

1 Running title: **ESOPHAGEAL TONSIL OF THE CHICKEN**

2

3 Title: **ESOPHAGEAL TONSIL; A NOVEL GUT-ASSOCIATED LYMPHOID**

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ORGAN

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26

ABSTRACT

26

27 The esophageal tonsil of the chicken is a novel, significant element of the
28 GALT. Its stable location and histological organization fulfills the meaning of
29 the term "tonsil". The six-eight isolated tonsillar units locate at the border of
30 the esophagus and the proventriculus. The number of tonsillar units is
31 identical with that of the esophageal folds. Each tonsillar unit consists of a
32 crypt lined by lymphoepithelium and surrounded by dense lymphoid
33 substance. The lymphoid substance is organized into T and B dependent
34 regions, like peripheral lymphoid organs. The excretory ducts of the mucosal
35 glands of the esophagus are frequently involved into the formation of the
36 lymphoepithelium. The esophageal tonsil anatomically locates before the
37 stomach, unlike the other parts of the GALT, therefore it is continuously
38 exposed to undigested environmental antigens, allergens, food and infectious
39 agents. The endeavor to develop oral vaccination further underline its
40 functional significance.

41

42

43 **Keywords:** GALT, esophageal tonsil, chicken, lymphoepithelial tissue.

44

INTRODUCTION

44

45 The avian gut-associated lymphoid tissue (GALT) has been extensively
46 studied (Oláh and Glick, 1978; Befus et al. 1980; Glick and Oláh, 1981; Oláh
47 et al.1984; Dolfi et al. 1988; Vervelde and Jeurissen 1993; Cortes et al. 1995;
48 Lillehoj et al. 1996; Nagy et al. 2001, Yasuda et al. 2002), and reviewed
49 (Payne, 1971; Glick 1988, Jeurissen et al. 1994). Along the entire digestive
50 tract small lymphoid accumulations, primary follicles occur besides the
51 classical lymphoid organs, like the bursa of Fabricius (Bockman and Cooper
52 1973, Glick 1988, Dasso et al. 2000), caecal tonsils (Oláh and Glick 1979),
53 Peyer's patches (Befus et al. 1980), Meckel's diverticulum (Oláh et al. 1984)
54 and diffusely infiltrated areas of the cloaca (Odend'hal and Breazile, 1980;
55 Gomez del Moral et al. 1998). There is very sketchy histological and no
56 immunological information on the lymphoid substance locating at the border of
57 the esophagus and proventriculus. In the available histological textbook of
58 fowls (Hodges, 1974) only one sentence has been devoted to this lymphoid
59 accumulation; "The junction between the esophagus and the proventriculus is
60 frequently heavily infiltrated with lymphatic cells and there are often many
61 large lymphoid foci lying in the tunica". This is the only reference that is related
62 with the existence of lymphoid tissue at the junction of the esophagus and
63 proventriculus.

64 The significance to deal with this remarkable lymphoid tissue is double; 1)
65 theoretical: it may be participated in the B cell development and (Ratcliffe
66 2002), 2) practical: this is the only substantial lymphoid accumulation which
67 locates in the digestive tract before the stomach. Because of its unique
68 location it is constantly exposed by undigested environmental antigens.

69 Therefore, to be familiar its morphological structure and immunological
70 function could be crucial to understand tolerance to undigested antigens, food
71 allergy and intestinal infection, like infectious bursal disease virus. So, in this
72 short paper we introduce the basic anatomical and histological structure of the
73 lymphatic accumulation at the border of esophagus and proventriculus, what
74 we called esophageal tonsil.

75

75

MATERIALS AND METHODS

76 ***Animals***

77 Fertilized White Leghorn SPF chicken eggs were obtained from CEVA-
78 Phylaxia Hungary and incubated at 37,7⁰C in humidified incubator.
79 Histological examinations were made from 4, 6, 8, 12 week old chickens. The
80 design and condition of the animal experiments were approved by the Animal
81 Ethical Committee of Semmelweis University, Budapest, Hungary
82 (TUKEB/2000). The animals were sacrificed by cervical distortion.

83

84 ***Antibodies***

85 To identify hemopoietic cells and B lymphocytes anti-chick CD45
86 monoclonal antibody (mAb, clone: HIS-C7; Jeurissen et al. 1988) and anti-
87 chick Bu1b mAb (clone: 11G2) were used. Anti-CD45 and Bu1b mAb(s) were
88 generous gift from Dr. Haymo Kurz, Albert-Ludwigs University, Freiburg,
89 Germany and Dr. Olli Vainio, Turku University, Finland), respectively. Anti-
90 CD3 mAb (clone: CT3) were obtained from Dr. Chen-Lo Chen, University of
91 Alabama at Birmingham, Birmingham, AL. Epithelial cells were visualized by
92 using a pan-cytokeratin mAb (clone: Lu-5)¹.

93

94 ***Histological procedures***

95 For cryostat sections the border of oesophagus and proventriculus was cut
96 out and snap frozen in liquid nitrogen. The ten-micrometer frozen sections
97 were collected on poly-L-lisine coated slides (Sigma)², fixed in cold acetone
98 and air dried. For histological examination the tissue samples were fixed in
99 buffered 4% glutaraldehyde (Merck)³ solution for overnight and postfixed in

100 1% osmium tetroxide (Polyscience)⁴ solution for two hours. After rehydration
101 in graded ethanol, the tissue samples were embedded in Polybed/Araldite
102 6500 mixture (Polyscience)⁴. The one-micrometer thick semithin sections were
103 stained with toluidine blue. For hematoxylin-eosin staining the specimens were
104 fixed in buffered formalin and embedded in paraffin.

105

106 ***Immunocytochemistry***

107 Immunocytochemistry was made on cryostat sections according to
108 standard techniques. Briefly, after rehydration in PBS, the acetone fixed
109 sections were incubated with primary antibodies for 45 minutes, followed by
110 biotinylated horse anti-mouse IgG (Vector)⁵ and avidin-biotinylated peroxidase
111 complex (ABC, Vectastain Elite ABC kit, Vector)⁵ at room temperature.
112 Endogenous peroxidase activity was quenched before ABC incubation by
113 treatment of the sections with 3% hydrogen peroxid (Sigma)² for 10 minutes.
114 The binding sites of the primary antibodies were visualized by 4-chloro-1-
115 naphthol (Sigma)².

116

117

118

119 **Footnotes:**

120 ¹BMA, BIOMEDICALS AG, Rheinstrasse 28-32; CH-4302 Augst, Switzerland,

121 ²Sigma- Aldrich Kft. Nagydíófa str. 7, H-1072 Budapest, Hungary.

122 ³Merck Kft., Talpas str. 3, H-1116 Budapest, Hungary.

123 ⁴Polysciences, Inc. 400 Valley Road, Warrington, PA 18976.

124 ⁵Vector Laboratories, Inc. 30 Ingold Road, Burlingame, CA 94010

125

RESULTS

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126 The esophagus forms six-eight longitudinal folds, which create a stellate-
127 shaped appearance on cross-section. The lymphoid tissue is localized at the
128 distal ends of the folds, before the esophagus turns to proventriculus (Fig. 1a).
129 This area is about three-eight millimeter long. At this region the tunica propria
130 and the lymphoid tissue are covered by stratified squamous epithelium but the
131 proventricular glands are already emerged. Cylindrical, proventricular
132 epithelium occurs only above the most distal part of the lymphoid tissue
133 (Fig.1b). The lymphoid tissue is associated with the bottom of the folds, where
134 it forms isolated units. Although the lymphoid units appear in circumferential
135 in the wall of the esophagus, it does not form a continuous ring. The number
136 of the units is identical with that of the longitudinal folds of the esophagus
137 (Fig.1a). This finding suggests, that the esophageal folds are not randomly
138 formed during swallowing, but they are anatomically determined i.e. they are
139 stable structures of the esophagus.

140 The real tonsillar lacuna or crypt starts at the bottom of the folds and
141 reaches the muscular layer of the tunica mucosa. The tonsillar unit consists of
142 a crypt and the surrounding lymphoid tissue (Fig.1c). Unlike the Peyer's
143 patches, where the lymphoid tissue occupies the tunica propria and
144 submucosal layers of the intestine, these tonsillar units are restricted to the
145 tunica propria (Fig. 1b). In the borderline of the esophagus and proventriculus
146 the proventricular glands gradually fill up the submucosa of the esophagus,
147 while towards the proventriculus the tunica muscularis mucosae breaks up. It
148 is apt to notice, that the proventricular glands reveal scattered primary follicles
149 (Matsumoto and Hashimoto, 2000), which might be involved into the

150 pathogenesis of a new variant of the infectious bursal disease virus resulting
151 in proventriculitis (Dormitorio et al. 2001; Giambrone, 2002). These primary
152 follicles do not show anatomical relationship with the esophageal tonsil.

153 Blood-borne lymphoid tissue surrounds the crypts (Fig.1d.) and infiltrates
154 the stratified squamous epithelium (Fig.2a). The infiltration of the surface
155 epithelium turns it to lymphoepithelium (Fig.2b). The heavily stained B
156 lymphocytes are localized in the germinal centers, but many weakly stained
157 Bu-1b positive cells also occur in the T dependent interfollicular areas (Fig.2c).
158 In addition to the T cell regions significant number of T cells also occur in the
159 germinal centers (Fig.2d).

160 Mucosal glands of the esophagus are frequently associated with the
161 lymphoid substance. The secretory acinus is generally free of mobile cells, but
162 the excretory ducts are involved in the formation of lymphoepithelium.
163 Interesting, that the wall of the duct is covered partly by cylindrical and partly
164 by stratified squamous epithelium, and only the latter part of the duct is
165 infiltrated with lymphoid cells. (Fig.2e). Occasionally the lumen of the duct is
166 dilated and filled with detritus.

167

DISCUSSION

167

168 The gut-associated lymphoid tissue of the chicken consists of solitary and
169 aggregated lymphoid nodules. The latter appears as lymphoid organs. Now,
170 we add to the GALT a novel organ, which has not yet got enough attention.
171 Possibly, it has been thought, that this lymphoid tissue accumulation
172 represents only small, variable, insignificant lymphoid substance. However,
173 the location of this lymphoid substance is anatomically strictly determined,
174 therefore it is a stable, consistent lymphoid structure, like the caecal tonsil. We
175 named this lymphoid substance as esophageal tonsil.

176 Generally, the correct term of the tonsil covers a complex organ, which
177 consists of an epithelium lined crypt surrounded by dense lymphoid
178 substance. The epithelium is infiltrated with mobile lymphoid cells creating
179 lymphoepithelial tissue. The lymphoid substance shows a well-organized
180 structure, namely in the interfollicular space mainly T cell are accumulated,
181 while the B cells form germinal centers. The described structural unit is
182 identical with the simplest tonsillar structure, occurring in the human lingual
183 and rabbit palatine tonsils. So, the term "tonsil" to characterize this lymphoid
184 accumulation perfectly fulfills the histological postulate of the tonsil.

185 Each isolated tonsillar unit is associated to the bottom of two neighboring
186 folds, which suggests, that each unit has separate blood- and lymphatic
187 circulation and form a functional and/or pathological unit.

188 The esophageal tonsil locates before the stomach, unlike the major parts
189 of the GALT, so it is continuously exposed by large amount of undigested
190 environmental antigens, food and allergens. Due to its location, its function
191 could be delicate comparing with other parts of the GALT, which can be found

192 in the alimentary tract distal to the stomach. This unique anatomical location
193 may be taken into consideration for developing oral vaccination.

194 The mucosal glands of the lower esophagus frequently associated with
195 the lymphoid substance. The excretory ducts of the glands are partly lined by
196 stratified squamous epithelium, which is transformed to lymphoepithelium. The
197 significance of this association is unknown, but we speculate, that the
198 lymphoepithelial tissue of the tonsil functions as "gate" for environmental
199 antigens, through which antigens and/or allergens continuously stimulate the
200 immune system. The secretion washes away the excess of antigens and
201 allergens. Possibly, the insufficient cleanup function of the gland results in
202 obliteration of the excretory duct, and consequently formation of "salivary
203 bodies".

204

204

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207

208

APPENDIX

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211

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LEGEND OF FIGURES

299

300

301 Fig 1a.

302 HE-stained cross-section of the esophagus shows seven longitudinal folds. At
303 the bottom of the esophagus seven tonsillar units are present in the lamina
304 propria. Mucosal glands of the esophagus (arrow). Mag: x13

305 Fig 1b.

306 HE-stained longitudinal section through a tonsillar unit, which is covered by
307 stratified squamous epithelium. A mucosal gland (MG) is embedded into the
308 lymphatic substance. The arrow marks the border of the stratified squamous
309 and cylindrical epithelium. Mag: x32

310 Fig 1c.

311 HE-stained tonsillar unit. The lymphoid substance is organized to germinal
312 centers (GC) and interfollicular areas. Mag: x30

313 Fig 1d.

314 Anti CD-45 mAb (hemopoietic cell marker) clearly outlines the tonsillar units at
315 the bottom of the esophageal folds. A primary follicle is also present in one of
316 the proventricular gland (arrow). Mag: x8

317 Fig 2a.

318 Anti-cytokeratin stained cryostat section shows the stratified squamous
319 epithelium of the e esophagus and the associated mucosal glands (arrow).
320 The epithelial cells of the proventricular glands (PG) also express keratin
321 intermediate filaments. Mag: x8

322

322 Fig 2b.

323 One micron thick section stained with toluidin blue. Surface epithelium is
324 heavily infiltrated by lymphoid cells, that transform the stratified squamous
325 epithelium to lymphoepithelium. Mag: x40

326 Fig 2c.

327 Bu-1b mAb identifies a heavily- and a lightly stained cell populations in the
328 germinal centers and in interfollicular areas, respectively. Mag: x8

329 Fig 2d.

330 Anti CD3 (T cell) mAb heavily stains the interfollicular areas and reveals
331 significant number of T cells inside the germinal centers (GC). The surface
332 epithelium also shows CD3 positive cells. Mag: x30

333 Fig 2e.

334 HE-stained section shows the duct of a mucosal gland. One part of the duct is
335 covered by stratified squamous epithelium which is infiltrated by lymphoid
336 cells, while the other part of the duct is lined by cylindrical epithelium. LE,
337 Lymphoepithelium. Mag: x144