

ACUTE OBSTRUCTIVE PARASTERNAL LYMPHEDEMA PRODUCED IN RATS WITHOUT SURGERY

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ABSTRACT

Although rat red blood cells (RBC) were altered by pretreatment with glutaraldehyde, they nonetheless were well absorbed into mediastinal lymphatics of Lewis rats after intraperitoneal injection. Unlike normal RBC, they did not pass freely through the draining mediastinal lymph nodes. Instead, clumps of free and phagocytosed glutaraldehyde-treated RBC distended and obstructed the afferent lymphatics and the sinuses of draining lymph nodes. The lymphatic obstruction caused edema in a clearly defined compartment of fatty connective tissue between the muscle layers of the ventral chest wall. The obstructive lymphedema interfered with the immunizing effect of small doses of sheep RBC injected intraperitoneally. This study emphasizes the potential utility of the rat's mediastinal lymphatics for studies of lymphatic leakage and obstruction.

Except by extensive surgical procedures (1,2), it has been difficult to reproduce obstructive lymphedema in experimental animals because of the availability of collateral routes for lymph flow. We describe here a simple, nonsurgical method to produce acute obstructive lymphedema in the rat by a single intraperitoneal (IP) injection of glutaraldehyde-fixed erythrocytes. The "fixed" cells are absorbed into the draining mediastinal lymph nodes which become obstructed. As a result, there develops lymphedema of the ventral chest wall. This experimen-

tal model may be unique in its utilization of the rat's mediastinal lymph nodes and parasternal lymphatics in a completely nonsurgical approach for producing obstructive lymphedema.

MATERIALS AND METHODS

Lewis rats of either sex, from 100 to 300g in weight, were housed in hanging cages with wire mesh floors and were fed Purina Rodent Chow 5001 and tap water *ad libitum*. They were fasted the night before inoculations to minimize accidental penetration of the gastrointestinal tract.

Rat blood was collected by aortic puncture and it was defibrinated by swirling around wooden applicator sticks. Normal red blood cells (RBC) were prepared by centrifugation, washing in sterile saline and resuspending in nine volumes of saline. Alternatively, the washed RBC sediment was suspended in an equal volume of saline and the suspension was mixed with an equal volume of 1% glutaraldehyde (Kodak or Polysciences) in phosphate buffered pH 7.4 saline. After incubation at 37°C for 30 minutes, the suspension was centrifuged, washed twice in large volumes of saline and resuspended in nine volumes of saline. The efficacy of the treatment was verified by the development of a brown color, by tight packing of the cells after centrifugation, and by the absence of hemolysis when an aliquot of cells was diluted in distilled water and left overnight at 4°C. Each recipient was given 5ml/100g body weight

of the treated or untreated RBC suspensions IP, which corresponded to a dose of packed cells of 0.5ml/100g of body weight. Variations of these procedures are described under Results.

A chemical peritonitis was produced in advance in some recipients because this procedure enhances absorption of blood and spleen cells and other particulates (3,4). Specifically, household bleach (5.25% NaOCl) was diluted 1:100 or 1:200 in saline, and 5ml/100g were injected IP one week beforehand.

Necropsies were usually done one day after injection of cells. Anesthetized rats were exsanguinated, abdomen and thorax were opened, and note was taken of residual inoculum in the peritoneal cavity or greater omentum, edema of the ventral chest wall ("parasternal edema," for brevity), dilation of the parasternal lymphatics (also known as internal thoracic lymphatics) and color changes in the mediastinal (parathymic) lymph nodes. Tissues were fixed in Bouin's fluid or picric acid-formic acid-formalin for simultaneous decalcification. Paraffin sections of diaphragm, omentum, ventral chest wall (sternum with costal cartilages and contiguous soft tissues), mediastinal lymph nodes and spleen were stained with hematoxylin and eosin. The lymph nodes were dissected clean and weighed in some experiments but not in others where preservation of the afferent lymphatics was desired. Serial 2mm blocks of the ventral chest wall were cut transversely in order to study the entire extent of the parasternal lymphatics in cross sections.

RESULTS

One day after IP injection of normal rat RBC, there was little or no residual inoculum in the peritoneal cavity, even on microscopic study of the omentum. The mediastinum and ventral chest wall had no edema or only microscopic traces (Fig. 1). The parasternal lymphatics were not visible grossly but blood had leaked out of some of them, as reported previously (5). The mediastinal nodes draining the peritoneal cavity were sometimes red in

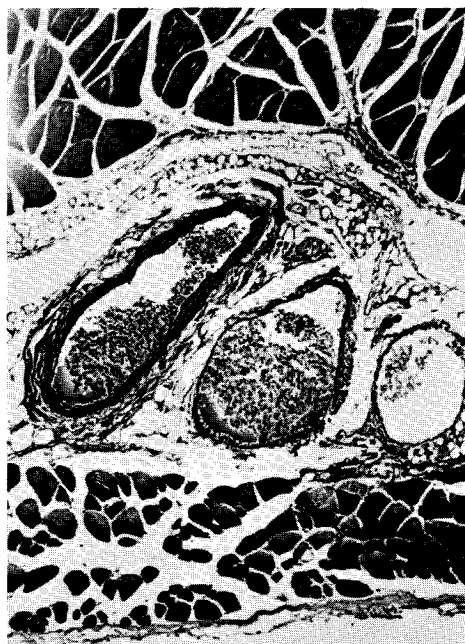


Fig. 1. The normal parasternal connective tissue between intercostal muscle (above) and transversus thoracis muscle and pleura (below) contains (from left to right) artery, vein, and lymphatic. Hematoxylin and eosin, x100.

color because their sinuses still contained some of the inoculated RBC.

Gross and microscopic observations were quite different one day after IP injection of glutaraldehyde-treated rat RBC (hereafter "glut-RBC"). The following descriptions are based on 220 rats in 22 experiments. Residual inoculum was usually observed clinging to the omentum, the falciform ligament, the caudate lobe of liver and the peritoneal folds around the spleen. Microscopically, some of the glut-RBC were already within phagocytic cells. Whether intra- or extracellular, the glut-RBC were distinguished by their marked refractility, strong eosinophilia, increased diameter, and conspicuous biconcave disc shape. The contrast with the rat's own normal RBC was exaggerated in some specimens because the Bouin's fixative fluid often lysed normal RBC but it had no effect on the injected glut-RBC.



Fig. 2. The parasternal connective tissue compartment is widened after IP injection of glutaraldehyde-treated RBC. It contains edema fluid and greatly dilated parasternal lymphatic vessels completely filled with darkly stained fixed RBC. The artery and vein are smaller than the lymphatic and they contain RBC with normal tinctorial property. Hematoxylin and eosin, $\times 100$.

The pleural side of the ventral chest wall and sometimes the adjacent mediastinal tissues (pre-pericardial, perithymic) were edematous, and the dilated parasternal lymphatics were grossly visible as gray or black lines. Microscopically, the edema fluid was a unilateral or bilateral homogeneous, amorphous, eosinophilic material which expanded the fatty connective tissue that separates the transversus thoracis (subpleural) and intercostal muscle layers of the ventral chest wall. Blood and lymphatic vessels in this adipose layer were surrounded by edema fluid (Fig. 2). The parasternal and diaphragmatic lymphatics were dilated by masses of glut-RBC with variable numbers of macrophages. Extravasates of glut-RBC and polymorphonuclear neutrophils sometimes surrounded the parasternal lymphatics. The neutrophilic reaction was often absent, and when present it was usually scanty. The edema occasionally extended into the septa of the intercostal muscles, among the fibers of the transversus thoracis muscle and into the diaphragm.

The cause of the edema and the site of the obstruction were found in the mediastinal lymph nodes which appeared tan or brown because of their content of

glut-RBC. They were not enlarged (fresh weight of mediastinal nodes averaged $33.5\text{mg} \pm 10.2$ S.D., $N=14$, compared to control rats averaged $30.4\text{mg} \pm 13.0$ S.D., $N=8$). Microscopically, the lymph node sinuses, especially the subcapsular, were stuffed with closely packed glut-RBC, mostly within macrophages and sometime in polymorphonuclear neutrophils (Figs. 3,4). The afferent lymphatic vessels just outside the nodes, like the more distal parasternal afferents described above, were dilated and contained glut-RBC in transit. Similar changes were found in some celiac, renal and lumbar nodes and in retroperitoneal lymphatic vessels. Smears made from teased lymph nodes, 24 hours after inoculation, had many clumps of glut-RBC and many very large macrophages that contained 20 or more glut-RBC. No clumps developed in an aliquot of the same glut-RBC inoculum that had been held *in vitro* at 37°C for 24 hours.

The changes described above were seen in rats of either sex, from 6 to 16 weeks of age (100 to 300g in weight) and also in mice and in rats subjected to pretreatment of the peritoneal cavity with sodium hypochlorite so as to produce a



Fig. 3. The subcapsular sinus of a draining lymph node is distended and obstructed by masses of darkly stained, clumped glutaraldehyde-treated RBC, either free or in large macrophages. (The afferent lymphatics were similar.) Hematoxylin and eosin, $\times 100$.

chemical peritonitis one week before injection of the glut-RBC. This procedure is known to prevent retrosternal hemorrhage (5) but it did not prevent the ob-

structive lymphedema in any of 14 rats in three experiments. In fact, these rats had obstruction of sinuses in mesenteric as well as mediastinal nodes, indicating a more widespread effect of the glut-RBC in the post-peritonitis state. Other procedures that did not affect the ability of glut-RBC to produce lymphedema are indicated in *Table 1*.

The changes produced by glut-RBC were present at 6 hours and at 1 or 2 days after inoculation. Dilated lymphatics were still present but were much less conspicuous after 4 or 7 days, and edema was slight or absent. The lymph nodes had few or no intact glut-RBC; instead there were many large macrophages with foamy and granular cytoplasm. Some of the dilated afferent lymphatics still had clumps of such macrophages and residual glut-RBC attached to the mural or valvular endothelium, and there was some thickening of the lymphatic wall.

The histologic evaluation of parasternal edema was confirmed by analysis of water content. One day after IP inoculation of glut-RBC or saline, the rats were anesthetized and exsanguinated. Skin, pectoral muscles and diaphragm were removed and the ventral thoracic cage wall was separated by cutting above the sternum and through the costochondral junctions and the sternoclavicular joints. It was weighed immediately and re-

Table 1
Procedural Changes that did not Affect the Ability of
Glutaraldehyde-Treated RBC to Produce Parasternal Lymphedema

1. Incubation of RBC in 1.0% or 0.25% glutaraldehyde instead of 0.5%^a
2. Incubation overnight at 4°C instead of 30 minutes at 37°C
3. Washing treated RBC five times instead of twice
4. Washing treated RBC five times over a four day period
5. Dose of treated RBC one-fifth or one-tenth usual dose
6. Dose of treated RBC suspended in twice or one-fifth usual volume of saline
7. Sheep RBC or human RBC instead of rat RBC
8. Freezing treated RBC at -40°C
9. Admixture of equal volume of normal rat RBC^b

^a0.125% was less effective; 0.05% had no effect and the treated RBC caused retrosternal hemorrhages like untreated RBC.

^bRetrosternal edema was accompanied by retrosternal hemorrhages indicating that each component had its usual effect, independent of the other.

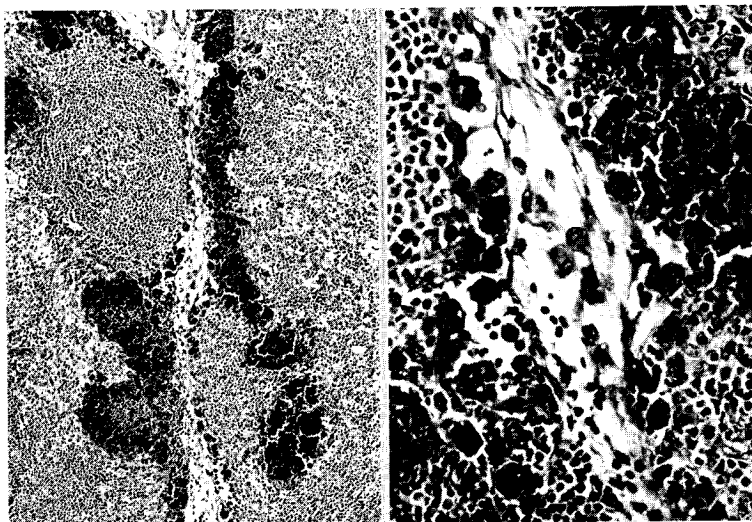


Fig. 4. Left--Parts of the subcapsular sinuses of two closely apposed lymph nodes are distended by masses of glutaraldehyde-treated RBC, while other parts are of normal caliber. Hematoxylin and eosin, x100; Right--Higher magnification of the center of the previous illustration shows a distended, obstructed segment of the subcapsular sinus in the lymph node on the right. The large RBC-filled macrophages are more readily discerned in the minimally dilated subcapsular sinus of the lymph node on the left. Hematoxylin and eosin, x400.

weighed after drying to constant weight (4 days at 60°C). The water content (weight loss divided by original fresh weight x100) was 67.90% ± 0.28 SD for the saline controls and 68.83% ± 0.21 SD for the glut-RBC injected rats, thereby confirming the presence of edema.

In view of the morphologic evidence of lymphatic obstruction, it was of interest to search for functional effects of glut-RBC blockade. Toward this end, small amounts of normal sheep red blood cells (SRBC) were added to the IP glut-RBC inoculum and serum was taken one week later for titration of anti-SRBC hemagglutinins (see reference 4 for methods). When the dose of packed washed SRBC was 5×10^{-4} ml, the resultant titer was 1:256 whether the SRBC were injected in saline or mixed with glut-RBC. A much smaller dose of SRBC, 5×10^{-6} ml, yielded titers of 1:32 or 1:64 when injected in saline but no detectable titer when mixed with glut-RBC. While other types of interaction between SRBC and glut-RBC have not been excluded, the inhibition of hemagglutinin formation is compatible with

failure of absorption of low doses of SRBC caused by lymphatic obstruction.

DISCUSSION

Although the chemical assay of water content confirmed the presence of edema, the histologic evaluation was preferred because the dissection that we devised to provide a reasonably reproducible specimen for assay yielded a rather large portion of chest wall which contained several tissues of widely varying water content, much of which was irrelevant (i.e., not edematous). In contrast, histologic study revealed the edema fluid precisely localized in the connective tissues between transversus thoracis and intercostal layers of muscle, the same compartment that harbors the lymphatics and blood vessels. In fact, the parasternal lymphatics of the rat are an excellent and underutilized subject for study of lymphatic leakage and lymphedema. The following advantages should be noted:

1. The sternum and costal cartilages provide a rigid framework during

fixation so that shrinkage artifacts are minimized.

2. The lymphatics do not have to be visualized or dissected in advance.

3. Fixation can be accomplished *in situ* by intrathoracic injection of fixative.

4. Serial transverse blocks of the ventral chest wall provide cross-sections of the lymphatics over virtually their entire course.

5. The initial lymphatics in the diaphragm and their termination in mediastinal lymph nodes are easily added to the study.

In addition to these technical advantages, our study was made possible by the enormous absorptive capacity of the diaphragmatic lymphatics for materials injected into the peritoneal cavity. The absorption of large amounts of either normal or glut-RBC injected IP was attended by leakage through the walls of the parasternal lymphatics. Leakage has been documented previously under other conditions and in other lymphatic vessels (5-8). The leakage of normal RBC was massive, leading to grossly visible hemorrhages (5) while the remainder of the normal RBC reached and passed through the draining lymph nodes without causing any lesions. In contrast, only modest amounts of glut-RBC leaked into the tissue spaces and the bulk of the inoculum remained in the lumens, distending and obstructing the lymphatic vessels and lymph node sinuses. Red blood cells hardened by glutaraldehyde are capable of plugging narrow channels (9). However, our observations indicate that the basis for the lymphatic obstruction was the clumping of the glut-RBC and the large macrophages which were filled with phagocytosed glut-RBC. The clumping was observed best in smears made from unfixed, teased lymph nodes. Other manifestations of the damage to RBC caused by the glutaraldehyde treatment were the changes in RBC color, refractility, size, eosinophilia, and resistance to hemolysis. It is noteworthy that when normal RBC and glut-RBC were mixed and injected IP together, both parasternal hemorrhages

and obstructive lymphedema occurred, each type of RBC causing its own type of pathology independently. It may be important that the pathology produced by normal RBC, but not that produced by glut-RBC, was prevented by prior induction of a chemical peritonitis. Equally important was the observation that the lymphatic obstruction was sufficiently extensive to cause functional effects when simultaneous immunization with small doses of sheep RBC was attempted.

Obstructive lymphedema in the rat's parasternal tissues would be more valuable as a disease model if it could be more chronic, and our current efforts are directed toward that objective.

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