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APPENDIX IN THE RABBIT. MORPHOLOGY OF DEVELOPMENT AND REACTIVITY UNDER NATURAL AND EXPERIMENTAL CONDITIONS

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ABSTRACT

The rabbit appendix is an important intestinal segment, being considered a primary lymphoid organ. Appendix fragments from conventional rabbits were microscopically and immunohistochemically examined. Wheat germ agglutinin (WGA) and pig isolated *E. coli* strain O149 K88 were orally administered. The most important findings are the relationship between dome lymphocytes, macrophages and reticuloepithelial cells in adult rabbits; tingible body macrophages (S-100 + cells) were numerous in the dome and centre of nodules; horizontal intraepithelial lymphocytes migration was seen in follicle associated epithelium (FAE); appendix FAE permits transit of Eimeria spp. stages to the dome cells, up to the lymphoid nodules centre; FAE has a characteristic morphology in rabbit appendix as early as the first day of life; lymphoid nodules are well developed in the first day of life; anti-lambda and –kappa chain polyclonal sera reaction occurred at 7 days of age, labelled cells being placed in nodules, dome and FAE; administration of both *E. coli* and WGA stops glandular exfoliation; WGA and *E. coli* cause the same morphologic transformation in appendix structure. M cells rate is variable: 30.2% at 7 days of age; 16.4% at 30 days of age; 14% in control group, decreasing to 7%, 5.4%, 9.8% and 5.6% in experimental conditions.

INTRODUCTION

In the rabbit, gut-associated lymphoid tissue (GALT) consists of Peyer's patches, caecal patch (Sacculus rotundus) and caecal appendix. Morphological and functional features of these structures are known, the appendix being considered as the major lymphoid organ that functions as a mammalian bursal equivalent (Dasso & Howell, 1997; Gebert & Hach, 1993; Pospisil & Mage, 1998a; Pospisil &Mage, 1998b; Weinstein et al, 1994).

We studied the rabbit appendix to establish structural microscopic features and postnatal morphogenesis and compare with those of adult animals, to identify immunohistochemical cellular types in the appendix walls, to establish limits of local reactivity in natural conditions and after wheat germ agglutinin (WGA) and *E. coli* (pig isolated strain) administration.

MATERIAL AND METHODS

Appendices sampled from slaughtered animals were examined.

Animals: 74 clinically healthy rabbits (Table 1) of different strains (New Zealand White and hybrid rabbits), all of them kept in conventional environment. The animals originate from different farms near Bucharest and from the animal farms of some research institutes. They are

reared with commercial pellets, carrots and tap water *ad libitum*. The rabbits were slaughtered 100 hours after the administration of WGA and *E. coli*.

Table 1 – Rabbits used in investigations

No.	Number of rabbits	Age of rabbits	Used for:
1	37	2-6 – month-old	Preliminary examination, impression smear, semifine sections
2	3	1 - day – old	Postnatal morphogenesis
3	3	5 - day – old	Postnatal morphogenesis
4	5	7 - day – old	Immunohistochemical investigation (only two rabbits)
5	3	14 - day – old	Postnatal morphogenesis
6	3	21 - day – old	Postnatal morphogenesis
7	5	30 - day – old	Immunohistochemical investigation (only two rabbits)
8	3	2 - month – old	Group I – control group
9	3	2 - month – old	Group II – oral administration of WGA
10	3	2 - month – old	Group III – oral administration of *E. coli*
11	3	2 - month – old	Group IV – *E. coli* administration, then WGA 15 min. later
12	3	2 - month – old	Group V – WGA administration, then *E. coli* 15 min. later

Methods

The samples were fixed in neutral 10% formaldehyde, embedded in paraffin, sectioned (5 μm) and stained by: trichromic Masson, hematoxilin-eosin, van Gieson, Giemsa, Gömöri for reticulin fibres. Fragments of appendix were harvested and fixed in 2.5% glutaraldehyde solution, included in Epon and semifine sections were blue-toluidine stained. Impression smears from appendix wall were May-Grümwald-Giemsa (MGG) stained.

The samples for immunohistochemical studies were embedded in paraffin. Fine sections of 2-3 μm from two 7-day-old and two 30-day-old rabbits were labelled using monoclonal antibodies (mAb) and polyclonal antisera (PAS) (Table 2). The immunohistochemical method used by Dr. C. Ardeleanu is Avidin-Biotin-Peroxidase (ABP). The dewaxed sections were introduced into a 30% perhydrol solution diluted 0.3% in methyl alcohol for the endogenous peroxidase inhibition; following rehydration, the sections were washed in PBS, pH 7. The sections were incubated for 20 minutes with nonimmune normal serum (goat-derived when PAS were used and pig-derived when mAb were used) to avoid Fc nonspecific sites binding; the primary antibody was then added at working dilution, with overnight incubation. The next day, the sections were PBS-washed, incubated with the secondary antibody (the anti-rabbit one) for 30 minutes at room temperature and then with the ABP complex for another 45 minutes (Vector kit, Burlingame, USA). After washing in running water, the sections were developed in a solution consisting of 10 mg of 3.3% diaminobenzidine (DAB) in 88 ml of PBS, plus 0.025% hydroxide peroxide. The DAB (split by the free peroxidase in the ABP complex) produces a brown precipitate, clearly evidencing the target antigen. The nuclear counterstaining with Meyer's haemalun was followed by dehydration and mounting in Canada balsam.

Table 2 – Monoclonal antibodies (mAb) and polyclonal antisera (PAS) used

No.	mAb and PAS	Specificity / Reactivity	Clone	Source	Dilution
1.	anti-Vimentin (mAb)	57 kD intermediary fibres of the mesenchymal cells and specialized epithelial cells (M cells)	V_9	Dako, Glostrup, Denmark	1:30
2.	S-100 (PAS)	Specific protein for histiocytic cells, mononuclear phagocytic cells, interdigitated reticular cells, dendritic cells and all cells of neuroectodermic origin	-	Dako, Glostrup, Denmark	1:1500
3.	Kappa chains (PAS)	B lymphocytes and plasmocytes	-	Dako, Glostrup, Denmark	1:16.000
4.	Lambda chains (PAS)	B lymphocytes and plasmocytes	-	Dako, Glostrup, Denmark	1:16.000

Lectin

Each rabbit received 5 ml of a 4g/100 ml WGA solution. The lectin was obtained by affinity chromatography with chitin. The protein concentration was dosed by means of Lowry's method, and the haemagglutination tests were based on Allen's method (A. Pop et al., 1995). The solution was sterilised using 0.45 μm Millipore filters.

Escherichia coli

This experiment used an enterotoxigenic strain of *E. coli* (O149 K88 ac+ and β-hemolytic) isolated from pigs, originating from the Pasteur Institute collection. 2.5 ml of a 24 hours culture ($1x10^9$ CFU/ml) of the strain were given orally to each rabbit.

RESULTS AND DISCUSSION

Structure of the appendix: general features and age peculiarities

Lymphoid nodules occupy all the wall of the appendix, penetrating deeply into the lamina propria. The muscularis was thin, forming a continuous layer, characteristically arranged for the digestive tube. Perinodular stroma was similarly arranged with caecal and Peyer's patches. Lymphoid nodules appeared partially delimitated by a fibrilar capsule, with a large communication space between the nodules and afferent dome. It was demonstrated that fibrilar capsule had a basket-like lymphatic network, which envelopes the medium and basal portions of each single lymphoid nodule (Azzali et al, 1997). Vascular structures (capillaries, venules and arterioles) were only in the corona, dome and internodular spaces.

The dome epithelium generally had not goblet cells, presenting a homogenous population of cells with large, euchromatic nuclei in the crypts. One single case had a goblet cell in FAE, close to the crypt. FAE was irregularly infiltrated with lymphocytes (intraepithelial lymphocytes – IEL). The thickness of the FAE differed from case to case.

Several features were noticed in the semifine sections:

- migration of the lymphocytes and immunoblasts to the dome epithelium;
- corpuscular antigens (bacterial bodies) accumulation in the M cell cytoplasm;
- transfer of the corpuscular antigens to the dome macrophages.

The apical pole differentiation of the M cell from other type of cells was very difficult. The M cells seem to have a luminal pole without microvilli, having fine irregular prolongations instead.

Tingible bodies macrophages were detected in domes, under the basal membrane and in the central areas of lymphoid nodules. Some images proved the existence of macrophages cytoplasmic prolongations into the FAE. When bacteria were present in FAE, IELs were clustered. The lymphocytes seem to have direct contact with antigens.

In many cases Eimeria spp. intestinal infection without clinical signs was diagnosed. FAE were covered by an amorphous, thick, eosinophilic film with parasitic forms in it. Parasitic phases, with slightly modified forms (vacuoles) were seen in domes, delimitated by a cytoplasmic mass. In other cases parasitic multiplication in M cells was observed, parasitic vacuoles being beneath or above the nucleus (already described by Pakandl et al., 1996, in experimental conditions).

Vertical lymphocytes migration through the basal membrane was usually seen; in one case of parasitism with Eimeria spp., however, we could visualise lateral movement of a lymphocyte along the dome epithelium.

The impression smear from the appendix wall evidenced a poor immunoblastic and plasmoblastic reactivity, with numerous activated macrophages.

The microscopic structure of this intestinal fragment is presented in Table 3.

Table 3 – Morphological aspects of appendix in rabbits

No.	Age of rabbits	Morphological characteristics
1	One-day-old	• lymphocytes organised in lymphoid nodules or uniformly distributed without nodular pattern; • scattered lymphocytes in the typical cuboidal epithelium (dome epithelium).
2	5-day-old	• well represented lymphoid tissue with nodular arrangement; • scattered lymphocytes in the dome epithelium.
3	7-day-old	• cellular reorganisation (apoptosis and numerous mitoses) in the centres of nodules; • M cells labelled with anti-vimentin mAb represented 30.2%, IELs (identified from morphology) 37.4% and other cells (not anti-vimentin labelled) 32.4% of FAE (Tab 4); • Positive reaction to anti-lambda and anti kappa chain PAS appeared in 1% of the lymphoid cells; • Anti-S-100 PAS labelled 6 to 10% of nodular cells and 6% of the dome cells.
4	14-day-old	• Definition of the structure
5	21-day-old	• Definition of the structure
6	30-day-old	• M cells (labelled with anti-vimentin mAb) represented 16.4%, IELs 42.8% and other cells 40.8% of FAE (Table 4); • Positive reaction to anti-lambda and anti kappa chain PAS appeared in 1-3% of lymphoid cells, in 20% and 35% respectively in the dome and in 23% and 11% respectively in the internodular area; • Anti-S-100 PAS labelled 1 to 4% of nodular cells and 26% of the dome cells.

Table 4 – M cells, intraepithelial lymphocytes and other types of cells ratio in FAE.

Field	Cellular type	number and (percentage) of different types of cells / microscope field (ob. 100) in rabbits						
		7-day-old	30-day-old	Group I	Group II	Group III	Group IV	Group V
Field 1	M cells	7 (29%)	4 (22%)	3 (7%)	3 (8%)	2 (5%)	4 (11%)	1 (2%)
	IELs	11 (46%)	7 (39%)	35 (79%)	31 (79%)	29 (79%)	25 (70%)	43 (88%)
	other cells	6 (25%)	7 (39%)	6 (14%)	5 (13%)	6 (16%)	7 (19%)	5 (10%)
Field 2	M cells	3 (14%)	2 (7%)	3 (9%)	2 (6%)	2 (4%)	4 (9%)	2 (6%)
	IELs	8 (38%)	20 (74%)	24 (73%)	29 (85%)	41 (84%)	35 (78%)	26 (79%)
	other cells	10 (48)	5 (19%)	6 (18%)	3 (9%)	6 (12%)	6 (13%)	5 (15%)
Field 3	M cells	7 (35%)	4 (24%)	5 (24%)	2 (5%)	4 (10%)	3 (8%)	2 (4%)
	IELs	6 (30%)	5 (29%)	14 (66%)	31 (82%)	32 (80%)	30 (77%)	49 (87%)
	other cells	7 (35%)	8 (47%)	2 (10%)	5 (13%)	4 (10%)	6 (15%)	5 (9%)
Field 4	M cells	6 (37%)	2 (12%)	5 (11%)	3 (10%)	2 (4%)	3 (11%)	4 (8%)
	IELs	6 (38%)	4 (24%)	40 (85%)	22 (76%)	40 (89%)	22 (82%)	40 (80%)
	other cells	4 (25%)	11 (64%)	2 (4%)	4 (14%)	3 (7%)	2 (7%)	6 (12%)
Field 5	M cells	6 (36%)	4 (17%)	5 (19%)	2 (6%)	2 (4%)	5 (10%)	3 (8%)
	IELs	6 (35%)	11 (48%)	20 (74%)	28 (80%)	43 (90%)	41 (84%)	31 (77%)
	other cells	5 (29%)	8 (35%)	2 (7%)	5 (14%)	3 (6%)	3 (6%)	6 (15%)

The uniformity of the epithelial cells localised in the lymphoid nodules-associated crypt seems to confirm other researchers' hypothesis, whose opinion is that the M cells forerunner can be found in the lymphoid nodules-associated crypts (Jepson et al., 1993a).

It is known that oligomucosal cells originate from the stem cells located in the intestinal crypts (Jubb, Kennedy & Palmer, 1993). The majority of authors agree that FAE has not goblet cells (Geryk et al., 1992; Militaru, 1999; Polak et al., 1990). In our study we observed a goblet cell (maybe an oligomucosal cell) in the appendix FAE (probably a deficiency in the differentiation and cell migration). This aspect pleads for a common origin of the dome epithelium, enterocytes and goblet cells.

The transfer activity of the corpuscular elements by FAE to the structures able to realise an immune response is very intense in the appendix. We observed that the appendix FAE may uptake and transfer parasitic and corpuscular bodies to the specialised cells. This feature has

already been used in rabbit immunisation by oral administration of inactivated parasitic forms and drugs (Gebert & Bartels, 1995; Jebson et al., 1993b; Pakandl et al., 1996).

The rabbits appendix is a specialised organ that plays an important role in the generation of B cells (Griebel & Hein, 1996; Pospisil & Mage, 1998a; Pospisil & Mage, 1998b; Weinstein et al., 1994). The results of this experiment show the presence of lambda and kappa chains in 7-day-old conventional rabbits and the appendix ability to synthesise the immunoglobulins. A marked positive reaction occurred in 30-day-old rabbits in the dome and internodular area for both types of chains, maybe because of cell diversification.

At 30 days, many intraepithelial vacuoles and lymphocytes (42.8%) were seen in the appendix dome epithelium, suggesting an intensive transcytosis.

Apart from these findings at 7 days FAE does not have a clear activity, the lymphoid nodules being the place of important cellular transformation.

Structural changes caused by WGA and *E.coli* administration

Both WGA and *E. coli* administration produced several structural changes in the appendix; the FAE cells percentage is presented in Table 4.

- Group I (control) – the digestive (interdomes) epithelium was parasitized by Eimeria spp., eosinophils being observed in lamina propria. Apoptotic cells and a few cells with foamy, abundant cytoplasm were seen. M cells represented 14% of FAE cells.

- Group II (WGA) –interdome and glandular epithelia were strongly affected by Eimeria spp. Lamina propria presented oedema, clustered eosinophils and mononuclear cells. Stasis and microthrombi were present under the basal membrane epithelium. In some structures, eosinophilic, flat enterocytes - signs of necrobiosis - occurred. On the dome epithelium surface amorphous material appeared, with Eimeria spp. stages in it. The foamy cells were situated in the centre of the dome and some lymphoid nodules had broken lymphocytes, while others had many lymphoblasts. In one case, “foamy macrophages” were seen in the lymphoid nodules. M cells represented 7% of FAE cells.

- Group III (*E. coli*) – very thin granular material was attached on FAE surface. Mucosal fold enterocytes became smaller and eosinophilic. Near the basal membrane of the digestive epithelium, a slight oedema occurred. Lamina propria had many mononuclear cells. Clustered eosinophils were present only in the internodular spaces. An interesting feature of this group was the presence of some exfoliated cells in the crypts. Well developed lymphoid nodules had many apoptotic lymphocytes and the dome had a few “foamy cells”. M cells represented only 5.4% of FAE cells.

- Group IV (*E. coli* + WGA) – lymphoblasts and clustered foamy cells were seen, the general image being like that of a reactive lymphoid organ. Unfortunately, Eimeria spp. infection was found. M cells represented 9.8% of FAE cells.

- Group V (WGA + *E. coli*) – structure modifications did not occur, excepting parasitic stages of Eimeria spp. and a discreet infiltrate with eosinophils in lamina propria. Lymphoid nodules were reactive and well developed. “Foamy cells” were seen in and out the nodules. M cells represented 5.6% of FAE cells.

The only thing which suggested a special reaction in relation with *E. coli* was noted in Group III, because of glandular epithelium descuamation caused by the bacteria. The WGA-E.coli association or the administration of WGA alone did not produce similar lesions. The fact that M cells demonstrate affinity for some lectins (Gebert & Hach, 1993; Geryk et al., 1990; Jepson et al., 1993b) is the probable explanation of the lack in exfoliated cells in other groups. The more exact interpretation of the appendix reactivity was more difficult because of the natural Eimeria spp. infection. Another important modification consists in the increasing of the IELs percentage in Groups II, III, IV and V and the decreasing of M cells rates in the same groups.

The dome modifications ("foamy cells" with abundant eosinophilic cytoplasm) can be the proof for an intense secretory and endocytosis activity. It cannot explain the absence of a similar reaction in Groups IV and V even if we admit the direct intervention of the lectin or *E. coli*.

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