

INFLUENCE OF A CATIONIC TRIPHENYLMETHANE DYE ON GRANULATION TISSUE GROWTH IN VIVO

An Experimental Study in Rats

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Abstract. The influence of aqueous solutions of crystal violet, a triphenylmethane dye used topically as an antimicrobial agent, on granulation tissue formation was studied by subcutaneous implantation of viscose cellulose sponges in rats. The dry weight of tissue formed in the sponge and the breaking load of an incision in the sponge was recorded 10 days after implantation. Crystal violet in concentrations even below those used in patients almost completely inhibited the ingrowth of granulation tissue into the sponges.

Gentian violet exhibits in vitro high antibacterial and antifungal activities (1, 5, 10, 11). Topical application of gentian violet (0.1-2%) in aqueous solution is widely recommended in textbooks on dermatology for the control of superficial pyogenic, dermatophytic and monilial infections of the skin and mucous membranes (6, 13, 16). A few cases of sensitization to the dye have been reported (2, 9), but otherwise it is regarded as a safe and effective therapeutic agent (20).

Over the past years we have encountered a number of patients with necrosis of the skin following the application of 1% gentian violet in aqueous solution (3). Such a reaction was reproducible in normal humans and guinea pigs by painting the dye solution on stripped skin. By a tissue culture technique it could be demonstrated that cationic triphenylmethane dyes in clinical use have a cytotoxic effect on fibroblasts in dilutions as low as $1:10^6$ (15).

The purpose of the present study was to investigate the influence of gentian violet on the formation of granulation tissue in vivo. However, gentian violet is a poorly defined mixture of violet rosaniline dyes, essentially pentamethyl-

pararosaniline chloride (methyl violet) and hexamethylpararosaniline chloride (crystal violet) (14). Various companies may supply different compositions under this name. We therefore decided to choose for our study the hexamethylated component of gentian violet, crystal violet, which can be obtained in pure form. It is well known to bacteriologists and histopathologists for use in staining procedures.

MATERIAL AND METHODS

Animals. Fifty-eight adult male white Sprague-Dawley rats weighing between 300 and 400 g were used. The rats had free access to standard pellets and tap water. They were kept in separate cages at room temperature.

Implantation and removal of sponges. The method used was described by Viljanto (21). Viscose cellulose sponge (Visella®) was cut into pieces measuring $10 \times 10 \times 20$ mm and weighed in a dry state. They were paired so as to obtain sponges of almost equal weight in each pair. Each sponge was further cut into two equal sections across its length (creating two cubes $10 \times 10 \times 10$ mm), and the cut surfaces were apposed in their original position with a loop of braided silk. The sponges were sterilised before implantation.

Under ether anaesthesia the back of the animal was shaved with electric clippers. Two median incisions, each about 3 cm long, were made under sterile conditions through the skin and superficial fascia caudally from the angle of the shoulder blade. The distance between the incisions was 2 cm. By blunt dissection from each incision subcutaneous pockets were prepared in which the pair of sponges were implanted, one pair on either side (about 1 cm lateral to the incisions) (Fig. 1). The incisions were closed by continuous sutures with 000 black braided silk. The wounds were left undressed.

In four experiments (I-IV), each comprising 10 animals, one of the sponges in each pair was soaked in sterile water and the other in a sterile aqueous solution of

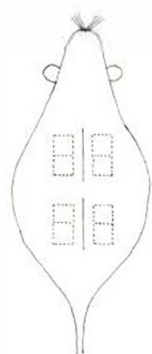


Fig. 1. Location of wounds and sponges.

crystal violet (C1 42555) in dilutions of 1:100, 1:1 000, 1:2 000 and 1:10 000 respectively (in per cent: 1, 0.1, 0.05, 0.01 w/v). In a fifth experiment (V), also 10 animals, the wound pockets were exposed for 5 min to a solution of crystal violet 1:2 000 and sterile water, respectively, and all sponges were soaked in sterile water. Before implantation of the sponges the fluid in the wound was allowed to escape.

Ten days after implantation the sponges were removed through separate incisions. The sponges were freed from the enclosing connective tissue capsule. Care was taken not to damage any of the connective tissue that had grown into the pores of the sponges. The loop of silk holding the two halves together was removed. In a few rats, stitch abscesses developed. These animals were discarded.

Determination of breaking load and dry weight. Immediately after the preparation of the sponges the breaking load (in newton units)¹ of the tissue bridging the narrow cleft between the two halves of each sponge was measured.

¹ N=newton, international force unit, 1 N=0.102 Kp (kilogram-force)=0.225 lb_f.

ured using the tensiometer described by Sandblom, Petersen & Muren (18). Then the dry weight of each sponge was determined after drying to constant weight in an oven at 70°C.

Histology. Another 8 rats, divided into 4 subgroups of 2 animals, were used for histological examinations. The sponges were implanted as in series I-IV, but each animal received only one pair of sponges. These were also removed after 10 days, fixed in 10% formalin and further prepared using standardized methods for paraffin embedding. The sections were stained with hematoxylin-eosin and according to van Gieson.

Skin lesions developed above the dye-exposed sponge in a few rats of series I. The lesions from 2 animals were excised, stretched to their original size and shape and then mounted on corkplates. Fixation and further procedures were the same as for the sponges.

Statistics. Comparison was made for each pair of sponges between the control and the experimental sponge. Difference in breaking load was expressed as a percentage of the value for the control sponge. The calculation of significance of differences was made using Student's *t*-test.

RESULTS

Amount of tissue contained in sponges. The mean dry weight of tissue in the sponges for each group of rats is given in Table 1. For sponges soaked in crystal violet 1:100, 1:1 000 and 1:2 000 there was no significant difference in the weight of tissue as compared with controls. In sponges treated with the dye in a concentration of 1:10 000, and when the wounds were treated with crystal violet 1:2 000 but sponges soaked in water (exp. V), the dry weight was significantly reduced.

Breaking load. The strength of the connection

Table 1. Dry weight of tissue and breaking load in sponges exposed to crystal violet or sterile water 10 days after subcutaneous implantation in rats

Experiment no.	Crystal violet conc.	No. of animals	Dry weight, gram (mean $\pm$ S.E.)		Breaking load, newton (mean $\pm$ S.E.)	
			Dye	Water	Dye	Water
I	1:100	7	0.134 $\pm$ 0.007	0.147 $\pm$ 0.015	0.02 $\pm$ 0.02	2.21 $\pm$ 0.56***
II	1:1 000	10	0.144 $\pm$ 0.007	0.162 $\pm$ 0.015	0.21 $\pm$ 0.05	2.51 $\pm$ 0.31***
III	1:2 000	9	0.140 $\pm$ 0.005	0.150 $\pm$ 0.015	0.39 $\pm$ 0.06	1.99 $\pm$ 0.21***
IV	1:10 000	10	0.124 $\pm$ 0.006	0.194 $\pm$ 0.011***	0.67 $\pm$ 0.12	3.81 $\pm$ 0.44***
V	1:2 000	9	0.116 $\pm$ 0.006	0.176 $\pm$ 0.017**	0.09 $\pm$ 0.04	3.05 $\pm$ 0.44***

** $p < 0.01$; *** $p < 0.001$.

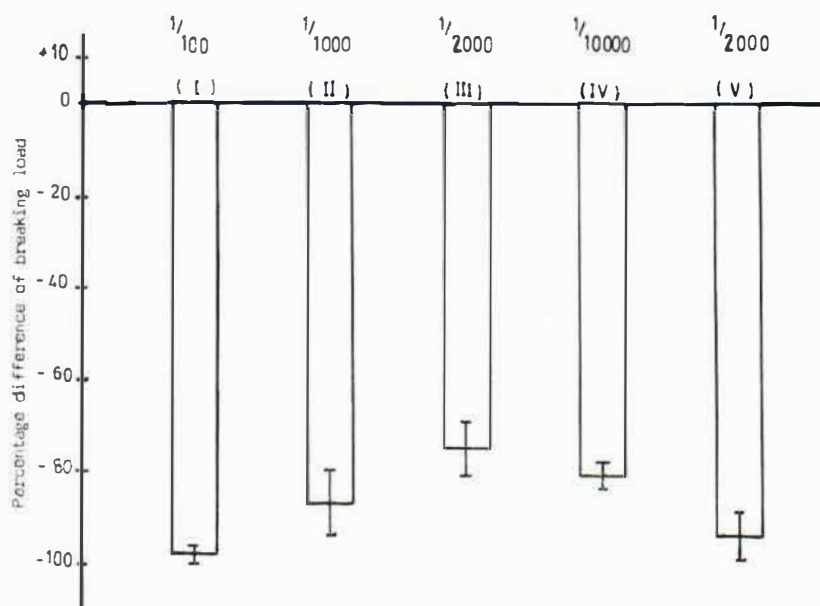


Fig. 2. Percentage deviation of breaking load of "wounds" in sponges exposed to various aqueous solutions of crystal violet as compared with control sponges.

between the two halves of the sponges was considerably lower in crystal violet exposed sponges at all dye concentration than in control sponges (Table I). The differences in breaking load, which are highly significant, are illustrated in Fig. 2, which also includes the standard error for each group of rats.

Histologic examination of sponges. In sponges soaked with water 10 days previously, connective

tissue ingrowth can be seen. Capillaries, fibroblasts and a network of collagen fibres penetrate the pores from a capsule of connective tissue surrounding the sponge (Fig. 3a). Some lymphocytes and plasma cells are seen in the newly formed connective tissue. There is a rather marked foreign-body reaction with numerous foreign-body giant cells along the walls of the sponge pores. This foreign-body reaction is noted only at the

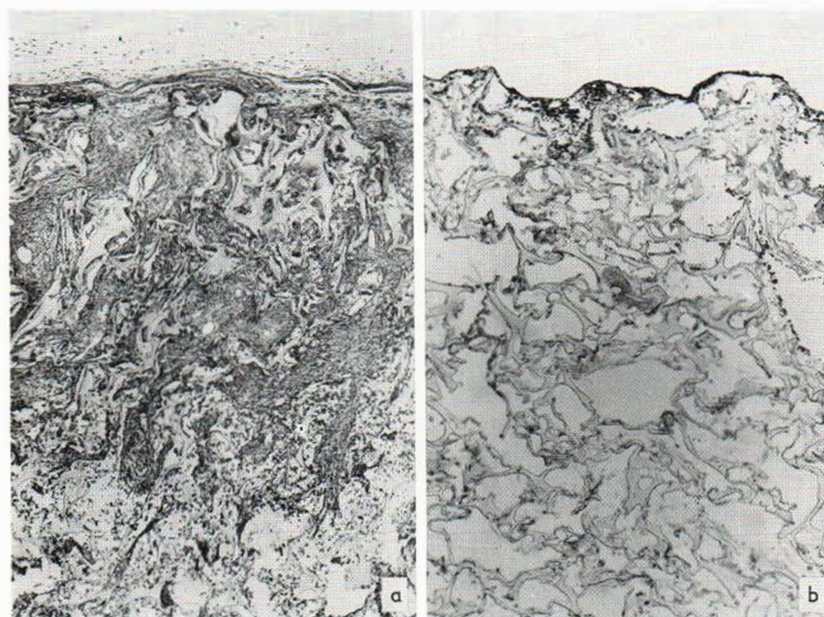


Fig. 3. Margin of a control sponge soaked with (a) water and (b) crystal violet 1:2000, illustrating the difference in formation of new tissue. Note complete loss of fibroplasia in the dye-treated sponge ($\times 7$).

periphery in the more mature connective tissue where the inflammatory reaction has disappeared and the healing process has reached the stage of fibroplasia. The centre of the sponges, not yet occupied by connective tissue, is filled with fibrin, polymorphonuclear leukocytes and monocytes.

In all dye-exposed sponges irrespective of dye concentration, no connective tissue can be seen, either on the surface or in the pores of the sponges. The pores are filled with a serofibrinous oedema and inflammatory cells (Fig. 3*b*). Polymorphonuclear leukocytes occur in the outermost pores. There is no eosinophilia.

Necrosis of skin overlying sponge. In 4 of the 10 rats in series 1 (crystal violet 1:100) necrotic areas could be seen on the skin overlying the dye-soaked sponges. They were probably not self-inflicted by the animal, as in no rat did the skin above control sponges or sponges exposed to lower concentrations of the dye exhibit similar skin lesions.

Microscopically there is a crusted ulceration of the skin, which involves the upper part of the corium. The crust contains fibrin and many polymorphonuclear leukocytes. In the surrounding tissues there is some increase in round cells and polymorphonuclear leukocytes. The pannicular muscle is intact. In the subpannicular layer, there is necrosis of the fat demarcated by fibrinous exudation and infiltrates of polymorphonuclear leukocytes. There is no eosinophilia.

DISCUSSION

The technique of subcutaneous implantation of synthetic sponges in order to harvest granulation tissue was originally described by Grindlay & Waugh (12). A later modification by Viljanto (21) using viscose cellulose sponges has been used by Rydberg (17) for assessing the influence of a cationic detergent on the formation of granulation tissue. According to Viljanto, during the first days after implantation, a saline-soaked sponge is filled with a fibrinous network and contains some blood cells. Then connective tissue cells and vessels grow in, so that about 1 week after implantation the sponge is evenly filled with fibroblasts, capillaries, acid mucopolysaccharides and collagen. The breaking load—dependent on the amount and quality of collagen—of the newly formed granulation tissue quickly increases.

By use of this method it is easy to separate newly formed granulation tissue from adjacent old connective tissue, and make a quantitative sampling of granulation tissue. The sponges have also been considered to constitute an experimental model of the healing processes in a wound (7, 8). However, foreign-body irritation from the sponge and friction between the sponge and the surrounding connective tissue capsule may influence tissue regeneration.

If a tissue-damaging agent causes a reduction of the surrounding tissues' capacity for new tissue formation and ingrowth into the sponge, the strength—as measured by the tensiometer—of the connection between the two halves of the sponge will be lower than in an untreated sponge. The weight of the tissue trapped in the sponge says nothing about its nature; it might be connective tissue and/or blood constituents. This can only be determined by histological and biochemical means.

The results of the present investigation show that exposure of subcutaneous tissue to crystal violet causes a pronounced reduction of the regenerative capacity as demonstrated by the reduced breaking load. In this study, where the cross-sectional "wound area" is standardized to 1 square cm, the value for breaking load is identical with that for breaking strength (i.e. breaking load per unit wound area). For the highest dye concentration (1:100) there is a nearly total lack of strength in the connection between the two halves of the sponge. With more dilute dye solutions, the breaking load is somewhat higher but still there is a highly significant reduction as compared with control sponges. This may indicate that the strength over an incision in a dye-exposed sponge is provided by a fibrin clot rather than by newly formed connective tissue. This was confirmed by the findings in the histological sections, fibrin and blood cells occupying almost all empty spaces in the sponge material. In the control sponges, on the contrary, fibroblasts penetrated into the pores of the sponges. The histological picture 10 days post-operatively of a dye-exposed sponge is similar to that which can be seen during the very first days after implantation in a water-soaked control sponge, before regeneration has started. The almost total inhibition of granulation tissue formation by crystal violet in this study is compatible

with a tissue injury, causing a lengthening of the initial phase of the healing process and a delay of the onset of fibroplasia and collagen formation. The present investigation, which is performed 10 days after implantation of the sponges, gives no indication as to when tissue regeneration starts or at what rate it proceeds.

Series IV and V are the only ones with a significantly higher dry weight of control sponges than in dye-treated sponges. The breaking load values of control sponges are also higher in series IV and V, where exposure to crystal violet in experimental sponges was less, than in series I-III. This may indicate a generalized effect of locally applied crystal violet or a leakage from side to side.

In tissue culture, high concentrations of serum and polyanions, e.g. heparin, partly neutralized the crystal violet and reduced its cytotoxicity (15). The marked tissue effects of the dye in the present investigation may partly be explained by the dye being administered immediately after traumatization when the inflammatory reaction still is weak. The concentration of the dye and the time the tissues are exposed to it probably also influence the severity of the tissue injury. In this study, dye concentrations below that recommended for topical use in patients resulted in significant tissue damage. The implanted dye-soaked sponge probably acts as a depot, resulting in a prolonged contact between the dye and surrounding tissues. Exposure of subcutaneous tissue to crystal violet for only 5 min, however, also inhibited tissue growth in this study (exp. V). This corresponds to the finding in vitro, that exposure of human skin fibroblasts to crystal violet (conc. $1:10^5$) for 5 min was sufficient to induce a cytotoxic effect (15). This similarity between fibroblastic growth characteristics in vitro and in vivo is in accordance with Schilling et al. (19).

As further evidence of crystal violet's capacity to induce severe tissue injury, necrotic areas were found in some rats on the skin overlying sponges soaked with the highest concentration of the dye (1%). Identical skin necroses have been seen after injecting crystal violet, also 1% in aqueous solution, intradermally into guinea pig skin (4).

A few clinical cases of immediate and delayed hypersensitivity to triphenylmethane dyes have been reported (8, 9). If such a reaction developed

in the dye-soaked sponges, it could profoundly influence the milieu for tissue regeneration. Edwards et al. soaked sponges with chick embryo extract before implantation (8). Later a sterile, intense inflammatory reaction of seemingly allergic actiology developed, histologically dominated by mononuclear cells in and around the sponges. In the present study, however, we saw no indications of allergic reaction to the dye.

The results of this study indicating a reduced capacity for tissue growth and repair after exposure to crystal violet are in agreement with those obtained in vitro (15). The experimental conditions in this study are, however, somewhat remote from dye application in clinical practice. Studies designed to evaluate the tissue effects of this and similar agents under conditions more close to the clinical situation are in progress.

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