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EFFECT OF TRIPHENYLMETHANE DYES (BRILLIANT GREEN, CRYSTAL VIOLET, METHYL VIOLET) ON PROLIFERATION IN HUMAN NORMAL FIBROBLAST-LIKE AND ESTABLISHED EPITHELIAL-LIKE CELL LINES

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Abstract. Some cationic triphenylmethane dyes—brilliant green, crystal violet and methyl violet—used topically as antibacterial and antimycotic agents, were studied in vitro for their effects on the multiplication of normal fibroblast-like and established epithelial-like human cells. All these dyes were strongly cytotoxic at concentrations as low as one-tenth of one percent of the concentrations used clinically. Exposure to crystal violet for 5 minutes hampered the multiplication of cells significantly. The negative effects of the dye were markedly counteracted by high concentrations of serum as well as by heparin and other polyanions. The results indicate that the necrotizing reactions occasionally seen in patients treated topically with cationic triphenylmethane dyes might be due to an inadequate inflammatory reaction and/or a defective mast cell system.

Therapeutically, gentian violet and brilliant green are frequently used as antibacterial and antifungal agents. They are recommended for topical use (0.1–2 % = 1–20 mg/ml in aqueous solution) on infected skin and mucosal lesions (30, 32).

Gentian violet, a poorly defined mixture of hexamethylpararosaniline chloride (crystal violet) and pentamethylpararosaniline chloride (methyl violet) (cf. 22) may, however, cause skin and mucosal necrosis in humans treated for mycotic infections (4). Similar necrotic reactions were reproduced regularly by painting stripped normal skin in humans and guinea pigs with crystal violet or brilliant green in clinical concentrations (whereas no such reactions occurred in painted unstripped normal skin), and intradermal injection of dye in guinea pigs results in necrosis within a few days (4, 5). Against this background it remains obscure why only a small proportion of patients show necrotizing reactions after treatment with gentian violet or other triphenylmethane dyes. We therefore considered it important to study the direct effect of such dyes on cells in vitro where no host reactions modify the result.

The aim of the present study was to assess the direct effect in vitro of some cationic triphenylmethane dyes currently used in the clinic with respect to the multiplication of human normal fibroblast-like cells from skin and epithelial-like cells of squamous origin, and how the effect of these dyes is influenced by the addition of heparin, some other polyanionic agents and by serum.

MATERIAL AND METHODS

Cell lines

A fibroblast-like cell line, GP 119-2, was used, which originated in the Cell Culture Laboratory, Department of Pathology in Göteborg (25), and was derived from skin tissue in an apparently healthy legally aborted foetus in the second foetal stage. It was studied in subculture generations 10 through 19, fairly early in phase II (19). Established epithelial-like HeLa cells, emanating from a squamous cell carcinoma (15) were used in one experiment.

The cells were fed MEM (9) with Earle's salts (Grand Island Biological Co., New York, USA) supplemented with 10% postnatal non-inactivated calf-serum, streptomycin (100 μg/ml) and penicillin (100 IU/ml). The medium was changed three times a week with the first change on the day after subculture.

All experiments were performed on cultures in approximately 20 cm^2 dishes with a bottom diameter of 50 mm (Falcon Plastics, Los Angeles, USA), at pH 7.2–7.4 and 37°C in a humidified CO_2–air atmosphere. The cell inocula used for experimentation comprised 200 000 ± 100 000 cells per dish, except for plating efficiency studies where only about 200 cells were seeded per dish. The

cell cultivation technique has previously been described in detail (25).

Reagents

Unless otherwise stated, 0.5 ml of the reagent in aqueous solution was added to each dish containing 4.5 ml growth medium 4–6 hours after subculture when the cells had settled and stretched on the substrate.

Brilliant green, tetraethylpararosaniline sulphate ($C_{27}H_{34}N_2O_4S$, mol.wt 482.7; Merck, Darmstadt, West Germany) was used in sterile aqueous solution at concentrations between 1 and 100 μg/ml growth medium (0.0001% and 0.01%).

Crystal violet, hexamethylpararosaniline chloride ($C_{25}H_{30}ClN_3$, mol.wt 408.0; Merck, Darmstadt) in sterile aqueous solution was used in concentrations between 1 and 100 μg/ml growth medium.

The osmolarity—measured by lowering of the freezing point—was 253 mOsm/kg both in 4.5 ml MEM_{90}-$serum_{10}$ admixed with 0.5 ml sterile water (controls) and in 4.5 ml MEM_{90}-$serum_{10}$ admixed with 0.5 ml aqueous dye solution with a final dye concentration of 10 μg/ml. The pH in air atmosphere at 1 and 100 μg dye/ml MEM_{90}-$serum_{10}$ was 7.25 and 7.35 respectively, whereas the controls showed a pH of 7.20.

Crystal violet constitutes an integral part of methyl violet and gentian violet.

Methyl violet 2B (Merck, Darmstadt) a mixture of tetra-, hexa- and penta-methylpararosaniline chloride in sterile aqueous solution was used at concentrations between 1 and 100 μg/ml growth medium.

Brilliant green, crystal violet and methyl violet are all cationic triphenylmethane dyes.

Heparin (mol.wt approx. 17 000) in aqueous solution, 10 000 IU (77 mg)/ml (Vitrum AB, Stockholm, Sweden) was used at concentrations of 0.1, 0.5, 10 and 200 IU/ml growth medium. This heparin preparation carries about 3.1 moles sulphate per gram dry weight and 2.5 sulphate groups per repeating disaccharide unit.

Chondroitin sulphate (minimum mol.wt approx. 26 000; 99% pure, mixed isomers, Sigma Chemical Co., St. Louis, Mo., USA) carrying 0.98 sulphate groups per repeating disaccharide unit was used at a concentration of 100 μg/ml growth medium.

Na-dextran sulphate (mol.wt approx. 500 000; a gift from Pharmacia AB, Uppsala, Sweden) containing approx. 17±1% sulphur and carrying about 5.6 moles sulphate per gram was used at a concentration of 100 μg/ml growth medium.

Heparin, chondroitin sulphate and dextran sulphate are polyanions and were diluted with MEM before addition to growth medium.

Sterile water, from the same source as that used for dissolving the reagents, was added (0.5 ml) to 4.5 ml MEM_{90}-$serum_{10}$ to the control cultures.

Cell counts

Monodispersed cell suspensions of cultures dispersed in 0.25% trypsin (Difco 1 : 250) admixed with 0.02% Versene (w/v) in Ca- and Mg-free buffer for 10 minutes at 37°C were fixed in medium containing formaldehyde and electronically analysed (17, 25) within 1 hour in a Celloscope 302 (AB L. Ljungberg, Stockholm, Sweden). The effect of heparin on cell counting has been studied previously (26).

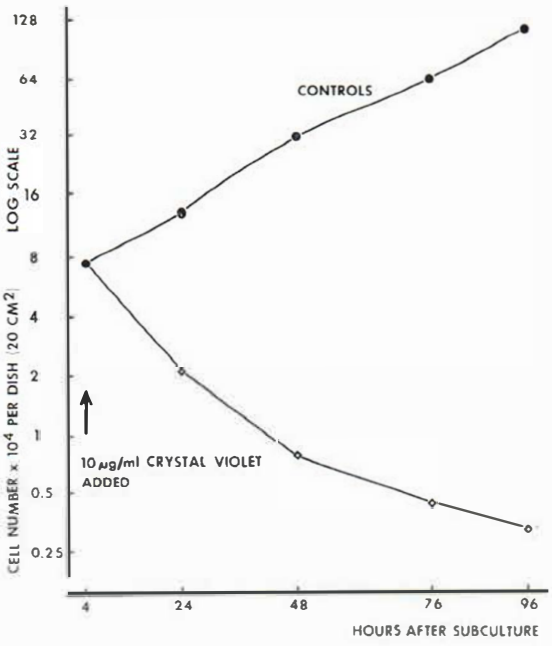

Fig. 1. Cell number in human normal fibroblast-like dermal GP 119-2 (12) cultures (each point of observation represents a mean of 10 cultures) 24, 48, 76 and 96 hours after subculture, exposed to 10 μg crystal violet per ml since 4 hours after subculture.

RESULTS

The first series of experiments were designed to ascertain how brilliant green, crystal violet and methyl violet affect the cell multiplication, as recorded by cell counts in normal human skin fibroblast-like cells as well as in epithelial-like cells of squamous origin, HeLa cells, after 3 days' culture. Table 1 shows that all the reagents at all concentrations significantly and very markedly reduced the cell number. Consistently the effect was not linearly correlated to the concentration of the test substance, the strongest effect regularly occurring at an intermediate concentration. This indicates a prozone effect.

For subsequent experimentation, a chemically defined dye—crystal violet—at a single concentration (10 μg/ml) was chosen to be tested on the skin fibroblast-like cell line GP 119-2.

In order to study the course of events of cell

Table I. *Cell counts in 3-day-old, normal foetal skin fibroblast-like cultures GP 119-2, and HeLa cultures of squamous origin, exposed to varying concentrations of brilliant green, crystal violet and methyl violet since 4 hours after subculture. Cell counts were made in samples of varying dilution*

Cell line (subculture generation)	Reagent	μg/ml	No. of cultures	Mean cell counts ±S.E.	Cell number in per cent of control	Total cell number × 10^3 per dish (20 cm^2)
GP 119-2 (10)	Control		6	10 407±547	(100)	520
	Crystal violet	100	4	489± 40	5	24
		10	4	167± 23	2	8
		1	4	2 637±137	26	132
	Methyl violet	100	4	1 052± 82	10	53
		10	4	122± 25	1	6
		1	4	2 363±144	23	118
(19)	Control		5	5 117±158	(100)	256
	Brilliant green	100	5	58± 16	1.1	3
		10	5	23± 4	0.4	1
		1	5	36± 7	0.7	2
HeLa	Control		6	8 308±244	(100)	415
	Crystal violet	100	4	965±173	12	48
		10	4	586± 95	7	29
		1	4	4 971±229	60	249

loss the cell count in cultures was established 24, 48, 76 and 96 hours after subculture. As seen in Fig. 1, the cell number in control cultures increased almost exponentially whereas the cultures exposed to crystal violet showed a progressive, very large reduction in cell number. Thus the reduced cell number recorded in cultures containing 10 μg/ml crystal violet 3 days after subculture (Table I) could not to any extent be explained by a prolonged lag phase.

The next step was to test the effect of crystal violet on dense, topo-inhibited cultures where the proportion of cells synthesizing DNA and dividing is markedly reduced (8) and where the close proximity between the cells somewhat resembles the situation in a tissue. Crystal violet added to 12-day-old cultures resulted in a very marked cell loss when analysed 2 days later (Table II). Thus the toxicity of crystal violet seems equally pronounced in dense and sparse cultures.

Crystal violet, being a cation, would be expected to be neutralized to some extent by serum proteins as well as by heparin, a strong polyanion. This was tested by adding crystal violet to cells in growth media containing varying serum concentrations—2.5, 10 and 40% (v/v)—and to cultures containing varying concentrations of heparin—0.1, 0.5, 10 and 200 IU per ml (Table III). Increase in serum up to 40% (MEM_{60}-$serum_{40}$) clearly reduced the toxic effect of crystal violet (Table III). The total protein content in MEM_{60}-$serum_{40}$ medium per dish was approximately 120 mg (2.4%, w/v), taking serum to contain approximately 6% proteins.

Heparin at all concentrations tested, when mixed with crystal violet and added to MEM_{90}-

Table II. *Cell counts in 14 day GP 119-2 (13) cultures exposed to 10 μg crystal violet per ml since day 12. Cell counts were made in samples of varying dilution*

	No. of cultures	Mean cell counts ±S.E.	Cell number in per cent of control day 12	Total cell number × 10^3 per dish (20 cm^2)	Total cell number × 10^4 per cm^2
Control, day 12	7	3 937±554	—	3 937	19.69
Control, day 14	9	2 523±372	128	5 046	25.23
Crystal violet, day 14	9	35± 4	2	70	0.35

Table III. *Cell counts in 3-day-old, normal GP 119-2 fibroblast-like cultures grown in media containing 2.5, 10 and 40% (v/v) serum and exposed to crystal violet (10 μg/ml) and to crystal violet admixed with heparin (0.1 to 200 IU/ml, 1 IU = 7.7 μg), chondroitin sulphate (100 μg/ml) or Na-dextran sulphate (100 μg/ml)*

"s" indicates a significant increase in cell number ($p \leq 0.02$), using Student's *t*-test for differences between two means

Cell line (subculture generation)	Growth medium	Reagent (μg/ml)	No. of cultures	Mean cell counts ±S.E.	Cell number in per cent of controls	Total cell number × 10^3 per dish (20 cm^2)
GP 119-2 (12)	$MEM_{97.5}$-$serum_{2.5}$	Control	5	6 058±815	(100)	324
	$MEM_{97.5}$-$serum_{2.5}$	Crystal violet (10)	5	88± 16	1	4
	MEM_{60}-$serum_{40}$	Control	5	12 530±558	(100)	695
	MEM_{60}-$serum_{40}$	Crystal violet (10)	5	4 242±305	31	222
	MEM_{90}-$serum_{10}$	Control	5	7 601±646	(100)	417
	MEM_{90}-$serum_{10}$	Crystal violet (10)	5	99± 20	1	5
	MEM_{90}-$serum_{10}$	Crystal violet (10) + 0.5 IU heparin/ml	5	9 864±518[s]	131	547
	MEM_{90}-$serum_{10}$	Crystal violet (10) + 10 IU heparin/ml	5	7 645±813	101	419
	MEM_{90}-$serum_{10}$	Crystal violet (10) + 200 IU heparin/ml	5	4 517±621	57	239
GP 119-2 (19)	MEM_{90}-$serum_{10}$	Control	5	5 080±384	(100)	254
	MEM_{90}-$serum_{10}$	Crystal violet (10)	5	153± 20	3	8
	MEM_{90}-$serum_{10}$	Crystal violet (10) + 0.1 IU heparin/ml	5	262± 21	5	13
	MEM_{90}-$serum_{10}$	Crystal violet (10) + 0.5 IU heparin/ml	5	602±111	12	30
	MEM_{90}-$serum_{10}$	Crystal violet (10) + dextran sulph. 100 μg/ml	5	834± 74	16	42
	MEM_{90}-$serum_{10}$	Crystal violet (10) + chondroitin sulph. 100 μg/ml	5	1 041± 30	20	52

$serum_{10}$ strongly neutralized the dye, as recorded by significantly increased cell numbers (Table III). In one experiment (subculture generation 12), a heparin concentration as low as 0.5 IU (3.85 μg/ml) seemed to neutralize the dye completely, resulting in a significant increase in cell numbers compared with the controls (MEM_{90}-$serum_{10}$). Heparin concentrations of 200 IU/ml, however, resulted in a reduced cell number, probably because of an excess of free heparin. Heparin at 10 IU/ml compared well with the controls. In another experiment (subculture generation 19) the addition of heparin at 0.1 and 0.5 IU/ml (0.77 and 3.85 μg/ml respectively) to the standard concentration of crystal violet (10 μg/ml) resulted in a significant increase in cell number compared with cultures exposed to crystal violet only. Still higher cell counts were recorded when chondroitin sulphate and Na-dextran sulphate at 100 μg/ml were admixed with crystal violet (Table III). However, the MEM_{90}-$serum_{10}$ controls contained more cells, which indicates that full neutralization of the dye by the anions tested might not have been achieved in this experiment, but there is also a possibility of an excess of free anions causing the reduction in cell number (26).

The effect of various durations of crystal violet exposure at 37°C was tested by adding crystal violet (10 μg/ml) 6 hours after seeding of a very small inoculum of approximately 200 cells per dish. Six cultures were used in each experimental group and 12 control cultures were used. The exposure was ended after 5, 20, 80, 320 and 1 280 minutes by removing the crystal violet-containing medium, rinsing the cultures once with 10 ml MEM containing 0.5 IU/ml heparin, followed by a second rinse with 10 ml pure MEM and adding 5 ml fresh growth medium (MEM_{90}-$serum_{10}$). Ten days later the cultures were fixed in acetic acid/methanol (1 : 3), stained according to May-Grünwald-Giemsa and microscopically examined for the number of multicellular colonies present. Colonies consisting of two or more morphologically well preserved topographically proximate cells were recorded. Most colonies consisted of tens or hundreds of cells. Table IV shows that

Table IV. *Number of multicellular colonies and relative plating efficiency in cultures 10 days after being exposed for varying durations to 10 μg crystal violet per ml*

Cell-line (subculture generation)	Minutes of exposure to 10 μg crystal violet per ml	No. of cultures	No. of multicellular colonies 10 days after exposure to crystal violet, mean ± S.E.	Differences from control $p \leq$	Relative plating efficiency
GP 119-2 (14)	0 = controls	12	11.2 ± 1.3		
	5	6	5.2 ± 1.1	0.01	0.46
	20	6	5.3 ± 1.1	0.02	0.48
	80	6	2.3 ± 0.8	0.001	0.21
	320	6	1.2 ± 0.4	0.001	0.10
	1 280	6	nil		

exposure of GP 119-2 cells to 10 μg/ml crystal violet even for 5 minutes significantly reduced the number of colonies.

DISCUSSION

Previous studies of the cytotoxic action in vitro of triphenylmethane dyes have concentrated on gentian violet. The somewhat divergent findings in the literature might be attributed to differences in experimental design, technical differences and different compositions of gentian violet. In 1912 Churchman (6) reported on gentian violet's selective bactericidal action on gram-positive bacteria. Two years later Russel (34) found that embryonic and adult frog tissue grew in the presence of 50 μg/ml of this dye (1 : 20 000), a concentration lethal to *Bacillus subtilis*. This greater resistance to the dye of tissue cells than bacteria led to its use in the treatment of infectious diseases. However, in the study of Lambert & Meyer (21) cells from fragments of rabbit spleen survived 4 μg gentian violet per ml (1 : 250 000) and were killed by 10 μg/ml (1 : 100 000), whereas the corresponding values for *Staphylococcus aureus* were 40 μg/ml (1 : 25 000) and 100 μg/ml (1 : 10 000). German (14) found that a concentration of 1.25 mg gentian violet per ml (1 : 800) totally inhibited the outgrowth of cells from explants of embryonic chick skin tissue fragments and in lower concentrations a partial inhibition occurred as compared with controls. Welch & Brewer (38) reported that concentrations of gentian violet higher than 100 μg/ml (1 : 10 000) inhibited the phagocytic action of leucocytes, participating in the body's defence against infections. Judging from these observations, one would clinically expect toxic effects on tissues to occur frequently since gentian violet is mostly used in concentrations as high as 10–20 mg/ml (= 1–2 %, aqueous solution). Clinicians, however, repeatedly reported on gentian violet's non-toxicity and safety for topical use (1–2 % in aqueous solution) (1, 12) and especially remarked that it did not interfere with the proliferation of tissue cells when applied to ulcers (37).

The cells used in the present study were fibroblast-like dermal cells in fairly early subculture generations and epithelial-like cells of squamous origin. Fibroblast-like cells derived from normal human tissues possess a series of features of normality (19, 25), produce collagen and are considered to have important functional similarities to fibroblasts in vivo (16, 36). The HeLa cells used certainly acquired new characteristics during their long cultivation. They are still epithelial-like but are not spinocellular. In the lack of spinocellularly differentiated, serially propagable human cell lines, the lines used in the present study are among the most representative available for the tissues clinically exposed to triphenylmethane dyes (skin, oral cavity and vagina).

According to Salle (35) single cells are more sensitive to germicides than cells in a composite tissue. Though most of the present study was performed on quickly multiplying single cells, a very marked cell loss was also noted when crystal violet was added to dense, topo-inhibited and somewhat "tissue-like" cultures.

Osmolarity and pH influence the viability of living cells (10, 28). Since there was no measurable difference in osmolarity between control medium and medium containing 10 μg crystal violet per ml such differences seem to be of negligible importance in the present study. The bulk pH in the cultures in CO_2–air atmosphere

could be slightly raised, but such a rise would be harmless (28, 33).

Since the effect of all dyes tested, in concentrations as low as 1 μg/ml, was very strong, we considered as unjustified a more penetrating study aiming at describing the effects on cell population kinetics and cell cycle phases. From the literature it is known that: the dyes are fat-soluble and diffuse easily into cells; crystal violet is bound to reactive acidic groups (29); crystal violet binds to free groups of DNA-phosphate (24); crystal violet inhibits the formation of the bacterial wall at a point different from that affected by penicillin (27), and it is suggested that the dyes act on bacteria by inhibiting the synthesis of glutamine precursors (13).

The most pronounced effect of the positively charged (cationic) dyes regularly occurred at an intermediate concentration, indicating a type of prozone effect. Such effects on cells seem to be produced preferentially by electrically charged substances (7).

In the 14-day-old GP 119-2 cultures the cell density was approximately $25 \times 10^4/cm^2$ which is more than twice the density at which chick embryo fibroblast-like cells reach confluence (33) and corresponds to about three confluence equivalents in human embryonic fibroblast-like cultures cultivated rather similarly to ours (2). The increase in cell number from day 12 to 14 was from 19.7 to $25.2 \times 10^4/cm^2$ corresponding to a population doubling time of about 110 hours which is about 5 times the population doubling time during the exponential growth phase (25, 26). Provided the generation time (intermitotic time) of individual cells and the death index (25) are similar in exponentially expanding and in dense, topo-inhibited cultures, the growth fraction (23) would be reduced to about 0.20 in the dense cultures. What the growth fraction between days 12 and 14 really is, remains unknown, however. Considering the loss of 98 % of all cells in cultures exposed to crystal violet (Table II), it seems reasonable to conclude that the dye affected not only DNA-synthesizing and dividing cells, but also topo-inhibited cells resting in the G1 (G0) cell cycle phase. This has some bearing when trying to explain the toxic skin reaction in patients, for in the skin, as in most other mature tissues, the majority of cells are in the G1 (or G0) phase (18, 31).

The plating efficiency experiments were designed to test the effect of different exposure times to crystal violet on the subsequent cell multiplication. In order to remove an excess of dye, a rinsing procedure was employed consisting of heparin in MEM followed by rinsing in pure MEM. The strongly negatively charged heparin should successfully compete with relatively weaker anionic sites on the cell surface for binding sites of the dye molecule. The fact that there was a negative correlation between exposure time and the number of developing colonies (Table IV) shows that the rinsing procedure actually removed the excess of dye. Therefore we believe that the results are valid and that an exposure to crystal violet even for 5 minutes harmfully affects the cells with regard to their proliferative capacity or integrity (20). Exposure in vitro of cells to cationic detergents for 10 minutes has been regarded as representative of the action time of such substances in a surgical wound (3).

The negative effect of crystal violet was significantly reduced by a high serum protein concentration. A marked neutralizing effect was also achieved by all polyanions tested, regardless of differences in chemical structure and molecular weight. On a basis of concentration, heparin, having the highest density of negative charge, seemed most efficient in neutralizing crystal violet. GP 119-2 (12) cultures exposed to the admixture of 10 μg crystal violet per ml and 3.85 μg heparin per ml had increased in cell number after 3 days whereas admixtures with higher heparin concentrations resulted in a reduced cell number (Table III). This is probably explained by heparin causing a shortening of the lag phase but also a concentration-dependent lengthening of the population doubling time during the exponential growth phase, due to increase in the effective death rate, i.e. a decreased effective survival time (26). Heparin is stored in mast cells together with histamine and serotonin and these substances are released in tissue injuries, causing, among other things, exudation of serum. Thus in vivo neutralization of the cationic dye may very well result, not only from chondroitin sulphate in the connective tissue ground substance, but also from heparin and plasma proteins. The fact that topical treatment with triphenylmethane dyes seems to create clinical complications in only a rather small proportion of all patients treated might de-

pend on an inadequate inflammatory reaction and/or a defective mast cell system in the patients affected. The number of mast cells in the skin and other tissues seems to vary among humans (cf. 11); this may be important in this respect.

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