Lymphology 18 (1985) 56-63

ENDOLYMPHATIC APPLICATION OF BLEOMYCIN OIL SUSPENSION IN DOG MODEL

P. Hirnle

Departments of Experimental Surgery and Gynecology, University of Bonn and Department of Gynecology, University of Tubingen, West Germany

ABSTRACT

To explore the potential usefulness of a cytostatic agent deposited directly in lymph nodes, 4ml Bleomycin oil suspension (Oil-Bleo) was injected over one hour into hind leg lymphatics of seven dogs. Five of these dogs received a second, identical dose one week later into lymphatics of the contralateral hind leg.

Peak serum concentration of Oil-Bleo after the first injection (7 dogs) was 12.3 μg/ml but after the second injection (5 dogs) was slightly lower (10.8 μg/ml). Maximum level of Oil-Bleo in blood was 12% of the endolymphatic dose and represented the "spillover" from lymph transport. In a control experiment, in which 60mg of aqueous Bleomycin was injected, the serum spillover was one-third higher.

Large amounts of Oil-Bleo were stored in popliteal and retroperitoneal lymph nodes for several weeks. Twenty-four hours after injection the weight of "treated" lymph nodes was 73% greater than "untreated" nodes and one month later treated lymph nodes were still 37% heavier. After 24 hours, 4.7% of Oil-Bleo instilled was distributed within extracted lymph nodes, and one month later 0.12% was still detectable. By contrast, after aqueous Bleomycin infusion, only 0.05% was detected in these lymph nodes after only six hours. In general, lymph nodal architecture was preserved after Oil-Bleo. Together the findings suggest that Bleomycin oil suspension may be a useful agent for treatment of lymph nodal metastases by endolymphatic infusion.

Malignant metastases in retroperitoneal lymph nodes are not readily amenable to therapy. Complete surgical excision is technically limited whereas the maximal allowable irradiation dose is often insufficient to eradicate tumor growth. On the other hand, systemic treatment with cytotoxic drugs is complicated by notable side effects, and therefore it is difficult to achieve a therapeutic concentration of these agents in diseased lymph nodes.

There are few reports (1-3) of treatment of regional lymph node metastases by endolymphatic infusion of cytotoxic agents. Their overall effectiveness appears limited but it is noteworthy that these drugs have usually been administered in aqueous solution. Unfortunately, these solutions diffuse readily into surrounding tissue after endolymphatic injection and reach lymph nodes only in small amounts (4).

In the following experiments, a cytostatic agent, Bleomycin, was injected as an oil suspension. It was anticipated that this suspension favored deposition in regional lymph nodes for a prolonged interval and thereby provides the basis for more effective endolymphatic treatment of lymph nodal metastases in patients.

MATERIALS AND METHODS

Eight young related bastard dogs with a weight of 13±1.5kg were used. The experimental plan is outlined in Table 1.

Dogs anesthetized with Pentobarbitone received a research sample of Oil-Bleo (generously supplied by the Nippon Kayaku Co., Tokyo) by endolymphatic injection.

Table 1.

Endolymphatic Injection of Suspension Oil Bleomycin Compared with Aqueous Bleomycin

Dog	Experiment	Time	Operation
1-7	I	Day of injection	Endolymphatic injection of 4ml Oil-Bleo (60 ml Bleomycin) on right side within one hour. During injection and 6 hours thereafter sampling of blood and urine.
1 (rapid experiment)	II	One day later	Collection of lymph from the thoracic duct. Extirpation of lymph nodes.
2-6 (intermediate experiment)	III	7 days later	Second endolymphatic injection of 4ml Oil-Bleo (60 mg Bleomycin) on left side within one hour. During injection and 6 hours thereafter sampling of blood and urine.
	IV	14 days later	Collection of lymph from the thoracic duct. Extirpation of lymph nodes.
7 (prolonged experiment)	V	28 days later	Collection of the lymph from the thoracic duct. Extirpation of lymph nodes.
8 (control)	VI	Day of injection	Endolymphatic injection of 60ml Bleomycin in 4ml physiological saline within one hour. During injection and 6 hours thereafter sampling of blood and urine. Subsequently collection of thoracic duct lymph and extirpation of lymph nodes.

The concentration of Bleomycin was 15mg/ml suspension. The needle was inserted into a lymphatic between the dorsal side of the foot and the shank. Correct anatomic placement of the needle (#55, supplied by Mathys and Sohn, Zurich, Switzerland) was confirmed by injecting 0.5ml of an oily contrast medium (Lipiodol Ultra-Fluid , Byk Gulden, Konstanz, West Germany) followed by an x-ray (Fig. 1). One dog (#8) received aqueous contrast medium (Iotasul , research sample, Schering, West Berlin) and aqueous Bleomycin (Bleomycinum Mack , Illertissen, West Germany). The rate of injection was 1mg Bleomycin/min.

To collect lymph from the thoracic duct, a reservoir was formed around the junction of the duct with the venous system in the neck. This "vein sac" filled with lymph and was drained by a catheter (5). In three instances, the thoracic duct was cannulated directly using a thin teflon catheter (6). At various intervals the lymphocyte count in the blood was determined.

The following three groups of lymph nodes were examined (7): popliteal, pelvic (internal iliacs, medial and lateral sacrals, deep inguinals; grouped together because of great anatomic variations) and external iliac. Half of each lymph node was fixed for

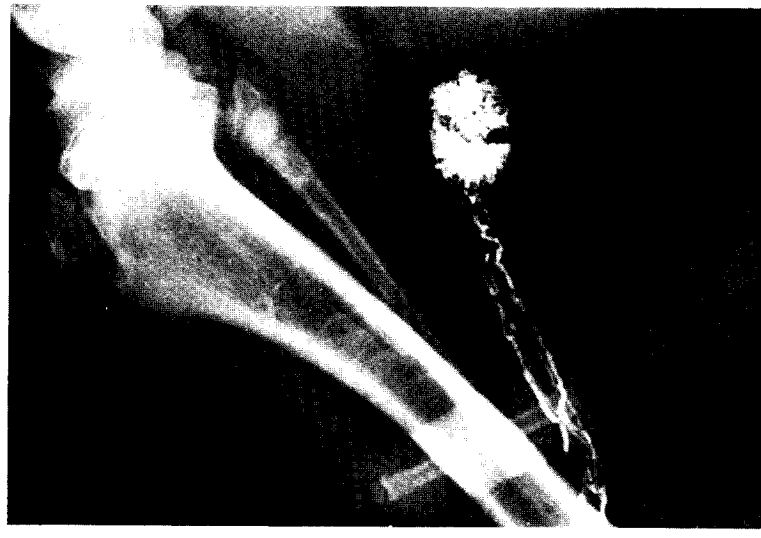


Fig. 1: X-ray confirmation of correctness of lymphatic cannulation. After injection of 0.5 ml Lipiodol, afferent lymphatics and a popliteal lymph node partially are seen. Contrast medium flows more proximally only when lymph node is filled.

histological examination and the other half was homogenized to determine the Bleomycin concentration. Tissue was pulverized in liquid nitrogen by a Micro-Dismembrator (B. Braun, Melsungen, West Germany). Homogenate was diluted 1:4 with PBS and centrifuged 60 min at 100,000g and 4° C. The concentration of Bleomycin in the supernatant was determined by radioimmunoassay with I-labeled Bleomycin based upon previously-published methods (8,9).

RESULTS

Concentration of Bleomycin in serum:

A rapid rise in serum concentration of Bleomycin occurred with both oil suspension and aqueous media. The highest serum concentration was obtained with aqueous Bleomycin (Table 1, Exp. VI), which had ⅓ higher average level than suspended Bleomycin (Table 1, Exp. I and III). Differences in the concentrations for Bleomycin are shown in Figs. 2 and 3. In each instance, the serum concentration of Bleomycin fell to < 1 μg/ml after four hours, and remained virtually constant for the next two hours.

From the peak concentration in serum, the total amount of Bleomycin in blood was computed. This quantity in blood represents a distinct fraction of the original amount instilled endolymphatically and is

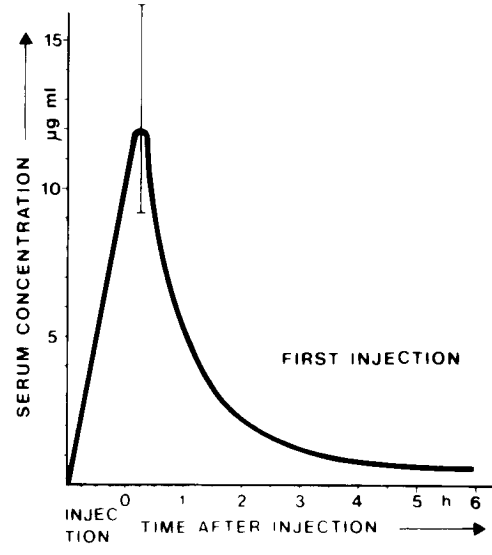


Fig. 2: Experiment I. Average concentrations of Bleomycin in serum up to six hours after endolymphatic injection of 4 ml Oil-Bleo (60 mg Bleomycin, n=7).

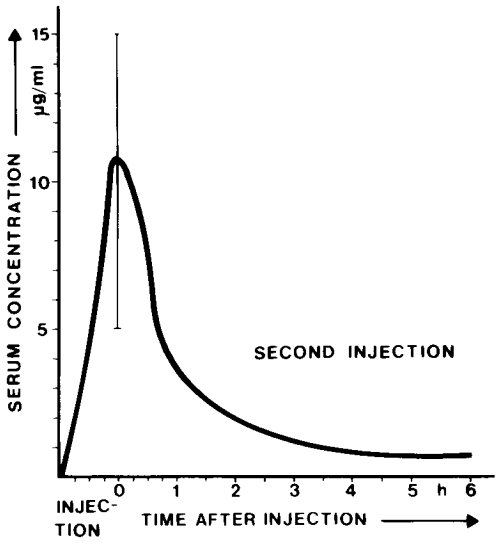


Fig. 3: Experiment III. Average concentration of Bleomycin in serum up to six hours after endolymphatic injection of 4 ml oil-Bleo (60 mg Bleomycin) on the left side (n=5) seven days after a similar endolymphatic effusion on the right leg (see Fig. 2).

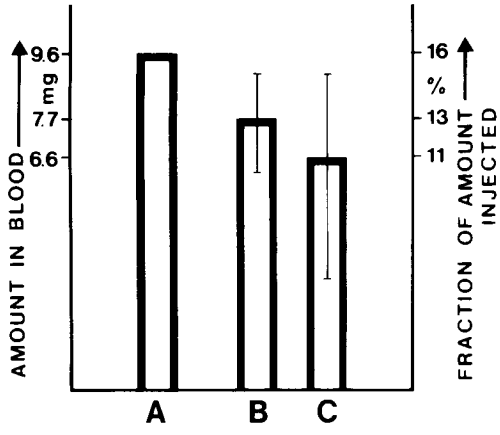


Fig. 4: The peak amount of Bleomycin in blood after endolymphatic injection of 60 mg Bleomycin — A: aqueous (control): n=1. B: in Oil-Bleo suspension (after the first injection): n=7. C: in Oil Bleo suspension (after the second injection): n=5.

designated as the "spillover" factor (Fig. 4).

Concentration of Bleomycin in urine:

A typical time course of the concentration of Bleomycin in urine (after Oil-Bleo injection) is depicted in Fig. 5. The peak concentration after the second injection (Table 1, Exp. III) is slightly less than that after the first injection (Table 1, Exp.1), but remains higher over the ensuing six hours.

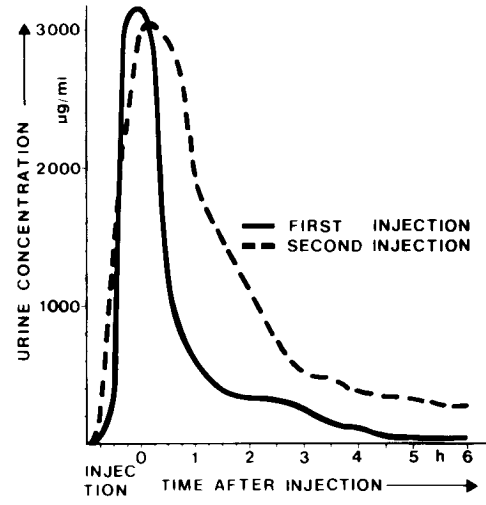


Fig. 5: Dog #2. Concentrations of Bleomycin in urine after two endolymphatic injections of 4 ml Oil-Bleo separated by seven days. The concentration after the second injection exhibits a slightly lower peak than that after the first injection, but remains greater over the ensuing six hours.

Concentration of Bleomycin in lymph:

Twenty-four hours after endolymphatic injection of Oil-Bleo, the concentration of Bleomycin in thoracic duct lymph was 0.45 µg/ml whereas the serum concentration was extremely low and beyond detection by the assay (i.e., less than 10ng/ml). In later experiments (Table 1,IV, V) Bleomycin was not detected above 10ng/ml either in serum or lymph. By comparison in dog #8 receiving Bleomycin in aqueous solution, the concentrations of Bleomycin in blood and thoracic duct lymph were much higher and nearly equal at 0.9 µg/ml.

Concentration of Bleomycin in lymph nodes:

These findings are depicted in Fig. 6-9, where the lymph node groups are identified by circles. The upper value denotes the

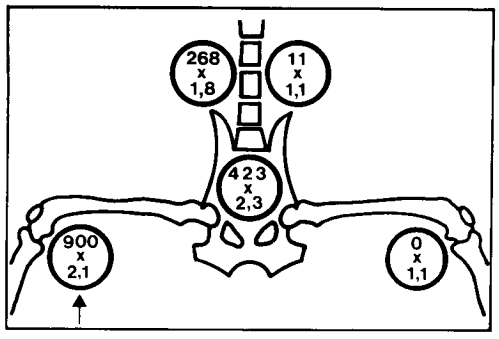
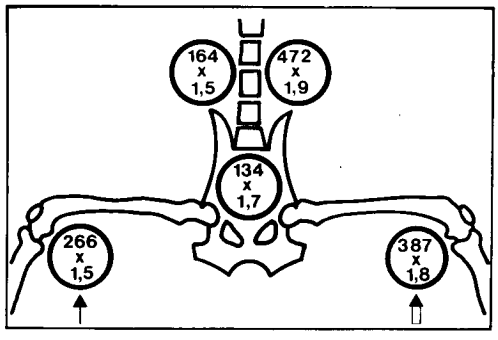


Figure 6



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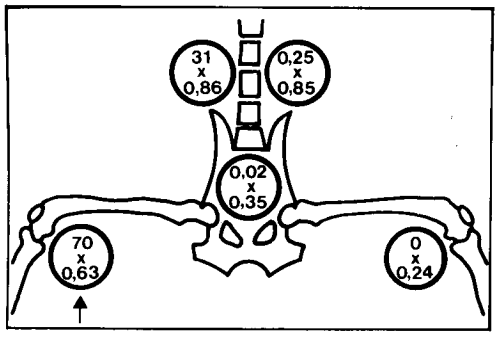

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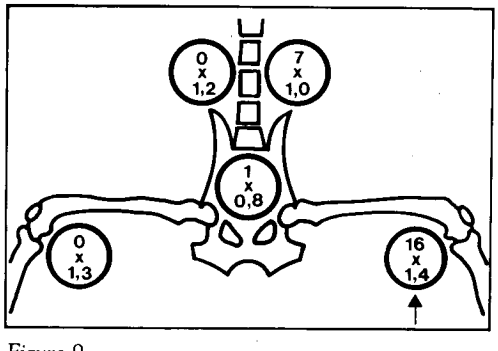

Figure 9

concentration (μg Bleomycin per g lymph node tissue) and the lower value the weight of the lymph node. The product of these two values yields the amount of Bleomycin in μg. The first injection is indicated by a thin arrow and the second dose by a broad arrow. The results are tabulated according to the number of experiments (see Table 1).

Experiment II: Values are shown in Fig. 6. Lymph nodes "treated" with Oil-Bleo (right) are 73% heavier than "untreated" nodes on the left. All together the lymph nodes contain 4.7% of Bleomycin injected.

Experiment IV: Average values from dogs 2 to 6 are shown in Fig. 7. Lymph nodes on the left (second injection) are 21% heavier than those on the right (first injection). The concentration of Bleomycin is 2x greater on the left than right.

Experiment V: Values are shown in Fig. 8. Lymph nodes on the right are 37% heavier than those on the left. Together the lymph nodes contain 0.12% of Bleomycin injected.

Experiment VI: Values are shown in Fig. 9. The infusion did not alter the weight of the lymph nodes. After six hours, only 0.05% of injected Bleomycin is detected.

Bleomycin concentrations in the popliteal lymph nodes of all dogs are presented in Fig. 10: after ¼ day, dog 8 (aqueous Bleomycin) after one day, dog 1 (rapid); after seven and 14 days, dogs 2 to 6 (intermediate); after 28 days, dog 7 (prolonged).

Toxic effects of Bleomycin:

Bleomycin exhibited limited toxicity with a maximal decrease of 25% in the blood lymphocyte count. Despite considerable filling of lymph nodes with suspension (see Fig. 11) there were no

Fig. 6-9: Lymph nodal distribution of Bleomycin after endolymphatic effusion of Oil-Bleomycin. The upper circled value depicts the concentration within node and the lower value the lymph node weight. The first injection is indicated by a thin arrow and the second injection by broad arrow. See Table 1 and text (RESULTS).

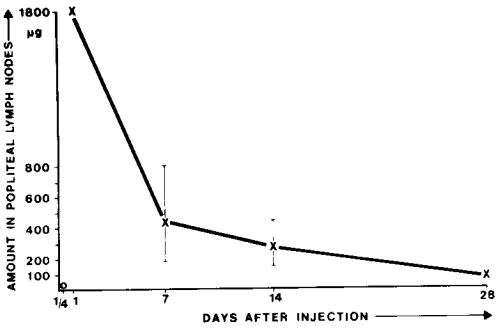

Fig. 10: Decrease of oil-suspension Bleomycin (Oil-Bleo) concentration in popliteal lymph nodes (X————X) over 28 days after endolymphatic injection of 4 ml Oil-Bleo (60mg Bleomycin). The concentration of aqueous Bleomycin six hours after endolymphatic injection of 60mg Bleomycin and 4ml physiological saline is represented by a circle (o) lower left corner.

deleterious changes in the basic architecture of the nodes, attributable to Bleomycin, an assertion confirmed by light microscopy (report in preparation). Oil droplets, however, compressed lymph node tissue

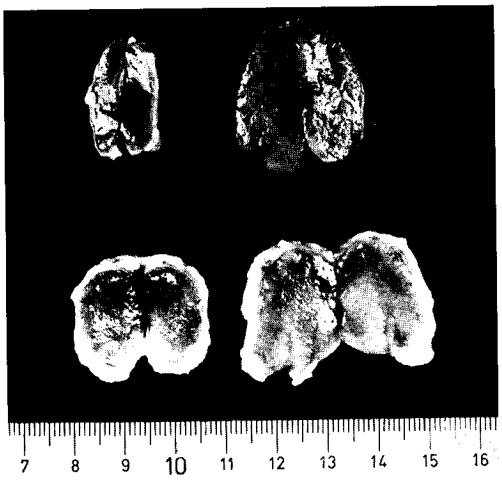

Fig. 11: Popliteal (lower) and external iliac (upper) lymph nodes: right — 14 days, left — 7 days after endolymphatic injection of 4ml Oil-Bleo.

Lymph nodes on the left (7.28g) are 72% heavier than those on the right. Moreover, lymph nodes on the left contain 61% more Bleomycin than those on the right.

(Fig. 12), and giant cell reactivity was detected on the boundary of the droplets. In over half of the lymph nodes examined, small clusters of lytically altered cells and fibrosis were seen. After four weeks, oil droplets were notably less in size and number and no further cell damage was visible. Numerous ribbon-like scars persisted in lymph nodal parenchyma.

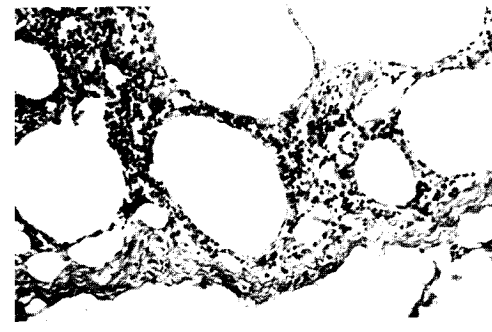

Fig. 12: Popliteal lymph node 14 days after endolymphatic injection of Oil-Bleo (Masson-Goldner).

DISCUSSION

Technical considerations:

The rate of endolymphatic injection must be carefully regulated so as not to exceed tissue pressures tolerated by lymph nodes, a consideration rarely addressed. In one study using rabbits (10) with an injections rate of 0.3ml/min, intralymphatic pressure reached 16mmHg. Under these conditions, rupture of lymphatics did not occur. Also in this report (10) the maximal injected rate tolerated in dogs was 1.8ml/min with a pressure level of 64mmHg.

In the present experiments, an injection rate of only 4ml/hour was used. This rate is similar to that previously shown to be noninjurious in rabbits (3.8ml/hour) (11) and pigs (3.3ml/hour) (12).

The volume injected is also important. With large volumes, excess fluid and emulsion enters the venous system with fat embolism in the lungs (13). A volume of 4ml emulsion for an average body weight of

13kg corresponds to the recommended dose of 0.3mg/kg (14).

The concentration of Bleomycin using our modified radioimmunoassay technique permitted detections of the drug as low as 10ng/ml.

Distribution of Oil-Bleo:

The amount of Oil-Bleo (4ml for each side) ensured complete filling of the lymph system while allowing determination of the fate of the excess. This amount (15mg/ml; 60mg/injection) exceeds that needed for therapy. Determination of the serum concentration of Oil-Bleo during and after endolymphatic injection allowed the maximal storage capacity of the lymph system to be calculated. Thus, the maximal blood concentration of Bleomycin was reached near completion of injection. From this value, the total amount absorbed in blood can be estimated. For example, in 13kg dogs injected with 4ml of emulsion, maximally 12% (on the average) was detected in blood. This "spillover" factor should rise as the volume of emulsion instilled is increased and decline as the volume is decreased, and therefore is a useful index for guiding endolymphatic therapy clinically.

Although the "spillover" factor was only ⅓ larger when aqueous Bleomycin was administered, (suggesting that the amount of aqueous Bleomycin stored in the lymphatic system was lower by this order of magnitude) in point of fact as shown in Fig. 10, the differences are actually much larger. Thus, aqueous solutions infused endolymphatically diffuse rapidly into surrounding tissue and the "missing" aqueous Bleomycin is initially localized in the interstitium. After diffusion into adjacent tissue, Bleomycin is readily taken up into the blood over several hours. A low concentration (0.9 μg/ml) of aqueous Bleomycin in thoracic duct lymph six hours after infusion suggests that the bulk of aqueous Bleomycin does not enter the bloodstream via the lymphatic system.

The biphasic time course after injection of Oil-Bleo suggests that several mechanisms are responsible for efflux of Bleomycin in oil emulsion from lymph. In the initial phase, the peak concentration is attained near completion of injection, and thereafter the concentration decreases rapidly. This portion of the curve corresponds to the "spillover" described earlier. The second or elimination phase commences an hour after completion of injection, lasts several days, and probably represents transport by the lymph system. Thereafter, Bleomycin gradually separates from the emulsion, diffuses throughout the lymph node, and subsequently drains to the bloodstream.

A comparison of concentrations of Bleomycin both in blood (Figs. 2-3) and in urine (Fig. 5) after the first and second injection discloses that the peak is slightly smaller and the decrease in the concentration more gradual after the second injection. Most likely, before the emulsion reaches the efferent lymph nodal lymphatic it is first transported via the afferent nodal lymphatic and throughout the node itself (Fig. 1). If the lymph node, however, is already loaded with emulsion from a previous injection, a partial barrier is created to passage of a second injection of Bleomycin. This physiologic blockage probably accounts for a lower peak and more gradual decline in the concentration of Bleomycin after a second dosage.

Total blockage of a lymph node (i.e. absence of transnodal lymph flow) seemingly renders endolymphatic therapy of this node useless. Even in such instances, however, endolymphatic delivery of cytostatic agent may still be useful. Although the agent fails to reach the blocked lymph node, it can nonetheless enter normal nodes immediately proximal and be stored there thereby retarding lymph nodal tumor spread more centrally.

Histologic investigations:

After standard lymphography with Lipiodol, typically damage of lymph nodes is observed (15, 16),and these changes can be compared to ascertain the effects of Oil-Bleo. With Lipiodol droplets there are early histologic changes but with time nodal

architecture gradually is restored.

After endolymphatic injection of Oil-Bleo, cytotoxic damage by Bleomycin at several nodal sites is seen, an observation in conformity with its effect on cultures of healthy lymphocytes (17).

Clinical application:

Possible future determination of the optimal dose of Oil-Bleo for clinical treatment could be extrapolated from the values obtained in this study. The amount of Bleomycin which reaches the blood in the initial phase should not exceed 30mg, a dose considered acceptable for intravenous administration. If this amount enters the bloodstream through the lymphatic system ("indirect-intravenous") then a situation exists which only superfically is equivalent to an intravenous injection of 30mg Bleomycin. Thus, after endlymphatic treatment (in contradistinction to the intravenous route) a much higher therapeutic concentration of Bleomycin reaches and is maintained in affected lymph nodes.

Address correspondence to:
Dr. Med. Peter Hirnle
Payerstrasse 20
D-7400 Tubingen
West Germany

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ACKNOWLEDGEMENTS

I wish to express appreciation to Prof. Dr. Norbert Lang for valuable and helpful suggestions.