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RETROSTERNAL HEMORRHAGE: AN EXPERIMENTAL MODEL FOR STUDY OF LYMPHATIC LEAKAGE

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ABSTRACT

Courtice and colleagues observed that blood injected into the peritoneal cavity of rats occasionally leaked from retrosternal lymphatics. The present work shows that this leakage is determined by volume as well as dose of inoculum. The uniform occurrence of visible retrosternal hemorrhage after injection of diluted blood suggests its use as a model for lymphatic leakage. Leakage was prevented when the blood was instilled during the healing phase of a chemical peritonitis.

In 1953, Courtice, Harding and Steinbeck published a classic paper in which they showed that the absorption of red blood cells (RBC) from the peritoneal cavity into the parasternal lymphatics was rapid, especially in the rat (1). They observed that "During the passage of the red cells along the parasternal lymph trunks, some of the red cells occasionally leaked out of the lymph tributaries of these trunks to give an extravasation of red cells beneath the pleura or into the pleural cavities." In the present work, we have defined the conditions to convert this "occasional" extravasation into a constant occurrence so that it might be used for further studies on lymphatic leakage.

MATERIALS AND METHODS

Lewis rats of either sex, 220-350g (Harlan Sprague Dawley, Inc., Indianapolis) were maintained in hanging wire mesh cages on Purina Rodent Show 5001 and tap water *ad libitum*. They were fasted overnight before intraperitoneal (IP) inoculations in order to avoid accidental penetration of the gastrointestinal tract. Rat or mouse blood was defibrinated by swirling over wooden sticks. Whole defibrinated blood or washed RBC, or dilutions prepared with sterile saline, were injected IP without anesthesia. The recipients rats were held vertically, head down, during injection, and then they were rapidly rotated three times to ensure wide distribution of the inoculum.

In the experiments to determine the effects of prior inflammation, a sterile chemical peritonitis was produced by IP injection of 50ml/kg sodium hypochlorite (NaOCl, household bleach) diluted 1:100 in saline, as described previously (2-4).

Rats were sacrificed by exsanguination from neck vessels while under ether anesthesia. The abdomen was opened in the midline. Residual fluid was absorbed into a pre-weighed 4 inch square gauze sponge which was pushed into all parts of the peritoneal cavity including lumbar gutters, pelvis and the subdiaphragmatic space. Testes and fluid in the scrotum

were pushed into the abdominal cavity by external pressure aided by gravity. While the sponge was being weighed, the abdomen was opened more widely with a horizontal incision, and the absorption of remaining fluid accomplished with a second gauze sponge. The second sponge usually absorbed only 1/10 to 1/20 as much as the first sponge and it left the peritoneum free of any visible blood. The procedure was carried out rapidly in order to minimize losses from evaporation. In each of two normal, uninoculated rats, 0.33g fluid was recovered in this manner; this "blank" value was subtracted form all recovery data. The same procedure was carried out in two rats that had been given NaOCl one week previously but no further inoculation. An almost identical "blank" value was obtained. The volumes of inoculated fluids were converted to grams (specific gravity of blood 1.05) and residual fluid was expressed as percent by weight of inoculum recovered in the sponges.

After removing residual blood, the thorax was opened and hemorrhages in the retrosternal area were noted and scored from 1+ to 4+, where 1+ was a minimal lesion, only a few mm in size, and 4+ was a maximal lesion covering a few cm^2. The parathymic nodes were weighed fresh.

Nodes and sternums were fixed in Bouin's fluid, the latter were decalcified, and both tissues were embedded in paraffin, sectioned and stained with hematoxylin-eosin-phloxine.

RESULTS

Retrosternal hemorrhages were observed 3, 5, 24, and 48 hours (but not 1 hour) after IP inoculation of blood. The hemorrhages usually occupied a triangular area, the apex behind the middle or upper third of the sternum and the base near the attachment of the diaphragm *(Fig. 1)*. Within this area, the hemorrhages were blotchy and irregular or they followed one or several costal cartilages. They never extended so far laterally as to reach the bony ribs. The

Fig. 1. Retrosternal hemorrhage in a rat, 5 hours after IP injection of blood. The heart and thymus have been reflected craniad and are visible at the top. Part of the hemorrhage follows the course of the costal cartilages.

term "retrosternal" was not strictly correct because a narrow vertical midline strip immediately behind the sternum was usually spared. The hemorrhages were frequently assymmetrical or even unilateral but without predilection for one side or the other.

Microscopically, the hemorrhages were subpleural or in the layer of fat that intervened between the thin strip of subpleural muscle and the costal cartilages with attached intercostal muscles. The main retrosternal blood vessels and lymphatics were located in this same layer of fat. The retrosternal lymphatics were filled with blood, presumably derived from the IP inoculum. In the midline, the epimysium of the subpleural muscle blended with the periostium of the sternum, which explained the inability of hemorrhages to penetrate to this midline site. Rats killed 8 days after inoculation had only hemosiderin deposits to attest to the occurrence of retrosternal hemorrhage.

Courtice et al had injected 20ml/kg of whole heparinized blood or washed RBC and observed occasional hemorrhages (1). In our rats, 10ml/kg of defibrinated blood produced retrosternal

Table 1
Volume and Concentration of IP Blood Inoculum Determine the Occurrence of Retrosternal Hemorrhages

Concentration of Blood[a]	Volume ml/kg	Total IP Dose of Blood ml/kg	Retrosternal Hemorrhages[b]
1	10	10	4,2,2
1/5	50	10	4,4,4,4
1	2	2	0,0,0,0
1/5	10	2	1,0,0,0
1/25	50	2	4,1,1,tr
1/25	10	0.4	0,0

[a]Rat blood was defibrinated. Concentration of "1" denotes undiluted blood. Note that concentration x volume = dose.
[b]Scored from 1 to 4 *(see text)* in individual rats. "tr" = trace.

hemorrhages in all subjects but some were mild. Uniformly severe hemorrhages were obtained when the same dose of blood was injected after dilution 1:5 in sterile saline *(Table 1)*. The importance of a large volume of inoculum was demonstrated even more clearly when a small suboptimal dose of defibrinated blood (2ml/kg) was injected with or without dilution *(Table 1)*. Washed RBC reconstituted with saline to original hematocrit and then diluted 1:5 produced the same results as the corresponding doses of whole blood recorded in *Table 1*. In subsequent experiments, 10ml/kg of blood or reconstituted RBC was the routine dose and it was diluted 1:5 in saline before injection. Hemorrhages were observed from mouse as well as rat RBC *(Table 2)* and rat blood from a different strain (LBN F_1 hybrids) was also effective. Rat blood injected into Fischer 344 rats produced hemorrhages similar to those in the Lewis rats that were used in all the other experiments.

Lymphatic absorption of dyes, metal powders, oils and cells is increased during the healing phase of a chemical peritonitis (2-4). Therefore, rats were given NaOCl IP one week before injection of blood or RBC. The NaOCl did not cause any obvious indisposition of the rats but it left them with fibrosis of liver and spleen surfaces and various degrees of obliteration of the greater omentum (2). The NaOCl pretreatment prevented retrosternal hemorrhages following IP blood *(Table 2)*.

The effect of NaOCl on absorption of the inoculum was studied *(Table 2)*. As a baseline for this study, two rats were inoculated with blood and immediately anesthetized and exsanguinated. Ninety percent of the inoculum was recovered from their peritoneal cavities. The missing 10% may have been absorbed during the process of obtaining the peritoneal contents and/or may have evaporated during the weighings. Rats killed at intervals after inoculation revealed a progressive diminution of peritoneal content but there was a wide spread of individual values and no clear difference between control and NaOCl treated rats.

Eight additional rats were given NaOCl 3 days instead of 7 days before challenge with the usual dose of rat blood or RBC. None of them had retrosternal hemorrhages when necropsied 3, 5, or 24 hours later in contrast to the controls. In an effort to overcome this inhibitory effect, two rats were given 50ml/kg of a rat RBC suspension (five times the usual dose) one week after NaOCl treatment, but to no avail, whereas the control rats (no NaOCl) developed the usual retrosternal hemorrhages.

Table 2
Healing Peritonitis Prevents Retrosternal Hemorrhage

Hours After IP Blood[c]	Retrosternal Hemorrhages[a]		Residual IP Blood, %[b]	
	Control	After NaOCl	Control	After NaOCl
0 (rat blood)	0,0	ND	90,90	ND
3 (rat RBC)	4,2,2	0,0	65,59,48	72,68
4 (rat RBC)	3	ND	52	ND
5 (rat blood)	4,3	1,1,0,0	33,21	60,39,33,18
5 (rat blood)[d]	4,3	1,1	5,3	15,9
6 (mouse RBC)	4	0	15	13
24 (rat RBC)	4	ND	0	ND
24 (rat blood)[d]	4,4	1,0	0,0	0,0
24 (mouse RBC)	4	0	3	5

[a]Scored from 0 to 4+ *(see text)* in normal rats with healing peritonitis due to injection of NaOCl one week previously. ND = not done.
[b]Weight of fluid recovered on gauze
[c]10ml/kg of whole blood or washed RBC reconstituted to original hematocrit, diluted 1:5 for injection
[d]Female recipients; all others were male

DISCUSSION

The original observations of Courtice, Harding and Steinbeck on retrosternal hemorrhages (1) were incidental to a detailed study of the absorption of RBC from the peritoneal cavity (1,5). The escape of RBC was considered to be basically similar to the escape of plasma into the retrosternal tissues and pleural cavity after IP injection of plasma (6,7). The same points were made without further elaboration in the monographs published 3 and 17 years later (8). There is considerable interest in the study of the permeability of lymphatic vessels in relation to function and ultrastructure (9-11), and special methods and approaches have been developed (12,13). Nevertheless, we have been unable to find any further application of this extremely simple way to produce and study leakage of cells from lymphatics, nor have we found any mention of the phenomenon in various monographs on lymphatics (10,14). The explanation may be the relative infrequency of its occurrence (1). However, the uniform occurrence of hemorrhages when we injected large volumes of diluted blood should make it useful as an experimental model. It should be noted that increasing the volume of fluid or the number of particles inoculated IP has been shown to increase the amount absorbed in other experiments (15,16).

We studied the effect on retrosternal hemorrhages of a subsiding peritonitis because of previous demonstrations that inflammation in the healing phase increased the absorption from the peritoneal cavity of various particulates (2-4). Courtice et al had attributed the leakage to partial obstruction within the parathymic lymph nodes and subsequent damming back of lymph flow (1). If this is valid, the slight enlargement of these lymph nodes following NaOCl peritonitis might have prevented the obstruction and thereby prevented the leak. Alternatively, the peritonitis might have prevented leakage by reducing the permeability of the lymphatics draining the inflammed cavity, but there is no evidence for this hypothesis. In fact, *increased* permeability has been noted in lymphatics draining a site of inflammation (albeit of a different location and type) (9). Nevertheless, the lymphatic vessels are not merely passive conduits (17); they are the actual site of leakage and they may be directly involved in the inhibition of leakage by NaOCl.

The data of *Table 2* provide a hint of a third explanation: NaOCl might decrease or slow the absorption of blood from the peritoneal cavity. Decreased absorption of amorphous glass particles has been found one day after inception of peritonitis by turpentine (16). Unfortunately, the amount of residual blood that was found was so variable that no conclusion could be drawn *(Table 2)*. Perhaps the inhibition of leakage of NaOCL involves some combination of retarded absorption and structural changes in the draining lymphatics and/or in the draining lymph nodes.

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