Bull Yamaguchi Med Sch 29 : 15-21, 1982

Mechanism of Neutrophil Accumulation in Splenic Marginal Zone after Bacterial Toxin Administration: A Light and Electron Microscopic Study

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(Received February 4; revised February 25, 1982)

Abstract Lipopolysaccharides (LPS) from *Salmonella typhosa* was injected into the peritoneum of ICR mice. Light and electron microscopic changes of neutrophils in both the systemic circulation and in the marginal zone of the spleen were studied, both without (Oh) and 0.5, 1, 2, 4, 6, 8, 12, 24, 36 and 48h after the LPS treatment. Sequestration of the circulating neutrophils into the marginal zone of the spleen was observed after the LPS treatment.

The neutrophils both in the circulation and in the marginal zone of the spleen were damaged by the LPS treatment, as evidenced by 1) toxic cytoplasmic granules, 2) degranulation and vacuolation, 3) dilatation of the rough- and smooth-surfaced endoplasmic reticulums and 4) dilatation of the nuclear envelope. The damage was most marked 12h after the LPS challenge. At 12h, 65% of the neutrophils in the circulation showed some or all of the above mentioned signs of damage, while 100% of the neutrophils in the marginal zone of the spleen did so. The degree of damage was more marked in the neutrophils in the marginal zone than in those of the circulation. It was concluded from these findings that the splenic marginal zone actively traps the neutrophils damaged by the bacterial toxin, rather than being invaded by, undamaged neutrophils from the circulation. The trapping most likely is done by the reticulum cell meshwork in the marginal zone, helped in part by phagocytes.

Key Words: Bacteria; toxin, lipopolysaccharide, Spleen; bacterial toxin, neutrophils

Introduction

The marginal zone of the spleen, which contains lymphocytes, plasma cells, macrophages, granulocytes and erythrocytes, lying in a reticular meshwork, is situated between the inner red pulp and the outer white pulp. Because of the presence of numerous vascular terminations, a tight reticular meshwork and phagocytic cells in the area, it has long been recognized that this area works as an important filtration center. Foreign particles, damaged erythrocytes and granulocytes, antigens, immune complexes and blood-borne materials are known to be trapped in this area[1]. Little is known, however, on the mechanism of "trapping". The question arises whether the "trapping" is passive, first

invaded by these components, and then the invading components are gradually processed by the local tissue.

Clark and Weiss[2] observed sequestration of circulating neutrophils into the marginal zone of rat spleen after systemic administration of a typhoid vaccine. Based on the findings on both light and electron microscopy, they concluded that the neutrohils damaged by the vaccine are sequestered in the spleen, particularly in the marginal zone.

We studied light and electron microscopic changes of the neutrophils both in the systemic circulation and in the marginal zone of the spleen of laboratory mice after a peritoneal administration of lipopolysaccharides from *Salmonella typhosa*. The study was designed to determine the mechanism of sequestration of neutrophils into the spleen.

Materials and methods

A total of 30 male ICR mice, aged 6 to 7 weeks, were intraperitoneally injected with 50μg of LPS of *Salmonella typhosa* (Sigma) in 0.2ml of saline. Each three mice were sacrificed at 30min, 1, 2, 4, 6, 8, 12, 24, 36 and 48h after the injection. Animals were bled by heart puncture under ether anesthesia. Another three mice, intraperitoneally injected with 0.2ml of saline solution, served as controls (Oh).

Light microscopy

a) Spleen: The spleens were fixed with 5% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin.

b) PB smears: Peripheral blood smears were stained in Wright's stain. A total of 200 white cells were analysed in each of the mice studied. The PB total leukocyte count was performed.

Electron microscopy

a) Spleen: Spleens were removed, fixed in Mizuhira's tannic acid fixative[3], and then in 1% OsO_4. Ultrathin sections were prepared and doubly stained with uranyl acetate and lead citrate. Each of the mice studied was examined for electron microscopy of the spleen.

b) PB neutrophils: The buffy coats of the peripheral blood samples obtained from three mice at 12h following LPS treatment were fixed in tannic acid fixative, and then in 1% OsO_4, and processed in the same manner as in spleen.

Determination of cytoplasmic granules/cytoplasm ratio in neutrophils

Electron microscopic photographs (18,000X) of PB buffy coat as well as the marginal zone of the spleen from mice at Oh and 12h after LPS treatment, 20 neutrophils in each sample were screened. The dimensions of cytoplasms of the neutrophils in an enlarged photograph were measured by Digiplan (Kontron). The number of cytoplasmic granules in each cm^2 was calculated.

Results

Light microscopy

a) Counts of leukocytes in systemic circulation:

The mean counts of the total leukocytes, neutrophils and lymphocytes were plotted up to 48h, the duration of observation period, expressed as ratios of the counts of the controls (Oh) (Fig. 1). The total leukocyte count during the first 4h period after the LPS treatment lower than controls. The decrease was followed by transient increase was seen peaking at 8h. As for the neutro-

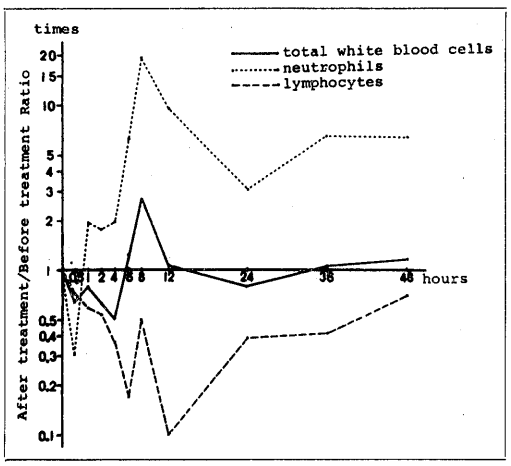


Fig. 1 Chronological changes of the numbers of ciuculating blood leukocytes, neutrophils and lymphocytes, expressed as ratios of the values in the controls. Each point represents means of values of three mice studied.

phils, a transient decrease was seen 0.5h after the LPS challenge, followed by a marked increase, as much as 20 times the control level, peaking at 8h. The lymphocytes decreased for the first 12h, then gradually increased to return close to the control level. The first decrease of neutrophils may reflect their removal from the systemic circulation to the marginal pool, i.e., the pool other than the systemic circulation.

b) PB smears

i) Light microscopy: Toxic granules were

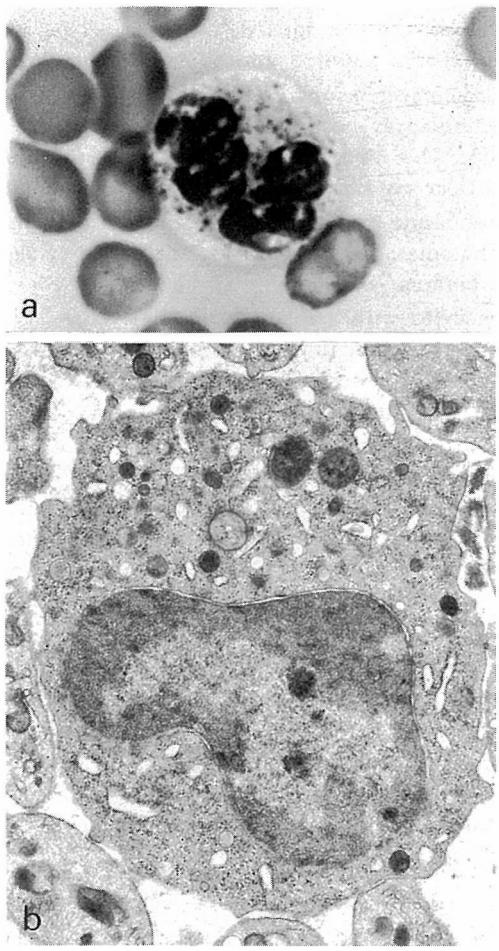


Fig. 2 Circulating blood neutrophils. **a**; A neutrophil 12h after LPS treatment, with many large toxic granules. **b**; Electron microscopic photograph (×12,600) of a neutrophil at 12h, with cytoplasmic small granules, a few large azurophilic granules, and dilated rough- and smooth-surfaced endoplasmic reticulums.

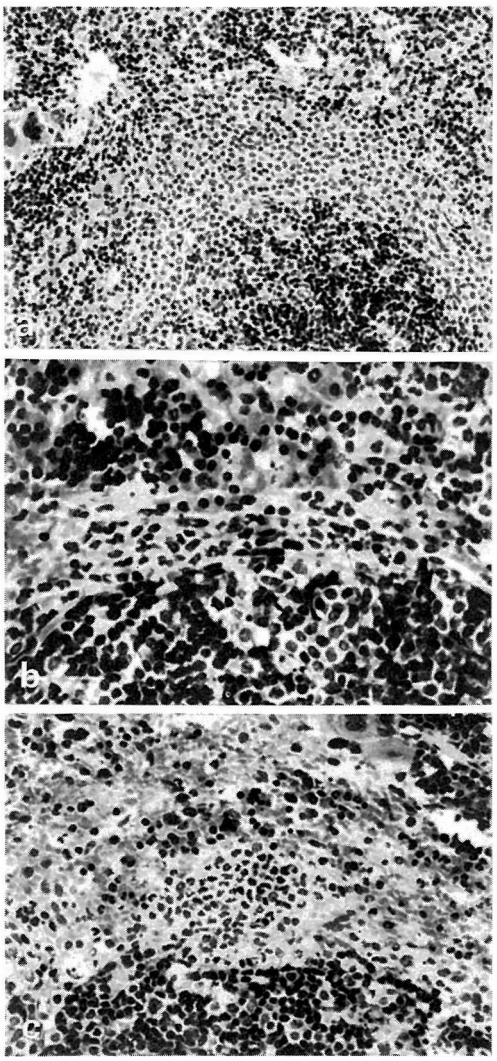


Fig. 3 Light microscopic photographs of the splenic marginal zone. **a**; Control mouse. A clear band-like zone, 60μm in width, surrounding the white pulp. **b**; 12h after LPS treatment. Few lymphocytes and diffusely accumulated neutrophils in a thin marginal zone. **c**; 12h after LPS treatment. Focal aggregation of neutrophils.

not observed in any of the control mice studied, while 2–25% neutrophils at 0.5–48h had the toxic granules (Fig. 2a). Their rate was highest (13–25%; mean 17%) at 12h. A range of 1–2.5% of neutrophils in the controls was band forms, while 19–24.5% of those at 12h were band forms.

ii) Electron microscopy: The neutrophils at 12h showed no remarkable electron microscopic changes, except for a slight decrease of cytoplasmic granules, the appearance of vacuolated granules and swollen azurophilic granules (Fig. 2b).

c) Spleen

i) Light microscopy: The splenic marginal zone in control ICR mice was a clear, band-like zone, about 60μm in width, surrounding the white pulp, separated from the latter by marginal sinuses or reticulum cells (Fig. 3a). The reticulum cells had a basement membrane-like extracellular reticulum. The marginal zone contained many lymphocytes and a few macrophages, neutrophils and erythrocytes, all lying in a reticular meshwork. Although light microscopic studies were done in each of the 30 mice studied, those at 12 h will be described in detail.

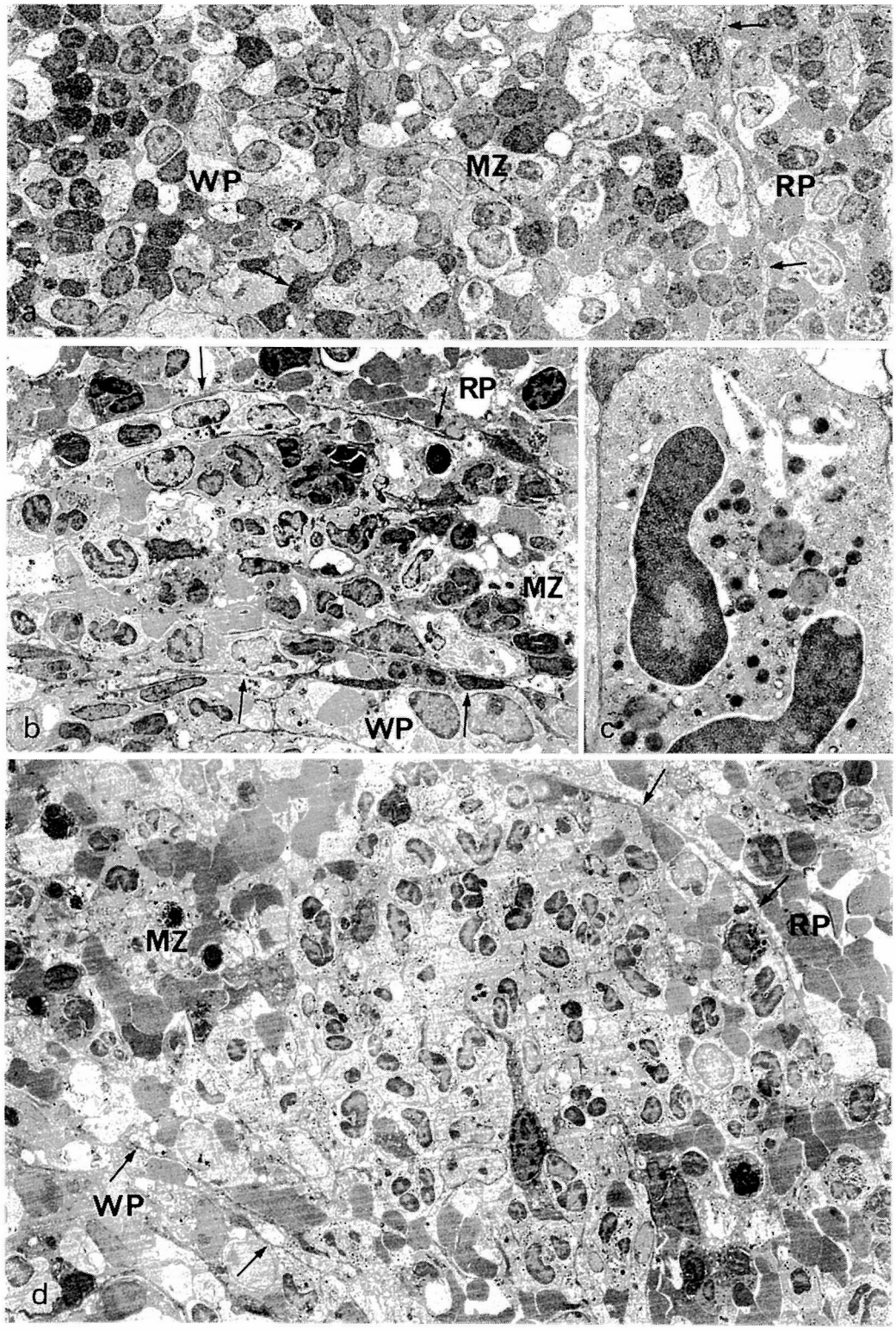
At 12h, the number of the lymphocytes in the marginal zone was much lower than that in controls. The marginal zone was thin(Fig. 3b). The reticular meshwork was crowded, and the reticular cells were close to each other. The border between the marginal zone and the white pulp was clear. Accumulation of the neutrophils with areas of aggregates interspersed with the background with more or less evenly scattered neutrophils. A few tingible-body macrophages were noted. The aggregates were composed of closely packed neutrophils and protruded into the adjacent tissues.

ii) Electron microscopy: In control mice, the reticulum cells that form reticular meshwork in the splenic marginal zone were spindle-shaped, had a nucleus with a dispersed chromatin pattern, and long, slender cytoplasmic extensions (Fig. 4a). Two kinds of lymphocytes were founds in the marginal zone. One was small lymphocytes with their nuclear chromatin lying close to the nulear membrane, scanty cytoplasm with many ribosomes, a few mitochondria and Golgi apparatus. The other was medium-sized lymphocytes with a dispersed nuclear chromatin pattern, abundant cytoplasm, many ribosomes, and a few rough endoplasmic reticulums and mitochondria.

The neutrophils in the marginal zone had a lobulated nucleus and a cytoplasm with many cytoplasmic granules, glycogen granules, rough- and smooth-surfaced reticulums, mitochondria, and Golgi apparatus. The macrophages had a round nucleus with a dispersed chromatin pattern and abundant, clear cytoplasms with lysosomes, mitochondria and ribosomes. Some had a few phagocytic residues.

Although electron microscopic studies were done throughout the 48h study period, those at 12h, with the most marked changes, will

Fig. 4 (overleaf) Electron microscopic photographs of the splenic marginal zone. **a**; Control mouse (×1.000). The marginal zone is demarcated by reticulum cells (arrows) from both the red pulp (RP) and the white pulp (WP). Many medium-sized lymphocytes, small lymphocytes, and a few erythrocytes, macrophages and neutrophils, all lying in a reticular meshwork. **b-d**; 12h after LPS treatment. **b**; Many neutrophils, erythrocytes and a few macrophages with phagocytic residues in the marginal zone (×1,300). **c**; Dilatation of the nuclear envelope and endoplasmic reticulums of neutrophils, small cytoplasmic granules and a few large azurophilic granules. Some of the granules are vacuolated, while some others are of low density (×13,000). **d**; A focal aggregate of neutrophils is bounded by reticulum cells (×1,200).


WP
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be described in detail (Fig. 4b-d). The neutrophils in the marginal zone had clear cytoplasm with a few dilated rough- and smooth-surfaced endoplasmic reticulums. The average of cytoplasmic granules were significantly low both compared with those in control spleens (Table 1: $p<0.01$) and with the PB neutrophils ($p<0.01$). Vacuolation and low density of the granules were also noted. Together, these changes accounted for 6.4-33% (mean 17%) of the neutrophils at the marginal zone at 12h.

Table 1 Average Counts of Cytoplasmic Granules in Neutrophils*

	Controls	12h after LPS treatment
Peripheral blood neutrophils	1.64±0.14	1.34±0.24
Neutrophils in splenic marginal zone	1.73±0.51	0.98±0.29

*Twenty randomly sampled neutrophils form three mice were photographed, printed at 18,000X, and the number of cytoplasmic granules per cm^2 of cytoplasm was calculated in each neutrophil. Mean ± S.D.

Focally aggragated areas of neutrophils in the marginal zone: The neutrophils in the aggregates had a sparse cytoplasm and 1-8 endoplasmic reticulums, the latter were dilated to an extent unseen in the non-aggregated neutrophils in the marginal zone. Macrophages, some of which had phagocytized neutrophils in their cytoplasm, were rarely seen in the periphery of the aggregates, stretching out their cytoplasmic extensions among the surrounding neutrophils.

Discussion

Both the degree and the rate of damage were more marked in the neutrophils in the marginal zone of the spleen than those in the peripheral blood, when both were compared at the peak of damage both 12h after the LPS treatment. This would indicate that the sequestration of neutrophils as observed in our study resulted from trapping of damaged neutrophils by the reticular meshwork of the marginal zone, rather than its invasion by undamaged neutrophils and their subsequent degeneration after the invasion. If the latter were the case, one would have observed both undamaged and damaged neutrophils in the marginal zone.

As for the signs of damage or degeneration of neutrophils, we used the following four criteria: 1) toxic cytoplasmic granules, 2) degranulation and vacuolation, 3) dilatation of the rough- and smooth-surfaced endoplasmic reticulums, and 4) dilatation of the nuclear envelope. Both dilatation of the endoplasmic reticulums and focal dilatation of the nuclear envelope have been observed in autolyzed hepatic cells, excised left at room temperature[4]. Degranulation has been described in neutrophils in severe bacterial infection[5].

The distribution of the neutrophils was not even in the marginal zone of mouse spleen in our experiment. Areas with aggregated neutrophils were seen interspersed with the background area with more or less evenly distributed neutrophils. This aggregation of neutrophils has not been reported in the literature. The neutrophils in the aggregated area were closely packed, and sparse reticular meshwork were seen among them. Macrophages were seen in the outskirts of these aggregations, and they phagocytized neutrophils. These findings indicate that the neutrophils in the aggregated areas were intercepted by reticulum cells that form reticular meshwork of the marginal zone, rather than by macrophages. The intercepted neutrophils in turn are phagocytized by macrophages. It seems that the neutrophils thus altered cannot pass through the marginal zone or migrate from the marginal zone to the red pulp because of their impaired

mobility. The ability of the reticulum cells to trap neutrophils may result from their physical structure, or, alternatively, from their biological property.

The author is indebted to Prof. F. Uchino for his kind guidance and encouragement throughout this study. Thanks are also due to Dr. N. Mastumoto for his kind advices and review of this manuscript. I am grateful to Drs. T. Iwata, T. Ishihara, M. Takahashi for their advices, and Messers M. Yamashita and Y. Murakami for excellent technical assistance.

This work was presented at the 21th Annual Meeting of the Japan Society of the Reticuloendothelial System in Ube, May, 1981.

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