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FLUORESCENCE MICROSCOPIC STUDIES ON HEMAL LYMPH NODES IN RATS: A NEW IMMUNOBIOLOGICAL CONCEPT

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ABSTRACT

Hemal lymph nodes are characterized by a high content of blood cells most of them in different stages of erythrophagocytosis. These peculiar structures are not well understood up to now regarding their functional morphology. Above all, their biological relevance, especially to the phenomenon of disintegration of one's own blood cells, has eluded conclusive explanation so far. In the present study, hemal lymph nodes of 45 rats of the perirenal group were investigated by means of confocal laser fluorescence microscopy (CLSM) in combination with three fluorescent markers: latex standard particles, liposomes and autologous erythrocytes. Each marker briefly entered the hemal lymph nodes when injected into the kidney, whereas no notable migration took place after intravenous injection. Besides direct connections between hemal lymph nodes and the homolateral kidney, the study also revealed lymphatic communications with the contralateral kidney. Each marker was ingested by nodal macrophages, most of them surrounded by red blood cells (rosette formation) and laden with the by-products of cellular disintegration (erythrophagocytosis). Intimate contact of lymphocytes with macrophages as an expression of special interaction (emperipolesis) between both types of cells was frequently observed. A new concept is proposed, which ascribes to the hemal lymph nodes an important immunobiological role for the recognition of antigenic properties of one's own red blood cells permanently released by the kidney. The information macrophages obtain from these cells is presented to lymphocytes, which, in turn, initiates suppresser immune reactions. Under normal conditions, this mechanism of cellular identification and surveillance serves to preserve self-tolerance of the defense system against permanent renewal of one's own red blood cell population during a life time. In this way, an autoaggressive immune anemia is circumvented.

Hemal lymph nodes are characterized by a high content of red blood cells, which account for their peculiar brown or reddish color. Using light and electron microscopy, erythrophagocytosis is a prominent phenomenon in these organs. In many species like rodents and ruminants, hemal lymph nodes are constant elements. In rats, a group of hemal lymph nodes is located on both sides near the kidney *(Fig. 1)*. Since their first description in the last century (1) hemal lymph nodes have raised several general and special issues, which remain controversial. Although their basic morphology have been elucidated in rats and other species (2-10), the route which the red blood cells take to infiltrate the nodes and, above all, the biological relevance of intranodal erythrophagocytosis is elusive.

In a previous study using scanning electron microscopy (11), we showed that

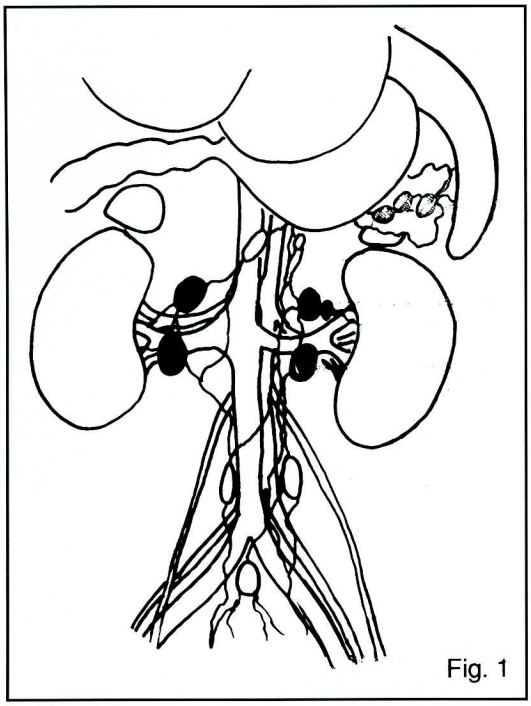

Fig. 1. Topography of the hemal lymph nodes in rat (perirenal group, marked in black).

hemal lymph nodes in rats were supplied with a dense capillary system and an extraordinarily high number of postcapillary venules controlled by sphincter-like mechanisms. Moreover, afferent vessels and a highly spacious intranodal sinus system were apparent. The findings were interpreted as a highly specialized histiocytic system in hemal lymph nodes and, with regard to the special organization of the post-capillary venular system, as a highly effective base for the homing of lymphocytes. In the present study, we attempted to gather further information on erythrophagocytotic activity and other mechanisms of cellular interaction of the nodal macrophages. Using modern fluorescence microscopy based on confocal laser microscopy (CLSM) and intrarenal injection of three different kinds of markers — polystyrene standard particles, liposomes and autologous red blood cells — we hoped to determine the functional significance of the hemal lymph nodes in general and the phenomenon of erythrophagocytosis in particular.

MATERIALS AND METHODS

In 45 adult rats (Wistar strain) of both sexes, weighing 250 - 350 g, the region of the left and additionally (in 3 rats), the region of the right kidney was exposed under deep anesthesia (Trapanal, 100 mg/kg body weight, intraperitoneally). 0.5 - 0.2 ml of the marker substance was injected beneath the capsule into the outer renal cortex of each exposed organ. The following markers were used: polystyrene standard particles of 1.0 µm stained with FITC (Fluoresbrite®, Polysciences, Oregon, USA) (13 experiments), liposomes of 1.0 µm manufactured on lecithin base and stained with fluorescent yellow or pyronin G (12 experiments), and autologous red cells stained with the cell linker PKH 26 GL (Sigma Chemie, Deisenhofen, Germany) (8 experiments). For this purpose, blood was drained via the femoral vein and processed with the cell linker 30 minutes before operative preparation. In other cases, 0.3 ml of the marker (4 experiments for each marker) were injected into the femoral vein.

One group of the treated rats was killed by thoracotomy and opening of the cardiac ventricles at different time intervals ranging from 10 minutes to 6 hours after injection. A second group of rats was killed 1, 3, 6, 15 and 42 days after injection. The blood circulation was rinsed with 38°C heparinized Ringer's solution and thereafter with 4° C cold 2.5 % glutardialdehyde solution via a cannula introduced into the aortic arch. The renal hemal lymph nodes on both sides were removed from the opened abdominal cavity and cut under the dissecting microscope with a razor blade in 100 - 300 µm thick slices. Some specimens were counterstained with azure and ethidium bromide; others remained unstained. The slices were mounted on object holder glasses and sealed under a cover glass with resin. Prepared undried specimens were

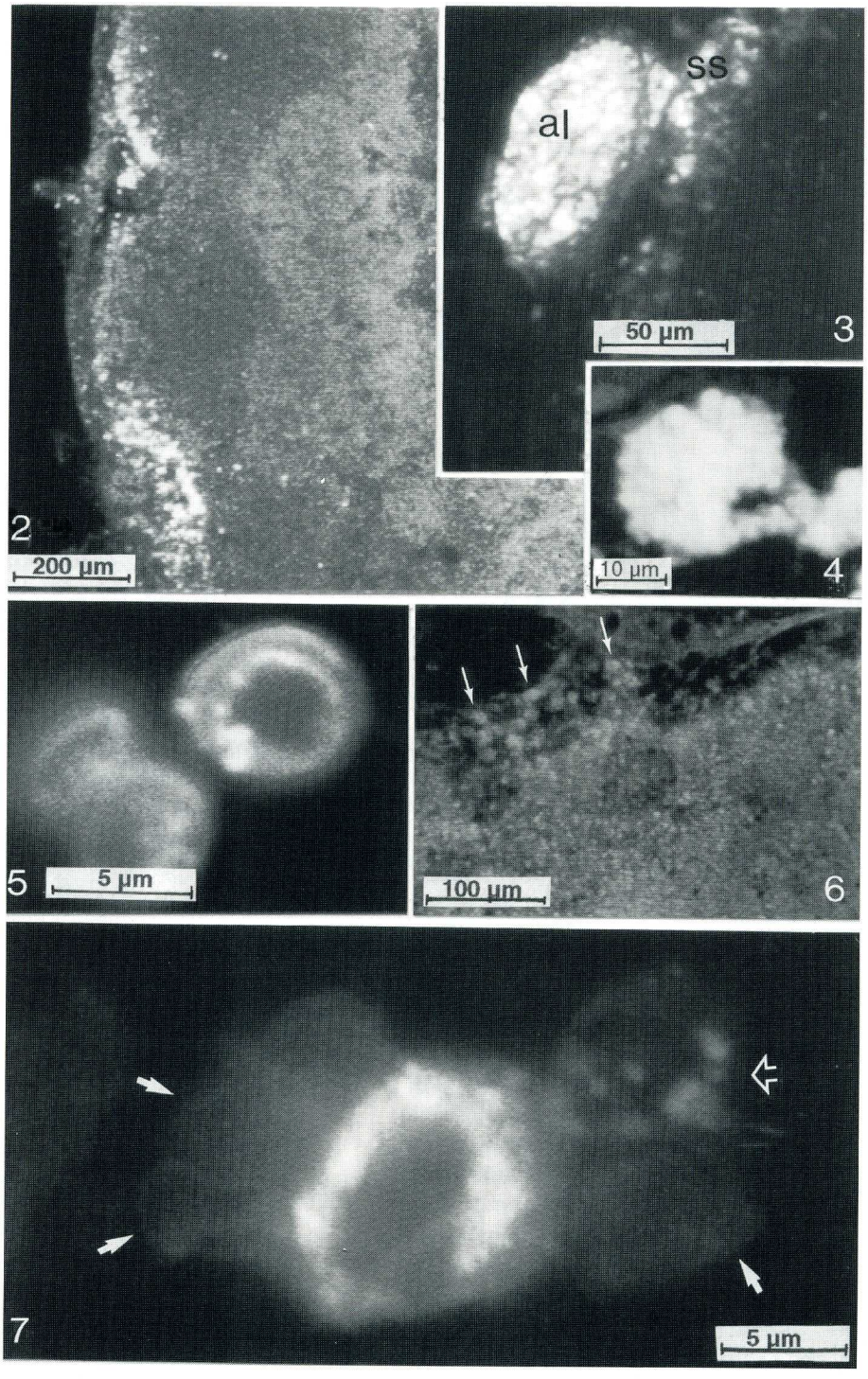

Fig. 2. Bright fluorescence appears within the subcapsular zone of a hemal lymph node two hours after injection of polystyrene microparticles into the kidney.

Fig. 3. Close to the nodal capsule, an afferent lymphatic (al) filled with polystyrene microparticles is shown in that specimen. Some microparticles have already migrated to the subcapsular sinus (ss).

Fig. 4. Macrophage of the subcapsular sinus from the specimen shown in Fig. 2 laden with injected microparticles.

Fig. 5. Red blood cells freshly stained with the cell linker PKH 26 before intrarenal injection. Note the spot-like pattern of fluorescence appearing on the surface of these cells.

Fig. 6. Hemal lymph exhibiting fluorescence (arrows) from stained autologous red blood cells, which have infiltrated the subcapsular sinus three hours after their injection into the kidney.

Fig. 7. Macrophage of a hemal lymph node with attached red blood cells (light arrows). The specimen was removed $4^1/_2$ hours after intrarenal injection of stained autologous red blood cells. Bright fluorescence with an excitation closely corresponding to the stained red blood cells is seen in the cytoplasm. One attached red blood cell contains a fluorescence pattern (dark arrow) similar to that shown in Fig. 5.

examined in the CLSM (Leica, Bensheim, Germany). Photographic records were made from the monitor screen of the CLSM.

RESULTS

Each marker (polystyrene microparticles, liposomes and stained autologous red blood cells) was detected in the renal hemal lymph nodes when injected into the kidney. In the initial phase of such an experiment, the area of fluorescence produced by each marker was confined to the subcapsular sinus *(Figs. 2,3,6)*; in some experiments, also to the afferent lymphatics *(Fig. 3)*. On the other hand, an accumulation of fluorescent markers in these spaces or other corresponding nodal compartments was never found when the markers were injected intravenously. In these latter experiments, the markers appeared only as sporadic elements randomly distributed in the cortical parenchyma near blood vessels. Although the highest concentration of the markers was noted in the hemal lymph nodes related to the homolateral kidney, in which the deposit was set, a weak fluorescence signal was noted also in the nodes of the contralateral side. Consequently, when two different markers, liposomes stained with fluorescent yellow and those with pyronin G, for example, were injected separately into the kidney of both sides, each homolateral hemal lymph node contained both marker fractions. The periods of time the three markers took to reach the hemal lymph node from the kidney differed slightly. Fluorescent liposomes were detected in hemal lymph nodes as early as 10 minutes after intrarenal injection, whereas stained autologous red blood cells and fluorescent polystyrene particles needed one hour and longer.

In the further course of an experiment, 1 - 8 days after intrarenal injection of the markers, the zone of fluorescence within the hemal lymph nodes gradually extended and then included the radial sinuses and the outer zone of the medullary sinus *(Fig. 8)*. The fluorescence caused by the markers faded after 2 - 4 days, when autologous red cells were used, and remained nearly unchanged up to 42 days in specimens which had taken up polystyrene particles. Fluorescence created by liposomes was well recognized in most samples until 15 days after injection. In late stages of an experiment, fluorescence emanated from single cellular elements of the cortical parenchyma and disappeared in the sinuses at the same time.

High power imaging of CLSM revealed that each marker was captured soon after entering the hemal lymph node by large macrophages of the sinuses *(Fig. 4,7, 9)*. Hence, free liposomes, polystyrene microparticles and individually stained red blood cells could be clearly recognized only in the initial phase of an experiment. Two to three hours after intrarenal injection, most of the markers were already identified as ingested elements within the cytoplasm of the sinus macrophages *(Fig. 4)*. A large amount of markers was also taken up by macrophages, which exhibited a rosette-like formation with unstained blood cells *(Figs. 7,9)*. The cytoplasm of the macrophage contained both the products of the applied fluorescent marker and those of disintegrated red blood cells. CLSM images recorded on the base of optical sectioning and taken from counterstained specimens allowed us to distinguish the different structures relating to the process of ingestion. Disintegration of liposomes was characterized by a loss of the ball-like shape of these particles *(Figs. 10, 11)*. Formation of crystalline structures in reference to the dye component (fluorescent yellow) occurred at a later stage. Similarly, stained autologous red blood cells, once captured by macrophages, soon disintegrated producing a spotted fluorescence within the macrophage cytoplasm *(Fig. 7)*. Those cells treated with the cell linker PKH 26 exhibited some features which facilitated their identification. A fine-spotted pattern of fluorescence marked the cellular surface best seen in freshly stained cells spread on a slide *(Fig. 5)*. This pattern was also seen in stained cells infiltrating

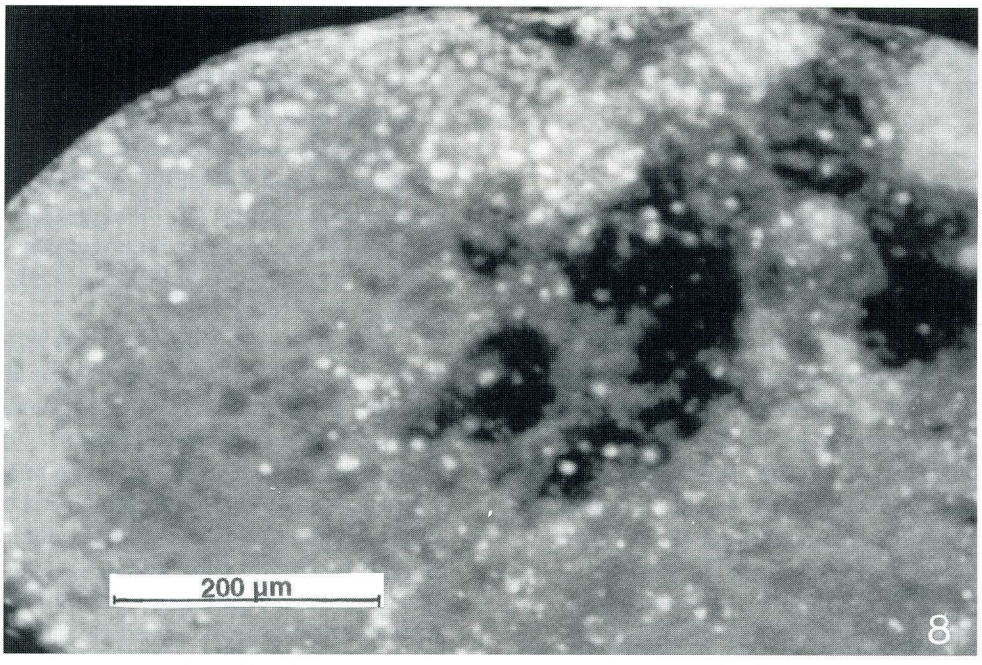

Fig. 8. Fluorescence emanating from different topographic areas of a hemal lymph node removed 5 days after intrarenal injection of fluorescent liposomes.

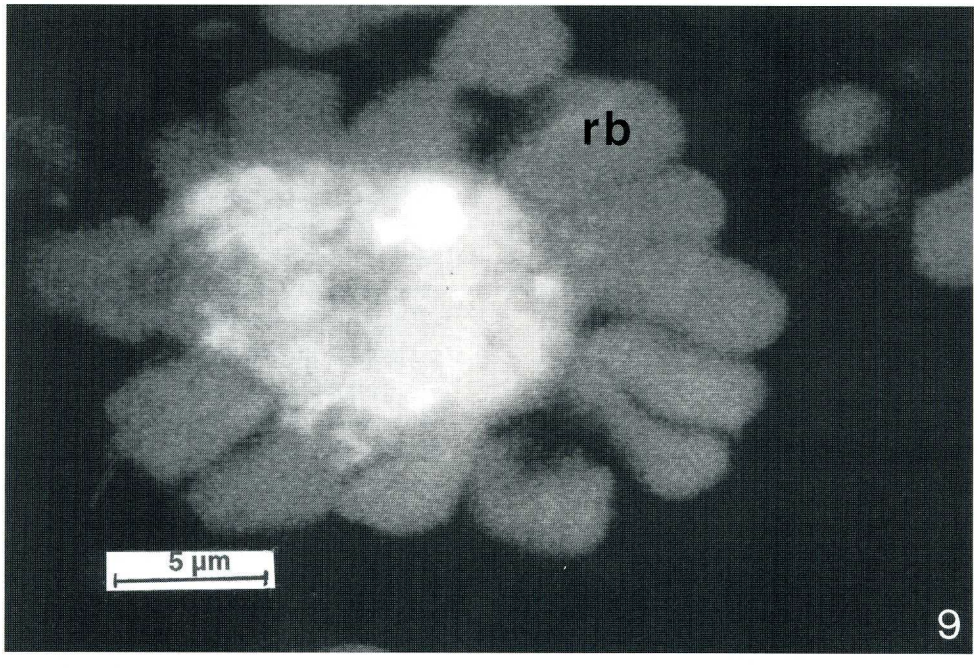

Fig. 9. The cytoplasm of a macrophage forming a rosette-like structure with red blood cells (rb) containing many ingested fluorescent liposomes. This high power micrograph was taken from the specimen shown in Fig. 6 after counterstaining with azure.

hemal lymph nodes, although their general fluorescence was diminished. A few hours after intrarenal injection, red blood cells provided with typical spot-like fluorescence were found attached to a macrophage, which, at that time, revealed fluorescence produced by ingested cells as well *(Fig. 7)*. Fluorescent polystyrene microparticles withstood ingestion by the macrophages for more than 42 days, and probably longer, without notable alteration in shape and fluorescent properties. In late stages of an experiment, only single macrophages of the cortex parenchyma were filled with these particles.

Another finding concerned the relationship of the sinus macrophages to lymphocytes. In examining counterstained specimens of hemal lymph nodes, it became apparent that in many specimens single lymphocytes were in close contact to numerous sinus macrophages of rosette-like configurations *(Fig. 10)*. High power micrographs of CLSM showed that certain lymphocytes seemed to be deeply pressed into the macrophage, although no true ingestion of these cells was detected. Fragmentation of lymphocytes or other nuclei-containing cells in a stage of interaction with macrophages was not found. The phenomenon of cellular interaction between macrophages and lymphocytes was less apparent in phagocytic cells which were neither laden with red blood cells nor marker substances.

DISCUSSION AND CONCLUSIONS

This experimental approach demonstrates that three different kinds of markers—artificial microparticles and liposomes as well as reinjected autologous red blood cells—reach the renal hemal lymph nodes after subcapsular injection into the kidney within a short timeframe. The supposition of some investigators (13) that there are direct connections between the kidney of one side and adjacent hemal lymph nodes is corroborated. In a previous SEM study, we were able to demonstrate afferent lymph vessels as joining links in corrosion casts (11). The present study also provides evidence for interconnections between hemal lymph nodes of both sides. Under normal conditions, red cells escaping from the renal blood circulation apparently use these lymphatic pathways to enter the hemal lymph nodes.

The special topographic relationship of hemal lymph nodes to the kidney is considered a strategic site, which contributes to the surveillance functions of the immune system (10). Migration of red blood cells into the interstices of the kidney and from there into the adjacent hemal lymph node is caused or, at least, stimulated by stress (14). Perhaps the high intrarenal blood pressure level from which renal microvessels generate urine formation, is an additional factor that facilitates the escape of red blood cells from the blood circulation.

After entering the intranodal sinuses, red blood cells are quickly captured by nodal macrophages. The large number of rosette-like cellular complexes, each consisting of a large macrophage laden with many attached and engulfed red blood cells, suggests high histiocytic activity within hemal lymph nodes. During rosette formation, two phenomena are distinctly visible. One is attachment of red blood cells to the surface of the macrophage; the other is the degradation of engulfed cells within the macrophage cytoplasm. These processes have commonly been interpreted as two stages of erythrophagocytosis. Recently, some investigators have studied the single steps of degradation of red blood cells by transmission electron microscopy (5,7,15,16). In fact, the phagocytic activity of the nodal macrophages appears extremely high. As shown in this study, polystyrene microparticles, liposomes and autologous artificially stained red blood cells are phagocytized in similar fashion. This could be also confirmed in our previous study using transmission electron microscopy (12).

Some investigators (17) propose that hemal lymph nodes are generally "hypoactive."

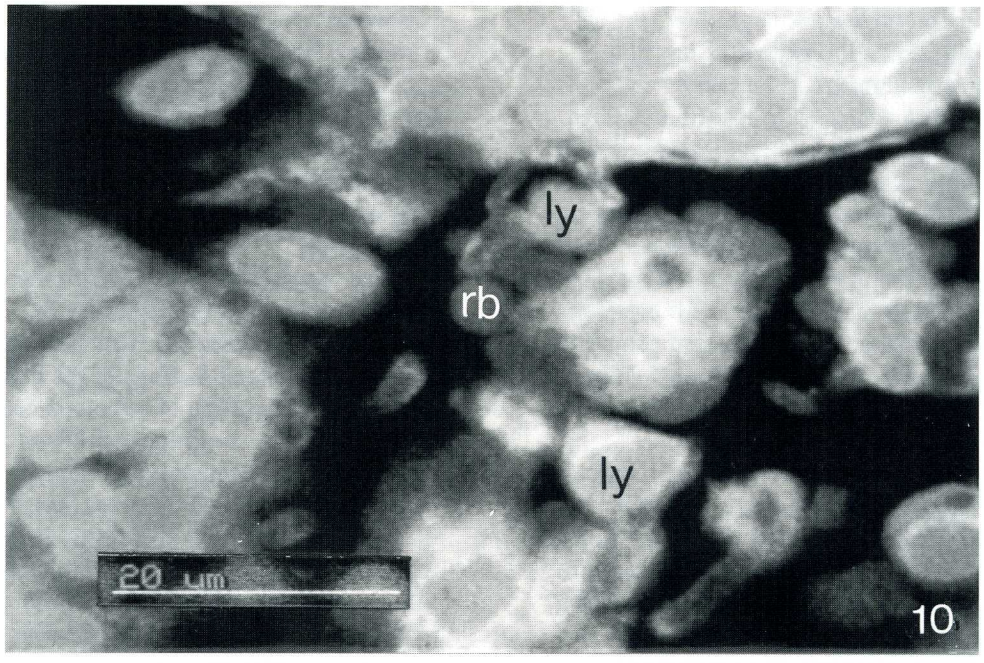

Fig. 10. View into the radial sinus of a hemal lymph node. In this experiment, liposomes were injected into the kidney 24 hours earlier. The sinus macrophages exhibit weak fluorescence with their body caused by ingested liposomes. Red blood cells (rb) appear as dark contours at the macrophage's surface. With counterstaining of the specimen with ethydium bromide and azure, many lymphocytes (ly), are visible in close contact with the macrophages.

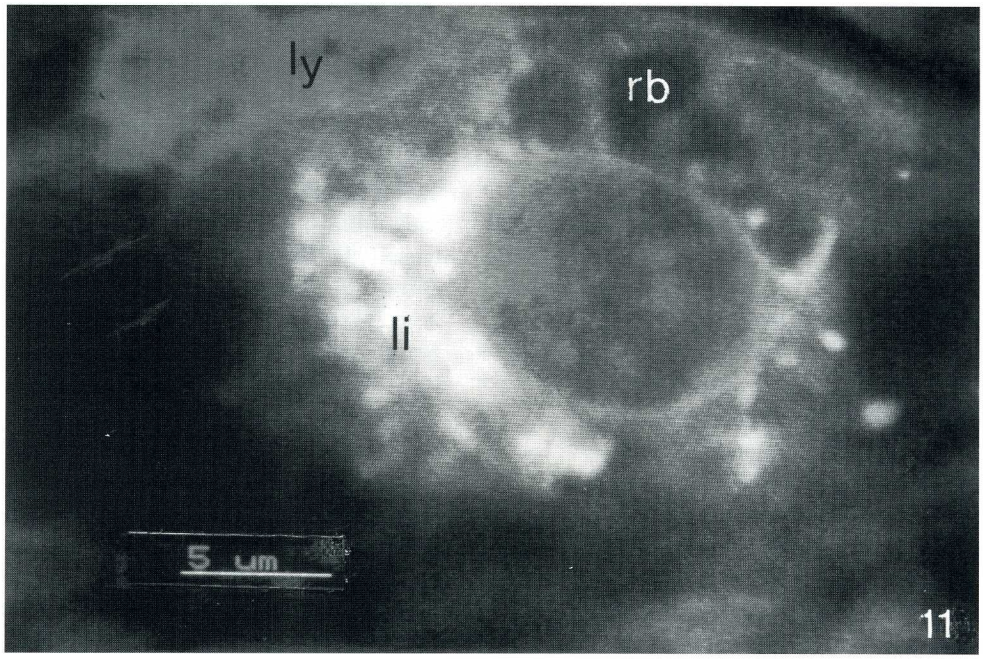

Fig. 11. High power image of a macrophage (from the specimen of Fig. 8) containing degraded erythrocytes (rb) along with ingested liposomes (li) in its cytoplasm. A lymphocyte (ly) is intimately associated with the macrophage surface (emperipolesis).

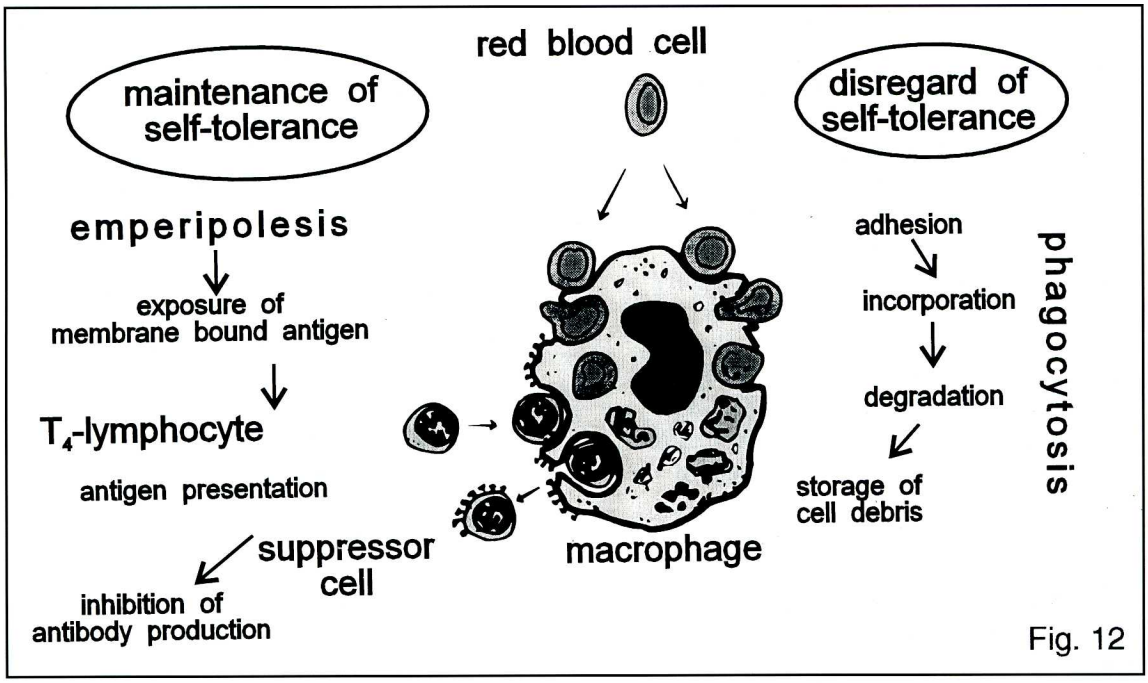

Fig. 12. Diagram demonstrating two different mechanism of interaction of red blood cells and macrophages in hemal lymph nodes. One, shown on the right, displays phagocytotic activity of the macrophage, which, by disregarding self-tolerance, leads to degradation of the red blood cells. The other shown on the left, takes into consideration self-tolerance and related processes such as emperipolesis and antigenic exposure of red blood cells to the macrophage and sequentially the antigenic presentation of T4-lymphocytes. By this sequence, defense system steadily gives information on the antigenic properties of red blood cells and, by initiating suppression functions, avoids autooveraggressive reactions against own red blood cell resulting in hemolytic anemia.

Whereas this supposition may be true with regard to lymphopoesis, the histiocytic system with its great number of large macrophages clearly surpasses the phagocytic capacity often found in other lymph nodes. Some questions, however, remain. Is the histiocytic system of hemal lymph nodes capable of only achieving non-specific endocytotic reactions, or does it also possess specific immune mechanisms? Why are red blood cells permanently captured and eventually destroyed by hemal lymph nodes? Does this process affect only effete cells or does it pertain to non-effete cells as well?

Inasmuch as corpuscular markers like polystyrene microbeads and liposomes are concerned, their ingestion and disintegration can be interpreted as a defense mechanism against foreign particulates. Similarly, the capture and rapid disintegration of extracted stained and reinjected red blood cells may be conceived of as a correspondent reaction against cells no longer recognized as "self." At the same time, effete red blood cells that normally migrate from the kidney into hemal lymph nodes, may be phagocytosed seemingly showing complete disregard of the principle of self-tolerance *(Fig. 12, right)*.

The large amount of intact red blood cells arranged around an individual macrophage seen in the light microscope suggests that some of these cells fulfill other functions than being simply precursors of degraded cells. Accordingly, we suggest that when coming into firm contact with the macrophage surface, red blood cells initiate an exchange of their antigenic properties thereby enabling presentation of such

information to other immunocompetent cells. In this regard, the contact of the macrophages with lymphocytes needs to be emphasized. Apparently, an intense contact base exists between the macrophage and the lymphocytes in the sense of "emperipolesis" (18) and "peripolesis" (19). These particular processes are interpreted as a special kind of cellular interaction, which provides both temporary engulfing and releasing of the lymphocyte by the host cell without disintegration. On that basis, information on the antigenic properties of red blood cells is processed. Besides this mechanism, transfer of immune information by messenger lymphocytes on the basis of afferent cell migration via blood circulation has been ascribed to hemal lymph nodes (5).

Our biological conception derived from these phenomena primarily concerns the immunobiological relevance of erythrophagocytosis and emperipolesis. Hemal lymph nodes are organs of major immune functions providing first and foremost surveillance of the body's red blood cell population. This major function among others characterizes hemal lymph nodes as structures which are especially sensitive to the status of the erythrocyte mass at any time. Red blood cells escaping from the blood circulation in the kidney present their antigenic properties to the nodal macrophages by surface contact and possibly by degradation of effete red blood cells. With subsequent interaction of the macrophages with other cells such as T-4 lymphocytes (emperipolesis), these cells absorb the information on the properties of red blood cells, and further steps such as stimulation of suppressor cells or inhibition of antibody production by plasma cells are initiated *(Fig. 12, left)*. In this way, hemal lymph nodes prevent autoaggression of the defense system against native red blood cells and thereby enable the body's defense system to respect the principle of self-tolerance.

The hypothesis seems highly plausible if the rapid renewal of the red blood cell population and the subsequent loss of information of antigenic properties is taken into account. Hence, the body's defense system must constantly refresh its knowledge about that particular cell type over its entire life cycle.

In human clinical experience, erythrophagocytosis dominates in axillary lymph nodes with breast disease and disorders of the histiocytic system (20). Under these conditions, however, the phenomenon of erythrophagocytosis has to be related to a reactive process and less to an immunobiological control mechanism as realized by rat hemal lymph nodes independent of a pathological disorder. Further experimental studies using immunofluorescence techniques in basic research and clinical experience in patients with a variety of hemolytic anemias associated with autoimmune intolerance may shed further light on these ideas.

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