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THE EFFECT OF PYOCYANIN ON HUMAN SKIN CELLS AND LEUCOCYTES

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THE dressings of burns infected with *Pseudomonas pyocyanea* often have a blue-green colour, which is due to the presence of pyocyanin and other pigments in the exudate. There is evidence that *Ps. pyocyanea* may cause the failure of skin grafts on burns and delay healing (Jackson, Lowbury and Topley, 1951). To find whether pyocyanin may play a part in causing these adverse results, we have studied the effect of different concentrations of the compound on human epithelial cells and leucocytes, and have attempted to assess the concentration of pyocyanin in green exudate from burns. These studies and a few animal experiments to determine the toxic rôle of pyocyanin in infection with *Ps. pyocyanea* are described in this paper.

Effect of Pyocyanin on Tissue Cultures of Human Skin.

Materials and methods.

The method of tissue culture, a modification of one described by Medawar (1948), was previously used by the authors in studying the effect of antibiotics on human skin (Cruickshank and Lowbury, 1952).

Solutions of pyocyanin (pyocyanine dihydrochloride, supplied by S. A. F. Hoffmann-La Roche & Co., Ltd.) were made in Ringer's solution at concentrations of 24·0, 2·4, 0·8, 0·24, 0·08, 0·024 and 0·008 mg. per ml. The culture medium consisted of 5 parts fresh homologous serum, 2 parts Krebs-Ringer phosphate, 1 part 5 per cent glucose in distilled water, 1 part pyocyanin solution and 1 part dihydrostreptomycin solution (500 units/ml. in Ringer's solution) to prevent the growth of contaminants. In the control medium the pyocyanin was omitted and the amount of Krebs-Ringer phosphate was increased to 3 parts. The medium had a pH of 7·2–7·4.

The excess of skin removed for storage at grafting operations was obtained, a specimen of medium thickness cut on the same day being used in each experiment. It was divided into pieces of about 1–2 sq. mm., which were floated on the surface of 0·5 ml. of culture medium in 5 ml. bottles with rubber stoppers, 3 bottles of each test dilution under study and of the control being set up at each experiment. The cultures were incubated at 37° in an atmosphere of oxygen, being rocked through 30 degrees about 5 times a min. on a platform.

After 3 days' incubation, the explants were fixed in formol saline, dehydrated, cleared and embedded in paraffin. Median sections were cut and stained with haematoxylin and eosin.

Results.

The appearance of sections is shown in Fig. 1–3. In the control culture epithelium has migrated from the edge of the explant to cover the cut surface of the dermis, which becomes encysted with new epithelium two or three cells thick. The dermis has the same appearance as that of newly cut skin. Two degrees of damage to the skin culture are described : (1) *Inhibition* : In these cultures the cells of the epidermis are still histologically normal but the amount of migration is less than in the controls. (2) *Destruction* : In this group there is no migration of epithelium, and the staining properties of cells are abnormal. The cultures are on occasion completely necrotic.

The effects of pyocyanin are shown in Table I. It will be noted that the highest concentration at which consistently good cultures were obtained was 0·0024 mg./

TABLE I.—*Effect of Pyocyanin on Cultures of Human Skin.*

Dilution of pyocyanin.	Number of explants showing			Ratio : normal/ abnormal.
	Normal growth.	Inhibition.	Necrosis.	
0·24 mg./ml.	—	—	9	0/9
0·08 ,,	—	—	3	0/3
0·024 ,,	—	2	9	0/11
0·008 ,,	5	4	—	5/4
0.0024 ,,	12	—	—	12/0
Controls	15	—	—	15/0

ml. At concentrations from 0·008 to 0·24 mg./ml. the results varied from minor inhibition to complete destruction ; at concentrations above 0·024 mg./ml. death of the explant was usual.

Effect of Pyocyanin on Human Leucocytes.

Materials and Methods.

The technique used by Abraham, Chain, Fletcher, Gardner, Heatley, Jennings and Florey (1941) for determining the toxic action of penicillin on leucocytes was adopted.

Drops of blood were allowed to clot at 37° on cover slips in a Petri dish containing moist filter paper. After about 30 min. the Petri dish was taken from the incubator and the blood clots were removed from the cover slips with forceps. Erythrocytes were washed away with warm Ringer's solution, and the cover slips were inverted over well-slides containing dilutions of pyocyanin in a special salt solution consisting of urea 0·3 g., glucose 1·0 g., $NaHCO_3$ 1·61 g., $NaH_2PO_4H_2O$ 0·425 g., NaCl 4·95 g., KCl 0·625 g., $MgCl_2$ 0·24 g., $CaCl_2$ 0·31 g., in 1 l. distilled water, with 10 per cent fresh human serum added at the time of the experiment. The dilutions of pyocyanin tested were 0·24, 0·08 and 0·024 mg./ml., 3 cover slips being prepared for each dilution and for controls without pyocyanin. The cover slips were sealed with paraffin wax.

Observations were made at hourly intervals for 4 hr. These were carried out with the microscope in an incubator box at 37° using an oil immersion objective. While not under observation the slides were kept at 37°. The motility and cytoplasmic movements of the granulocytes in a large number of fields were noted. Three experiments were made on different occasions.

Results.

When leucocytes were maintained in the presence of the " control " solution cytoplasmic movement remained vigorous for 4 hr. In pyocyanin 0·08 mg./ml.

and 0·024 mg./ml. the movement after 4 hr. was not impaired. In 0·2 mg./ml., however, the number of leucocytes active after 1 hr. was obviously reduced, and at 4 hr. all the cells appeared to be dead.

The Concentration of Pyocyanin in Exudate of Burns.

An approximate measure of the concentration of pyocyanin was obtained by extracting with a measured volume of chloroform a piece of gauze containing a known weight of exudate soaked up from a burn. The red colour reaction (acid pyocyanin) obtained on extracting the chloroform solution with acid was matched against a standard, and from the concentration of pyocyanin thus estimated to be present in the chloroform extract it was possible to assess the approximate concentration of pyocyanin in the exudate.

Method.

Absorbent gauze pads, 8 in. by 4 in. (20 × 10 cm.), were weighed, marked with black thread, wrapped in paper and sterilised by dry heat for 1 hr. at 160°.

Patients whose burns were colonised with *Ps. pyocyanea* and covered with green-stained dressings were selected for the investigation. Weighed gauze pads were included in the dressing under the cotton wool. The pads were removed when dressings were next changed (usually on the 3rd or 4th day), and if they were soaked with green exudate they were collected in glass ointment jars with screw-caps and re-weighed.

For the extraction and estimation of pyocyanin, the gauze pad was cut into small squares and returned to the ointment jar. A measured volume (50 ml.) of chloroform was then added, and the pieces of gauze were thoroughly extracted by stirring with a thick glass rod and shaking. Supernatant fluid was removed and centrifuged in small screw-cap bottles. It was then shaken with an equal volume of 0·1 N HCl, which extracted the pyocyanin as a red pigment (acid pyocyanin), the colour of which was matched against a range of doubling dilutions of acidified pyocyanin.

Results.

The approximate concentration of pyocyanin in the exudates from 5 burns (Table II) varied with the staining of the gauze and reached 0·31 mg./g. when

TABLE II.—*Approximate Concentration of Pyocyanin in Exudate from Five Burns.*

Patient.	Appearance of dressing.	Pyocyanin concentration (mg./g.)
1	Diffusely pea-green	0·07
2	Green and greenish yellow over part of gauze	0·008
3	Blue-green over most of gauze	0·22
4	Ditto	0·31
5	Patches of deep and pale green over most of gauze	0·14

the colour was bluish green, which is common in burns yielding heavy growths of *Ps. pyocyanea*.

These concentrations were similar to those obtained from 8-day agar slope cultures by extraction with chloroform (0·037 to 0·15 mg./ml.).

The Effects of Intradermal Inoculation of Guinea-pigs with Ps. Pyocyanea *and Pyocyanin.*

Fox and Lowbury (1953) have shown a difference between the size of small lesions produced on intradermal injection of rabbits with a strain of *Ps. pyocyanea* against which the animal had been immunised, and the larger lesions produced at the site of inoculation of unrelated strains of *Ps. pyocyanea.* By comparing the sizes of the lesions produced by strains of *Ps. pyocyanea* which did and which did not produce pyocyanin, it was hoped to obtain evidence of the rôle of pyocyanin in deeper infections. To the same end a series of intradermal inoculations of pyocyanin in Ringer's solution was made, and the effects observed.

Method.

Twelve strains of *Ps. pyocyanea* were used, of which 6 produced abundant pyocyanin and 6 produced no pyocyanin after 42 hr. culture on nutrient agar plates (18 hr. at 37° and 24 hr. on the bench) ; presence or absence of pyocyanin was confirmed by chloroform extraction of the fluid obtained after freezing and thawing the agar culture.

Three (in one case 2) intradermal injections in epilated guinea-pigs were made with undiluted and 10^{-1} dilutions of suspensions of each strain made by re-suspending washed broth culture in an equal volume of Ringer's solution. The red lesions, many of which had a necrotic and haemorrhagic centre, were examined and 2 diameters were measured with dividers after 24 and 48 hr.

The guinea-pigs were killed after 48 hr. and 1 lesion produced by each of the strains was excised, cut into small pieces, and extracted with chloroform to detect the presence of pyocyanin.

Four guinea-pigs were each given intradermal inoculations of 4 tenfold dilutions of pyocyanin in Ringer's solution (2·4, 0·24, 0·024 and 0·0024 mg./ml.). The guinea-pigs were examined after 24 hr., and lesions were excised for histological study.

Results.

There was no consistent difference in the size of lesions from the strains that produced pyocyanin and from those that did not produce the pigment.

Chloroform extracts of all lesions remained colourless : no evidence was therefore obtained of production of pyocyanin in the lesion.

The guinea-pigs given intradermal inoculations of pyocyanin showed slight macroscopic oedema, but negligible histological changes ; some infiltration with round cells was found at the sites of inoculation of the most concentrated solution but most of the other inoculation sites appeared normal.

DISCUSSION.

These experiments show that pyocyanin is toxic to human epidermis cultivated *in vitro*, at concentrations above 0·0024 mg./ml. Schoental (1941) demonstrated that pyocyanin inhibited the growth of chick embryo fibroblasts at concentrations above 0·025 mg./ml. although some growth took place at concentrations up to 0·1 mg./ml. From our experiments the leucocyte test would appear

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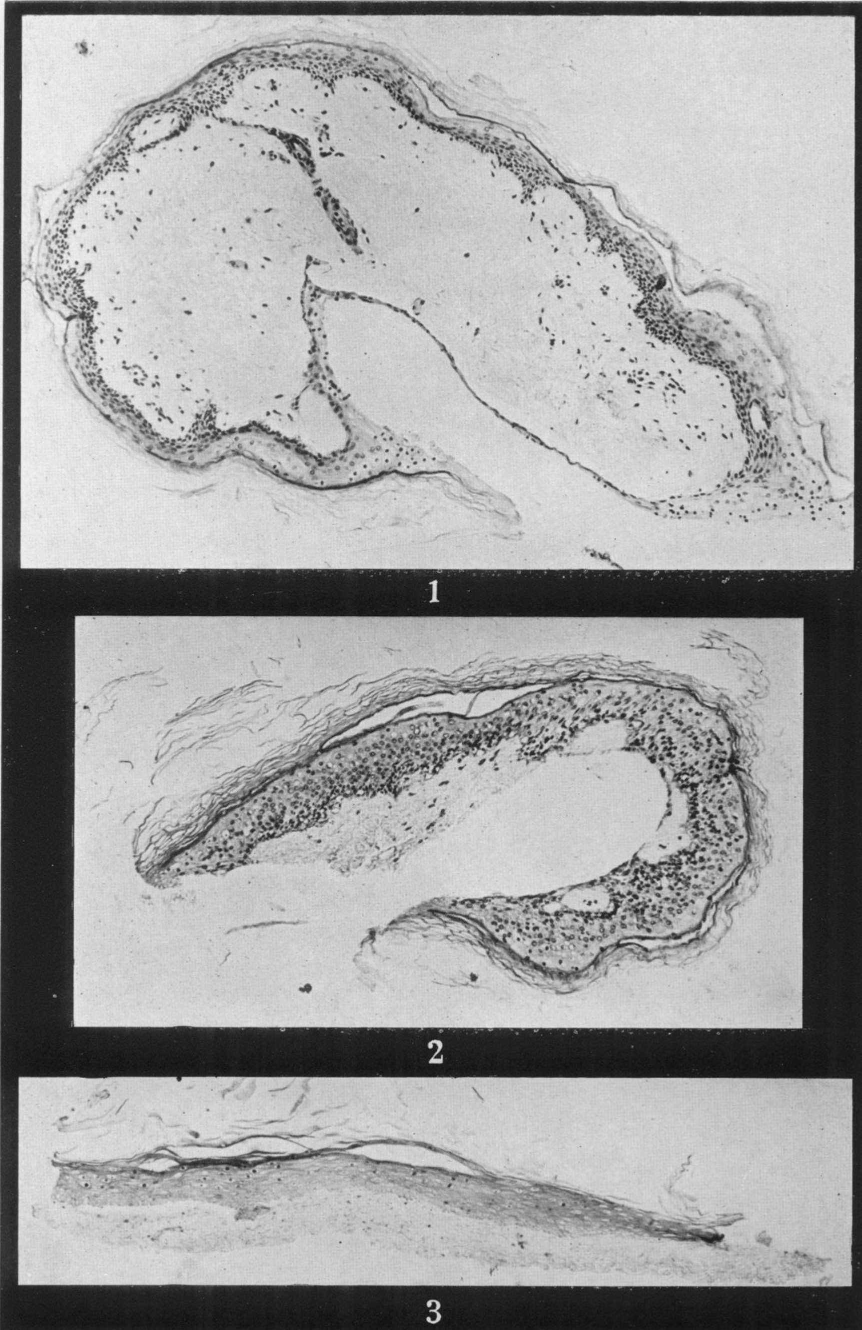
DESCRIPTION OF PLATES.

FIG. 1.—Normal culture. Epidermis with normal appearance, and a continuous layer of migrating epidermal cells over the cut surface of the dermis.

FIG. 2.—Inhibited culture. Almost complete failure of migration. Numerous vacuoles are present in the epidermis.

FIG. 3.—Necrotic culture. Absence of migration, and degeneration of epidermal cells. There is partial separation of epidermis from dermis.



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to be less sensitive than tissue culture, as no abnormal effect was noted at concentrations of 0·08 mg./ml. and toxicity was apparent only at 0·24 mg./ml.

The main interest in the findings is their relation to the amounts of pyocyanin found on burns colonised with *Ps. pyocyanea*. Our experiments showed that in burns producing green discoloration of dressings the concentration of pyocyanin in the exudate was between 0·008 mg./g. and 0·31 mg./g. In each instance the concentration was above that which might be expected to produce toxic effects upon the skin *in vitro*. It seems reasonable to suppose that dressings soaked with the green pigment may act as a reservoir of pyocyanin which hinders the repair of burned skin and the success of skin grafts. Moreover the reservoir is continuously replenished by the growth of *Ps. pyocyanea*, so that absorption into the circulation and dilution by fresh exudate will not necessarily diminish the concentration of pyocyanin on the burned surface.

It must be emphasised that the rôle of the local toxicity of pyocyanin in the pathogenicity of *Ps. pyocyanea* is postulated only with regard to surface infections. The animal experiments suggested that in deeper infections the local production of pyocyanin was not of significance. This conclusion is based on the absence of any response to the intradermal injection of pyocyanin and the fact that there was no difference in the size of the lesions caused by pyocyanin-producing and by non-pyocyanin-producing strains.

SUMMARY.

Experiments were made to determine the toxicity of pyocyanin to tissue cultures of human skin and to human leucocytes. Pyocyanin was found to be toxic to skin *in vitro* at levels above 0·0024 mg./ml. and to leucocytes at levels above 0·08 mg./ml.

Estimates were made of the concentration of pyocyanin present on 5 burns infected with *Ps. pyocyanea*. The concentrations ranged from 0·008 to 0·31 mg./g.

The concentrations found on human burns were all capable of causing toxic effects on skin *in vitro*. The leucocytes were less sensitive, being affected only by the highest concentrations found in burn exudates.

Intradermal injection of pyocyanin into guinea-pigs caused no local lesion. The lesions produced by the intradermal injection of pyocyanin producing and non-pyocyanin producing strains of *Ps. pyocyanea* did not differ in size.

These findings are discussed in relation to the observed ill-effects produced by *Ps. pyocyanea* on human burns.

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