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Preoperative Lymph-node Staining with Liposomes Containing Patent Blue Violet. A Clinical Case Report

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Abstract

A case study is reported using patent blue violet entrapped in liposomes to localize lymph nodes before surgery.

Liposomes containing 44 mg patent blue violet were injected into foot lymphatics of a patient due to undergo retroperitoneal staging-lymphonodectomy. Lymph nodes were readily visualized and removed, and urine and serum was collected for assay of the dye. The maximum concentration of patent blue violet in serum was 2·7 $\mu g\ mL^{-1}$, 1·5 h after the termination of the injection; almost no dye was detectable by the time of the operation 24 h later.

In the first urine sample (2 h after the termination of the injection) the concentration of patent blue violet was 1·3 $\mu g\ mL^{-1}$, and subsequently diminished, with the last urine sample to contain detectable dye being collected 20 h later. A total of 0·8 mg dye (2% of the injected amount) was excreted in the urine suggesting 10% of the circulating amount of dye was renally excreted.

Endolymphatic injection of patent blue violet simplifies the often difficult process of finding the lymph nodes and additionally reduces side-effects. All surgery cases demanding a radical lymphonodectomy may profit, including testicular or bladder cancers and many gynaecological tumours.

For thirty years there has been much research on the staining of lymph nodes before surgery. The retroperitoneal lymph nodes, for example, are the primary filter of metastases spreading from malignant tumours of pelvic organs. The inaccessibility of these retroperitoneal lymph nodes for therapy is still one of many unsolved clinical problems. The staining of lymph nodes prior to surgery would improve the radicality and selectivity of lymphonodectomy because lymph nodes are very often difficult to be located since they are covered by fat tissue of similar colour and consistency.

The method for staining lymph nodes (chromolymphography) has not yet become part of the clinical routine because of technical problems. The lymph nodes can not be stained directly, and the dye has to be injected into the small lymph vessels and transported from there to the higher lymph nodes. Another problem is that water-soluble dyes disappear from the lymph vessels into the surrounding tissues, so that insufficient amounts of dye reach the lymph nodes. Additionally, all lipid-soluble dyes cause disadvantages such as compression of lymph node tissue or general side-effects, such respiratory distress (Koehler 1977).

The requirements of a substance without these disadvantages and which stains the lymph nodes sufficiently may be achieved by liposomes carrying a water-soluble blue dye, such as patent blue violet, a dye routinely used in diagnostic lymphography.

Materials and Methods

Preparation of dye-liposomes

The liposomes were prepared from egg yolk lecithin and cholesterol in a molar ratio of 4 : 1 by modified detergent dialysis, using sodium cholate as detergent. A solution containing 20 mM lipids and 35 mM sodium cholate in phosphate-buffered saline pH 7·4 was sterile-filtered through 0·2-μm pores. The resulting mixed micelle solution was then dialysed with a capillary dialysis cartridge (Travenol) against a continuous flow of buffer until vesicles formed. The vesicles were homogenous with a diameter of 85–95 nm.

In a second step the dye was entrapped into the liposomes by detergent-induced liposome loading. Known amounts of patent blue violet and sodium cholate (Hirnle & Schubert 1991; Schubert et al 1991) were added to the liposome suspension. The detergent causes transient membrane holes, used by the dye to flow into the liposomes. To remove the detergent and the non-enclosed dye, the liposome suspension was dialysed again and stable patent blue violet-liposomes were formed. To concentrate the liposome suspension it was dialysed against a solution of 2% polyethylene glycol (mol. wt 40 kDa) in buffer. As a result of several different methods of preparation we were able to obtain 50–80 mL liposome solution containing approximately 1·8 mg mL^{-1} patent blue violet and 55 mg mL^{-1} lecithin (Pump 1994).

The resulting patent blue violet-liposomes were sterile. They were also stable for more than a month at room temperature.

Application of patent blue violet-liposomes

One of our preparations (Hirnle & Schubert 1991; Pump 1994) was used for preoperative lymph-node staining on a patient with testicular cancer scheduled for a retroperitoneal staging-lymphonodectomy. The left testicle had been removed one week earlier. The patient was 30 years old, 173 cm tall and weighed 86 kg. The total amount of patent blue violet entrapped in liposomes was 44 mg.

The patent blue violet-liposomes were injected into both foot lymphatics using the established lymphographic method of Kinmonth (1977), already used with patent blue violet-liposomes in animal studies (Hirnle 1991; Hirnle & Schubert 1991). The injection was over a period of approximately 100 min.

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During the injection and over the following 24 h blood and urine samples were obtained to measure the dye concentration. Twenty four hours after the injection of patent blue violet-liposomes, the lymphonodectomy was performed.

Intraoperatively the para-aortal lymph nodes were found to be stained deep blue, and could be easily removed.

One of the lymph nodes was examined histologically, and another prepared for measurement of dye. The content of patent blue violet in the body fluids and the lymph node was measured by HPLC 630 nm.

Analysis of blood, urine and lymph nodes for patent blue violet

The body fluids were mixed with an equal volume of 6 M guanidium chloride, transferred onto a C18 BondElut column (Analytichem Inc.) and eluted with 2 × 200 μL 40% acetonitrile. The column was equilibrated with methanol : water (1 : 1) before sample injection. The solid tissue was homogenized (Micro-Dismembrator, Braun, Germany) diluted with volumes of buffer and centrifuged (100 000 *g*, 60 min, 6°C). The separated pellet was diluted with 40% acetonitrile (1 : 5) to dissolve the dye. After another centrifugation the supernatant was diluted with distilled water (1 : 100) and was also measured by HPLC.

A 12·5 mm × 46 mm column Spherisorb ODS-2 (5 μm) was used for HPLC measurement. The solvent was distilled water : acetonitrile : 85% phosphoric acid (75 : 25 : 0·1). Samples (20 μL) were injected and eluted with a flow rate of 2·0 mL min^{-1}.

Results

The maximum concentration of patent blue violet in serum was 2·7 μg mL^{-1}, 1·5 h after the termination of the injection. Later the concentration dropped nearly continuously until at 24 h, when the operation began, almost no dye was detectable (Fig. 1).

In the first urine sample (2 h after the termination of the injection) the concentration of patent blue violet was 1·3 μg mL^{-1}, and subsequently diminished, with the last urine sample to contain detectable dye being collected 20 h later (Table 1). A total of 0·8 mg dye (2% of the injected amount) was excreted in the urine suggesting 10% of the circulating amount of dye was renally excreted.

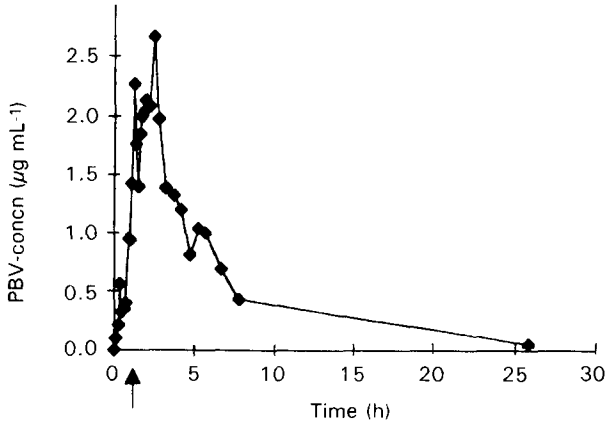

Fig. 1. Concentration of patent blue violet in human serum during and after endolymphatic injection of liposomes containing 44 mg patent blue violet.

Table 1. Concentration of patent blue violet in human urine after endolymphatic injection of liposomes containing 44 mg patent blue violet.

Time after end of chromolymphography (h)	Concn (mg mL^{-1})	Amount of patent blue violet (mg)
3	1·3	0·2
4	1·2	0·2
5	0·5	0·1
6	0·4	0·1
11	0·3	0·15
19	0·2	0·06

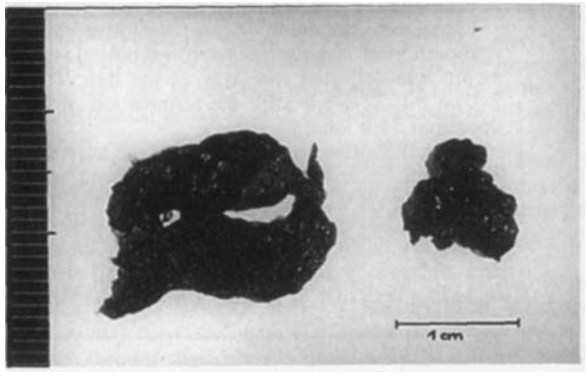

Fig. 2. Deeply blue stained para-aortal human lymph node removed 24 h after injection of liposomes containing 44 mg patent blue violet.

Deeply blue coloured para-aortal lymph nodes could easily be found 24 h after the endolymphatic injection of patent blue violet-liposomes. Thus they could be removed in a radical but selective way. The amount of patent blue violet in the 400-mg lymph node (Fig. 2) was 93 μg, which is equivalent to a concentration of 234 μg (g tissue)$^{-1}$. This concentration was sufficient to stain this lymph node an intense blue. The histological findings are shown in Figs 3 and 4. In the haematoxylin-eosin-stained preparations, blue dye could not be seen. By this technique all fat-soluble material is washed out. In the preparations there can be seen small, empty, round areas where drops containing liposomes may have accumulated. No compression of tissue or damage of cells can be seen. The histological examination of the preparations was not affected since

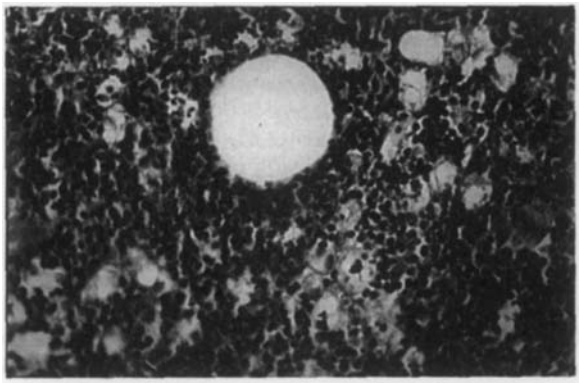

Fig. 3. Histological image from the centre of a para-aortal human lymph node, macroscopically blue, removed 24 h after injection of patent blue violet-liposomes. Haematoxylin-eosin-staining. The white central spot results from a liposomal suspension which was washed out by the haematoxylin-eosin-staining.

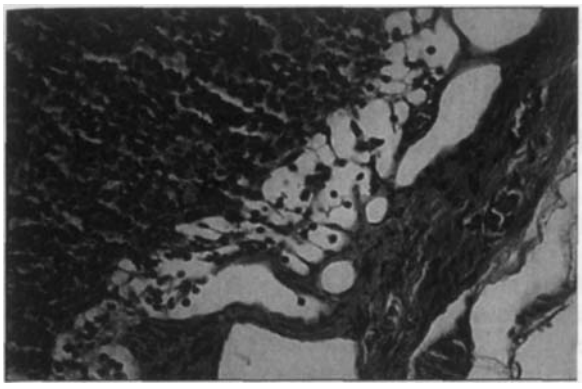

Fig. 4. Histological image from the edge of the same lymph node as in Fig. 3. The white areas represent depots of liposomes washed out by the haematoxylin-eosin staining.

the dye was washed out.

Based on the maximum patent blue violet concentration of 2·7 $\mu g\ mL^{-1}$, the estimated maximum amount of patent blue violet in blood can be calculated to be 8·6 mg, giving an estimate of spillover (Hirnle & Schubert 1991) of the dye via the ductus thoracicus of about 20% of the injected amount of 44 mg liposomal patent blue violet.

Discussion

In previous studies different dyes have been tried for chromolymphography (Zerbino et al 1975), focussing on a green dye, chlorophyll, dissolved in lipiodol, an oily contrast medium, injected into lymph vessels (Rummelhardt 1965; Elema et al 1967). Other substances such as carbon (black) with lipiodol (Ludvik & Kindl 1968) or guajazulen (blue) with lipiodol (Gregl et al 1969) have also been tested, with notable side-effects, including reversible or irreversible damage of lymph node tissue caused by the dye (Rummelhardt 1965; Elema et al 1967) or by lipiodol (Gregl et al 1969). Lipiodol is well-known to cause microembolism in lung capillaries (Gerteis & Greuel 1967; Koehler 1977). Another disadvantage of many dyes was insufficient staining of the more cranially situated lymph nodes.

In animal testing it has already been demonstrated that the lymph nodes were stained deep blue by liposomes containing isosulfan blue or patent blue violet, and that no damage of the lymph nodes could be detected (Hirnle 1991; Hirnle & Schubert 1991).

Even in this case we were able to demonstrate clinical application. No side-effects were observed. The para-aortal lymph nodes could be removed without complications because of their intense blue colour.

The endolymphatical injection of patent blue violet containing liposomes, leads to blue stained lymph nodes. This is a remarkable advantage in the surgery of cancer. In many cancer patients lymphonodectomy is required because of the spreading of malignant cells into lymph nodes. Endolymphatical injection of patent blue violet simplifies the often difficult process of finding the lymph nodes and additionally reduces side-effects. All surgery cases demanding a radical lymphonodectomy may profit, including testicular or bladder cancers and many gynaecological tumours.

Chromolymphography can be regarded as a model for different uses of endolymphatical injection of liposomes filled with drugs. Other substances could be transported to lymph nodes, such as cytostatics for direct treatment of cancer-affected lymph nodes.

The advantages would be fewer general side-effects and aspecific treatment of the lymph nodes since only a smaller amount of dye will reach the blood vessels. Only a minor dose of the applied substance is needed to obtain the same effect as obtained intravenously. Additionally, a depot of the drug entrapped in the liposomes residing in lymph nodes is achieved resulting in a longer effect. This is the only way to reach such a high concentration of the substances in lymph nodes.

References

Elema, J. D., van Minden, S. H., Oldhoff, J. (1967) Some experience with chromolymphography. Arch. Chir. Neer. 19: 291–302

Gerteis, W., Greuel, H. (1967) Kontrastmittelembolie der Lunge bei Lymphographie (Untersuchungen bei physiologischem Lymphabfluss) Fortschr. Geb. Röntgenstr. Nuclearmed. 108: 565–576

Gregl, A., Kienle, J., Steizner, J., Yu, D., Schoen, H. (1969) Morphologische Veränderungen in den Lymphknoten nach Lipiodollymphographie. Fortschr. Geb. Röntgenstr. Nuclearmed. 110: 297–306

Hirnle, P. (1991) Histological findings in rabbit lymph nodes after endolymphatic injection of liposomes containing blue dye. J. Pharm. Pharmacol. 43: 217–218

Hirnle, P., Schubert, R. (1991) Liposomes containing blue dye for preoperative lymph node staining: distribution and stability in dogs after endolymphatic injection. Int. J. Pharm. 72: 259–269

Kinmonth, J. B. (1977) Lymphography 1977. A review of some technical points. Lymphology 10: 102–106

Koehler, P. R. (1977) Complications of lymphography. In: Mayall, R. C., Witte, M. H. (eds) Progress in Lymphology, Plenum Press, New York, London pp 209–213

Pump, B. (1994) Optimierung der Herstellung von mit Farbstoff gefüllten Liposomen für die klinische Anwendung. Shaker, Aachen

Rummelhardt, S. (1965) Lymphographie mit lipiodol-chlorophyll. Z. Urol. Neph. 58: 609–612

Schubert, R., Wolburg, H., Schmidt, K.-H., Roth, H. J. (1991) Loading of preformed liposomes with high trapping efficiency by detergent-induced formation of transient membrane holes. Chem. Phys. Lipids 58: 121–129

Zerbino, D., Gelfot, M. M., Kobenstved, C. (1975) Chromolymphographie und Vitalfärbung von Lymphgefässen und Lymphknoten. In: Lüning, M. (ed.) Lymphographie bei Malignen Tumoren. Thieme, Stuttgart, New York, pp 281–284