences in antigen expression by lymphocyte subpopulations in both the IEL and LPL were not observed between juvenile and adult cats. Phenotypic results, therefore, reflect the findings of the combined juvenile and adult groups. Isolated IELs were examined for expression of T cell antigens including CD3, CD4, CD5 and CD8 (alpha and beta chains) (Table 3). Quantitation of expression, distribution within the intra-epithelial compartment and cytologic features of IELs were investigated using flow cytometry, immunohistology and cytology. The majority of IELs, including both granular and non-granular lymphocytes, expressed CD3. Fewer IELs expressed CD5. Both CD8α+ and CD8β+ lymphocytes consistently predominated in number over CD4+ T cells such that the ratio of CD4:CD8α lymphocytes was 0.25. Although increased numbers of CD8+lymphocytes were present in the IEL pool, a discrepancy in expression of CD8α and CD8β was observed. As many as 40% of CD8α+

Table 3
Flow cytometric analysis of the phenotype of feline IELs and LPLs[a]

Antigen	IEL	*N*	LPL	*N*
CD3ε	86.9±6.8[b]	22	IH	9
CD4	10.0±3.6	22	27.2±10.1[c]	9
CD5	44.5±11.9	22	43.5±10.8	9
CD8α	40.6±10.0	22	28.1±10.0[c]	9
CD8β	23.4±8.0	22	20.5±6.7	9
CD3+/CD4+	11.0±3.3	12	n.d.	
CD3+/CD8α+	49.0±7.3	12	n.d.	
CD3−/CD4+	0.6±0.2	12	n.d.	
CD3−/CD8α+	3.5±2.1	12	n.d.	
CD4+/CD8+	2.1±1.1	12	n.d.	
CD4−/CD8−	43.9±11.7	12	n.d.	
CD11d	5.9±3.2	22	0.9±0.2[b]	9
CD11d+/CD4+	0.9±0.5	12	n.d.	
CD11d+/CD8+	2.4±1.2	12	n.d.	
CD18	78.5±17.6	22	68.9±6.2	9
CD49d-like	74.2±14.1	22	67.2±5.3	9
MHC class II	33.4±26.5	22	43.7±24.4	9

[a] IH: Phenotypic analysis performed only by immunohistology; n.d.: not done; *N*: number of cats.
[b] Values are represented by mean percentage±S.D. on gated lymphocytes.
[c] Significant differences ($p<0.05$) detected between IEL and LPL subpopulations.

IELs did not express the CD8β chain and were interpreted as CD8αα IELs. Dual staining of IELs with CD3/CD8α and CD3/CD4 by flow cytometry detected very few CD3−/CD8α+ and extremely rare CD3−/CD4+ lymphocytes. Dual staining of IELs with CD4/CD8 by flow cytometry demonstrated a substantial population of CD4−/CD8− (DN) lymphocytes (44%) was present, while CD4+/CD8+ (DP) lymphocytes (2%) were rare.

3.3. LPL expression of T cell antigens

Isolated LPLs were also examined for antigen expression using the same panel of T cell mAbs (Table 3). CD3ε expression was investigated only by immunohistology and immunocytology since permeabilization caused major lysis of LPC leukocytes. However, expression of CD5, CD4, CD8α and CD8β antigens was investigated with both flow cytometry and immunohistology. As in the IEL population, the majority of LPLs were CD3+ T cells with fewer cells expressing CD5. However, CD4 and CD8α expression differed from the IEL population. LPLs were represented by a three-fold increase in CD4+ T cells and a three-fold decrease in CD8+ T cells, both of which were significant changes ($p<0.05$) compared with the IEL population. This variation in percentages of CD4 and CD8α lymphocytes resulted in a LPL CD4:CD8α ratio of 1.0, which was increased over that observed in the IEL pool. In contrast to the increased expression of CD8α over CD8β antigens as observed in the IEL pool, CD8+ cells in the LPL pool were composed of similar percentages of CD8α and CD8β cells. This equivalent level of CD8α and CD8β expression was more consistent with the majority of CD8 lymphocytes in the

LPL compartment expressing CD8αβ heterodimers, and fewer CD8 lymphocytes expressing CD8αα homodimers.

3.4. Expression of B cell antigens

Few B cells (CD21+ and CD22+) were detected in both the IEC and LPC. Fewer than 1% B cells in each compartment were detected by flow cytometry. In concordance with this, B cells were not observed by immunohistology in the IEC. However, occasional CD79a+ B cells were observed in the LPC by immunohistology and largely appeared as plasma cells in the deep portion of intestinal glands.

3.5. Expression of adhesion molecules

Intestinal lymphocytes were also assessed for expression of CD18, CD11b, CD11d and CD49d. A large percentage of IELs expressed CD18 (Table 3). However, antigen expression as determined by flow cytometry, occurred as a continuum which ranged from low to high density of expression. This variable expression was also observed by immunohistology where variation in intensity of staining occurred. A majority of LPLs also expressed CD18. In addition, immunohistology demonstrated CD18 expression on macrophages, neutrophils and antigen presenting cells. With the exception of rare neutrophils and macrophages in the LPC, CD11b was not expressed by IELs or LPLs. CD11d, the fourth α chain of the β2 integrin family, was expressed by a small subpopulation of IELs (Table 3). Dual label staining demonstrated that CD11d cells largely co-expressed CD3. However, although 40% of CD11d+ cells co-expressed CD8α and 15% of CD11d+ cells co-expressed CD4, as many as 45% of CD11d+ cells proved to be negative for both CD4 and CD8 antigens (Table 3). Evaluation of CD49d expression on IELs and LPLs demonstrated that a majority of IELs (75%) and fewer LPLs (67%) expressed this antigen, similar to that observed for CD18.

3.6. Expression of MHC class II antigen

MHC class II expression on both IELs and LPLs was the most variable parameter analyzed by flow cytometry (Table 3). Antigen expression in the IEL population was either low or had a continuous distribution such that a broad range of expression from 3 to 74% was observed. Although the lamina propria contained increased numbers of MHC class II lymphocytes, a similarly broad range of expression from 5 to 77% was also detected. Immunohistology demonstrated marked variation in staining on IELs and LPLs confirming this variation in density of cell surface expression. Intestinal epithelial cells did not express MHC class II.

4. Discussion

The study of diffuse MALT has been hampered by the difficulty of accessing intra-epithelial (IEC) and lamina proprial (LPC) leukocyte populations in sufficient purity and

yield to allow meaningful studies. In this study, adaptation of existing methods originally developed for the study of diffuse MALT in humans and rodents, resulted in consistent cell yield and purity from feline intestine. Feline IELs were enriched for granular lymphocytes, this was especially so in juvenile cats. The majority of feline IEL were T cells, which consistently expressed CD3, but exhibited reduced frequency of expression of CD5. Feline IEL predominantly expressed CD8α and less frequently CD8β, but a large population of lymphocytes, which lacked expression of CD4 or CD8, was also prevalent. In contrast, feline IEL, which co-expressed CD4 and CD8 were rare. In these respects feline IEL differed markedly from IEL of rodents (Vaage et al., 1990; Fangmann et al., 1991; Lefrancois, 1991; Maloy et al., 1991; Takimoto et al., 1992; Guy-Grand and Vassalli, 1993; Santhi and Ramanadham, 1995; Kearsey and Stadnyk, 1996; Helgeland et al., 1997) and humans (Spencer et al., 1989; Jarry et al., 1990; Guy-Grand and Vassalli, 1993; Machado et al., 1994; Lundqvist et al., 1995). Unexpectedly, feline IEL exhibited broad variation in MHC class II expression between individuals, in contrast to the consistent high frequency of MHC class II expression by peripheral blood lymphocytes of all lineages in cats. Also, the expression of CD11d by a minor subset of feline IEL appeared to be a unique feature. Feline LPLs were more difficult to assess due to poor viability following prolonged incubation necessary for their release. Feline LPL populations were broadly similar to IEL populations with the exception of higher frequency of CD4 expression by T cells and lower frequency of CD8α expression. In contrast to IEL, granular lymphocytes were infrequently encountered, and CD11d expression was minimal in the LPL population.

Assessment of differential cell counts and cytology, in conjunction with quantitation by flow cytometry, demonstrated that the isolation techniques used in this study provided representative populations of leukocytes from the IEC and LPC of the feline small intestine. Immunostaining of tissue sections and cytocentrifuge preparations qualitatively validated the lymphocyte phenotypes on IELs and LPLs that were established by quantitative flow cytometry. Although consistent leukocyte yields were obtained in both juvenile and adult cats, juvenile cats had significantly reduced IEC yields compared with adults. However, despite this reduced cell yield in juvenile cats, differential cell counts demonstrated that granular lymphocytes represented a significantly increased percentage of cells in the IEC. Although this has been observed in other species, the reasons or mechanisms by which this age-associated variation in yield and lymphocyte morphology occurs are not well understood. Aging has been associated with elevation in IEL numbers in man (Machado et al., 1994) and mouse (Maloy et al., 1991) and is hypothesized to be driven by continuous antigen stimulation. This increased cell yield which was consistently detected in adult cats suggests that a similar situation occurs in this species. The variation in lymphocyte morphology is also reported in other species. Granular IELs are reported in the rat where they represent a significant percentage of the CD3+ IEL population (Vaage et al., 1990). However, despite this observation, the function and significance of granular lymphocytes remains unknown.

Although the majority of feline IELs expressed CD3, only 50% of IELs expressed CD5. This contrasts with CD5 expression on peripheral T lymphocytes in peripheral blood and tissues and the thymus where CD5 is expressed by virtually all peripheral T cells and mature thymocytes (Van de Velde et al., 1991). A similar reduction in

the expression of CD5 on IEL subsets have been described in mouse (Lefrancois, 1991; Mosley et al., 1994), rat (Fangmann et al., 1991; Takimoto et al., 1992) and man (Jarry et al., 1990). Although, the functional significance of CD5− T cells is not well understood, it is hypothesized that the lack of CD5 may derive from the absence of interaction between T and B cells in the intestinal epithelium (Van de Velde et al., 1991).

Marked differences in CD4 and CD8 expression were observed in the phenotypic comparison between the IEL and LPL populations. Few feline IELs expressed CD4 compared with the LPL population where a significant increase in CD4+ LPLs was observed. Conversely, CD8α+ lymphocytes constituted a major subpopulation of feline IELs but were a reduced component of the feline LPL pool. This variation in CD4 and CD8 expression was assessed by examination of the CD4:CD8 ratio in each compartment. Increased percentages of CD8α+ lymphocytes (40%) and low percentages of CD4+ lymphocytes (10%) resulted in an IEL CD4:CD8α ratio of 0.25. This contrasted with the LPL CD4:CD8α ratio of 1.0 associated with increased percentages of CD4+ lymphocytes (27%) and reduced percentages of CD8α+ lymphocytes (28%). However, despite the increase in CD4+ LPLs in the feline intestine, the CD4:CD8 ratio in this compartment remained low compared with peripheral T cells in which a CD4:CD8 ratio of 2.5 is routinely observed in specific pathogen free juvenile cats (Woo et al., 1997). Similar findings are reported in the mouse (Lefrancois, 1991; Camerini et al., 1993; Boll and Reimann, 1995) and man (Lundqvist et al., 1995) where CD4+ cells represent a markedly reduced subpopulation of IELs, and an increased subpopulation of LPLs in contrast with CD8 intestinal lymphocytes.

Although CD8+ lymphocytes constituted a major subpopulation of feline IELs, a marked discrepancy between CD8α and CD8β chain expression was observed. This discrepancy implied that a significant percentage of CD8 IELs expressed CD8αβ homodimers, rather than CD8αβ heterodimers. This contrasts with the predominance of CD8αβ lymphocytes observed in other peripheral lymphoid tissues, peripheral blood and thymus. This discrepancy between CD8α and CD8β chain expression was not observed in the feline LPL population where both CD8α and CD8β chains were expressed in similar percentages, more consistent with conventional CD8αβ chain expression. The predominance of expression of CD8αα chains by feline CD8+IELs is similar to that described in mouse (Lefrancois, 1991), rat (Kuhnlein et al., 1994; Helgeland et al., 1997) and man (Jarry et al., 1990; Latthe et al., 1994) where they represent a substantial subpopulation of CD8+ IELs. However, despite the predominance of the CD8αα phenotype in the IEL population, the biology and specific functions of CD8αα IELs are not well understood.

This unusual phenotype has prompted the proposal that these cells are derived from extrathymic developmental pathways (Neuhaus et al., 1995; Taplin et al., 1996; Hamad et al., 1997) and their predominance in the intra-epithelial mucosa suggests that they perform a unique function in immune surveillance of the mucosal barrier. The localization of CD8αα lymphocytes in intraepithelial sites has also suggested that both the intestinal environment and epithelium play a critical role in expression of this phenotype (Reimann and Rudolphi, 1995). As a means of defining possible functions of CD8αα IELs in immune surveillance, further investigations have focused on T cell

receptor (TCR) expression and TCR-Vβ repertoire of this subpopulation. However, significant differences between CD8αα and CD8αβ IELs have not been described. Similarities between CD8αα and CD8αβ IELs include preferential co-expression of TCRαβ, (Latthe et al., 1994; Regnault et al., 1994; Neuhaus et al., 1995; Regnault et al., 1996; Sydora et al., 1996; Taplin et al., 1996) and minimal differences in TCR-Vβ repertoire (Regnault et al., 1996). Other indicators of function, including cytotoxic activity (Mosley et al., 1994) and cytokine response and production (Taguchi et al., 1991), have also not been found to differ between murine CD8αα/TCRαβ and CD8αβ/TCRαβ IELs.

A major fraction of feline IELs lacked expression of CD4 and CD8. This double negative (DN) subpopulation accounted for greater than 40% of the IEL pool. While this phenotype is unusual compared with lymphocytes described in peripheral blood and tissues, DN lymphocytes are consistently reported as a subpopulation of IELs. However, in contrast to our findings in the cat, DN lymphocytes represent minor IEL subpopulations accounting for less than 10% of IELs in both rat (Taguchi et al., 1991) and man (Spencer et al., 1989). This percentage of DN cells in this feline study may be exaggerated by the inclusion of natural killer (NK) cells or TCRγδ T cells which would not be expected to express either CD4 or CD8 antigens. More precise phenotypic analysis to determine TCR expression or the presence of NK cells within this DN population was not performed due to the lack of feline specific reagents. Also, CD3 expression on this DN subpopulation which would be expected on TCRγδ IELs was not directly determined. While murine studies demonstrate CD3 expression on intestinal TCRγδ T lymphocytes (Camerini et al., 1993; Kuhnlein et al., 1994), they are mostly negative for CD4 and CD8. By analogy, feline intestinal TCRγδ T lymphocytes are also be expected to be negative for CD4 and CD8. Converse to the findings with DN IELs, only a minor fraction of feline IELs co-expressed CD4 and CD8. This dual positive (DP) fraction accounted for only 2% of IELs which is, therefore, similar to the percentage of DP lymphocytes observed in peripheral blood. Again, this contrasts with reports in other species where DP lymphocytes represent much larger subpopulations of the IEL pool. This may range from 20 to 35% of IELs in rat (Fangmann et al., 1991; Kearsey and Stadnyk, 1996) and 4–30% of IELs in mouse (Lefrancois, 1991; Taguchi et al., 1991; Boll et al., 1995).

Investigation of β2 integrin expression by feline IELs demonstrated that a majority of lymphocytes expressed the beta subunit, CD18. Investigation of expression of the alpha subunits was restricted to CD11b and CD11d as feline specific reagents that recognize CD11a and CD11c are not available. A minor subpopulation of feline IELs expressed CD11d. The CD11d molecule is the most recently described alpha chain of the leukointegrin family, and its distribution has been examined most extensively in the dog (Danilenko et al., 1995). Canine CD11d is expressed by a minor subset of both splenic and peripheral blood lymphocytes, and there is no evidence of CD11d expression by intestinal lymphocytes by immunohistology. However, in humans, CD11d expression appears more diffuse and has been observed on mononuclear cells of the mucosa as well as deeper layers of the intestine (Bernstein et al., 1996). As CD11d expression in the cat is largely similar in its tissue distribution to that described for dogs (Danilenko et al., 1995), the finding of CD11d+ feline IELs was unexpected. Despite the presence of

CD11d+ lymphocytes in this unexpected location in the cat, many phenotypic similarities were noted between the feline IEL and canine splenic and peripheral blood CD11d+ lymphocytes. This includes the expression of CD3 by the majority of cells, the presence of a large percentage of DN cells, and the predominance of CD8 expression over CD4 as a component of this subpopulation. Also of note, were the presence of intracytoplasmic granules in 35% of canine splenic CD11d+ lymphocytes. As a significant population of feline IELs also exhibited granular morphology, it is likely that a subpopulation of granular IELs also express CD11d.

This preliminary morphologic and phenotypic investigation of the feline diffuse MALT demonstrates marked similarities with the murine, rat and human diffuse mucosal lymphoid tissue. As in these species, feline IELs were mostly CD3 T cells with reduced expression of CD5 and a predominance of CD8α expression over CD4. However, while T cell antigen expression was principally analogous to other species, quantitative differences were noted in the cat. This included increased numbers of DN lymphocytes, reduced numbers of DP lymphocytes and the presence of a minor subpopulation of CD11d+ IELs. As described for other species, the feline LPL population differed phenotypically from IELs. These differences included the reduced expression of CD8α and increased expression of CD4 and CD8β. However, further phenotypic investigation is clearly needed to better characterize mucosal T cell populations. In particular, investigation of TCR expression by both IEL and LPL subpopulations, expression of αEβ7 and α4β7 integrins associated with mucosal lymphocyte trafficking, and expression of markers of T cell activation are necessary to better understand the phenotypic basis of normal intestinal mucosal immune function in the cat. This would greatly enhance our ongoing investigations of perturbations, in the intestinal mucosal immune system, which are likely to accompany feline inflammatory bowel disease, feline intestinal lymphoma, and feline immunodeficiency virus infection.

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