

## **Morphological study of erythrocyte shapes in red pulp of mouse spleens revealed by an in vivo cryotechnique**

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**Summary.** The purpose of this study was to clarify erythrocyte shapes in splenic cords of living mouse spleens, using our in vivo cryotechnique followed by scanning (SEM) or transmission (TEM) electron microscopy. Some spleens of mice were quickly frozen by the in vivo cryotechnique while their hearts were beating under anesthesia. In contrast, other spleens were prepared by an in vitro freezing method after they were taken out from the abdominal cavity. They were routinely freeze-substituted, and prepared for SEM and TEM. A few mouse spleens were also routinely fixed and embedded in Quetol-812 to obtain conventional morphology. Erythrocytes in living mouse spleens showed a variety of shapes with narrow spaces between them, trapped among reticular fiber tissues. Similar various shapes of erythrocytes were kept in the red pulp even after blocking normal blood circulation, as prepared by the in vitro freezing method. In comparison to the above-mentioned findings, some erythrocytes were changed to biconcave discoid shapes by the conventional immersion fixation with chemical fixatives. They also showed wide spaces between each other among reticular fiber tissues. Such conventional morphological studies could hardly reveal the in vivo shapes of erythrocytes in functioning spleens with normal blood circulation. In contrast, the various shapes of erythrocytes in the functioning spleens were demonstrated by our in vivo cryotechnique. It is suggested that most erythrocytes congesting in spleens keep their original configuration in spite of microenvironmental alteration in splenic blood circulation.

**Key words:** Cryofixation, Erythrocyte shapes, Biconcave discoid, Spleen

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### **Introduction**

Erythrocytes in the circulating blood must have an ability to withstand some frequent deformation forces during lots of passages through the vascular system in their life span. It is also well-known that aged erythrocytes are selectively removed from the blood circulation by phagocytosis of splenic macrophage systems. In living animals, the spleen serves as a complex biological filter interposed in the blood circulation to clean the aged erythrocytes. Some investigators studied erythrocyte deformability in artificial microcirculation by various physiological methods which could be compared with that of erythrocytes flowing through spleens (Kubota et al., 1996; Suzuki et al., 1996). Others also performed physiological researches on altered properties of the aged erythrocytes (Kosower, 1993; Ghailani et al., 1995). Although some light microscopic studies of erythrocytes in the spleen were performed by high-resolution video-microscopy (MacDonald et al., 1987), there has been no detailed examination of erythrocyte shapes in the red pulp of spleens of living animals. Therefore, it is necessary to reveal the in vivo morphology of erythrocytes flowing in the spleens.

We have already developed an in vivo cryotechnique to examine erythrocyte shapes in large blood vessels and hepatic sinusoids in their functioning state (Terada et al., 1998; Xue et al., 1998). The erythrocytes flowing in the hepatic sinusoids and the abdominal aorta exhibited a variety of shapes including ellipsoid during normal blood circulation. However, biconcave discoid shapes of erythrocytes promptly appeared in the sinusoids after the mouse heart arrest, and were also seen in the inferior vena cava of living mice. In the present study, we have used the same in vivo cryotechnique to examine erythrocyte shapes in the red pulp of mouse spleens. For comparison with these findings, we also prepared other splenic specimens using an in vitro freezing method and the conventional immersion fixation

method.

### Materials and methods

A total of 11 BALB/c female mice, aged 6-8 weeks, were used for the present study and were divided into 3 groups. The 1st group (n=6) was prepared for the *in vivo* cryotechnique. The 2nd group (n=3) was prepared for the *in vitro* freezing method, and the 3rd one (n=2) was for the conventional immersion fixation. They were all intraperitoneally anesthetized with sodium pentobarbital (50 µg/kg body weight).

#### *In vivo* cryotechnique

The abdominal cavity was opened, and a spleen was placed on a small plastic plate without disturbing blood vessels. The *in vivo* cryotechnique was performed under the physiological condition of their heart beating, as previously described (Ohno et al., 1996). Briefly, the spleen was cut and simultaneously frozen by a cryoknife edge precooled in liquid nitrogen (-196 °C), while the heart was normally beating (Fig. 1a). The prepared liquid isopentane-propane cryogen (-193 °C) was immediately poured over the spleen, as reported before (Ohno et al., 1996). A large amount of the applied cryogen remained along the cryoknife edge and participated in the freezing process. This was followed by liquid nitrogen application to prevent a rise of the low temperature. The well-frozen splenic tissue parts were trimmed with a nipper in liquid nitrogen, preserved in the liquid nitrogen and then submitted to the following routine freeze-substitution, as described below.

#### *In vitro* freezing method

While the mouse heart was normally beating, the spleen was removed from the abdominal cavity, and then placed on a plastic plate (Fig. 1b). It was immediately cut and frozen with a cryoknife, as described above. The frozen specimen was also preserved in liquid nitrogen for the routine freeze-substitution.

#### Freeze-substitution method

Both frozen samples prepared by the *in vivo* cryotechnique and the *in vitro* freezing method were freeze-fractured with a scalpel in liquid nitrogen and transferred into absolute acetone containing 2% osmium tetroxide at -80 °C. They were routinely freeze-substituted at -80 °C for 20h, and then their temperature was raised up to -20 °C for 2h and 4 °C for 2h. Finally, they were raised to room temperature and washed in pure acetone 3 times, once every 10 min.

#### Conventional immersion fixation method

Spleens of 2 mice were removed from their abdominal cavity, and then cut into small pieces with

razor blades. They were prefixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (PB), pH7.3, at 4 °C for 2h and then washed with 0.1M PB 3 times, once every 10 min. They were post-fixed with 1% osmium tetroxide in 0.1M PB for 2h and routinely dehydrated in a graded series of ethanol (from 50% to 100%) at room temperature, once every 10 min.

#### Scanning (SEM) or transmission (TEM) electron microscopy

After the freeze-substitution method or the conventional dehydration steps, some specimens were transferred into t-butyl alcohol, freeze-dried in an ES-2030 apparatus (Hitachi Ltd., Japan), ion-sputtered with platinum/palladium (10-15nm in thickness) and examined in an S-4500 scanning electron microscope (Hitachi Ltd., Japan) at an accelerating voltage of 5kV. Other specimens were embedded in Quetol-812. Ultrathin sections were routinely prepared, stained with uranyl acetate and lead citrate and observed in an H-600 transmission electron microscope (Hitachi Ltd., Japan).

### Results

Scanning electron micrographs of the frozen splenic tissues showed relatively well-preserved surface areas, which had been freeze-fractured along the cut and frozen tissue surface by a scalpel in liquid nitrogen (Figs. 2b, 3, 4). The well-frozen areas were obtained in the initially contacted surfaces with the precooled cryoknife followed by the isopentane-propane mixture (-193 °C) during the *in vivo* cryotechnique procedure, as confirmed by crossly viewing on ultrathin sections (Fig. 2a).

An overall image of erythrocyte shapes in the red pulp of spleens, which had been prepared by the *in vivo* cryotechnique, was obtained in low magnified electron micrographs (Fig. 2). Various shaped erythrocytes were closely localized in the splenic pulp cords. In an electron micrograph of TEM (Fig. 2a), some ice crystal

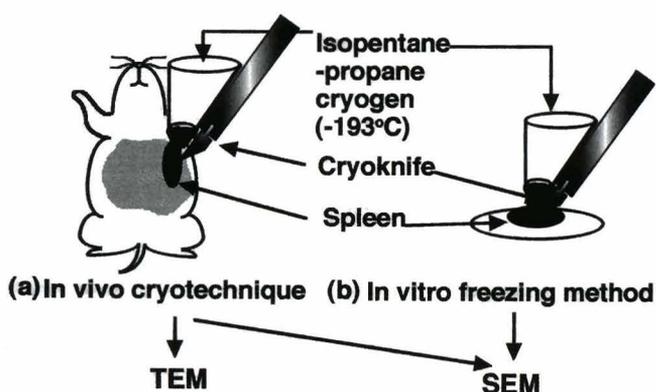


Fig. 1. Schematic representation of the *in vivo* cryotechnique (a) and the *in vitro* freezing method (b).

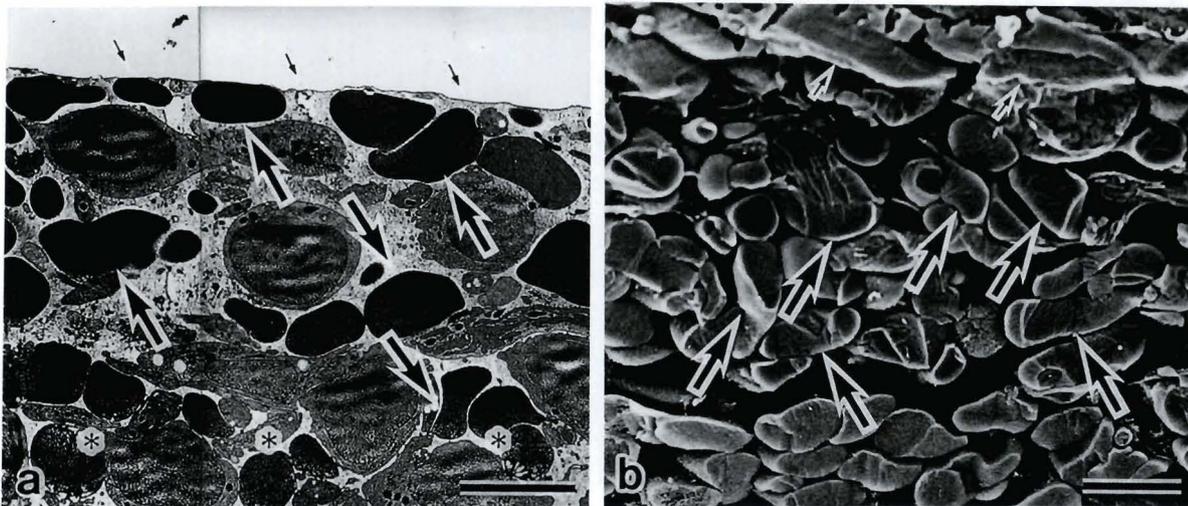
damage was recognized in deeper tissue areas beyond the well frozen part with a depth of 5-8  $\mu\text{m}$ . The 3-dimensionally viewed red pulp areas with SEM were obtained on the freeze-fractured splenic tissue surfaces (Fig. 2b). At high magnification, variously shaped erythrocytes were observed among elaborate parts of reticular tissues in the splenic pulp cords (Fig. 3). There were relatively fewer spaces among reticular cells, reticular fibers, and erythrocytes (Fig. 3a-c).

To compare such erythrocyte shapes under apparently normal blood circulation conditions, erythrocytes in spleens removed from the mouse body before the freezing step were also examined by SEM, as described in Figure 1b. Although the blood flow into the spleens was completely stopped, we obtained similar morphological findings to those revealed by the *in vivo* cryotechnique (Fig. 4). Various shaped erythrocytes were observed in the splenic pulp venule near endothelial cells (Fig. 4a) and among reticular tissues (Fig. 4b). The morphological feature of accumulating erythrocytes in the spleens indicated no typical biconcave discoid shapes, which were closely located near endothelial cells or reticular cells.

On the contrary, typical biconcave discoid shapes of erythrocytes were often detected in the splenic cord by the conventional fixation method (Fig. 5). Some erythrocytes appeared to be extravasated during the tissue preparation procedure, and other remaining erythrocytes were clearly seen in frameworks of bundled reticular fibers throughout the splenic pulp cords (Fig. 5a). The erythrocytes were loosely localized among the frameworks, and showed two types of morphological contours, such as typical biconcave discoid shapes and shrunken peculiar or irregular ones.

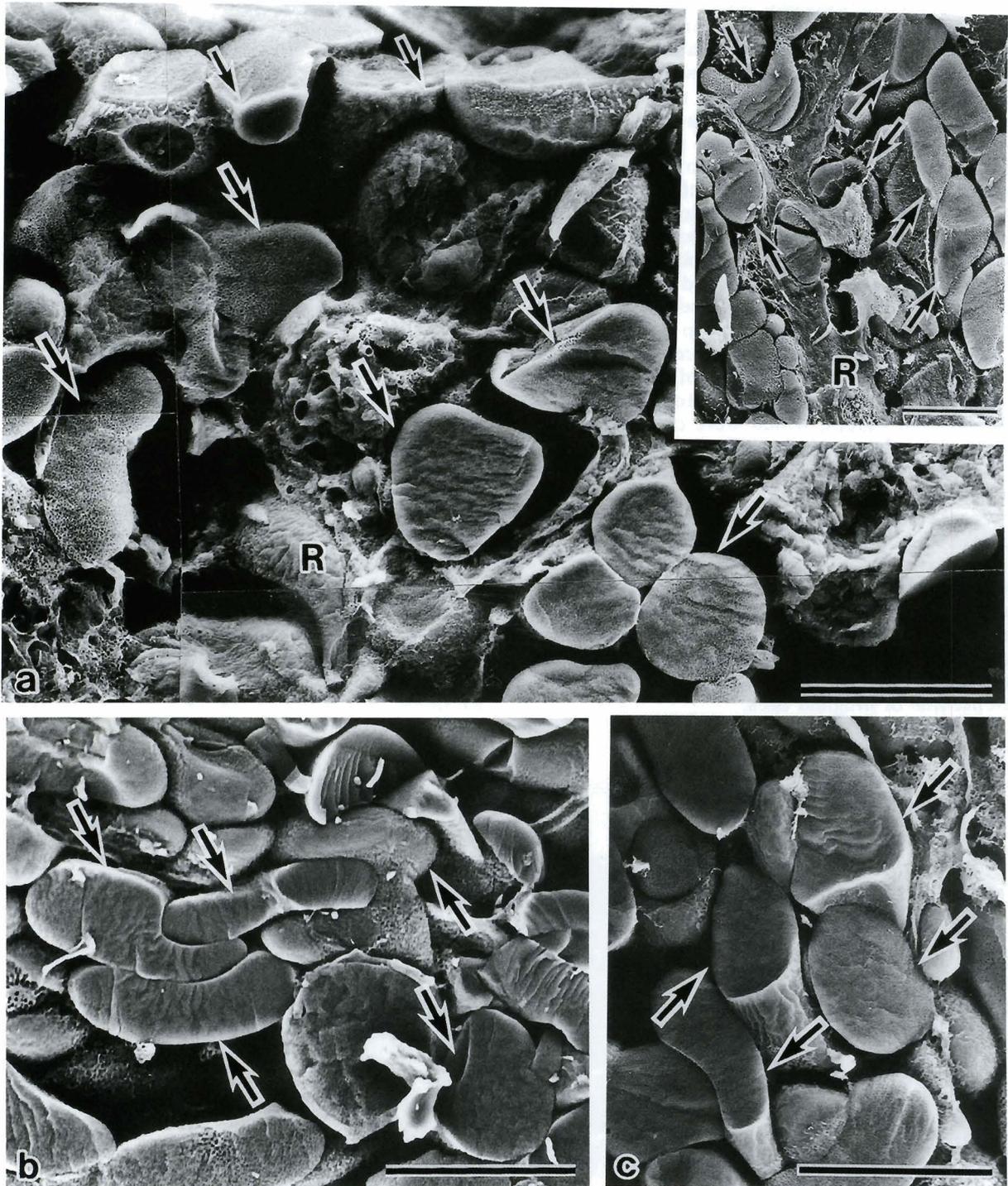
## Discussion

The 3-dimensional image of erythrocyte shapes in the red pulp of mouse spleens was obtained by the *in vivo* cryotechnique, which was compared with that revealed by the *in vitro* freezing method and conventional immersion fixation. First, it is necessary to discuss some advantages and disadvantages of these 3 methods. When the *in vivo* cryotechnique was performed by using a precooled cryoknife, a high cooling rate was critically necessary for freezing the living spleens. The higher the freezing rate was, the better the ultrastructure was preserved in biological specimens (Plattner and Bachmann, 1982; Bald, 1986). In the present study, the isopentane-propane mixture (-193  $^{\circ}\text{C}$ ) with a cryoknife was selected as liquid cryogen for freezing the spleens, because it remained liquid even at the liquid nitrogen temperature and possessed good thermal conductivity (Jehl et al., 1981; Murray et al., 1989; Cole et al., 1990). All biological processes in the living spleens were instantly stopped by the freezing step, and their structural components were usually maintained *in vivo*. Therefore, a morphological alteration induced by stopping the blood supply was avoided in the present experiment. Some well-frozen erythrocytes could be usually obtained up to a depth of about 5  $\mu\text{m}$  from the cut tissue surface, as shown in Figure 2a. The narrow spaces among erythrocytes, reticular cells and reticular fibers probably indicate functional significance of the erythrocyte deformability and aggregation. The network matrix structure can easily trap aged erythrocytes in the red pulp of spleens and help macrophages for their phagocytosis, even though the mechanism to initially determine such phagocytotic factors remains unclear in



**Fig. 2.** a. Montage of transmission electron micrograph of erythrocytes in the splenic red pulp, as prepared by freeze-substitution method after the *in vivo* cryotechnique. The depth of well-frozen areas is within 5-8  $\mu\text{m}$  from the frozen tissue surface (small arrows), but it is enough to observe various erythrocytes (large arrows). Ice crystal damage is detected in the deep areas (asterisks). Bar: 5  $\mu\text{m}$ . x 2,760. b. Scanning electron micrograph of erythrocytes in the splenic red pulp, as prepared by freeze-substitution method after the *in vivo* cryotechnique. At a depth of 15-20  $\mu\text{m}$  from the frozen tissue surface (small arrows), variously shaped erythrocytes are also observed (large arrows). Bar: 6.5  $\mu\text{m}$ . x 1,840

*Erythrocytes shapes in splenic red pulp*



**Fig. 3.** Higher magnified scanning electron micrographs of erythrocytes in the splenic red pulp, as prepared by freeze-substitution method after the in vivo cryotechnique. **a.** Widely viewed areas are obtained by a montage technique with a series of electron micrographs. Small arrows indicate the frozen tissue surface. There are fewer spaces between freeze-fractured reticular cells and variously shaped erythrocytes (large arrows). Bar: 5  $\mu\text{m}$ . x 5,700. Inset: Variously shaped erythrocytes (large arrows) in the splenic red pulp are observed among the reticular cells (R). Bar: 5  $\mu\text{m}$ . x 2,850. **b. and c.** Variously shaped erythrocytes. Bar: 5  $\mu\text{m}$ . x 5,700

### Erythrocytes shapes in splenic red pulp

vivo (Tang, 1997; Venerando et al., 1997).

In the present study with the *in vivo* cryotechnique, morphological changes of erythrocytes were observed in the living spleens, which are different from stable erythrocytes *in vitro*. So, many microenvironmental factors are probably responsible for alterations of variously shaped erythrocytes in the living spleens. Some of them include the weak deformability of aged erythrocytes (Tillmann et al., 1980; Mohandas and Groner, 1989; Marikovsky, 1996), the slower and intermittent blood flow and the narrow passage routes formed by reticular tissues (Blue and Weiss, 1981; Kashimura and Fujita, 1987). Moreover, a special pH condition was reported to be as low as 6.8 in the splenic red pulp (Levesque and Groom, 1976), which would lead to an increase in mean volume and membrane rigidity of erythrocytes. Such a physical change occurring in an acidic environment might be due to erythrocyte

metabolic changes, and may cause their short lifespan within the red pulp, indicating a postulated role of intrasplenic reduced pH for the erythrocyte destruction (Maeda et al., 1988; Baumann et al., 1994; Gedde et al., 1997).

The *in vitro* freezing method, which has often been used as a promising cryofixation, also arrests all biological reactions of cells and tissues within a very short time (Nicolas, 1991; von Schack et al., 1993). When the blood circulation is stopped in mouse livers, as in the case of the *in vitro* freezing method, heterogeneous erythrocyte shapes are suddenly changed to homogeneous biconcave discs in the hepatic sinusoid (Terada et al., 1998). Moreover, the circulating erythrocytes, which have a good deformability, adapted for various living microenvironments, easily changed their ellipsoid shapes in the abdominal aorta into biconcave discoid shapes in the inferior vena cava under normal blood circulation condition (Xue et al., 1998). In contrast, the blood supply was completely stopped after the removal of spleens, and various shapes of erythrocytes were still detected in the splenic specimens prepared by the *in vitro* freezing method. This finding may be due to the increased rigidity of membrane skeletal proteins in the aged erythrocytes (Abe and Orita, 1990; Kosower, 1993; Marikovsky, 1996; Bosman et al., 1996) and the high splenic hematocrit-concentrating blood (MacDonald et al., 1991). Moreover, the time needed for shape recovery of the aged erythrocytes has already been reported to be longer than that of young erythrocytes (Bronkhorst et al., 1995).

The conventional fixation method has often been used to examine the ultrastructure of animal spleens for SEM and TEM (Hataba et al., 1981; Blue and Weiss, 1981). However, it is well-known that it usually extracts splenic cell and tissue components and alters their structural organization *in vitro*. A rapid dehydration step in organic solvents at room temperature has been reported to induce their drastic shrinkage during the conventional preparation steps (Chan et al., 1993; Ohno et al., 1996). Such an ultrastructural alteration could be avoided by using slow and gentle dehydration of the freeze-substitution at a low temperature, as reported before (Parthasarathy, 1995; Takahashi et al., 1996). Chemical fixation with common glutaraldehyde and osmium tetroxide and radical dehydration with a series of ethanol have usually modified the natural ultrastructure of experimental mouse spleens. In the present study, we obtained an apparently native ultrastructural image of erythrocyte shapes in the splenic red pulp, as shown in Figures 2-3.

Erythrocyte shapes in the splenic red pulp are probably affected by various microenvironmental factors *in vivo*, including chemical metabolic state, osmotic pressures, hemodynamic state, hematological components, and also erythrocytes themselves. It is our goal to clarify the structure closely related to native flowing erythrocytes in various organs and to determine each critical factor in their microenvironment.

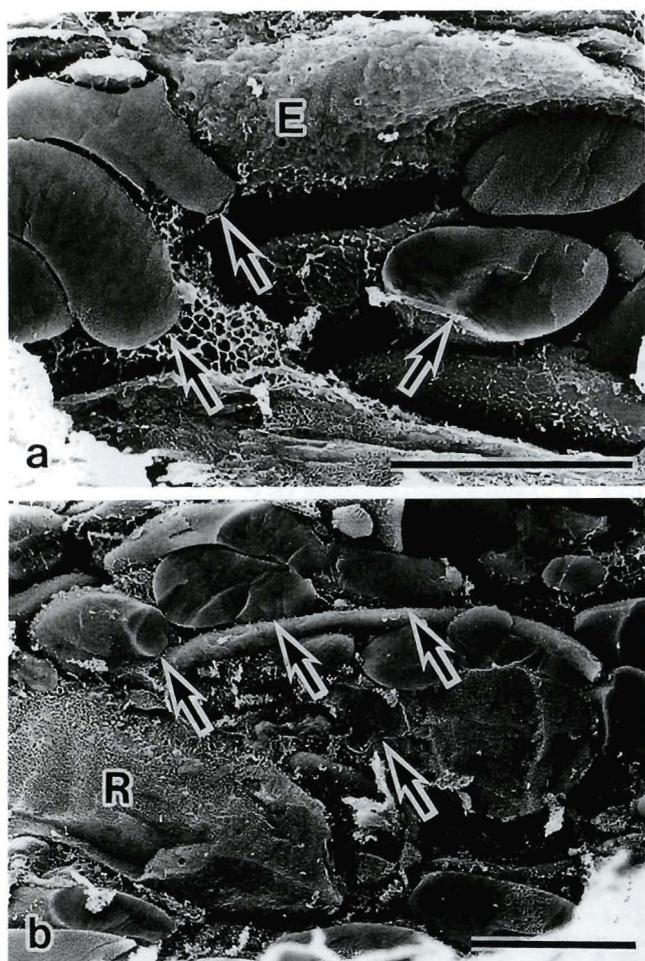
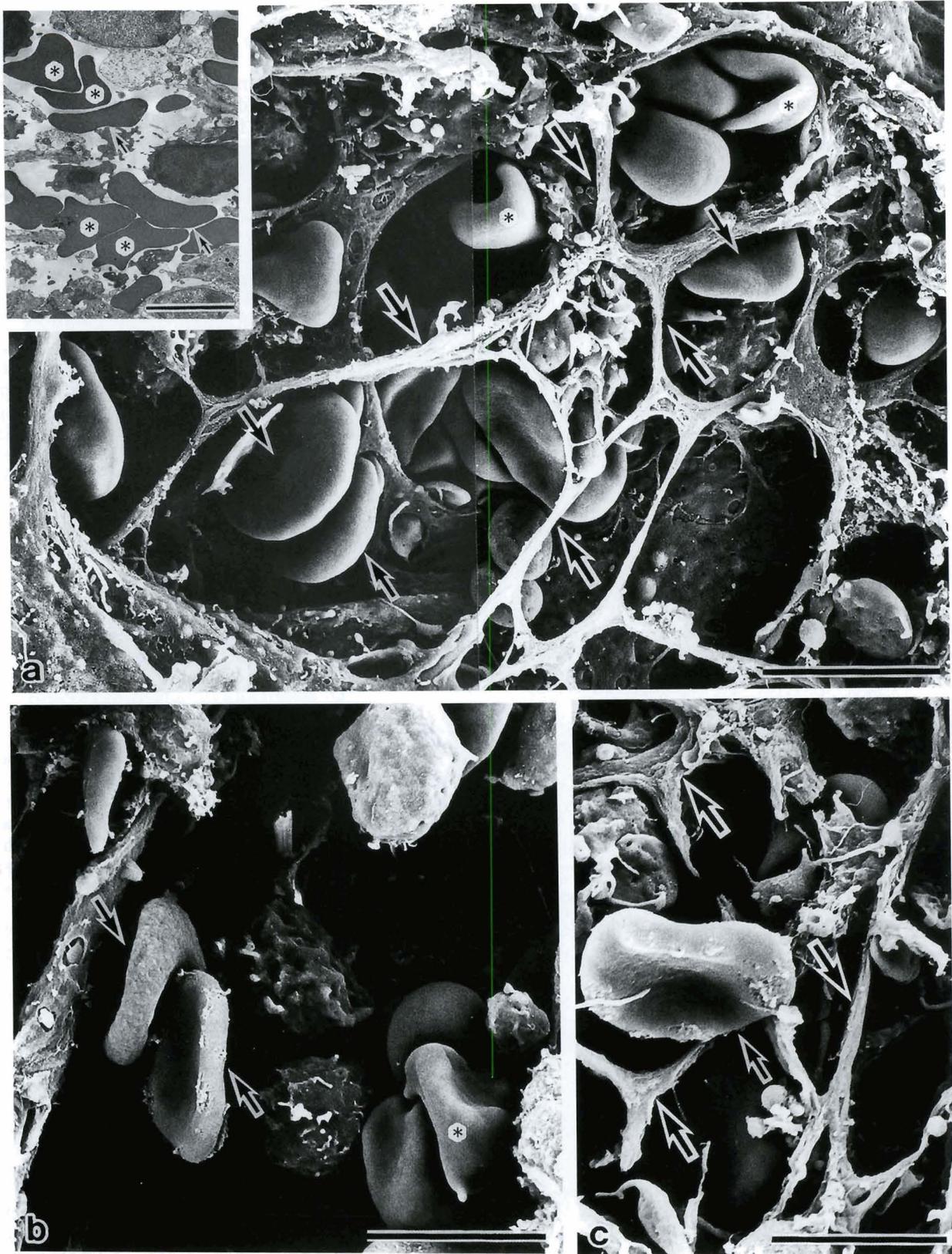


Fig. 4. Scanning electron micrographs of erythrocytes in the splenic red pulp, as prepared by freeze-substitution method after the *in vitro* freezing method. a. Variously shaped erythrocytes (arrows) are observed near endothelial cells (E). Bar: 5  $\mu$ m. x 5,980. b. Some erythrocytes are trapped among reticular cells (R). Bar: 5  $\mu$ m. x 4,140

*Erythrocytes shapes in splenic red pulp*

**Fig. 5.** Electron micrographs of erythrocytes in the splenic red pulp, as prepared by a graded series of ethanol dehydration after conventional immersion fixation. **a.** Widely viewed areas are obtained by a montage technique with a series of electron micrographs. **a, b and c.** The reticular fibers merge to form coarser bundled fibers (large arrows). There are biconcave disc shaped erythrocytes (small arrows) and some irregularly shaped erythrocytes (asterisks) loosely localized among them. Bar:  $5\ \mu\text{m}$ .  $\times 5,700$ . **Inset:** Transmission electron micrograph of erythrocytes in the splenic red pulp, as prepared with the conventional fixation. Bar:  $5\ \mu\text{m}$ .  $\times 2,850$

**References**

- Abe H. and Orita M. (1990). Age-related changes of erythrocyte membrane in the senescence-accelerated mouse. *Mech. Ageing Dev.* 51, 215-222.
- Bald W.B. (1986). On crystal size and cooling rate. *J. Microsc.* 143, 89-102.
- Baumann E., Linss W., Frohner M., Stoya G. and Richter W. (1994). pH-induced denaturation of spectrin changes the interaction of membrane proteins in erythrocyte ghosts. *Biochemical and electron microscopic evidence.* *Anat. Anz.* 176, 93-99.
- Blue J. and Weiss L. (1981). Vascular pathways in nonsinusoidal red pulp—an electron microscope study of the cat spleen. *Am. J. Anat.* 161, 135-168.
- Bosman G.J., Engbersen A., Vollaard C.H., Bartholomeus I.G., Pistorius A.M., Renkswek K. and DeGrip W.J. (1996). Implications of aging and degeneration-related changes in anion exchange proteins for the maintenance of neuronal homeostasis. *Cell. Mol. Biol.* 42, 905-918.
- Bronkhorst P.J., Streekstra G.J., Grimbergen J., Nijhof E.J., Sixma J.J. and Brakenhoff G.J. (1995). A new method to study shape recovery of red blood cells using multiple optical trapping. *Biophys. J.* 69, 1666-1673.
- Chan F.L., Inoue S. and Leblond C.P. (1993). The basement membranes of cryofixed or aldehyde-fixed, freeze-substituted tissues are composed of a lamina densa and do not contain a lamina lucida. *Cell Tissue Res.* 273, 41-52.
- Cole R., Mstuszek G., See C. and Rieder C.L. (1990). A simple pneumatic device for plunge-freezing cells grown on electron microscopy grids. *J. Electron Microsc. Tech.* 16, 167-173.
- Gedde M.M., Davis D.K. and Huestis W.H. (1997). Cytoplasmic pH and human erythrocyte shape. *Biophys. J.* 72, 1234-1246.
- Ghailani N., Guillemin C. and Vigneron C. (1995). Chronology of the formation of vesicles and membrane protein aggregates during erythrocyte aging. *Nour. Rev. Fr. Hematol.* 37, 313-319.
- Hataba Y., Kirino Y. and Suzuki T. (1981). Scanning electron microscopic study of the red pulp of mouse spleen. *J. Electron Microsc.* 30, 46-56.
- Jehl B., Bauer R., Dorge A. and Rick R. (1981). The use of propane/isopentane mixtures for rapid freezing of biological specimens. *J. Microsc.* 123, 307-309.
- Kashimura M. and Fujita T. (1987). A scanning electron microscopy study of human spleen: Relationship between the microcirculation and functions. *Scanning Microsc.* 1, 841-851.
- Kosower N.S. (1993). Altered properties of erythrocytes in the aged. *Am. J. Hematol.* 42, 241-247.
- Kubota K., Tamura J., Shirakura T. and Kimura M. (1996). The behaviour of red cells in narrow tubes in vitro as a model of the microcirculation. *Br. J. Hematol.* 94, 266-272.
- Levesque M.J. and Groom A.C. (1976). pH environment of red cells in the spleen. *Am. J. Physiol.* 231, 1672-1678.
- MacDonald I.C., Rafan D.M., Schmidt E.E. and Groom A.C. (1987). Kinetics of red blood cell passage through interendothelial slits into venous sinuses in rat spleen, analyzed by in vivo microscopy. *Microvasc. Res.* 33, 118-134.
- MacDonald I.C., Schmidt E.E. and Groom A.C. (1991). The high splenic hematocrit: A rheological consequence of red cell flow through the reticular meshwork. *Microvasc. Res.* 42, 60-76.
- Maeda N., Seike M., Suzuki Y. and Shiga T. (1988). Effect of pH on the velocity of erythrocyte aggregation. *Biorheology* 25, 25-30.
- Marikovsky Y. (1996). The cytoskeleton in ATP-depleted erythrocytes: The effect of shape transformation. *Mech. Ageing Dev.* 86, 137-144.
- Mohandas N. and Groner W. (1989). Cell membrane and volume changes during red cell development and aging. *Ann. N.Y. Acad. Sci.* 554, 217-224.
- Murray P.W., Robards A.W. and Waites P.R. (1989). Countercurrent plunge cooling: a new approach to increase reproducibility in the quick freezing biological tissue. *J. Microsc.* 156, 173-182.
- Nicolas G. (1991). Advantages of fast-freeze fixation followed by freeze-substitution for the preservation of cell integrity. *J. Electron Microsc.* Tech. 18, 395-405.
- Ohno S., Terada N., Fujii Y., Ueda H. and Takayama I. (1996). Dynamic structure of glomerular capillary loop as revealed by an in vivo cryotechnique. *Virchows Arch.* 427, 519-527.
- Parthasarathy M.V. (1995). Freeze-substitution. *Method Cell Biol.* 49, 57-69.
- Plattner H. and Bachmann L. (1982). Cryofixation: a tool in biological ultrastructural research. *Int. Rev. Cytol.* 79, 237-304.
- Suzuki Y., Tateishi N., Soutani M. and Maeda N. (1996). Flow behavior of erythrocytes in microvessels and glass capillaries: effects of erythrocyte deformation and erythrocyte aggregation. *Int. J. Microcirc. Clin. Exp.* 16, 187-194.
- Takahashi R., Okuyama T., Matsuo S. and Maeda S. (1996). Freeze substitution and freeze drying for stable, long-term preservation of cytologic specimens for immunostaining. *Acta Cytol.* 40, 396-400.
- Tang T.K. (1997). Free radical theory of erythrocyte aging. *J. Formos. Med. Assoc.* 96, 779-783.
- Terada N., Kato Y., Fujii Y., Ueda H., Baba T. and Ohno S. (1998). Scanning electron microscopic study of flowing erythrocytes in hepatic sinusoids as revealed by in vivo cryotechnique. *J. Electron Microsc.* 47, 67-72.
- Tillmann W., Levin C., Prindull G. and Schroter W. (1980). Rheological properties of young and aged human erythrocytes. *Klin. Wochenschr.* 58, 569-574.
- Venerando B., Fiorilli A., Croci G.L. and Tettamanti G. (1997). Presence in human erythrocyte membranes of a novel form of sialidase acting optimally at neutral pH. *Blood* 90, 2047-2056.
- Von Schack M.L., Fakan S., Villiger W. and Müller M. (1993). Cryofixation and cryosubstitution: a useful alternative in the analyses of cellular fine structure. *Eur. J. Histochem.* 37, 5-18.
- Xue M., Kato Y., Terada N., Fujii Y., Baba T. and Ohno S. (1998). Morphological study by an in vivo cryotechnique of the shape of erythrocytes circulating in large blood vessels. *J. Anat.* 193, 73-79.