Distribution of Chicken Globule Leucocytes Detected by Anti-Thymocyte Serum

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ABSTRACT. Chicken globule leucocytes (GL) were immunohistochemically detected in various organs using anti chicken thymocyte serum (ATS), which reacted specifically on thymocytes and peripheral T-cells. GL were distributed in the epithelium and the lamina propria of the digestive tract, Disse's spaces and the mesenchymal area of liver, renal tubules, the epithelium of the urinary ducts, the interfollicular epithelium and the epithelium covering the large diffusely infiltrated area of the bursa of Fabricius. There were two kinds of GL, ATS-negative and ATS-slightly positive. The reaction products on the cell surface were weaker than those of lymphocytes in the same areas. The granules of GL in the epithelium of the intestinal crypt were apparently larger than those in the villous epithelium. Spleno-GL were rarely seen as their granules were extremely fine. No GL were found in the thymus, bone marrow, respiratory organs, the pancreas and its ducts, the gall bladder, and the lymphoid follicles associated with the alimentary tract. The characteristic distributional patterns and the nature of the T-cell lineage of chicken GL, were similar to those of the natural killer cells.—**KEY WORDS:** anti-thymocyte serum, chicken, globule leucocyte, immunohistochemistry, natural killer cell.

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It is known that the mucosal immune system plays an important role in the defense against antigenic exposures caused by food and microorganisms. The alimentary tract and other mucous epithelia of intact mammals and birds contain varying numbers of migrating immunocompetent cells such as globule leucocytes (GL), lymphocytes, macrophages, granulocytes and plasma cells. GL have been found in various animal species as intraepithelial mononuclear cells containing characteristic acidophilic granules. These cells were described by Heidenhain in 1888 [21], and designated as "Schollenleukozyten" by Weill (1919) [52]. Then Keasbey (1923) [26] translated the German into "globule leucocyte." Since the first description by Heidenhain [21], many investigators have attempted to elucidate the origins and functional roles of these cells [24].

Several hypotheses concerning the cellu-

lar origin of mammalian GL have been proposed for the mast cells [9, 35, 50], lymphocytes [14, 27, 28, 52], plasma cells [53] and eosinophils [22]. However, in mammals, it is generally believed that GL are of lymphocyte or mast cell origin. In domestic fowls the lymphocyte was consistently confirmed as the origin for GL by morphological and ontogenetical investigations [3, 7, 23, 24, 49]. As a result of experiments using neonatal thymectomy or bursectomy, it has been also suggested that chicken intraepithelial GL in the alimentary tract were thymus-dependent [29]. However, there have been no reports definitely verifying the T-cell lineage of GL through the application of an anti thymocyte serum (ATS).

In the present study the direct immunohistochemical staining of chicken GL against ATS was demonstrated in various organs.

MATERIALS AND METHODS

Animals: Eight White Leghorn chickens (8 weeks old) from our colonies were used. They were homozygous at the major histocompatibility locus: blood types B9/B9 and B11/B11.

ATS: The procedures for preparation of polyclonal ATS were the same as those of Ogata *et al.* [36]. The rabbit antisera were incubated at 56°C for 30 min and then cross-absorbed three times with an equal volume of packed bursal cells and erythrocytes taken from 4 day-old chicken.

Immunohistochemistry: The following organs were fixed in periodate-lysine-paraformaldehyde fluid: thymus, bursa of Fabricius, spleen, alimentary canal (esophagus, glandular stomach, duodenum, Meckel's diverticulum, ileum, cecum, colon and cloaca), liver, gall bladder and bile ducts, pancreas, kidney, ureter, trachea and lung. Paraffin sections cut at 4 μ m were treated with 0.1% hydrogen peroxide and methanol for removal of the endogenous peroxidase activity, reacted with ATS as primary serum and then with horseradish peroxidase conjugated anti rabbit IgG goat serum (MBL, Japan). Then the sections were lightly counter-stained with hematoxylin and eosin (H-E). As a negative control, non-immunized rabbit serum was used for the primary antiserum. From the tissues embedded in epoxy resin, semi-thin sections were sliced, after which the resin was removed by saturated sodium ethoxide in absolute ethanol [15]. Then the sections were reacted with ATS and slightly counter-stained with H-E.

Preembedding procedures of the enzyme-labeled antibody method for electron microscopy were carried out on lymphocyte fractions collected from the mucosae of the small intestine, spleen and thymus using a mechanical cell separation technique.

RESULTS

By light microscopy, cells positive to ATS had a ring-shaped feature in that the reaction products were localized only on the cell surface membrane. GL were characterized by the presence of several eosinophilic cytoplasmic granules of various sizes and pale stained cytoplasm. Their cytoplasm/nucleus ratio was larger than that of agranular lymphocytes. The distribution of GL and thymus-derived lymphocytes in various organs was as follows:

The thymus cortex, which mainly consisted of a large number of thymic small lymphocytes, was intensely ATS-positive (Fig. 1a). The outermost region of the cortex was thickened as a layer of 2–5 cells consisting of both ATS-weakly positive medium-sized or large lymphocytes and a small number of ATS-negative large lymphocytes (Fig. 1b). In the medulla, only a small number of ATS-positive small lymphocytes were distributed to form small clusters preferentially in the areas beneath the cortex. GL were not detected in this organ.

In the bursa of Fabricius, the cortex of the lymphoid follicles contained no ATS-positive lymphocytes (Fig. 2a). A few ATS-positive lymphocytes were situated in the medullary tissue. GL were not detected in the follicular parenchyma. Though the follicle-associated epithelium contained neither ATS-positive lymphocytes nor GL, a small number of ATS-negative and slightly positive GL were found to infiltrate into the interfollicular epithelium (Fig. 2b). In the interfollicular connective tissue, a small number of ATS-positive small lymphocytes and a very small number of ATS-positive GL were sparsely distributed. A large ATS-positive area was detected at the stalk region of the bursa of Fabricius. This area was filled with small ATS-positive lympho-

Fig. 1. a) ATS-intensely positive thymic cortex and negative medulla. $\times 80$. b) ATS positive cells presented ring-shaped features in the cortex. The outer region has faintly positive medium or large thymic cells (arrow heads). Deepoxynized semi-thin section with slight H-E counter stain. $\times 630$.

Fig. 2. a) Lymphoid follicles of bursa of Fabricius. The cortex was ATS negative. $\times 90$. b, c) ATS-slightly positive GL (arrow heads) in the interfollicular epithelium (b), and in the epithelium covering a large ATS-positive area(c). Paraffin section with H-E counter-stain. $\times 630$.

cytes. The epithelium covering the area contained a small number of ATS-negative and slightly positive lymphocytes and GL (Fig. 2c).

Periarterial and perivenous lymphoid tissues of the spleen mainly consisted of ATS-positive lymphocytes, while periellipsoidal lymphatic tissue contained many ATS-negative lymphocytes (Fig. 3a, b). Both ATS-negative and positive GL with extremely minute granules were seen on rare occasions.

In the bone marrow, a small number of ATS-positive lymphocytes were occasionally found to form small clusters in adipose tissue. Solitary medium-sized and small-sized lymphocytes were scattered throughout the parenchyma. However, neither ATS-positive nor negative GL were detected in the parenchyma. In cells of the granulo-hematopoietic and erythropoietic series and other cellular elements, no ATS-reactive surface markers appeared.

The esophageal mucosa contained no GL.

Fig. 3. ATS positively stained periarterial lymphatic tissues (a: A), perivenous lymphatic tissues (b: V) and almost negatively stained periellipsoidal lymphatic tissues (a: E) in the spleen. Paraffin section with H-E counter-stain. $\times 150$.

Fig. 4. ATS-positive lymphocyte (small arrow head) and GL (large arrow heads) possessing fine acidophilic granules (a, b) in villous epithelium, and possessing large acidophilic granules (c, d) in crypt epithelium of the intestine. Paraffin section with H-E counter-stain. $\times 630$.

In glandular stomach, small diffuse aggregations of small lymphocytes consistently populated the convexes of the mucosa, which were located at the interglandular regions of deep propria glands. In the lamina propria, except for lymphocyte aggregation, there were few ATS-positive lymphocytes, and heterophils were relatively predominant compared with other areas of the alimentary tract. Both ATS-negative and slightly positive GL were rarely seen in the epithelium of superficial propria glands.

Large aggregations of lymphocytes with

ATS-positive surface markers in the lymphonodules were recognized at Meckel's diverticulum and the cecal tonsil. The number of diffused ATS-positive lymphocytes in intestinal mucous membrane other than the lymphonodules was larger than that in other mucous membranes. Furthermore, diffusely infiltrated ATS-positive lymphocytes in the duodenum, jejunum and cloaca were particularly numerous.

Both ATS-negative and slightly positive GL were located in the intestinal epithelium, but rarely in the lamina propria.

Reaction products of ATS-positive intraepithelial GL were weaker than those of ATS-positive intraepithelial lymphocytes (Fig. 4a, b). GL in the intestinal crypt epithelium consistently possessed larger granules than those in the villous epithelium (Fig. 4c, d).

In the pancreas, aggregations of small ATS-positive lymphocytes were infrequently seen in the mesenchyma. However, neither ATS-positive lymphocytes nor any GL were ever seen in the acini of the pancreas and in the pancreatic ducts.

In the liver, small clusters of ATS-positive lymphocytes were recognized around the central veins. The clusters occasionally contained ATS-positive GL (Fig. 5a). ATS-positive GL were also distributed in Disse's spaces (Fig. 5b).

In the gall bladder and bile ducts, diffuse aggregations of ATS-positive lymphocytes were invariably located at the lamina propria of the mucosal folds. In the intermucosal fold regions, no GL could be detected, but ATS-positive lymphocytes were scattered in the epithelium and the lamina propria.

In the kidney, small clusters of small ATS-positive lymphocytes were scattered

throughout the mesenchyma. ATS-negative and slightly positive GL and small lymphocytes existed in the epithelium of the renal tubules. In ureter, no cluster-formation of ATS-positive or negative lymphocytes was found. Both slightly positive and negative lymphocytes and GL were occasionally recognized in the epithelium.

In the trachea and lungs, large ATS-positive cell aggregations were rarely seen. Though a small number of ATS-positive lymphocytes were distributed in the epithelium and lamina propria of trachea and bronchus, ATS-positive or negative GL could not be recognized in either region.

Electron microscopic immunohistochemistry confirmed the ATS-positivity of GL in lymphocyte fractions from the spleen and the intestine, though the reaction products on the cell surface were weak (Fig. 6). Other cellular elements, except for the T-lymphocytes, were ATS-negative.

DISCUSSION

Since chickens possess two distinct central

Fig. 5. a, b) ATS positive lymphocytes (small arrow heads) and GL (large arrow heads) in the liver. V: central vein. Paraffin section with H-E counter-stain. $\times 630$.

Fig. 6. Immunoelectron microscopy of GL isolated from the intestine. ATS positive reaction products (small arrow heads) were demonstrated to be very weak and discontinuous. Large arrow head: cytoplasmic granule. Small arrow: multivesicular body. $\times 15,200$.

lymphoid organs (the thymus and the bursa of Fabricius), many active investigations have been carried out on the peripheral T- and B-cell systems *in vivo* and *in vitro*. Early investigations applying neonatal thymectomy and/or bursectomy suggested that periarterial and perivenous lymphatic tissues are T-dependent areas, and that periellipsoidal lymphatic tissue and plasma cell series were B-dependent in chicken spleen [25]. Thereafter, these areas were determined to be T- or B-cell areas by polyclonal anti T- and B-cell serum, respectively [8, 36]. The present anti chicken T-cell serum reacted exclusively with the lymphocytes in the periarterial and perivenous areas of the spleen, and never reacted with the cortex of the bursa of Fabricius. Furthermore, the serum reacted intensely with thymic cortex, including almost mitogenic large thymocytes in the peripheral cortex region, and never reacted with other cellular elements in the various organs. From these findings, the serum is considered to be specific for cells of T-cell lineage.

In healthy mammals, GL have been detected in the intestinal epithelium in cattle [26], sheep [26, 27, 54], pig [52], dog [52], cat [52], rabbit [52], rat [10, 28], mouse [52] and human [50]. In several species, GL have been also detected in various other organs, such as glandular stomach of cattle, sheep [26, 27], cat [45], dog [14], rat [28], respiratory organs of human [4, 51], sheep [35], rat [10, 17, 28], gall bladder of cat [14, 45], parotid gland of bovine [20], urinary bladder and ureter of rat [9, 10], genital tract of goat [48], rat [10], spleen and thymus of cat [45] and lymph node of dog [13, 45]. These previous results suggest that the GL show a wide range of organ distribution. In fowls, GL have been detected almost exclusively in the intestine and liver of chicken [7, 23, 24, 49] and some wild birds [12]. However, our present immunohistochemical study demon-

strated much wider distributions of GL in various organs, except for respiratory organs, gall bladder and thymus. Thus, the extensive localization of GL shows that GL appear to be migrating cells with an immunological survey role throughout the body.

Intestinal GL were also detected in the lamina propria. This suggests that GL in mucous epithelium may be derived from other hematopoietic organs via the circulatory system and then moved from crypt epithelium to villous epithelium.

GL (and cells corresponding to globule leucocytes) which earlier investigators described and named [21, 26, 52] were studied in non-infected, healthy animals. In healthy mammals, the cellular origin of GL has been attributed to various migrating cell populations. Although eosinophil origin was asserted by Heine & Schaeg [22], this hypothesis has few advocates. The plasma cell origin of GL [53] that was proposed in helminth-infected mammals, was denied by the immunohistochemical investigation of Whur & White [54]. Some cytochemical and ultrastructural investigations supported the mast cell origin of GL [9, 22, 35, 50]. However, doubt has been cast on this hypothesis histochemically and ultrastructurally [39, 45]. Weill [52] first confirmed that the GL were transformed from lymphocytes after a process of granulation on the basis of light microscopic observations in five mammalian species. Dawson [13] recognized the serial transitional stages from normal lymphocytes to GL. Kent and collaborators also emphasized the similarities of GL and lymphocytes in their serial ultrastructural, cytochemical and experimental studies [27, 28].

In healthy fowls, the light microscopical appearance of intestinal GL was not like that of mast cells, but more similar to that of intraepithelial lymphocytes [7]. Bäck [3] suggested that chicken intestinal GL were of

lymphocyte origin based on an ontogenetical study in which the kinetics of the cells was proportional to that of intraepithelial lymphocytes. Toner [49] and Holman [23, 24] have ultrastructurally pointed out that lymphocytes appear to be parent cells of chicken intestinal GL. Thus, lymphocytes have been hitherto considered to be a possible source of origin for GL in fowls and our present observations of GL support this view.

More recently, as a result of ultrastructural and immunohistochemical studies [5, 17], natural killer (NK) cells have been postulated to be a source of human GL. NK cells are characterized by a spontaneous cell-mediated cytotoxic activity (SCMC) in several types of tumor cells *in vitro*. The population possessing SCMC is generally identified as "large granular lymphocytes" (LGL) in human [40, 47], mouse [33] and dog [32]. LGL have a high cytoplasmic/nuclear ratio, effectuate numerous cytoplasmic processes, and display granulations of varying intensities in their pale cytoplasm [32, 47]. Both ATS-positive and negative GL in the present study were very similar to mammalian LGL.

NK cells (LGL) and NK activity have been readily detected in peripheral blood, lung [2, 40], liver [34], spleen [2, 40], and intestine [44], but not in thymus [2] in mammals. Though no attempt has been made to detect NK cells or NK activity in various organs throughout the body, NK cells seem to possess wide range of organ distribution and non-specificity for animal species. In chicken, the present distribution of GL is similar to the pattern of NK cells in mammals, except for respiratory organs.

Canine NK cells (LGL) express cell surface antigens Dly-6, 1A1 and E-11, suggesting T-cell lineage [32]. Rat NK cells also express Ox-8 and a few Thy-1 antigen [40], which a portion of T-cells possesses. In mouse, Thy-1 antigen appears in a portion

of NK cells [2, 6, 30, 31], and is expressed commonly on thymocyte and peripheral T-cells. Other cell surface markers on cells of T-cell lineage, Ly-1 [19], Ly-2 [2], T-200 [2], Qa-2 [30, 31], Qa-3 [31], Qa-4 [6, 31], Qa-5 [30, 31], H11 [41], 7.2 [41] and Nk-1 [2, 30, 31], are also coexpressed on a portion of NK cells. Human NK cells demonstrate sheep erythrocyte rosette forming capability and possess a positive activity of alpha naphthyl acetate esterase which cannot be inhibited with sodium fluoride [47]. Some cell surface markers of human NK cells which have been identified are OKT-3 [38, 55], OKT-8 [37], OKT-10 [37], OKT-11 [46, 55], Leu-4 [1], T-200 [38] and Lyt-3 [37], which are coexpressed in the T-cell lineage. Though NK cells of human and mouse coexpressed surface antigens with macrophages [37, 55], NK cells are considered to be furnished with surface antigens, suggesting cells of T-cell lineage in many animal species. The present immunohistochemical detection of a slightly positive surface marker against ATS on chicken GL, consists with the presence of T-cell markers on NK cells. It was considered that ATS was mainly recognized the surface markers which were added to the lymphocytes in thymic cortex and which postthymic lymphocytes possessed. This result suggests the possibility that chicken GL partially differentiate on postthymic T-cell lineage, on which cytotoxic T lymphocytes (CTL), helper T-cells and suppressor T-cells differentiate. Recently, natural cytotoxic T cell (NCTC), which was recognized in Peyer's patches of mouse intestine, was considered to be an intermediary cell from an antigen specific CTL to NK cell which was considered to be prethymic lymphocyte [18]. Natural cytotoxic (NC) cells were also recognized in mice [43]. The NC cells which possessed little surface marker of thymocyte in common, were considered to be an end stage differentiated from CTL *via* NCTC [18]. If

the subpopulations corresponding to NCTC and NC cells populates in chicken intestine, NCTC may be involved by ATS-slightly positive GL and NC cells may belong to ATS-negative GL.

On the other hand, there is a view that avian NK cells are non-T-cell in nature. This was suggested from results showing that NK activity of untreated chicken spleen cells was almost the same as the NK activity of ATS plus complement-pretreated chicken spleen cells, and also from the same results by means of immuno-effector cells, which enhanced the activity induced by the inoculation of Marek's disease virus [42]. However, in that study, as an average of 52.4% of normal spleen cells reacted with ATS, and both the target cell/untreated effector cell ratio and the target cell/ATS-plus-complement pretreated effector cell ratio were equal, the cytotoxic activity in target cells should have been enhanced if chicken NK cell were refractory to treatment with potent ATS. However, no enhancement or reduction of the cytotoxic activity was demonstrated after the treatment. Thus these results should be interpreted as demonstrating the limited effect of ATS on NK activity in chicken. This interpretation is consistent with our results showing that GL consisted of at least two populations; i.e., a slightly ATS-positive population and a negative one.

Some intraepithelial lymphocytes (IEL) of the mammalian intestine are morphologically similar to blood large granular lymphocytes (B-LGL) [11]. The granular IEL are effector cells of NK activity in the gut [44], though all granular IEL may not perform this function [16]. However, there are some reports that phenotypes of NK cells in intestinal epithelium are different from B-LGL [2, 17]. Their cytotoxicity and lytic efficiency are not rapid in comparison with those of B-LGL [16], and they possess larger granules than those in B-LGL [2]. On

the other hand, mouse intestinal NCTC, which is Thy 1.2⁺ and asialo Gm₁⁻, is distinguished from the NK cell population, which is Thy1.2[±] and asialo GM₁⁺, and also the NC population, which is Thy1.2⁻ and asialo GM₁⁻ [18, 43]. The present investigation clarified 2 subpopulations of GL, ATS-positive and ATS-negative cells. Therefore, GL also may be a heterogenous population belonging to the NK family which consisted of NK cells, NC cells and NCTC. The relations between GL and NC cells, and GL and NCTC should be further examined.

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要 約

抗胸腺細胞血清によるニワトリ globule leucocyte の検出：北川 浩・橋本善春・昆 泰寛・王藤規雄（北海道大学獣医学部家畜解剖学教室）——胸腺細胞および末梢T細胞と特異的に反応する抗ニワトリ胸腺細胞ウサギ血清（ATS）を用いて、ニワトリの諸臓器における globule leucocyte (GL) の分布を免疫組織学的に検索した。GL は腸管の上皮および固有層、肝臓の Disse 腔、腎尿細管、尿管上皮、ファブリキウス嚢の濾胞間上皮および頸部に存在する大型のリンパ球浸潤を覆う上皮中に分布し、それらの GL は ATS 弱陽性あるいは陰性であった。GL の ATS に対する反応性は同部位に存在する ATS 陽性のリンパ球のそれに比べて低かった。腸陰窩上皮内の GL 果粒は絨毛上皮内のそれに比べて明らかに大型であった。脾臓では極めて微細な果粒を有する GL がまれにみとめられたが、胸腺、骨髓、脾臓、脾管、胆管および腸管附属リンパ小節には見出されなかった。上記のニワトリ特有の GL の分布および抗胸腺細胞血清に対する反応は、natural killer 細胞のそれらに類似していた。