

BLUE LIPOSOMES FOR IDENTIFICATION OF THE SENTINEL LYMPH NODES IN PIGS

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ABSTRACT

Effective preoperative staining of regional lymph nodes improves their intraoperative identification and thus the selectivity of "sentinel node" lymphadenectomy. Unfortunately, aqueous Patent Blue V (PBV) often fails to provide the requisite intensity and duration of contrast (1,2). A comparative study of staining characteristics of PBV in aqueous solution with those of liposomally encapsulated PBV was carried out on 7 female pigs with an average weight of 40 kg. The liposomes, consisting of lecithin and cholesterol in a molar ratio of 3:1, were produced by an extrusion technique using membranes with thicknesses of 5µm, 1.2µm, 0.4µm and 0.2µm. In each instance, a 0.5-ml depot containing 25 ± 0.4 mg of PBV/ml was injected into each of the four upper and lower mammary glands in aqueous solution on the left side and in a liposomal preparation on the right side. Stained lymph nodes of the groin, pelvis and neck were identified after 3, 6, 12 or 24 hours, then excised and photographed. Their PBV concentrations were measured by spectrophotometry. In all cases, the liposome preparations provided greater intensity and a longer duration than the aqueous solution. Liposomal PBV compared with aqueous PBV can therefore be recommended for better preoperative lymph node staining and identification of "sentinel" lymph nodes.

Regional lymph node metastases are an important prognostic factor in cancer (3,4). Treatment with surgery, chemotherapy or irradiation often leads to suboptimal outcomes. The customary method by operation is the removal of all regional lymph nodes. Unfortunately, this technique results in a high rate of side effects including secondary lymphedema. A new method termed "sentinel node dissection" has been advocated as a minimally invasive alternative to conventional lymph node dissection. The "sentinel" lymph node is the first node draining the area of a tumor. It has been shown that for different solid tumors especially breast and melanoma, the histopathological examination of the sentinel node is representative of neighboring lymph nodes (5-7). Localization of the sentinel node is prepared by staining with different dyes (8,9), by radioactivity (10,11), or a combination of both (12,13). It has been shown, that the more expensive and complex method of sentinel node identification using colored dye and radioactive tracer shows little or no advantage over dye injection alone (14).

Another problem at operation is to distinguish between the lymph nodes themselves and the soft tissue which surrounds them. The customary solution is the removal of the lymph nodes together with adjacent fatty tissue and lymph vessels. Unfortunately, this technique raises the risk of side effects.

Optimal localization of the sentinel node is attainable only through effective lymph

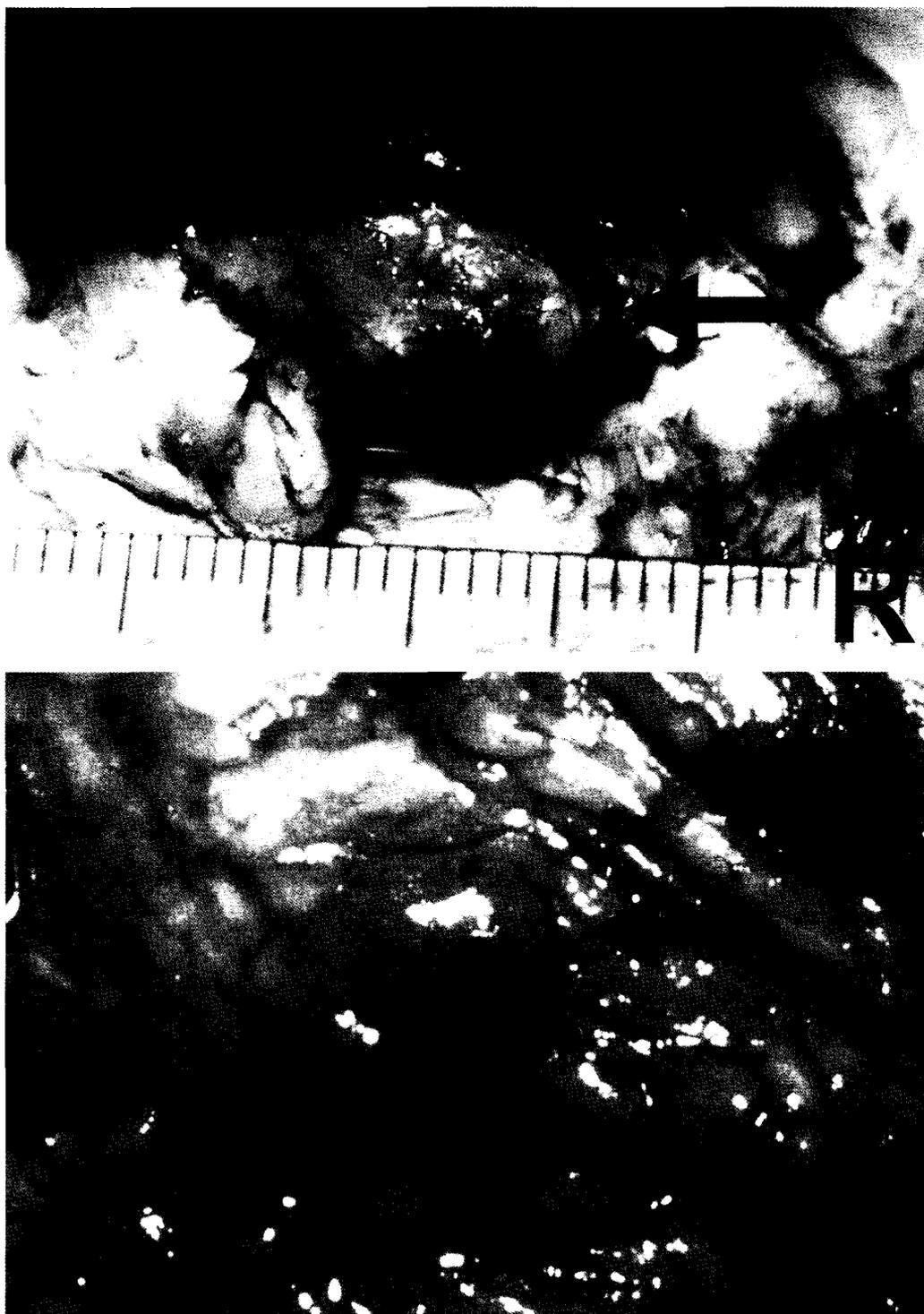


Fig. 1. R) Neck lymph nodes on right side 3h after injection of Patent Blue V (PBV) in liposomes compared with L) using PBV in aqueous solution. Note the more intense staining with liposomal PBV (arrows) (Fig. 1R).

node staining, which intensifies the color contrast between the nodes and surrounding tissues. Different substances have been tested for this purpose but, because of insufficient staining, toxicity, or both, have not been adopted clinically.

One recent trial, for example, used Guajazulen, a dark blue dye, which was dissolved in Lipidiol Ultra-Fluid (15). When injected endolymphatically, it afforded excellent blue staining of regional lymph nodes. However, it also resulted in local necrosis, tissue compression due to oil droplets, and lung involvement with potential respiratory distress. It was therefore abandoned in a search for a less aggressive method of endolymphatic staining. The search led to trials with PBV in lipoidal carriers.

PBV is a blue dye often used subcutaneously in aqueous solution to stain lymph vessels temporarily prior to lymphography. PBV in aqueous solution is also a commonly used dye for the localization of the sentinel node. Water-soluble dyes when injected interstitially are mainly removed by blood capillaries, and reach the lymph nodes only in very low concentrations (16). The results improve dramatically when PBV is encapsulated in lipoidal carriers: staining improves, nodal uptake increases, and diffusion of the drug out of the lymph vessels is minimized.

Best results with this technique are achieved with direct lymphography (17) since the entire amount injected reaches the sentinel node. However, direct lymphography also has its drawbacks; it is time-consuming and must be handled by trained experts.

Because the mammary glands are organs with a highly developed lymphatic drainage network, it was anticipated that indirect lymphography of these glands with blue liposomes would lead to improved staining of regional lymph nodes, which forms the basis of this study.

MATERIALS AND METHODS

The study was conducted with 7 female pigs weighing an average of 40kg. In each case, a 0.5-ml depot containing 25 ± 0.4 mg of PBV/ml was injected into each of the four upper and lower mammary glands in aqueous solution on the left side and in a liposomal preparation on the right side. The liposomes used for the study consisted of lecithin and cholesterol in a molar ratio of 3:1 and were produced by extrusion using membranes with thicknesses of $5\mu\text{m}$, $1.2\mu\text{m}$, $0.4\mu\text{m}$ and $0.2\mu\text{m}$. 7.4% of the total lipid concentration was 41 mmol/l , and the liposome diameters had a Z-average of 172 nm .

The pigs were sacrificed at different time intervals: 1 pig after 3 hours, 2 pigs after 6 hours, 4 pigs after 12 hours, and 1 pig after 24 hours. The lymph nodes of the groin, pelvis and neck were immediately photographed in situ and then removed. The excised lymph nodes were re-photographed and weighed. Areas with intense staining adjacent to unstained areas were weighed

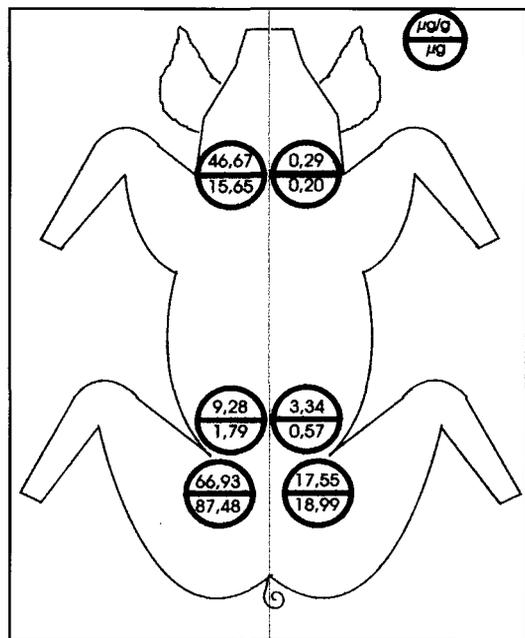


Fig. 2. Patent Blue V (PBV) concentration ($\mu\text{g/g}$), amount (μg), and ratio left/right in lymph nodes of the neck, the pelvis, and the groin 3h after injection.

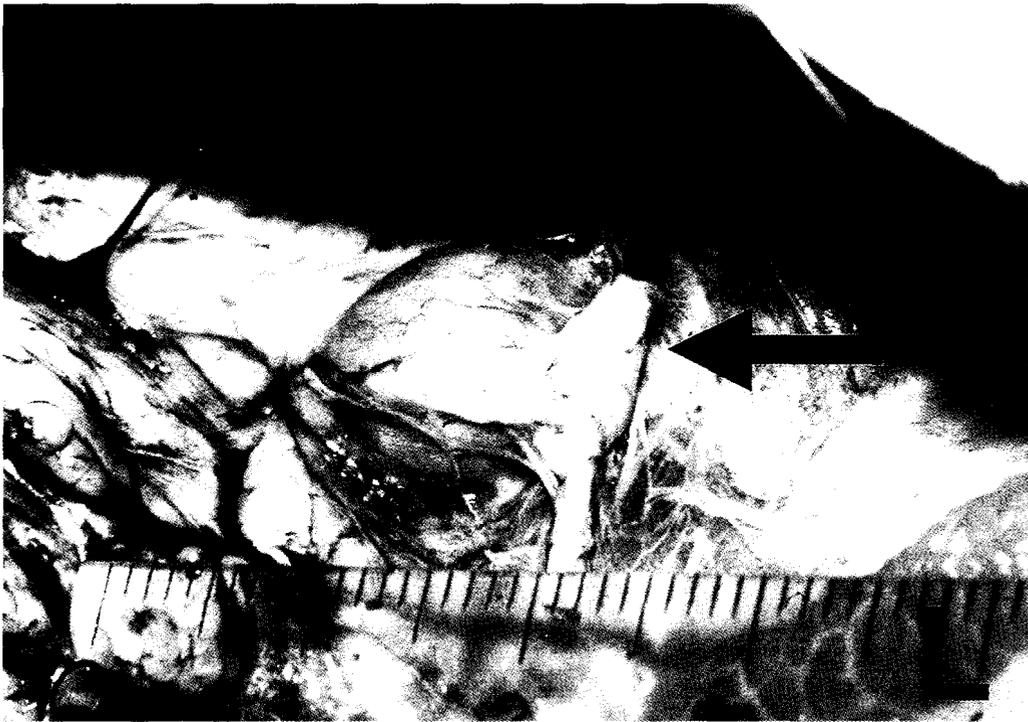
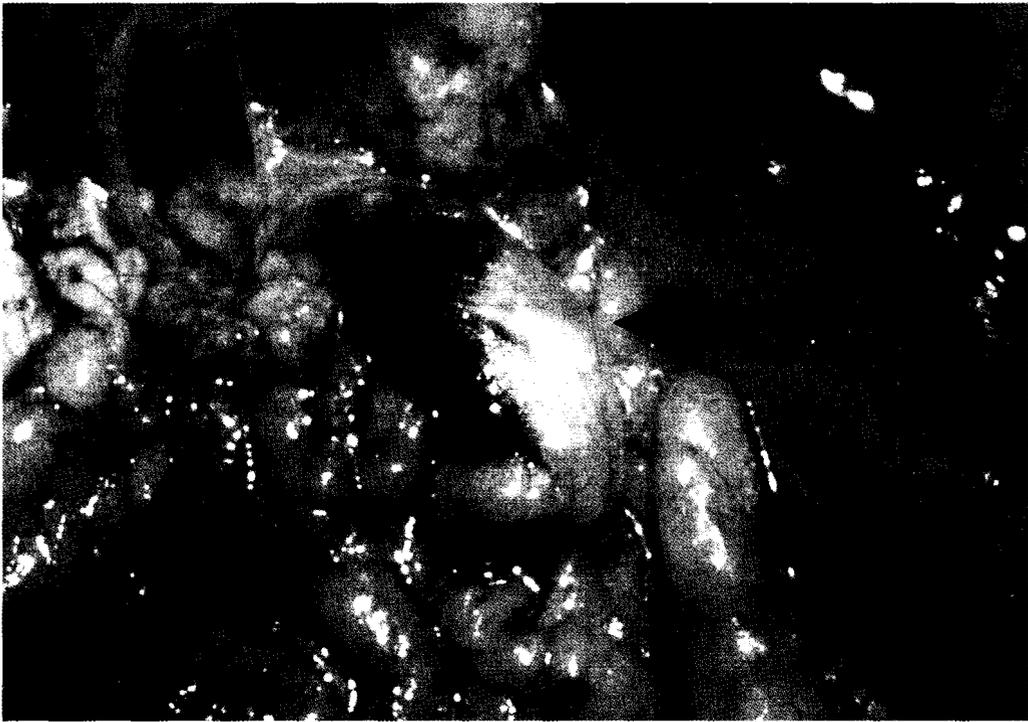


Fig. 3. R) Neck lymph nodes on right side 6h after injection of PBV in liposomes compared with L) using PBV in aqueous solution. Note the more intense staining with PBV and liposomes (arrows) (Fig. 3R).

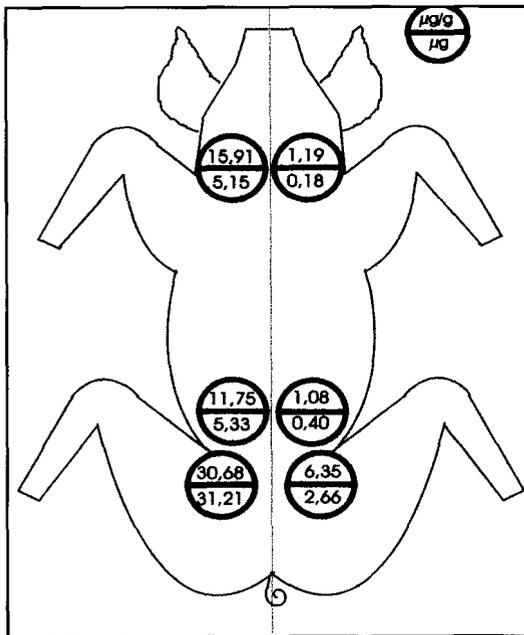


Fig. 4. Patent Blue V (PBV) concentration ($\mu\text{g/g}$), amount (μg), and ratio left:right in lymph nodes of the neck, the pelvis, and the groin 6h after injection.

separately; dye was liposomally encapsulated by the end of the preparation process. Representative parts of the lymph nodes were examined histologically. Each lymph node specimen was placed in ethanol and minced to a homogeneous cell suspension. This suspension was centrifuged at 1000xg for 30 minutes. PBV concentrations in the specimens were measured at 635 nm by spectrophotometry.

RESULTS

The lymph nodes of the pelvis and groin on both sides of each pig were well stained three hours after injection. The neck lymph nodes on the right side (Fig. 1R) were easily identified due to good color contrast, whereas identification on the left side was more difficult and time-consuming due to less intense contrast (Fig. 1L). The amount of PBV on the right side was 4.55 times higher compared with the left side (Fig. 2).

Six hours after injection, only the lymph nodes on the right side of the neck were stained (Fig. 3R). There was no visible staining on the left side of the neck (Fig. 3L). The amount of PBV on the liposomal side was 12.85 times higher compared with the side with the aqueous preparation (Fig. 4).

Twelve hours after injection, the neck lymph nodes remained stained on the side with the liposomes (Fig. 5R) but not on the side with the aqueous solution (Fig. 5L). The amount of PBV on the right side was 10.03 times higher compared with the left side (Fig. 6).

The lymph nodes of the neck were no longer localizable twenty-four hours after injection, and the pelvic lymph nodes were poorly stained on both sides. Lymph nodes in the groin remained stained only on the side with the liposomes. The total amount on the right side was 4.60 times higher compared with the left side (Fig. 7).

In every instance, staining with liposomes introduced much greater quantities of PBV into the lymph nodes than staining with an aqueous solution (Fig. 8).

There were no overt toxicities of liposomal PBV and histological examination showed no alterations which might have reduced the diagnostic value of this technique in detecting metastases.

DISCUSSION

Sentinel node dissection is gradually becoming the procedure of choice in regional staging of melanoma and breast cancer patients (11). The sentinel node concept shows a high sensitivity for nodal metastasis in melanoma, breast and penile malignancies (18,19). But the sentinel node concept is not consistent in patients with vulvar carcinoma or colorectal cancer because of a high false negative rate (20,21).

Clear localization of the sentinel node is attainable only through effective lymph node staining. Successful, persistent staining of lymph nodes has been achieved in the past after experimental direct injection of blue

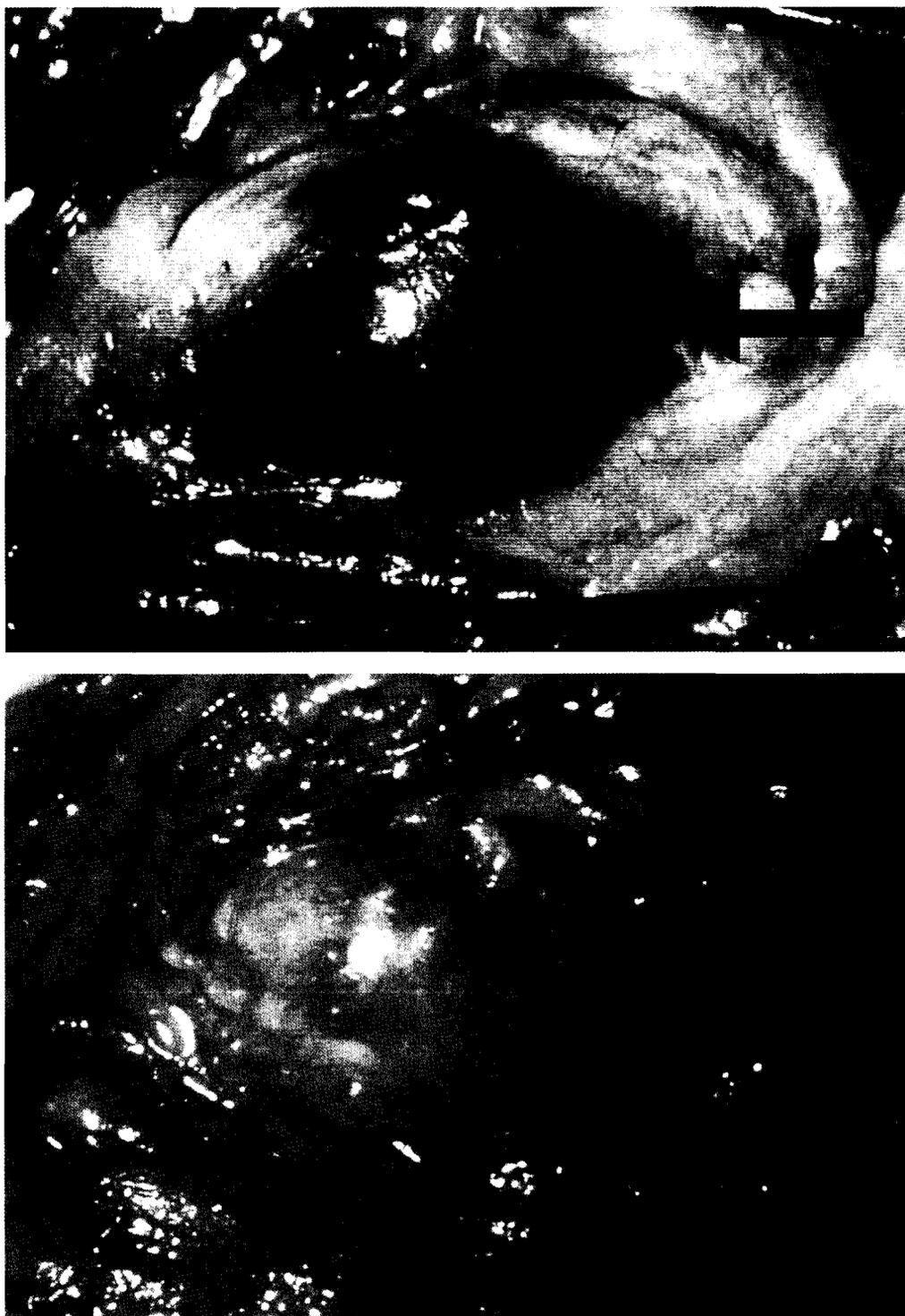


Fig. 5. R) Neck lymph nodes on right side 12h after injection of Patent Blue V (PBV) in liposomes compared with L) using PBV in aqueous solution. Note the more intense staining with liposomal PBV (arrows) (Fig. 5R).

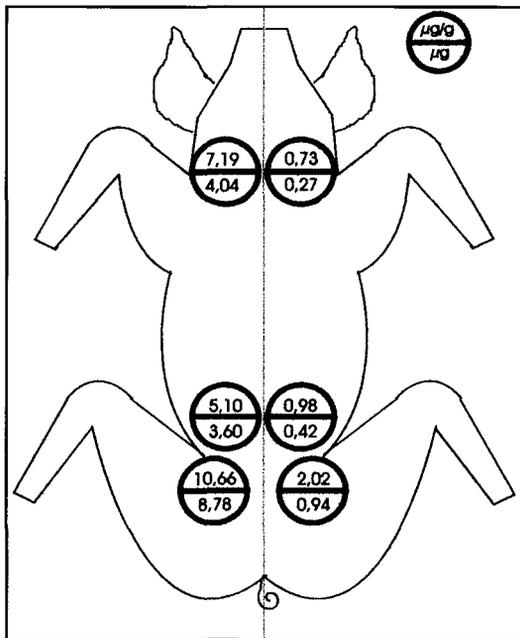


Fig. 6. Patent Blue V (PBV) concentration ($\mu\text{g/g}$), amount (μg), and ratio left/right in lymph nodes of the neck, the pelvis, and the groin 12h after injection.

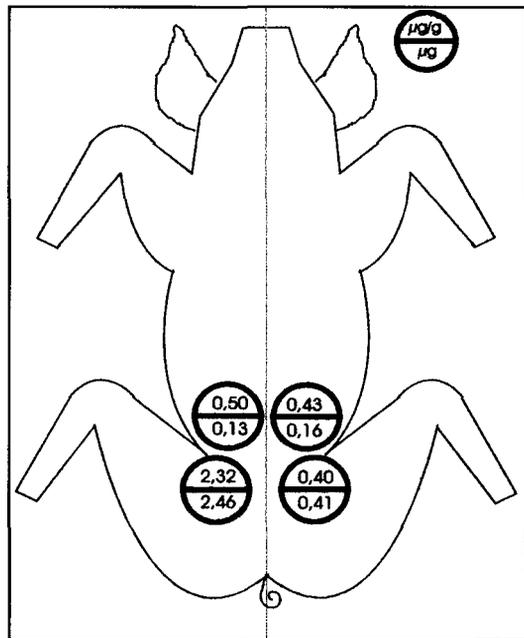


Fig. 7. Patent Blue V (PBV) concentration ($\mu\text{g/g}$), amount (μg), and ratio left/right in lymph nodes of the neck, the pelvis, and the groin 24h after injection.

liposomes into animals (2,22) and in clinical use (23). In these cases, concentrations of PBV in lymph nodes reached $100\mu\text{g/g}$ or more after direct lymphography. Indirect lymphography of the dorsal hind foot of dogs resulted in concentrations not exceeding $15\mu\text{g/g}$ (1). However, experiments with liposomes containing 6-carboxy-fluorescein in rats (16) and rabbits (24) offered improved detection.

As shown in the present study, PBV concentrations of up to $67\mu\text{g/g}$ in lymph nodes are possible after subcutaneous injection. Concentrations exceeding $7\mu\text{g/g}$ suffice for clear identification of lymph nodes.

No adverse reactions were observed after the use of liposomes. This is probably due to the fact that liposomes consist of substances already physically present in large amounts in lymph. Liposomes also have many other advantages over other lipoidal carriers. They are not deposited in the lungs and cause no embolization of blood capillaries. They are

non-toxic for lymph node parenchyma and cause neither pain nor fever (1).

The present study shows a clear advantage of lymph node staining using PBV in liposomes versus PBV in aqueous solution.

Liposomal PBV can be therefore recommended for preoperative lymph node staining and identification of "sentinel" lymph nodes.

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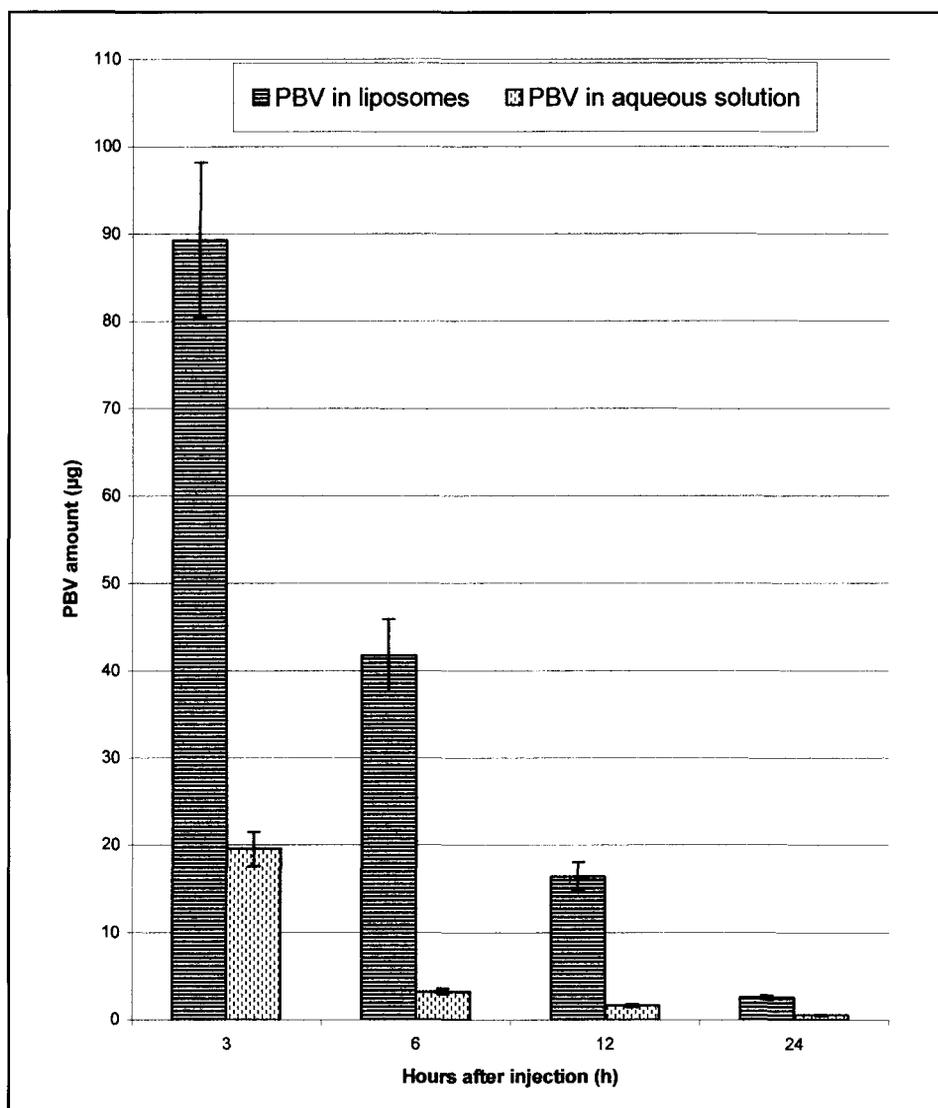


Fig. 8. Amount of Patent Blue V in regional lymph nodes after injection of PBV in the mammary glands.

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