

## STRUCTURE AND FUNCTION OF THE HEMOLYMPH NODE IN RATS

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### ABSTRACT

Hemolymph nodes (HLs) are unique lymph nodes, in that their lymphatic sinuses contain numerous erythrocytes. In this study, we compared the internal structure and immunologic function of HLs with those of ordinary lymph nodes (OLs) and the spleen. Electron microscopy revealed erythrocytes passing through the walls of blood vessels in the intermediate sinus area (IMSA) of a HL between expanded endothelial cell junctions. However, no direct communication was found between lymphatic sinuses and blood vessels. Numerous carbon particles appeared in the IMSA of HLs on 5 days after intravenous carbon particle injection, while OLs lacked particle deposition. Immunohistochemical studies showed that lipopolysaccharide (LPS) reached the IMSA of HLs and extravasated into medullary cords 4 hours after intravenous LPS injection, resulting in the appearance of more IgM-stained lymphocytes in the IMSA of HLs than in that of OLs on day 5. The ability of organs to produce antibodies was determined by counting the number of plaque forming colonies after intravenous injection of sheep red blood cells (SRBC). The HLs antibody-producing ability was between that of OLs and the spleen. These results suggest that HLs possess functionally open blood vessels in the IMSA and their immunologic capability is between that of OLs and the spleen. These findings suggests that HLs are lymphoid organs that have characteristics between those of the OLs and the spleen, both ultrastructurally and functionally.

Key Words: hemolymph node, rat, immunohistochemistry, lipopolysaccharide, ultrastructure

### INTRODUCTION

Hemolymph nodes (HLs) are histologically different from ordinary lymph nodes (OLs), in that their lymphatic sinuses and medullary cords contain numerous erythrocytes, producing a red macroscopic appearance. Gibbes first described HLs in humans near the renal arteries in 1884.<sup>1)</sup> Robertson named these structures "hemolymph nodes" in 1890.<sup>2)</sup> HLs have also been more often demonstrated in ruminants (including cows and sheep as well as various rodents) than in humans.<sup>3-6)</sup> The concept of HL is currently established in those animals, but poorly defined in humans. HL can be stimulated with intravenous injections of bovine serum albumin to produce reactions similar to those seen in other lymphoid organs.<sup>7)</sup> Therefore, HLs appear to belong to the reticuloendothelial system which includes the spleen and lymph nodes functionally. On the other hand, HLs considerably resemble OLs as a whole. However, erythrocytic

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infiltration in lymphatic sinuses of HLs is also similar to red pulps of spleens under the microscope. In spite of these findings, comprehensive studies concerning HLs have not yet been performed. The goal of this project was to characterize the structure and function of HLs by comparing HLs with other organs of the reticuloendothelial system.

## MATERIALS AND METHODS

### *Animals*

Male Wistar rats eight weeks old (body weight 260 to 360 g) obtained from Nippon SLC (Shizuoka, Japan) were studied. Experimental procedures were performed under diethyl ether anesthesia.

### *Carbon particle administration*

A carbon particle solution was produced by dissolving 120 mg of particulate carbon in distilled water. An amount of this solution containing 20 mg of carbon particles was intravenously injected into the tail vein six times on one day using a different site for each injection.

### *Lipopolysaccharide and its administration*

Lipopolysaccharide (LPS) was extracted from *Klebsiella pneumonia* strain LEN-111(O3:K1)-KO3 LPS by Westphal's phenol-water method<sup>8)</sup> at the Department of Bacteriology, Nagoya University School of Medicine. KO3 LPS was diluted in distilled water to produce a 1 mg/ml solution, and 0.3 ml of this solution containing 0.3 mg of LPS were intravenously injected into the tail vein.

### *Tissue preparation*

HLs, mesenteric lymph nodes, and spleens were excised from animals either 4 hours or 5 days after LPS administration. HLs and mesenteric lymph nodes were excised 5 days after injection of carbon particles. HLs, mesenteric lymph nodes, and spleens were excised from untreated animals served as controls. Excised organs were fixed in 10% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Portions of organs excised from untreated and LPS-injected animals were fixed in periodate-lysine-4% paraformaldehyde. Frozen sections were used for immunohistochemistry. Another portions of HLs excised from untreated animals were observed by electron transmission microscopy.

### *Antibody*

The anti-LPS monoclonal antibody IC6H was kindly provided by Dr. Yokochi (Department of Bacteriology, Aichi Medical University) and used undiluted. Anti rat-IgM monoclonal antibody MCA189 purchased from Serotec (Oxford, UK) was diluted 1:600 with 1% bovine serum albumin in phosphate-buffered saline.

### *Immunohistochemistry*

Immunostaining was performed by using the horseradish peroxidase labelling streptavidin-biotin method. Biotinylated rabbit anti-mouse immunoglobulin absorbed by 5% normal rat serum was used as the secondary antibody. Endogenous peroxidase activity was blocked by immersing sections in 0.3% H<sub>2</sub>O<sub>2</sub> in absolute methanol. Diaminobenzidine (DAB) solution was used as chromogen.

### *Transmission electron microscopy*

Sections for ultrastructural examination were produced by routine methods and examined with a JEM1200EX electron microscope (Japan Electron Optic Laboratory, Tokyo, Japan).

### *Antibody production after sheep red blood cell injection*

The ability of organs to produce antibodies after the injection of sheep red blood cells (SRBC) was estimated using Cunningham's method.<sup>9)</sup> SRBC were diluted in a physiologic salt solution to produce a solution containing  $10^8$  erythrocytes per milliliter. HLs, mesenteric lymph nodes, the spleen and cervical lymph nodes were excised from an animal 4 days after injection of 1 ml of the SRBC solution into the tail vein. The excised organs were minced in a balanced salt solution, and a lymphocyte suspension (LS) was produced. The chamber was created by placing a paraffin sheet with a punched out 1 cm hole on to a glass slide. Target erythrocyte solution containing guinea pig complement was mixed with the LS in the chamber. The mixture was incubated at 37°C for 2 h and the number of plaque forming colonies (PFCs) in the chamber was determined. The procedure was also performed on the LS of untreated animals. The ability of organs to produce antibodies was represented as a ratio of the number of PFCs of SRBC-injected animals to those of untreated animals.

## RESULTS

### *Histologic findings*

Macroscopically, HLs were identified by their red edges (Fig. 1), and were constantly located in three distinct sites: the edge of the thymus, between the spleen and the pancreatic head, and bilaterally near the aorta at the level of the renal artery. These HLs were temporarily named the ventro-mediastinal lymph node (VML), the pancreato-splenic lymph node (PSL), and the lumbar lymph node (LL), respectively (Fig. 2). The dominant vessels supplying these nodes were the common carotid artery, the splenic artery, and the renal artery, respectively.

Using light microscopy, numerous erythrocytes were seen in the lymphatic sinuses of HLs, specifically, in the marginal and in intermediate sinuses, in the medullary cords, and in the

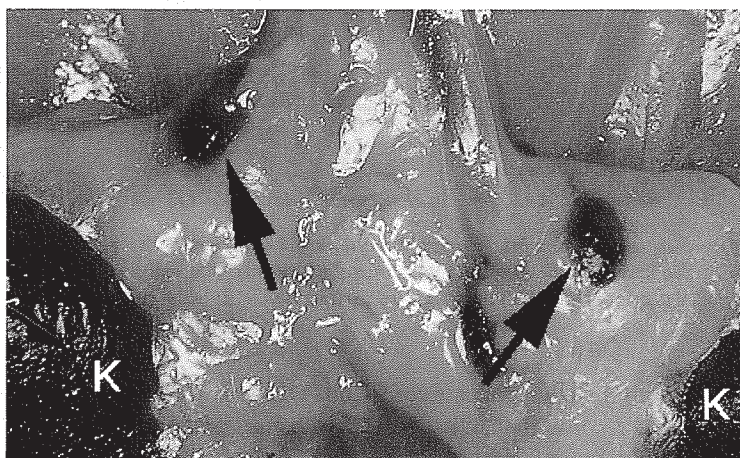


Fig. 1. Gross appearance of a HL. The arrows indicate a pair of lumbar lymph nodes. K; kidney.

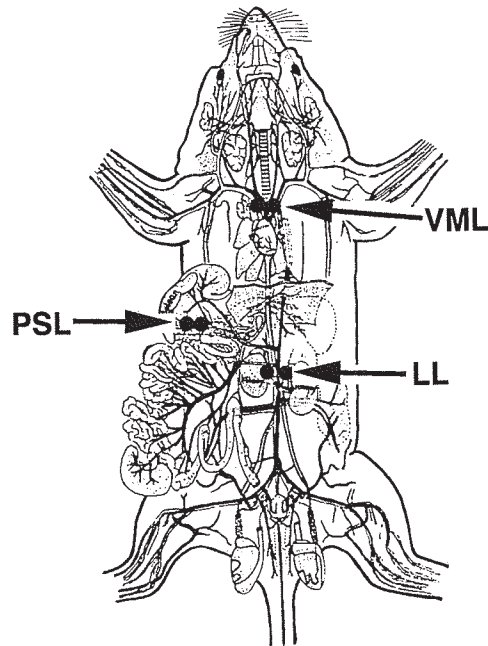


Fig. 2. Location of HLs in rats. VML; ventro-mediastinal lymph node, PSL; pancreato-splenic lymph node, LL; lumbar lymph node.

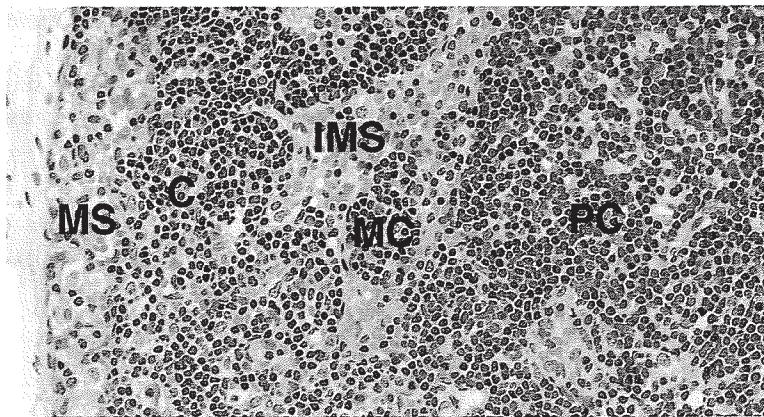


Fig. 3. Histologic appearance of a HL. Numerous erythrocytes are found in lymphatic sinuses, and some are seen also in medullary cords and the cortex (HE,  $\times 100$ ). MS; marginal sinus, IMS; intermediate sinus, C; cortex, MC; medullary cord, PC; paracortex, HE; hematoxylin and eosin staining.

cortices (Fig. 3). Few afferent lymphatics were found. HLs had much larger numbers of dilated vessels in their medullary cords than OLs. No direct communication was found between lymphatic sinuses and blood vessels.



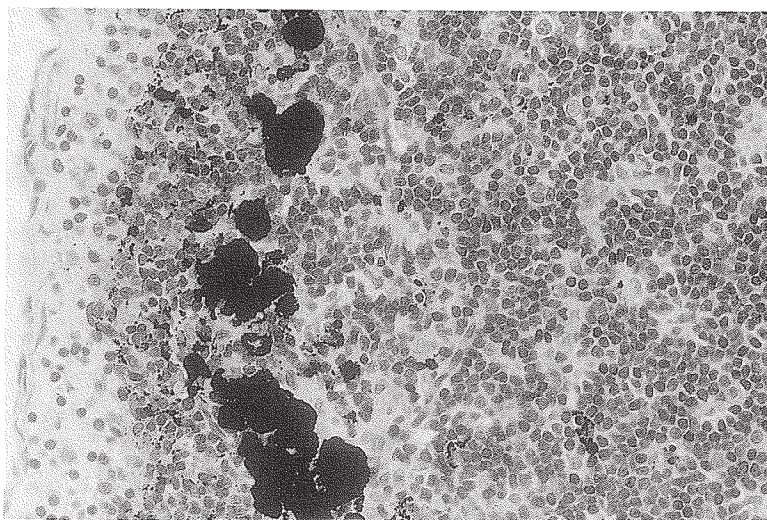


Fig. 4. Microscopic features of a HL on day 5 after carbon injection. Numerous carbon particles collected in the blood vessels of the IMSA, and a few have extravasated into the medullary cords and cortex. Carbon particles were rare in the marginal sinus (HE,  $\times 100$ ). IMSA; intermediate sinus area.

#### *Carbon particle injection*

Carbon particles collected in the blood vessels of HL medullary cords in an area consisting of the intermediate sinus and adjacent medullary cords (intermediate sinus area, IMSA). A small amount of carbon particles was found in medullary cords and cortices, and rare uptake of carbon particles was seen in the marginal sinuses (Fig. 4). In contrast, no uptake of carbon particles was recognized in OLs.

#### *LPS administration*

In LPS-injected animals, immunostaining with the anti-LPS antibody IC6H demonstrated clusters of positive cells in the medullary cords of IMSA 4 hours after injection (Fig. 5a). No reactive cells were found in the marginal sinus. LPS-positive cells were not found in any OLs. Numerous plasma cells and plasmacytoid cells appeared in HLs IMSA 5 days after LPS administration (Fig. 5b). Significantly fewer numbers of these cells were found in OLs. In day 5 HLs, numerous IgM<sup>+</sup> cells appeared, mainly in IMSA (Fig. 5c). The number of IgM<sup>+</sup> cells was much smaller in OLs than in HLs. The distribution of IgM<sup>+</sup> cells corresponded to that of IC6H in HLs. Scarce IgM<sup>+</sup> cells were found in the HL marginal sinuses (Fig. 5d).

#### *Transmission electron microscopy*

Erythrocytes could be seen passing through blood vessel walls via expanded endothelial cell junctions in the HL IMSA (Fig. 6a). The basement membrane of blood vessels of medullary cords in the HL IMSA were thinner and more disrupted than those in the OL IMSA (Fig. 6b).

#### *Antibody production after sheep red blood cells injection*

The number of PFCs after SRBC injection and the antibody-producing ability of each organ are shown in the Table 1. The antibody-producing ability of HLs was intermediate between that



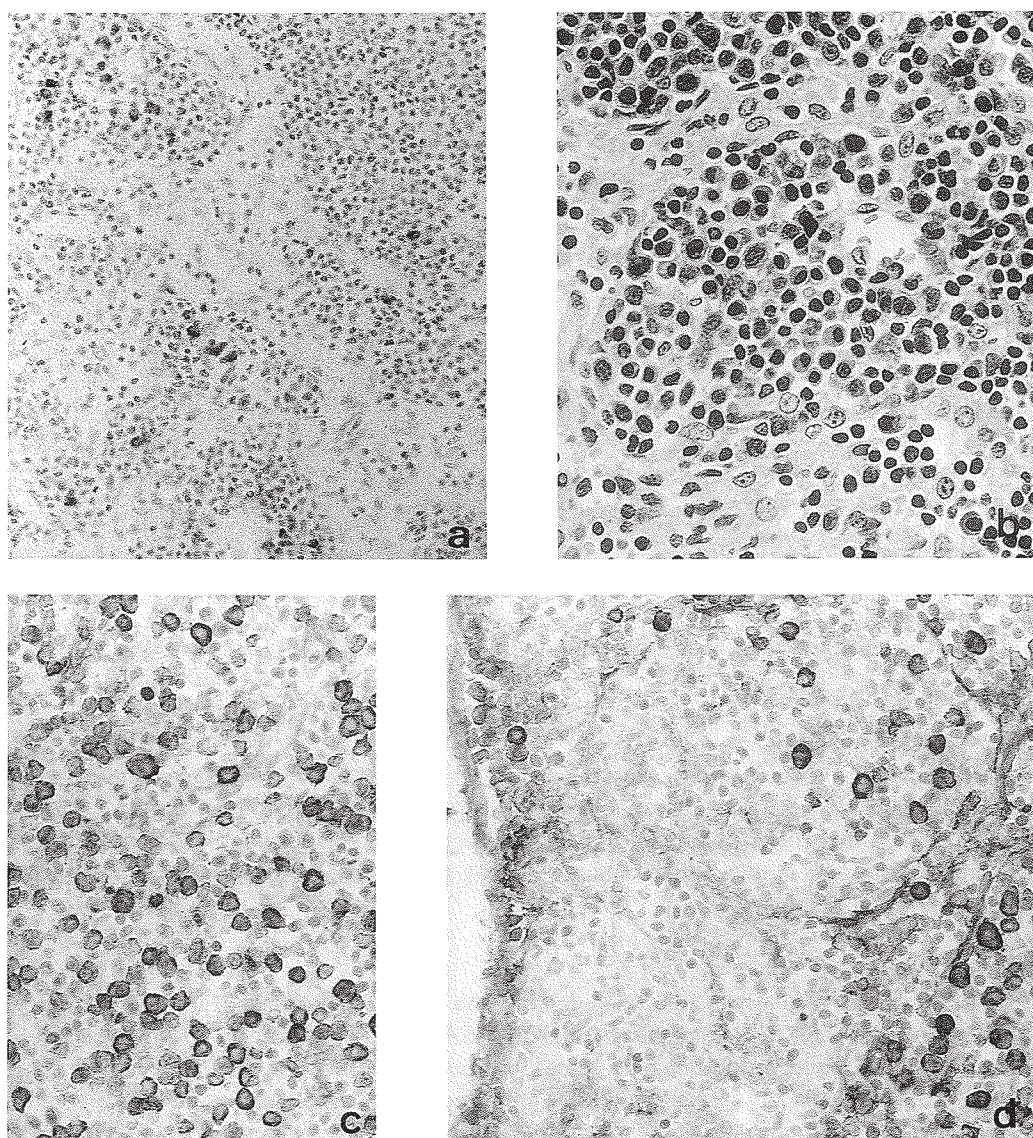


Fig. 5. Microscopic features of a HL after LPS injection. (a) Immunostaining with anti-LPS antibody IC6H 4 h after injection. Clusters of LPS-positive cells appeared in medullary cords of the IMSA (ABC method,  $\times 50$ ). (b) Numerous plasma cells and plasmacytoid cells appeared in medullary cords of the IMSA on day 5 after injection (HE,  $\times 132$ ). (c) Immunostaining with anti-rat IgM antibody MCA189 on day 5. Numerous IgM<sup>+</sup> cells appeared in the IMSA (ABC method,  $\times 100$ ). (d) Immunostaining with anti-rat IgM antibody MCA189 on day 5. The number of IgM<sup>+</sup> cells in the marginal sinus was much smaller than in the IMSA (ABC method,  $\times 100$ ). ABC; avidin-biotin complex.

of OLs and spleens with the exception of LLs. PSLs showed the highest antibody-producing ability of the HLs.



## STRUCTURE AND FUNCTION OF HEMOLYMPH NODE

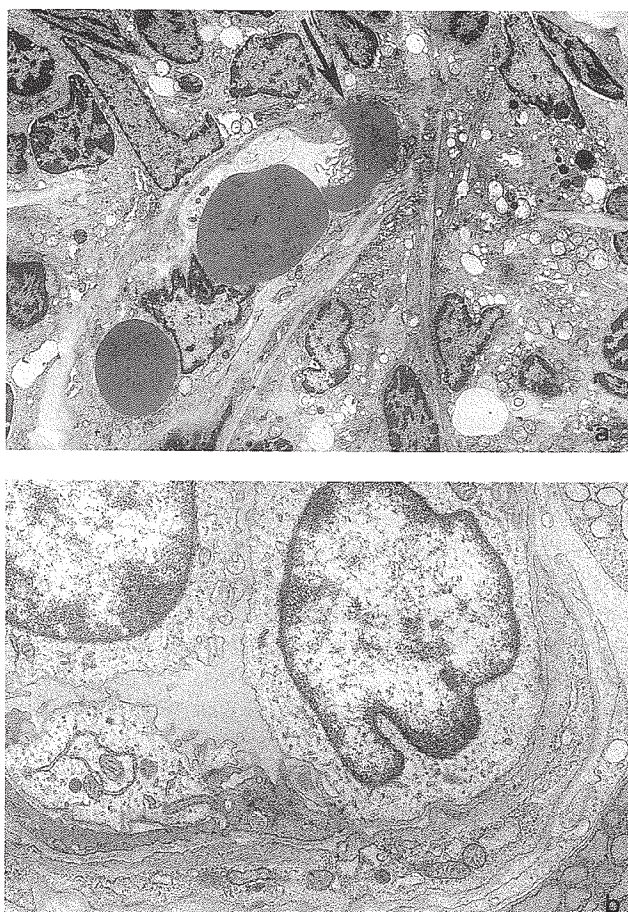


Fig. 6. Electron micrographs of a HL. (a) An erythrocyte passing through the wall of a blood vessel between endothelial cells in the IMSA (arrow)  $\times 4500$ . (b) The basement membrane of medullary cord blood vessels in the IMSA are disrupted  $\times 13000$ .

Table 1. The number of plaque forming colonies in chambers 4 days after SRBC injection and the antibody-producing ability of each organ

Organ		A (SRBC injection)	B (control)	C <sup>1)</sup> (ratio of A to B)
HL	VML	8.6	1.5	5.7
	PSL	96.9	1.2	80.8
	LL	9.2	5.0	1.8
OL	ML	0.9	0.4	2.3
	CL	1.8	0.8	2.2
Spleen		951.6	2.5	380.4

- 1) The antibody-producing ability of each organ is represented as the ratio of the number of PFCs of SRBC-injected rats to that of untreated rats.

HL, hemolymph node; OL, ordinary lymph node; VML, ventro-mediastinal lymph node; PSL, pancreato-splenic lymph node; LL, lumbar lymph node; ML, mesenteric lymph node; CL, cervical lymph node; PFC, plaque forming colonies; SRBC, sheep red blood cells

## DISCUSSION

HLs have characteristic morphology which includes erythrocytic infiltration of the lymphatic sinus. This phenomenon suggests that HLs have specialized vascular connections with the lymphatic sinus that are absent in ordinary lymph nodes. Erythrocytes appeared to enter the intermediate lymphatic sinus of HLs through a special connection between blood vessel wall and the lymphatic sinus. Abu-hilleh found that carbon particles injected intravenously into rats first appeared in the marginal sinus area and that no carbon particles crossed the walls of blood vessels.<sup>10)</sup> He concluded that sinus erythrocytes of HLs may be extrinsic and enter the node like injected carbon particles. However, in our study, a large number of carbon particles collected in the intravascular space and few carbon particles were found in the HL marginal sinuses. These differences may be partially explained by the possibility that a considerable number of carbon particles can leak out of the blood vessels at the time of injection and enter the lymphatics, and reach the HL via the afferent lymphatics.

Our findings reveal that HLs possess an immunologic functional activity intermediate between that of OLs and spleens in response to SRBC injection. Moreover, LPS extravasates into medullary cords of IMSA immediately after LPS injection. LPS is a well-known T-cell independent antigen that produces differentiation of B-lymphocytes to plasma cells without intervening lymphoid follicles 3 to 5 days after intravenous injection.<sup>11)</sup> Alternatively, SRBC is a T-cell dependent antigen that induces the differentiation of B-lymphocytes through lymphoid follicles.<sup>11)</sup> HLs responded more strongly than OLs to both of these antigens with different mechanisms. Carbon particle injection of HLs showed that carbon particles penetrate into medullary cords of IMSA like LPS. These results indicate that HLs may react more strongly to intravascular antigens than OLs, and that the site of carbon particle and LPS extravasation is the IMSA. HLs may react more strongly to intravascular antigens than intralymphatic antigens because HLs possess much smaller numbers of afferent lymphatics than OLs.<sup>12)</sup> The marginal zone of the spleen is a functional space open to the blood stream that allows the erythrocytes to enter the venous sinus without disturbance.<sup>13)</sup> Although the blood vessels of the HL IMSA possess no direct communication with the lymphatic sinus, erythrocytes can pass through expanded endothelial cell junction and disrupted basement membrane. Therefore, the IMSA of HLs correspond to the marginal zone of the spleen. Overall, HLs are quite similar to OLs under light microscopy, but appear to more closely resemble the spleen under ultrastructural examination.

The spleen is an immunoreactive organ centrally situated in the greater circulation that provides an efficient immunologic defense against circulating blood antigens. Lymph nodes are located in the lymph circulation and mediate immunologic responses to only a limited portion of the body.<sup>14)</sup> HLs are also immunoreactive organs that are predominantly located near central body cavities and appear to provide immunological defense against blood antigens passing through each dominant blood supplying artery.

IMSAs of HLs appear to correspond to the marginal sinus of the spleen in that they possess functional communication with the blood stream. Based on the findings of Fujino, the primary immunologic primary response to extrinsic antigens occurs in the areas from the marginal to the intermediate lymph node sinuses.<sup>11)</sup> HLs generally resemble OLs under light microscopy except that erythrocytes occupy lymphatic sinuses of HLs. The results of our experiment showing that HLs possess functional open spaces through which intravascular antigens can pass in the IMSA support the Fujino hypothesis that primary immune responses occur in the areas between the marginal and intermediate sinuses. Our findings also suggest that the IMSA of OLs functionally corresponds to that of HLs.

VMLs are closely situated on edges of the thymus and PSLs are located near the spleen. The two groups of HLs are situated near by large lymphoid organs. Therefore premordia of those



HLs and large lymphoid organs including thymuses and spleens are supposed to be common during embryogenesis although observations of rat embryos were not performed in our studies. Recently the aorta-gonads-mesonephros (AGM) region was proposed as the site where hematopoietic activity occurs earliest during embryogenesis.<sup>15)</sup> Moreover, because the presence of lymphocyte precursors in AGM regions was confirmed by molecular evidences such as RAG-1 gene expression, DNA rearrangements of D, and J genes of IgH locus emergence, AGM regions appear to be important sites of lymphocyte development during embryogenesis.<sup>16)</sup> LLs development during embryogenesis is supposed to be associated with the AGM region on the basis of their location. Overall, it may be possible to argue that many lymphoid organs including HLs develop from the AGM region during embryogenesis. In the future, it would seem important to perform a study of HL development during embryogenesis from this point of view.

### ACKNOWLEDGEMENTS

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### REFERENCES

- 1) Gibbes, H.: On some structures found in the connective tissues between the renal artery and vein of the human subject. *Quart. J. Micr. Sc.*, 24, 186–190 (1884).
- 2) Robertson, W.F.: The prevertebral haemolymph gland. *Lancet*, 2, 1152–1154 (1890).
- 3) Vincent, S. and Harisson, H.S.: On the haemolymph glands of some vertebrates. *J. Anat. & Physiol.*, 31, 176–198 (1897).
- 4) Warthin, A.S.: Normal histology of the human hemolymph glands. *Am. J. Anat.*, 1, 63–80 (1901).
- 5) Sabin, F.R.: The development of the lymphatic nodes in the pig and their relation to the lymph hearts. *Am. J. Anat.*, 4, 355–389 (1905).
- 6) Meyer, A.W.: The hemolymph glands of the sheep. *Anat. Rec.*, 2, 62–64 (1908).
- 7) Turner, D.R.: Immunological competence of the haemal node. *J. Anat.*, 110, 17–24 (1971).
- 8) Westphal, O. and Jann, K.: Bacterial lipopolysaccharides: Extraction with phenol water and further application of the procedure. In *Methods in Carbohydrate Chemistry*, vol. 5, edited by Wistler, R.L., pp. 83–91 (1965) Academic Press, New York.
- 9) Cunningham, A.J.: A method of increased sensitivity for detecting single antibody-forming cells. *Nature*, 207, 1106–1107 (1965).
- 10) Abu-Hijleh, M.F. and Scothorne, R.J.: Studies on haemolymph nodes. IV. Comparison of the route of the entry of the carbon particles into parathymic nodes after intravenous and intraperitoneal injection. *J. Anat.*, 188, 565–573 (1996).
- 11) Fujino, M., Ito, M., Nakagawa, A. and Asai, J.: Kinetics in the regions around lymphatics sinuses caused by antigenic stimulation. *J. Jpn. Soc. R.E.S.*, 36, 187–200 (1996) (in Japanese).
- 12) Nopajaroonsri, C., Luk, S.C. and Simon, G.T.: The structure of the hemolymph node: a light, transmission, and scanning electron microscopy study. *J. Ultrastruct. Res.*, 48, 325–341 (1974).
- 13) Sasou, S., Satodate, R. and Katsura, S.: The marginal sinus in the perifollicular region of the rat spleen. *Cell Tissue Res.*, 172, 195–203 (1976).
- 14) Mori, M.: The ontogeny and basic structure of lymphoid organs. In *Medical Immunology*, 3rd edn. edited by Kikuchi, K., pp. 27–35 (1989), (in Japanese), Nankodo, Tokyo.
- 15) Medvinsky, A.L., Samoylina, N.L., Muller, A.M. and Dzierzak, E.A.: An early pre-liver intra-embryonic source of CFU-S in the developing mouse. *Nature*, 364, 64–67 (1993).
- 16) Marcos, M.A.R., Morales-Alcelay, S., Godin, I.E., Dieterlen-Lievre, F., Copin, S.G. and Gaspar, M.-L.: Antigenic phenotype and gene expression pattern of lymphohemopoietic progenitors during early mouse ontogeny. *J. Immunol.*, 158, 2627–2637 (1997).