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Enhanced Phagocytic Activity of Lymph Node Macrophages After Intranodular Injection of Autologous Red Blood Cells

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Summary. Rabbits were killed 1 h to 12 days after injection of washed autologous red blood cells (RBC) into the paratracheal cervical lymph nodes. Microscopic, electron-microscopic, and histochemical techniques were used to study the time-dependent ingestion and digestion capacity of phagocytes in the lymph nodes. One hour after intranodular injection of RBC, a greater portion of the target cells had already attached to the surface of and were partly ingested by sinusoidal phagocytes in the marginal zone and medulla. After 6 h, degradation of erythrocyte hemoglobin into hemosiderin could be demonstrated in a few of the cells. After 9 days, erythrocytes, erythrophages, and hemosiderophages had disappeared and could no longer be observed in the lymph nodes. Comparative morphological and histochemical studies showed these phagocytes to be macrophages. The possibility of an opsonizing or a humoral factor in lymph serum which stimulates phagocytosis was excluded in in vitro studies (peritoneal macrophages incubated with lymph serum and RBC) and the possibility of phagocytosis induced by a T-cell-macrophage interaction, was excluded in in vivo studies with athymic nude mice. Comparable enhanced phagocytic activity of lymph node macrophages could be observed in vitro after injection of RBC into isolated lymph nodes of rabbits. The findings presented here indicate that marginal zone and medullary macrophages are primarily activated and that they eliminate autologous RBC from the sinuses without any additional stimulus by mechanisms which have not yet been clarified.

Key words: Lymph nodes enhanced, phagocytic activity – Macrophages, lymph nodes

Zusammenfassung. Es wurden tierexperimentelle Untersuchungen an Kaninchen zur Erfassung der phagozytotischen Aktivität von Lymphknoten-Phagozyten durchgeführt. Die Kaninchen wurden zu unterschiedlichen Zeitpunkten, von 1 h bis zu 12 Tagen nach Injektion autologer Erythrozyten in paratracheale

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cervicale Lymphknoten getötet. Es folgten lichtmikroskopische, elektronenmikroskopische und histochemische Untersuchungen zur Erfassung der zeitabhängigen Phagozytose und des Abbaues durch Phagozyten im Lymphknoten. Eine Stunde nach intranodulärer Injektion von Erythrozyten haftete bereits ein großer Teil der Zielzellen an der Oberfläche von sinusoidalen Phagozyten der marginalen und medullären Zone der Lymphknoten bzw. war zum Teil bereits durch sie inkorporiert. Bereits nach 6 h war ein Abbau des Erythrozytenhämoglobins zu Hämosiderin in einzelnen Zellen nachweisbar. Nach 9 Tagen waren Erythrozyten, Erythrophagen und Hämosiderophagen aus den Lymphknoten verschwunden. Vergleichende morphologische und histochemische Untersuchungen zeigten, daß es sich bei den Phagozyten um Makrophagen handelt. Die Möglichkeit einer Opsonination bzw. eines humoralen Faktors im Lymphserum, der die Phagozytose stimuliert, wurde durch In-vitro-Versuche ausgeschlossen (Peritonealmakrophagen wurden mit Lymphserum und Erythrozyten inkubiert); die Möglichkeit einer Induktion der Phagozytose durch T-Zellen wurde durch In-vivo-Versuche mit athymischen, nackten Mäusen ausgeschlossen. Eine vergleichbare, beschleunigte phagozytotische Aktivität der Lymphknotenmakrophagen konnte schließlich auch durch In-vitro-Versuche nach Injektion autologer Erythrozyten in isolierte Lymphknoten von Kaninchen festgestellt werden. Die wiedergegebenen Befunde lassen die Schlußfolgerung zu, daß die marginalen und medullären Makrophagen offenbar primär aktiviert sind und daß sie autologe Erythrozyten aus den Lymphknoten mit Hilfe von bisher unbekannten Mechanismen extrem schnell eliminieren.

Schlüsselwörter: Lymphknoten, phagozytische Aktivitätssteigerung – Makrophagen, Lymphknoten

Introduction

While extensive literature is now available on the morphological and immunologic differentiation of various mononuclear nonlymphoid cells in lymph nodes (LN) (e.g. Van Furth 1970, 1975; Carr and Daems 1980), studies on time dependency and mode of phagocytosis for LN macrophages are rare. Kettler (1936) was the first to observe iron in the regional LN 4 days after s.c. injection of autologous red blood cells in rabbits, an indication of the degradation of injected RBC. In similar experiments with mice, Masshoff (1947) observed iron in the regional LN within the first 24 h after injection of autologous and heterologous RBC; the iron disappeared completely from the lymph nodes after 200 and/or 250 h. Three to 6 min after injection of colloidal carbon into the hamstring muscle of rats, Nopajaroonsri and Simon (1971) observed carbon particles in the subcapsular sinuses. A few of the particles had already been ingested by local macrophages. After 30 min, partly phagocytized carbon particles could be identified in the marginal, cortical, paracortical, and medullary sinuses. Our pilot studies demonstrated an amazingly rapid phagocytosis of autologous and heterologous RBC in LN macrophages (Oehmichen and Wiethölter 1980).

This study was set up to examine the phagozytic activity of rabbit LN macrophages at predetermined intervals after intranodular injection of autologous RBC and therefore was limited to observations of marginal and medullary macrophages. By modifying the experiment, we attempted to determine the reason for the rapid ingestion and digestion of autologous RBC. A detailed description of the mode of RBC destruction/degradation will be published elsewhere (Wiethölter and Oehmichen, in prep.).

Material and Methods

Animals

German Giant Breed rabbits (no inbred breeds; sex: male and female; age: 6–12 months; body weight: 2,000–2,500 g), mice [inbred breed NMRI (Ivanovas, Kißlegg-Allgäu, FRG); sex: female; age: approximately 2 months; body weight: 20–30 g], and athymic nude mice [breed, Han : NMRI nu/nu (Central Institute for the Breeding of Laboratory Animals, Hannover, FRG); sex: female; age: approximately 2 months] were used in the experiment. All animals were fed with Altromin and water ad lib.

Cell Preparation Methods

Autologous RBC were acquired by puncture of the ear veins of the rabbits. Five milliliters of whole blood from each rabbit was centrifuged at 3,000 rpm for 15 min. The sediment was then washed three times with physiologic saline, and a 5% RBC suspension was obtained.

Surgical Procedure

All surgical interventions were carried out under combined ether/pentobarbital anesthesia. The animals were killed by an i.v. injected overdose of sodium pentobarbital.

Those symmetric deep paratracheal cervical LN of the rabbits were exposed which were impressive because of their size. Careful, slow injection of RBC directly into the center of the LN resulted in an acute increase of LN volume. The skin incision was then closed under sterile conditions with single button sutures.

RBC were injected into the foot pads of the mice, and the popliteal LN were subjected to histological examination.

Histological Procedures

Following cardial perfusion with a 4% solution of phosphate-buffered paraformaldehyde, some of the LN were embedded in paraplast for routine light microscopy and some in araldite for electron microscopy. After sectioning, the tissue embedded in paraplast was stained with hematoxylin and eosin (HE), Goldner's trichrome stain, or Prussian blue reaction. The tissue embedded in araldite was stained with toluidine blue for light microscopy. Ultrathin sections were stained with lead hydroxide for electron microscopy and examined on a Zeiss EM (EM 9A, Carl Zeiss, Oberkochem, FRG).

In addition to the methods described above, LN were also isolated and removed without fixation, frozen, and sectioned in a commercial cryostat (Slee, London, GB).

The following enzymes were anlyzed: α-naphthyl butyrate esterase (cf. Ansley and Ornstein 1970; Goud et al. 1975); α-naphthyl butyrate as substrate and pararosanilin as stain; acid phosphatase (Löffler and Berghoff 1962); diNa-α-naphthyl phosphate a substrate and para-rosanilin as stain; alkaline phosphatase (Pearse 1968): Na-α-naphthyl phosphatase as substrate and fast garnet red as stain.

Experimental Procedures

The following experiments were carried out to answer various questions.

Series 1. 0.05 ml autologous washed RBC was injected into the deep paratracheal cervical LN of each living anesthetized rabbit. Two animals were killed for each predetermined interval after the injection (1, 2, 3, 4, 5, 6, 12, and 24 h; 2, 3, 4, 5, 6, 9, and 12 days). After fixation, the LN were dissected out and treated according to the histological methods described above.

Series 2. Rabbits which survived the autologous RBC injection for 3 h were killed. The LN used for histochemical analysis were removed without fixation, frozen, and stored at −70° C under airtight conditions until sectioning. A total of three animals were examined.

Series 3. Paraffin-oil induced rabbit peritoneal macrophages (Gesner and Howard 1967) were collected and washed three times (Oehmichen 1978). A final 5% cell concentration was prepared in Hank's solution. The macrophage suspension together with a 5% RBC suspension was agitated for 60 min at 37° C and incubated under various conditions. Autologous lymph was additionly applied as incubation solution; the lymph was obtained by puncture of the cisterna chyli. After centrifugation of the lymph (5,000 rpm for 10 min), the sediment was discarded and the lymph serum applied.

The following in vitro experiments were carried out:

1. Prior to the addition of the RBC, the macrophages were incubated together with the lymph serum for 30 min at 37° C and then washed (macrophage/lymph serum).
2. Prior to the addition of the macrophages, the RBC were incubated together with lymph serum for 30 min at 37° C and then washed (RBC/lymph serum).
3. Untreated macrophages and RBC suspensions were each incubated for 60 min with concentrated lymph serum.
4. Untreated macrophages and RBC suspension incubated with Hank's solution served as a control.

Series 4. Washed sheep RBC were injected into the foot pads of five nude mice. The popliteal lymph nodes were examined after the mice had survived for 1, 2, or 3 h. Identical experiments in mice (NMRI breed) served as a control.

Series 5. The deep paratracheal cervical LN were removed from four untreated rabbits. Autologous RBC (0.05 ml in vitro) were injected in the LN stored in a damp chamber. The lymph nodes were fixed 1–3 h later by immersion in phosphate-buffered paraformaldehyde.

Control Experiments

In addition to the controls already mentioned, the following experiments were undertaken: In experimental series 1, 2, 4, and 5, the untreated contralateral deep cervical LN was also subjected to histological examination.

Results

Series 1

Killing the rabbits at different predetermined intervals after intranodular injection of autologous RBC provided information on how quickly phagocytosis and digestions of RBC occurs in LN phagocytes.

One hour after the RBC injection, most of the RBC attached to the surface of free nonlymphoid mononuclear cells within the sinuses of the marginal zone and medulla (Fig. 1); RBC, however, rarely adhered to the lining cells on the sinusoidal surface. A great number of RBC had already been ingested at this time. Occasionally, early signs of degradation (i.e., fragmentation and hemoglobinolysis —partly of noningested cells) were observed (Figs. 2, 3).

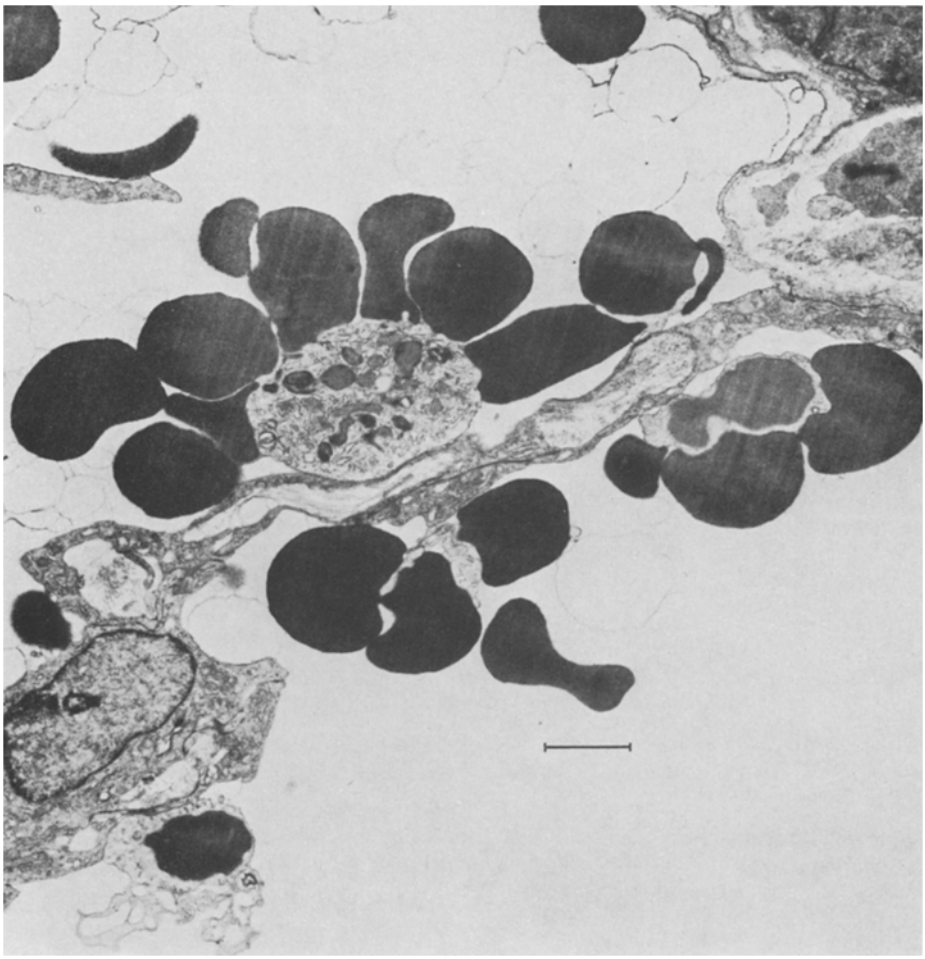

Fig. 1. Red cell attachment to macrophage surfaces 1 h after blood injection into rabbit lymph nodes. Within the sinus there is a great number of extracellular red cell ghosts (*bar* = 1 μm)

The number of nonphagocytized RBC declined within the next few hours; practically all RBC were attached to the surfaces of or ingested by free intrasinusoidal cells after 6 h. Degradation progresses as hemoglobin is lost, thereby producing RBC ghosts. More and more pseudomyelin-like figures (Fig. 3) as well as RBC fragmentation (Fig. 2) were recognized.

A faint blue staining of the cytoplasm was first observed with the Prussian blue reaction after a 6-h period; the staining became more pronounced in an increasing number of cells after a 9- to 12-h period (Fig. 4), apparently due to the ferric ions (i.e., hemosiderin) resulting from RBC degradation. Granulated hemosiderin was already demonstrable in isolated cells after 24 h.

The number of RBC- and hemosiderin-containing phagocytes declined in the days following, while the number of phagocytized RBC and the amount of

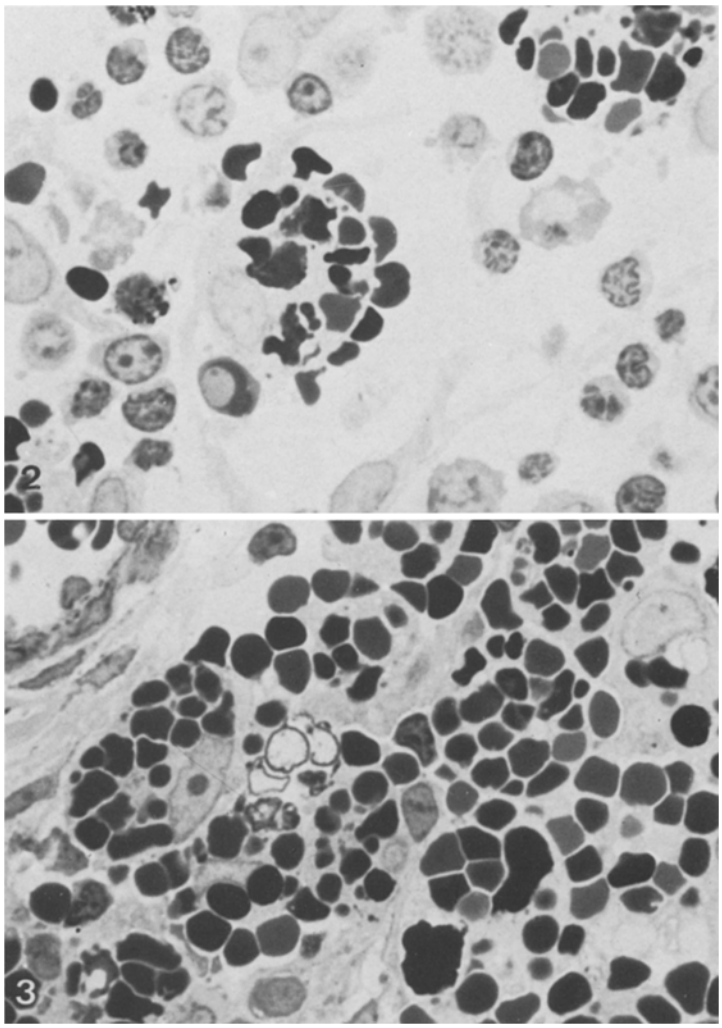

Fig. 2. Red cell attachment, ingestion, and digestion by lymph node macrophages 3 h after intranodular injection of blood. The figure especially demonstrates the intracellular process of fragmentation (toluidine blue; × 960)

Fig. 3. Red cell ingestion and digestion by lymph node macrophages 6 h after blood injection. Especially the phenomenon of extracellular hemoglobinolysis and the development of pseudomyelin body development is stressed (toluidine blue; × 960)

hemosiderin per phagocyte increased. RBC- and RBC- or hemosiderin-containing phagocytes were no longer demonstrable in the LN after 9 days.

The localization of the phagocytes within the marginal and medullary sinuses as well as the lack of morphological connections with the sinus-lining cells (i.e. free cells) tend to indicate that the phagocytes are best interpreted as macrophages. The predominantly round shape and ruffled membrane as well as the eccentric orientation of the nucleus (see below) supports this interpretation. Clear-cut

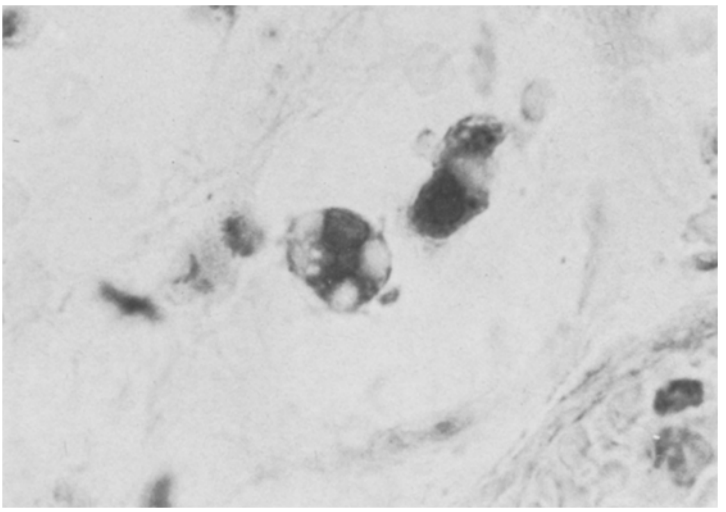

Fig. 4. Red cells- and hemosiderin-containing macrophages 9 h after the blood cell injection within a lymph node sinus (Prussian blue reaction; ×960)

ingestion of RBC by the sinus-lining cells, however, was not observed. Extremely fine Prussian blue-positive granules were found 3 days after the RBC injection and even later in some sinus-lining cells. The distinctly different phagocytosis capacity tends to indicate that two different cell types are represented here.

Series 2

Both the localization and the morphology of the phagocytically active cells tend to indicate that these cells are macrophages. This observation is additionally substantiated by the histochemical findings.

The histochemical demonstration of enzymes established a distinct acid phosphatase and α-naphthyl butyrate esterase activity (Fig. 5) as well as a negative alkaline phosphatase activity in all RBC-containing cells 3 h after the injection. In the control preparation comparable activity was identifiable only in the free nonlymphoid cells within the sinuses (i.e. macrophages) but not in the sinus-lining cells.

Series 3

The definitely rapid phagocytic capacity of the cells described here as macrophages raises the question of whether soluble (i.e. humoral) factors of the lymph serum could possibly influence the phagocytic capacity of these cells by altering the surfaces of the targets and/or the phagocytes. In vitro experiments with cell suspensions from rabbit peritoneal macrophages and autologous RBC were carried out in an attempt to answer this question. The cell suspensions were incubated together under different conditions, and the appearance of erythro-

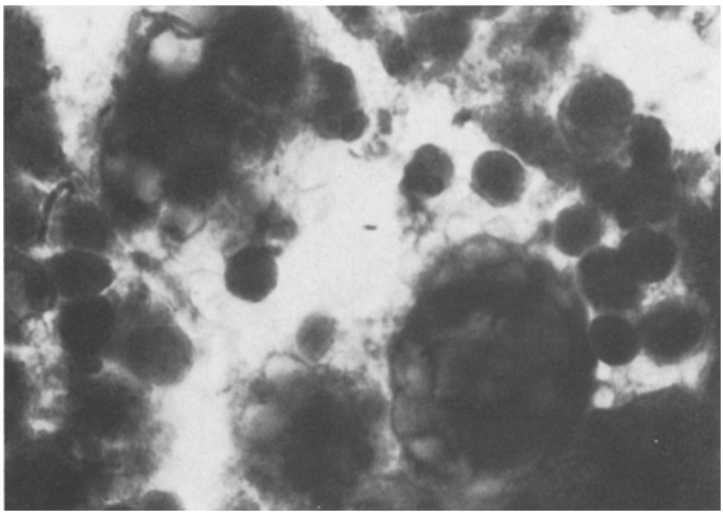

Fig. 5. α-Naphthyl butyrate esterase activity as an indication of the macrophage origin of the red cell phagocytes within the lymph nodes (α-naphthyl acetate esterase—hemalun; × 960)

phagocytosis was studied. Ingestion of target cells by more than 2% of the macrophages could not be observed after either incubation in Hank's solution or incubation in concentrated lymph serum. Altering the cell surfaces by pretreatment of RBC or macrophages with lymph serum was just as unsuccessful.

Series 4

Since the lymph serum does not seem to induce phagocytosis, the question arises as to whether a cell interaction of LN cells, especially the interaction of macrophages and T-lymphocytes, could possibly influence the phagocytic activity. The absence of T-cells in nude mice therefore could result in a reduction of RBC phagocytosis.

After injection of SRBC into the foot pads of control mice which contained T-cells, free RBC as well as single attached and ingested RBC could be observed in the marginal macrophages already after 1 h. A great many ingested RBC were found in the marginal zone as well as in the medullary macrophages after 3 h. The findings after foot pad injection in nude mice were identical: Comparison with the control animals revealed no quantitative differences.

Series 5

Since the experiments indicated that phagocytes were not stimulated by either soluble factors or cellular interaction, macrophages in the isolated LN probably also ingest autologous RBC in vitro. One hour after the injection and incubation of LN at 37°C, a great many RBC adhering to the surface of the cell and single ingested RBC were to be identified. The quantity had increased markedly after 3 h; autolysis, however, was already quite pronounced by this time.

Table 1

	Acid phosphatase	Nonspecific esterase	Alkaline phosphatase
Interdigitating cells	Weak activity[f]	Weak activity; sometimes focal, sometimes diffuse[f]	Negative[f]
Dendritic cells	Small clusters of positive granules close to nucleus[a,d,e]	Weak activity[a,b,d]	High activity[b]
Macrophages	High activity[c]	High activity[c]	Negative[c]

[a] Eikelendoom (1978), [b] Heussermann et al. (1980), [c] Leder (1967), [d] Li et al. (1972), [e] Steinman and Cohn (1973), [f] Veldman and Kayserling (1980)

Discussion

Lymph nodes have two basic functions:

1. Specific immunologic reaction (i.e. response to antigenic stimulation) by the production of specific antibodies or the generation of effector cells;

2. Nonspecific filtration of lymph by the framework of the reticulo-endothelial system, which is apparently formed by dendritic cells (Steinman and Cohn 1975), interdigitating cells (Veldman and Kaiserling 1980) as well as by fibroblasts, sinus-lining cells, and endothelial cells; the filtration function is supported by macrophages within the sinuses which clear up the lymph by phagocytosis (Hoefsmit et al. 1980).

The macrophages differ from other nonlymphoid mononuclear cells of the LN not only by their localization within the sinuses and their phagocytic function, but also by their morphology (Hoefsmit 1975): They are characterized by a lack of connections with the fibrous skeleton, a rounded form with a ruffled membrane, a eccentric nucleus, a central Golgi area, and lysosomes (Hoefsmit et al. 1980). Because of their marked acid phosphatase and nonspecific esterase activity with no alkaline phosphatase activity, phagocytes can be characterized histochemically as macrophages (Table 1).

Our observations therefore indicate that, after injection of autologous RBC, the LN macrophages in the marginal and medullary sinuses rapidly ingest and digest the target cells. A great many RBC were already phagocytized and degraded intracellularly after 1 h. The digestion of single RBC had already progressed far enough after 6–7 h that RBC degradation pigment (i.e. hemosiderin) was demonstrable.

A comparison of the RBC ingestion and digestion rates observed here with RBC degradation in other organs and/or tissues and cells shows the rate to be surprising. As a rule, amazing quantities of erythrophages are observed after 12–24 h; siderin is first observed after 72–96 h (Moritz 1942). This observation also holds true for blood cell degradation in rabbits (Vaughan 1965a, b; Oehmichen et al. 1980). In vitro experiments with cultured macrophages also indicated that

macrophages only ingest opsonized erythrocytes through the Fc or complement receptors (Schwartz 1967; Fedorko et al. 1973; Bianco et al. 1975; Van Furth et al. 1979; Van der Meer 1980).

The attachment and the ingestion by the LN macrophages are apparently not related to humoral factors in the lymph serum. This observation is supported by determination of immunoglobulin G and complement concentrations in lymph serum. The concentrations in different species were distinctly lower than in the blood serum (Oehmichen et al. 1980).

Allison (1978) established that phagocytosis is possible through macrophage-lymphocyte interaction. In our experiments, the rapidity of the phagocytic process as well as the absence of relevant immunologic stimulation tend to speak against a comparable process. The possibility of a B-cell-macrophage interaction being important for the observed processes therefore was excluded from the very beginning. The experiments with T-cell lacking nude mice eliminated any influence from a T-cell-macrophage interaction.

Our observations partially confirm the findings of other authors: Masshoff (1947) demonstrated an amazingly rapid erythrocyte degradation in the LN; Nopajaroonsri and Simon (1971) observed an equally rapid ingestion of i.v. applied colloidal carbon by LN macrophages. The team associated with Simon (1980) observed a comparably rapid phagocytosis in the spleen macrophages (Burke and Simon 1970) and in bone marrow (Luk and Simon 1974; Ogawa 1975). Burke and Simon (1970) observed the proximity of macrophages and platelets aggregating around carbon particles. Like Luk and Simon (1974), these authors attributed an important role to the platelets in marrow phagocytosis.

We did not observe aggregated platelets around target cells in the sinusoids or any other cytomorphological phenomenon which would adequately explain the rapid phagocytosis. Our study therefore established no external factors which could alter the surface of the target cells in any way. No factors were observed which would adequately explain an actual stimulation of the phagocytes themselves. Our findings therefore indicate that the marginal and medullary macrophages we observed are primarily highly activated. The accelerated capacity for recognition as well as attachement and digestion would tend to support this observation. The abundance of untreated macrophages at the intracytoplasmic organellae in the control animals could be a morphological indication of the primary activation.

The following observations also tend to substantiate this assumption: Based on completely different experiments, Humphrey (1978) characterized the marginal zone macrophages as especially large and phagocytically active cells and differentiated them from other LN macrophages. All authors who observed the intranodular kinetics of macrophages or targets in LN following i.v., s.c., or i.m. injection of cells or other tracers, concluded that primarily the marginal zone macrophages and then the medullary macrophages ingested tracers (Napojaroonsri and Simon 1971; Hoefsmit et al. 1980).

In addition to a primary activation of the macrophages, mechanical factors may possibly play an additional role, e.g. slow flow of tracers in the parallel maze of smaller, interconnected sinuses. Some authors (Pictet et al. 1969a, b; Simon 1980), however, observed phagocytosis most frequently. Even though phagocytes

with ingested RBC found in the small sinuses during the first few hours after the injection were impressive in our experiments, they were also always found in the large sinuses.

The question of how macrophages recognize autologous RBC as foreign matter, but usually do not phagocytize autologous lymphocytes, has not been clarified. Equally difficult to explain is the phenomenon of the so-called hemolymph node in whose sinuses autologous RBC regularly appear under physiologic conditions, which are also destroyed in the LN (Nopajaroonsri et al. 1974). The mechanism leading to primary stimulation of local phagocytes is also unclear. The highly activated state of LN macrophages seems to be important from a biologic perspective insofar as it promotes optimal nonimmunologic filtration of the peripheral lymph.

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