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The Red Pulp of the Rabbit Spleen Studied under the Scanning Electron Microscope

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Recent views on the fine structures of the sinus of the spleen are very divergent. Since the studies by WEISS (1957, 1963) some electron microscopists have believed that the sinus wall is formed by longitudinally elongated cells lying against one another's lateral surfaces and that potential gaps between them may allow migration of blood cells through them. WEISS (1957, 1963, 1966), JUNG (1958) and THOMAS (1967) recognized occasional gaps filled neither by a blood cell nor by an endothelial cell, whereas many other authors showed none of such fenestrations. SIMON and PICTET (1964), PICTET and his co-workers (1969) indicated a more constant occurrence of fenestrations through which the sinus lumen is continuous to the cord of Billroth. "Mild interdigitation" between adjacent endothelial cells of the sinus was described by HIRASAWA and TOKUHIRO (1970). GALINDO and FREEMAN (1963), denying the occurrence of intercellular gaps, said that the plasma membranes of the sinus lining cells "interdigitate in much the same manner as the plasma membranes of intestinal and renal tubular epithelial cells." RAPPAPORT (1970), in his recent review article, acknowledges the openings in the sinus wall solely as artifacts.

The views on the structural relation of the sinus to the cord of Billroth are also controversial. WEISS (1957) declared the latter to be nothing but collapsed sinuses, though this opinion was revised in his later paper (WEISS, 1963). Some authors recognized no difference between the sinus and cord channels with regard to the nature and arrangement of their lining cells (GALINDO and FREEMAN, 1963; ROBERTS and LATTA, 1964), although this view was opposed by other authors (BURKE and SIMON, 1970; HIRASAWA and TOKUHIRO, 1970). PICTET and his co-workers (1969), and HIRASAWA and TOKUHIRO (1970) stressed that the endothelial cells of the sinus and the reticulum cells of the cord are cells with distinctly different structures and functions.

Most electron microscopists seem to have ignored the excellent studies by light microscopists on the structure of the splenic pulp (see Discussion) and to have caused more confusion than elucidation of problems. Thus a large gap has occurred between the light and the electron microscopic knowledges.

This gap is thought to be filled by scanning electron microscopy which provides the three-dimensional visualization of structures which has been lacking in the conventional methods using thin sections and affords magnifications ranging from those of the magnifying glass to those of the electron microscope.

The present authors, observing the cut surfaces of the kidney with the scanning electron microscope, succeeded in revealing the fine structures of the glomerular

podocytes (FUJITA, TOKUNAGA and MIYOSHI, 1970), and this method seemed fully applicable also to the cut surfaces of the spleen, provided that the blood be eliminated from the organ beforehand.

Among various problems expected to be studied with this methodology is the one reported in this paper, the structure of the sinus wall and its relation to the cords of Billroth in the spleen.

In this paper, the lining cells of the sinus (rod cells) will be called endothelial cells for the convenience of description, though they may be situated between endothelial and reticular cells in their nature.

Materials and Methods

Spleens observed in this study came from adult rabbits weighing 2–3 kg. All animals were placed under surgical anesthesia by the intraperitoneal administration of 10 ml of Nembutal.

To remove the blood plasma and free cells and to fix the splenic tissue in situ, the spleen was perfused by the lienal artery with 300 ml of warmed Ringer solution and then with 100 ml of fixative.

The lienal vein was cut when the perfusion was begun. During the perfusion of Ringer solution, the spleen was gently massaged to ensure a complete removal of blood. After ligation of the lienal vessels, the whole spleen was removed and left in fixative for additional 2–6 days at room temperature. The fixative used in perfusion and immersion was 2.5% glutaraldehyde adjusted to pH 7.4 with 0.1 M phosphate buffer containing 50 ppm of CaCl_2 .

The fixed spleens were cut with a razor blade into small cubes of tissue (about $5 \times 3 \times 3$ mm) and their cut surfaces were washed by a gentle jet of 0.1 M phosphate buffer. All the specimen cubes were dehydrated with a series of graded acetone, dried in air (BARBER and BOYDE, 1968; FUJITA, INOUE and KODAMA, 1968) and kept in a desiccator containing Sillica gel granules.

Under a binocular stereoscope an adequate surface was selected on every specimen. Carbon and gold were evaporated at an angle of 45° against the samples. During the evaporation, the plate sustaining the samples was motor-driven into a combined rotating and rocking movement in order to attain an even metal coverage.

Observation and photography were made with a scanning electron microscope, Type JSM-2 of the Japan Electron Optics Laboratory Co. Accelerating voltage used was 9–25 kV.

Observations

Scanning electron microscopy of the cut surfaces of the rabbit spleen revealed winding furrows and caves of various sizes and shapes and areas of spongy structure among them (Fig. 1). The walls of the former were smooth in surface and regularly perforated. It was obvious that they corresponded to the splenic sinuses cut open, whereas the spongy structure corresponded to the cords of Billroth.

The wall of the splenic sinus (Fig. 2, 6) appeared lattice-like with oval perforations of variable sizes. The longitudinal and main component of this lattice was formed by flattened rods or, more precisely, belts which measured 2μ wide and 0.2μ thick (Fig. 2, 3, 6). These rods ran generally in parallel to each other and to the long

Fig. 1. A low-power scanning electron micrograph of a cut surface of the red pulp of the rabbit spleen. Winding furrows or caves with their wall of a lattice-like appearance are sinuses cut open (S), among which the cords of Billroth (C) show a spongy profile. $\times 360$

axis of the sinus, keeping a certain distance between adjacent ones. The transverse component was represented by processes of different width, issued rectangularly from the opposed lateral margins of the adjacent rods and connected with each other. In many places the processes of the rods were arranged in regular transverse rows forming a typical lattice (Fig. 6, 7). It was evident that the longitudinal rods with their side processes represented the endothelial cells of the sinus, as a spindle-like protrusion corresponding to their nuclei occurred on each rod (Fig. 4).

The nuclear protrusions were elongated and measured about 8μ long, 4μ wide and 3μ thick (Fig. 2, 4). They were mostly situated sporadically on the sinus wall. Sometimes, however, they were grouped at a certain area of the sinus wall, especially in a sinus of relatively large dimension (Fig. 3). Here, the nuclear protrusions of adjacent cells tended to line up alternately in two transverse rows. Thus the tapered ends of the spindles of elevations appeared interdigitated with each other (Fig. 4).

Fig. 2. A part of the red pulp showing larger and smaller sinuses (S) and splenic cords (C). The nuclei of the sinus endothelium are seen as spindle-shaped protrusions disseminated over the sinus wall. Arrows indicate orifices of the interconnecting channels on the sinus wall. $\times 1,100$

As mentioned above, the rods of the endothelial cells were loosely arranged and connected by side bridges. It was frequently seen, however, that neighboring rods were fused for a considerable length or a tapered rod was fused aslant with an adjacent one (Fig. 6, 7). These fused rods were recognized as such, as their axial portions were slightly elevated and intervened by a faint furrow. The occurrence and extent of the lateral fusion of the neighboring rods varied among sinuses, or even among areas of a sinus wall.

The side bridges or the lateral connections of rods occurred segmentally at a fairly constant interval of a few micra. In contrast to the rods of a relatively similar size, the side bridges were quite variable in thickness (Fig. 6, 7). Most of them were thin plates, a half micron wide. Occasionally there were bridges which were far wider than the longitudinal rods. A faint crest traversing the middle of the bridge was sometimes recognized (Fig. 7).

As the corners formed by the rod sides and the bridges were rounded by a lip-like attenuation of cytoplasm, the perforations delineated by them also were rounded in shape (Fig. 6, 7). The perforations were generally elongated and roughly measured $3 \times 1.5 \mu$, though they greatly varied in shape and size among areas of sinus wall or even among neighboring openings. There often occurred slits of 3μ length or small pores of 0.5μ diameter. The diversity of the shape and size of the openings appeared to correspond to that of the side bridges.

The luminal surface of the endothelial cells was delicately uneven. Tiny, wart-like projections, $200 \text{ m}\mu$ in diameter occurred sporadically on the cell surface (Fig. 4, 7). Slender projections $150 \text{ m}\mu$ thick and mostly $500 \text{ m}\mu$ long, also occurred but they were rare on the nuclear elevation. With a thickened and rounded tip these may be called drumstick microvilli. There seemed to be graduated forms between the

Fig. 3. A part of the red pulp showing a corridor of sinus wall extended from the upper right corner to the lower left, on which three groups of nuclei are observed. In the upper left, the reverse or cordal surface of two sinuses (*) is seen attached by the feet of reticular cells (rc) forming the network of the cord of Billroth. In the lower right, a labyrinthine meshwork of the splenic cord is seen. $\times 1,300$

warts and the drumsticks. Besides, a few cell projections as long as 3μ or more and less than $150m\mu$ thick were extended over the neighboring rods. These long threads mainly occurred on the lateral sides of the nuclear elevation (Fig. 4, 5).

Fig. 4. A closer view of the nuclear group in the center of Figure 3. The protrusions of adjoining cells are interdigitated alternately. Transverse processes of endothelial cells and the perforations formed by them are also obvious. Thread-like, drumstick-shaped and wart-like cytoplasmic projections are numerous. $\times 3,900$

Furthermore, it was seen in high-power micrographs (Fig. 5, 7) that the luminal surface of the endothelial cell was pitted here and there as if pricked by a needle. The pits were sometimes conspicuous on the nuclear elevation.

The inner surfaces of the sinuses showed orifices of smaller and larger sinal branches (Fig. 2, 8). These round, dark tunnels, mainly 10μ in diameter, were also lined by the lattice-like endothelium. As they were obliquely ramified from the sinus, a sharp, arched crest was formed on one side of the orifice (Fig. 9). On the opposite side, the sinus wall gradually sank into the branch wall, and the rods on the former were extended straightly into the latter. Towards the arched crest, however,

the rods turned almost parallel to the arch, and only one or two of them gently twisted and entered the tunnel to join its lining (Fig. 9A, 10A). However, in cases of very small branches, rods at their orifices were not twisted. A large rod on the sinus wall was bifurcated to form the crest of the orifice and further extended straightly to join the rows of general rods of the sinus, while a few of the latter smoothly entered the small tunnel (Fig. 9B, 10B).

Fig. 5. A high-power view of nuclear protrusions and their neighboring portions of the endothelial cells. Some small pits (arrows) and wart-like projections are seen on the protrusions. Note many drumstick-like processes at the margin of the rods and thread-like ones emerging from the lateral side of the nuclear protrusions. *R* a cell in the reticular tissue. $\times 13,000$

Sometimes, a crescent-shaped membranous layer was seen close behind the fenestrations of the sinus (Fig. 7). Its luminal surface was much smoother than that of the endothelial layer. This layer seemed to represent a part of a reticular cell which was closely attached to the back side of the endothelium.

Spongy structure with labyrinthic meshwork represented the Billroth's cord. Three-dimensionally visualized were the reticular cells of stellate form anastomosed

with each other (Fig. 3, 11A). Occasionally in the cord a few rough-surfaced blood cells were encountered (Fig. 11B, 12).

In contrast to the sinus endothelial cell, the reticular cell had strand-like cytoplasm so slender, about $0.8\text{--}1\mu$ thick, that it was difficult to define the perikaryal portion of the cell (Fig. 11A, 12).

The cord space just around the sinus was septated by the foot-like processes of the reticular cells, extended almost rectangularly to the sinus wall with a fairly constant interval of from 2 to 3μ (Fig. 11B). The reticular cell feet ended in a sole-like expansion which was applied to the outer surface of the sinus endothelial lattice (Fig. 11B, 12, 13).

Fig. 6 Inner surface of a sinus showing a typical lattice structure. Slender rods of endothelial cells of similar size run parallel with each other, keeping a fairly constant interval. The rods are connected by side processes of various width. A tapered endothelial cell (e_1) is fused obliquely with the adjacent cell (e_2). Perforations are aligned along the extension of the endothelial cells. $\times 6,100$

Fig. 7. A close-up of the central portion of Figure 6. A shallow furrow intervenes the fused two cells (e_1 and e_2), and faint crests (arrows) are noticed at the middle of some side bridges. In three perforations at the bottom, their margin is duplicated by the occurrence of the reticular cell cytoplasm attached from outside (R). $\times 13,200$

Discussion

Clear demonstration of the structure of the sinus wall is attained only after the spleen is washed away through blood vessels and then perfused with a fixing solution under certain pressure as was ascertained by early histologists (Woronin, 1898; Mollier, 1911). In the case of scanning electron microscopy this procedure is inevitable. The washing should be very thorough as even a small amount of blood plasma remaining in the sinus, when dried, covers its surface fine structure. Among laboratory animals the rabbit is known as most adequate for complete perfusion, because in this species the sinus network is extensively developed as compared with the Billroth's cords (v. Herrath, 1935; Tischendorf, 1969).

In the preliminary study the present authors perfused the spleens of the dog and the monkey for scanning electron microscopy, but a complete elimination of

blood elements, which was not very difficult in the rabbit, was not attained. In the paper by SAKAGUCHI and WATANABE (1969), which, as far as is known, contains the only hitherto published scanning electron micrographs of the spleen, the blood elements are not completely eliminated from the sinus wall of the rat they observed, in spite of their efforts in perfusion of the organ.

A lattice structure of the endothelial cytoplasm of the sinus as shown in the

Fig. 8. Sinus wall showing three orifices of connecting channels (arrows). In the upper right, the cordal side of a sinus wall is seen (*), which also has a lattice-like appearance with perforations C splenic cord. $\times 1,320$

present scanning electron microscope study may appear strange to those who are accustomed to the histology textbook descriptions of the sinus wall as being formed by longitudinally extended rod-shaped cells running in parallel with each other and leaving spaces in between, and by transverse reticulum fibers (ring fibers) binding them from outside (POLICARD, 1950; AREY, 1963; TOGARI, 1964; WEISS, 1966; BLOOM and FAWCETT, 1968; HAM, 1969; OSOGOE, 1969; BUCHER, 1970). Only some German-written textbooks (STÖHR, v. MÖLLENDORFF and GOERTTLER, 1963; BARGMANN, 1964) describe the sinus endothelial cells as having transverse processes of cytoplasmic nature connected in the form of a lattice. This discrepancy of views is based simply on species differences of the sinus wall structure which is briefly mentioned in the textbook of STÖHR, v. MÖLLENDORFF and GOERTTLER.

Fig. 9. Closer views of the orifices of the sinus branches in Figure 8. **A.** The endothelial cell e_1 and some other cells are twisted to form the crest of the orifice seen in the upper left. In the lower orifice two cells (e_2 and e_3) are fused and extended along the crest of the orifice, while another neighboring cell (e_4) is bent at the orifice to form the roof of the side branch. Arrows indicate the cut ends of endothelial cells. In **B**, a large endothelial cell (e_5) is seen bifurcated at the border of a small orifice to form the lateral margins of the orifice. Another small cell (e_6) is fused with the large one. $\times 3,960$

Fig. 10. Wall structure in the orifice of the side branch of splenic sinus. The spaces between the rods are dotted. The side branches and the nuclear swellings of the rod cells are omitted in this diagram.

It seems generally forgotten that 60 years ago MOLLIER (1911) clearly demonstrated in the dog a rectangular lattice pattern of endothelium formed by rods and their side processes. In some other animals, such as sheep and cattle, he saw a reticular pattern of sinus endothelium caused by random cellular processes. MOLLIER revealed that only in man and monkey did the cytoplasmic processes of the sinus endothelial cells disappear

during ontogeny and did the cells become literally rod-shaped, the transverse element being represented only by ribbon-like reticulum fibers or ring fibers. (MOLLIER's way of description was partly different from this as he regarded the rods not as individual cells, but as forming a large syncytium.)

The present scanning electron micrographs of the rabbit sinus clearly represent the lattice pattern of endothelium, i.e., the dog type of MOLLIER. Though MOLLIER himself gave no description on the rabbit sinus, it was later known to have an endothelial structure similar to that of the dog (HARTMANN, 1930). It may now be of high interest to examine the sinus wall of human type under the scanning electron microscope, especially to reveal whether the rod cells are devoid of even the finest processes traversing the interspaces of the rods. Although the transmission electron microscope study of HIRASAWA and TOKUHIRO (1970) treats with the sinus wall of the human spleen, these problems are not answered from their micrographs, neither are they aware of the peculiarity of the sinus structure of man in light microscopy.

The pattern of termination of the ends of the rod cell has not previously been demonstrated by the light or the electron microscope as pointed out by HARTMANN (1930) and TISCHENDORFF (1969). The aslant fusion of rods shown in the present study may represent at least one of the modes of rod termination.

The endothelial cells of the rabbit sinus showed wart-like microprojections and drumstick-shaped microvilli under the scanning electron microscope. These seem not to have been described clearly in previous transmission electron microscope studies but may correspond to the cytoplasmic projections shown in the paper of HIRASAWA and TOKUHIRO (1970). The significance of these conspicuous structures of the cell is not known. Neither is known the nature of the hitherto unknown threads of enormous length issued from the nuclear portion of the cell. A possibility cannot be eliminated that some granular substances on the cell surface may represent trace of blood plasma coagulated by fixation and attached to the surface by drying. The tiny pits found on the cell surface may possibly represent pinocytic caveolae, which have been recognized by transmission electron microscopy (WEISS, 1963; HIRASAWA and TOKUHIRO, 1970).

Some light microscopists believe that the endothelial lattice of the splenic sinus represents a syncytium (MOLLIER, 1911; LEWIS, 1957). From the present view-point of cytology, however, it seems probable that the transverse bridges in the sinus

lattice contain an intercellular contact, though this was suggested in the present micrographs by a slight surface ridge in only a few places. It is further likely that this contact place of cells, if present, may have a special attachment structure such as desmosome, as this site appeared highly resistant to tension caused by perfusion

A

B

Fig. 11. A. A closer view of the center of Figure 1 showing the labyrinthine meshwork of splenic cord intercalated between the sinuses (S). The feet of reticular cells are extended to the sinus wall. **B.** The feet of reticular cells (arrows) stand perpendicular to the endothelial wall of a sinus (S). BC blood cells with granular surface. **A:** $\times 2,400$, **B:** $\times 3,200$

12

13

Fig. 12. A scanning electron micrograph showing a large and a small sinus (S) surrounded by reticular tissue. Note delicate cytoplasmic feet of reticular cells, septating the perisinus space with a constant interval. A free cell with spherical cell body is seen near a possible perikaryon of reticular cell (*pk*). $\times 3,600$

Fig. 13. A micrograph showing the cordal surface of sinus wall. The perforated endothelium is attached by the feet of reticular cells (*rc*) and their stumps (arrows). $\times 3,000$

with saline and fixative. As far as the authors know, this was recognized in none of the previous transmission electron microscope studies (WEISS, 1963; GALINDO and FREEMAN, 1963; THOMAS, 1967). It should be taken into account, however, that most, if not all, transmission electron microscopists have been influenced by the erroneous view that the sinus wall of the laboratory animals which were used was formed by a simple palisade of rod cells without transverse connections, i.e. by human type endothelium.

Diversity in the interpretations of transmission electron microscopists on the intercellular spaces or perforations in the sinus wall was shown in the Introduction. The present study strongly suggests that the openings of the sinus wall of the rabbit are permanent structures, though it is likely that their size and shape may vary in accord with alterations of blood pressure in the sinus (OBERNIEDERMAYER, 1926; KNISELEY, 1936; BJÖRKMAN, 1947). A possible contractility of the endothelial cells (WEIDENREICH, 1901; FOOT, 1925; v. HERRATH, 1935) may also change them. It is also evident in the present micrographs that the sinus lumen is directly connected to the spaces of the Billroth's cord as has been known since MOLLIER (1911). Since WEIDENREICH (1901) some authors have believed in the existence of a thin membrane covering the endothelial fenestrations but this now seems to be an illusion.

As was suggested by light microscopy (HARTMANN, 1930) and confirmed by transmission electron microscopy, the sinus endothelium is attached from outside by reticulum fibers and the latter are covered, in their turn, by the processes of reticulum cells of the Billroth's cord. Thus, a reticulum fiber is sandwiched by an endothelial and a cord cell (WEISS, 1957, 1963; STOECKENIUS, 1958 and others). The lip-like structure which appeared close behind the endothelial fenestration as shown in Figure 7 probably corresponds to one of those cord cells backing the sinus wall.

The present scanning electron microscope study did not reveal an arterial capillary running through the cord of Billroth. Further studies with this microscope are expected, however, to be able to tackle the problem of whether the arterial endings are "open" or "closed." It is also expected that this methodology may throw light on the problem of the sphincter mechanism postulated at the connections between the sinus and its branches (KNISELEY, 1936). As shown in this study the twisted endothelial cells in the arched border between the sinus and its branch may be related to the spiral arrangement of endothelial cells in the corresponding site suggested by BJÖRKMAN (1947), HERRLINGER (1949) and TISCHENDORFF (1959) in their study under the light microscope.

Summary

The spleen of the rabbit was thoroughly washed with physiological saline via the lienal artery and then perfused with 2.5% glutaraldehyde. After ligation of the lienal vessels the excised spleen was further fixed for several days. Observation under the scanning electron microscope of small cubes cut from the spleen gave the following results on the fine structure of the red pulp:

1. The wall of the splenic sinus revealed the structure of a perforated lattice formed by longitudinally extended rod cells and by their transverse cytoplasmic processes.
2. The nuclei of the rod cells were conspicuously projected into the sinus lumen

and tended to be grouped in certain areas of the endothelium.

3. The transverse processes of neighboring rod cells were connected into intercellular bridges; the possible cell boundary was suggested only occasionally by an inconspicuous crest on the surface.

4. A characteristically twisted course of the rod cells was found at the corner of sinus ramifications and was thought to be related to the sphincter mechanism of the splenic sinus.

5. The luminal surface of the rod cells was provided with irregular microprojections, some of which were drumstick-shaped microvilli while others were cytoplasmic threads of conspicuous length.

6. The perforations in the lattice of the sinus represented direct continuations of the sinus lumen to the spaces in the cord of Billroth which contained a few blood cells.

7. The reticulum cells of the cord sent their foot-like processes from outside to line the sinus lattice. No naked reticulum fibers could be recognized in the red pulp.

走査電子鏡によるウサギの赤脾髄の観察 (内容自抄)

ウサギの脾臓に脾動脈から生理食塩水を環流して血液を十分に洗いだし、続いてグルタルアルデヒドを注入して固定した。この脾臓の切断面を走査電子鏡で観察して次の結果を得た。

1. 脾洞壁には多数の孔をもつ格子様構造が見られた。この構造は縦方向に走る杆状細胞と、これを横方向に結ぶ側突起から成る。

2. 杆状細胞の核は洞腔に強く突出していた。細胞核は洞内面に散在して見られるが、場所によって集団をなすことがあった。

3. 隣りあう杆状細胞は側突起による細胞間橋で結合されていた。この間橋を横切る低い高まりを認めることがあった。おそらく細胞境界を示すものであろう。

4. 脾洞の分枝がみられ、その分れ口の壁には、特徴あるねじれた走行を示す杆状細胞があった。この位置での杆状細胞の捻転は脾洞の血流調節に働く括約機序に関係あるものと考えられる。

5. 杆状細胞の表面にはいろいろの大きさの細胞突起があった。ほとんどは微絨毛とよぶべきものであるが、きわめて長い紐状の突起もあった。

6. 脾洞腔は格子様構造の孔を通して脾索の細胞間腔と直接交通していた。この孔に血球が見られることがあった。

7. 脾索の細胞は突起を伸ばして洞壁の外側面についていた。細網細胞に包まれず、むき出しになっている細網線維は認められなかった。

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