A SELECTIVE LETHAL EFFECT OF PENICILLIN ON SARCOMA CELLS GROWING WITH NORMAL TISSUE IN ROLLER TUBE CULTURES*

By IVOR CORNMAN

(From The Wistar Institute of Anatomy and Biology, Philadelphia)

PLATES 1 AND 2

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The clinical use of penicillin at Walter Reed General Hospital during 1942–43 made available small amounts of this substance for experimental study. Accordingly, at the suggestion of Lieutenant Colonel Carl J. Lind and Captain Jack M. Evans, some of the drug was taken to The Wistar Institute of Anatomy and Biology in Philadelphia, for use in tissue culture experiments. The early observation of what appeared to be a specific effect on sarcoma cells (2) prompted the following study. We are indebted to Dr. Margaret Reed Lewis and Dr. Warren H. Lewis for the use of their laboratory facilities and for their help throughout these studies.

Material and Method

Rats of the King A[1] and of the Wistar albino strains, and mice of the black (C_{57}) and the Bagg Albino (B.A.) strains were used. Each of these inbred strains had proved to be 100 per cent susceptible to the grafts of sarcomata that had been induced in the strain (3). Six rat sarcomata (King A No. 11, No. 89, No. 104, No. 120. and No. 132 and Wistar No. 304) and two mouse sarcomata (C_{57} No. 350 and B.A, No. 37) were used in the cultures. These spindle cell sarcomata had been induced by subdermal injection of dibenzanthracene or benzpyrene (5). The normal fibroblasts were derived from fragments of muscle from rats or mice, 1 to 2 days old, of the tumor host strain.

Roller tube cultures (4) with usually eight to ten fragments of a tumor and an equal number of muscle fragments 1 to 2 mm. in diameter were grown in a medium composed of 2 drops of chicken plasma, 2 drops of chick embryo extract, 5 drops of human placental serum, and 7 drops of Locke's saline solution. The pipettes used measured 18 to 20 drops to the cubic centimeter. Extensive outgrowth was obtained in 24 to 72 hours, and at this time a record was made of the extent of growth. The initial medium was then replaced with a medium of 7 drops of Locke's solution, 5 of serum, and 2 of plasma, and in the experimental tubes, 1 to 3 drops of penicillin solution were substituted for an equal quantity of Locke's solution. This penicillin

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[1] Kindly supplied by Dr. Helen Dean King.

solution was prepared from Squibb or Reichel sodium salt of penicillin dissolved in 0.85 per cent sodium chloride, and filtered through a sterile Seitz filter. Such pharmaceutical penicillin preparations contain substances in addition to the penicillin.

The duration of exposure was varied, dosage usually being continued until a definite selective effect was observed. After the effect of the drug had been studied, the penicillin medium was replaced with a Locke's solution + serum + plasma medium, and the recovery processes were studied. In those instances where the injured tumor explants showed renewed growth, half the explants were implanted into an animal of the strain native to the sarcoma to test whether the viable cells were malignant or stromal cells. In all, 38 tubes, treated and untreated, were studied.

RESULTS

The malignant cells were consistently more injured than the normal ones (Figs. 1 and 2, 4 and 5). With adequate dosage it was found possible to damage and kill the outgrowing cells of all six rat tumors and one of the mouse tumors without killing the cells which grew from the fragments of normal muscle. These malignant cells first reacted to the penicillin by assuming a granular, opaque appearance, with or without vacuoles. This was the initial response to a heavy dose of the drug or the full extent of response to a threshold dose. If the penicillin was removed at this point, all cells recovered. In the higher concentration, along with increasing granularity and darkening of the cytoplasm, there was a retraction of the elongate processes, producing cells irregularly rounded. Upon prolonged exposure the cells disintegrated. Even if penicillin was removed after the cells had rounded up, some of the cells never recovered. This sequence of changes is not peculiar to penicillin, but is the usual response to many cytotoxins and moderately toxic compounds. In the higher concentrations of penicillin, the normal fibroblasts followed the same sequence of changes. However, there was the very important difference that a concentration of penicillin sufficient to cause the rounding up of some of the fibroblasts, in most instances caused the death of all of the malignant cells. A dose too weak to produce any visible cytological changes was nevertheless selective in that it inhibited growth from the malignant explants, while growth of the normal explants was unaffected. In the untreated control tubes the outgrowth of the sarcoma equalled and usually exceeded the outgrowth of the normal cells.

To obtain a quantitative statement of the results, damage to the cells can be classified as incipient (granularity of 50 per cent or more of the cells, and increased irregularity and refractility of the cell boundary), marked damage (rounding, coagulation, or disintegration of the cells, short of 100 per cent), or lethal (no living cells visible). Table I shows the totals of explants classified according to their damage. A further subdivision of the comparisons better reveals the extent of the selective effect. In those tubes in which the 112 normal colonies were not at all affected, there were 29 of sarcoma which

showed incipient effect, 114 which showed marked damage (Figs. 2 and 5), and 23 which were dead. In tubes showing incipient effects upon 92 normal colonies, 70 tumor growths showed marked damage and 46 were completely killed. In tubes in which the 57 normal colonies showed marked damage, 24 colonies of tumor were markedly damaged, and 35 were killed. The overall effect was clear cut. Not only was the malignant tissue damaged more than the normal throughout the series, but in numerous instances the malignant cells were killed when there was no visible effect upon the normal.

These figures include results obtained with tube cultures of the rat tumors and of mouse C_{57} No. 350. Rat tumor 120 was less affected than the others, but the selective effect of penicillin upon the malignant cells of this tumor was unquestionable. The behavior of the mouse tumor B.A. No. 37, on the contrary, proved to be so like that of normal mouse tissue in its reaction to penicillin that the presence of a selective effect was doubtful. In three tubes there were 10 muscle colonies showing incipient effect, 16 showing marked

TABLE I

Number of Explants of Sarcoma Cells and of Normal Fibroblasts Showing Different Grades of Damage. Combined Totals of All Experiments

	None	Incipient	Marked	Lethal	Total
Colonies of normal tissue	112	92	57	0	261
Sarcoma	0	29	208	104	341

damage, and 4 dead, as against the cultures of No. 37 which showed 18 markedly damaged colonies and 7 dead. A dose heavy enough to kill the malignant cells had also killed some of the normal, and the slight advantage of the normal is of questionable significance.

The time at which damage appeared varied with the different cultures and doses, but typically, using a dose at the selective lethal level, an incipient damage to tumor cells could be detected at 12 hours. There was marked damage at 24 hours, and complete killing of the growth zone at 48 hours. By 48 hours, however, there was sometimes a new growth of tumor cells, already pushing out from the explants. If this reviving growth was then given fresh medium free from penicillin, the tumor cells grew vigorously. If the fresh medium contained penicillin, however, this new growth was in turn killed off. Four to six days (*i.e.* two to three changes of penicillin medium) were usually sufficient to eliminate all tumor cells from explants 2 mm. in diameter. Then when the medium free from penicillin was added no tumor cells grew out, or if the sarcoma explants were implanted into rats, no tumors formed. With explants of 1 mm. diameter, however, 2 days sufficed to kill the malignant cells, as determined microscopically and by implantation.

The stroma included in the explants of the various tumors responded much the same as did the fibroblasts growing from the muscle fragments, but was perhaps slightly more susceptible than the normal, and grew more slowly than either muscle fibroblasts or malignant cells. In some tube cultures it was possible, without killing the normal cells, to kill all cells in the tumor explants (rat tumors 104, 132, 304, and mouse tumors C_{57} 350), but there may not have been any stroma included in these explants. More frequently, apparently dead explants (initial migration zone disintegrating and no new cells migrating from the explants during treatment) recovered enough in a medium free from penicillin to send forth at least a few stromal cells. These long, fusiform cells resembled fibroblasts rather than the stout, multipolar malignant cells.

As a final safeguard against classifying viable malignant explants as killed, those pieces which showed no growth and those which showed a growth of stroma or of some doubtfully malignant cells (Figs. 2 and 5) were implanted in young rodents of the corresponding inbred strain. The failure of these cells to grow into tumors showed that the estimation of lethal effect had been conservative in that some malignant explants graded as merely damaged failed to produce tumors. Colonies in four tubes graded as 100 per cent lethal, failed to produce tumors when implanted into animals; and out of 21 tubes graded as probably containing surviving tumor cells, only six contained tissue capable of producing tumors. It is worthy of note that only a few untreated cells are necessary to produce a tumor *in vivo*. To verify the susceptibility of the animals, they were given implants of untreated as well as the penicillin-treated tumor tissue.

In two cultures the dosage in Oxford units was determined by bacteriological assay of the penicillin solution.[2] Rat tumor 304 was killed at a level of 59 units per cc. of Reichel lot 1C533. The muscle fibroblasts in these cultures showed marked damage. The malignant cells of rat tumor 132 were markedly damaged (without any damage to the normal fibroblasts) by 75 units per cc. of Squibb control 87225 and by 73 units per cc. of Squibb control 91478.

DISCUSSION

The evidence is fairly conclusive in showing that the agent producing the selective damage was in the penicillin preparations. The effect increased with the increase in dosage, and in control tubes identical except for the lack of penicillin, the malignant cells grew at least as well as the fibroblasts.

The revival of growth from the tumor explants after 2 days treatment suggests the possibility of a synergistic effect of penicillin along with some com-

[2] We are indebted to the Bacteriological Division of the Food and Drug Administration for the assays.

ponent of the medium. The hydrogen ion concentration is one such possibility, since fresh medium is more alkaline than medium in which tissues have been growing. However the pH alone is not responsible for the selective lethal effect since indicator dyes showed no difference between treated and control tubes, and no changes upon addition of penicillin. More probably the recovery of the malignant tissue resulted from breakdown of the penicillin. This possibility was checked by transferring the penicillin medium from one tube, in which it had killed tumor cells, to an untreated culture.[3] The malignant cells in the second culture were uninjured, clearly indicating that the medium had lost its potency.

Consideration must be given to the possibility that the medium favors the growth of the cells derived from the muscle, and that penicillin acts by merely lowering the life-supporting powers of the medium, whereupon the sarcoma succumbs first. The sustained superiority of growth of the malignant cells over the normal, however, indicates that the medium was entirely adequate. Omitting plasma or adding embryo extract during the penicillin treatment did not eliminate the selective lethal effect. Tests with different media may prove fruitful, however, in revealing whether penicillin acts upon an intrinsic peculiarity of malignant cells or merely upon a susceptibility created *in vitro*.

Although malignant growths have well established peculiarities of metabolism, few substances have been demonstrated to have a selective effect upon neoplastic cells *in vitro*. Chambers, Cameron, and Kopac (1) have reported injury to malignant lymphoid cells by three phenylenediamines. N,N,N′,-N′-tetramethyl-*o*-phenylenediamine was toxic to leukemic cells at concentrations one-seventh that which affected normal lymphoid cells.

Bacteriostatic action apparently does not necessarily carry with it a sarcoma-damaging activity. The sulfa drugs, for example, are even used routinely in cultures of neoplastic tissue to reduce infection, and no selective effect has been reported. Indeed, we have no proof that the results reported here are effects of the bacteriostatic agent in the mixture rather than of some substance not eliminated during the purification of the penicillin preparations used in these studies.[4]

SUMMARY

An agent present in pharmaceutical Squibb and in Reichel penicillin preparations was found to exert a selective lethal effect upon rat and mouse sarcoma cells growing with normal cells in tissue cultures.

[3] M. R. Lewis, unpublished observations.

[4] Subsequent studies by Dr. M. R. Lewis have shown that the selective effect is not exerted by highly purified colorless penicillin, but rather that the effect is due to some substance present in the less highly purified samples along with the bacteriostatic factor (*Science*, 1944, **100**, 314).

REFERENCES

1. Chambers, R., Cameron, G., and Kopac, M. J., Neoplasm studies. XI. The effect in tissue cultures of N,N,N′,N′-tetramethyl-*o*-phenylenediamine and other compounds on malignant lymph nodes, *Cancer Research*, 1943, **3**, 293.
2. Cornman, I., Survival of normal cells in penicillin solutions lethal to malignant cells, *Science*, 1944, **99**, 247.
3. Lewis, M. R., and Lichgenstein, E. G., Studies on the transplantability of induced and spontaneous tumors occurring in mice of pure inbred strains, *Growth*, 1937, **1**, 375.
4. Lewis, W. H., Rat malignant cells in roller tube cultures, and some results, *Carnegie Institution of Washington, Pub. No. 150, Contrib. Embryol.*, 1935, **25**, 161.
5. Lewis, W. H., Dibenzanthracene mouse sarcomas. Histology, *Am. J. Cancer*, 1939, **37**, 521.

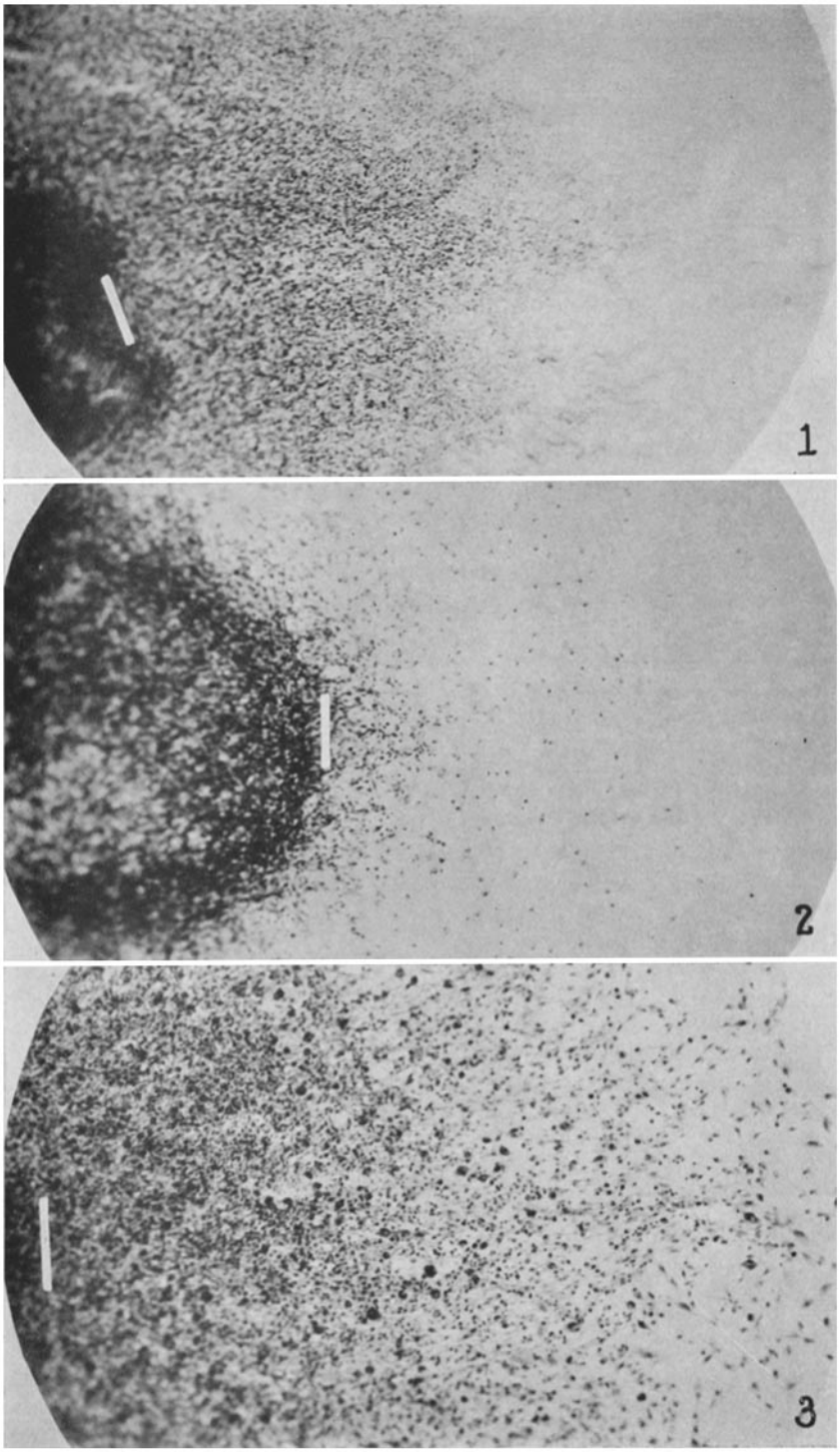
EXPLANATION OF PLATES

PLATE 1

The photomicrographs were prepared at the Army Medical School.

FIGS. 1 and 2. Muscle (Fig. 1) and sarcoma 132 (Fig. 2) growing in the same tube after 12 days' exposure to penicillin. The vigorous growth of the muscle forms a migration zone as broad as the diameter of the original explant, whereas the sarcoma shows only a sparse fringe of cells and scattered, rounded, moribund cells. A white bar indicates the edge of the explant. × 45.

FIG. 3. Sarcoma 132, untreated. The 5 days growth is equal to the 12 days' growth of the muscle in Fig. 1. × 45.



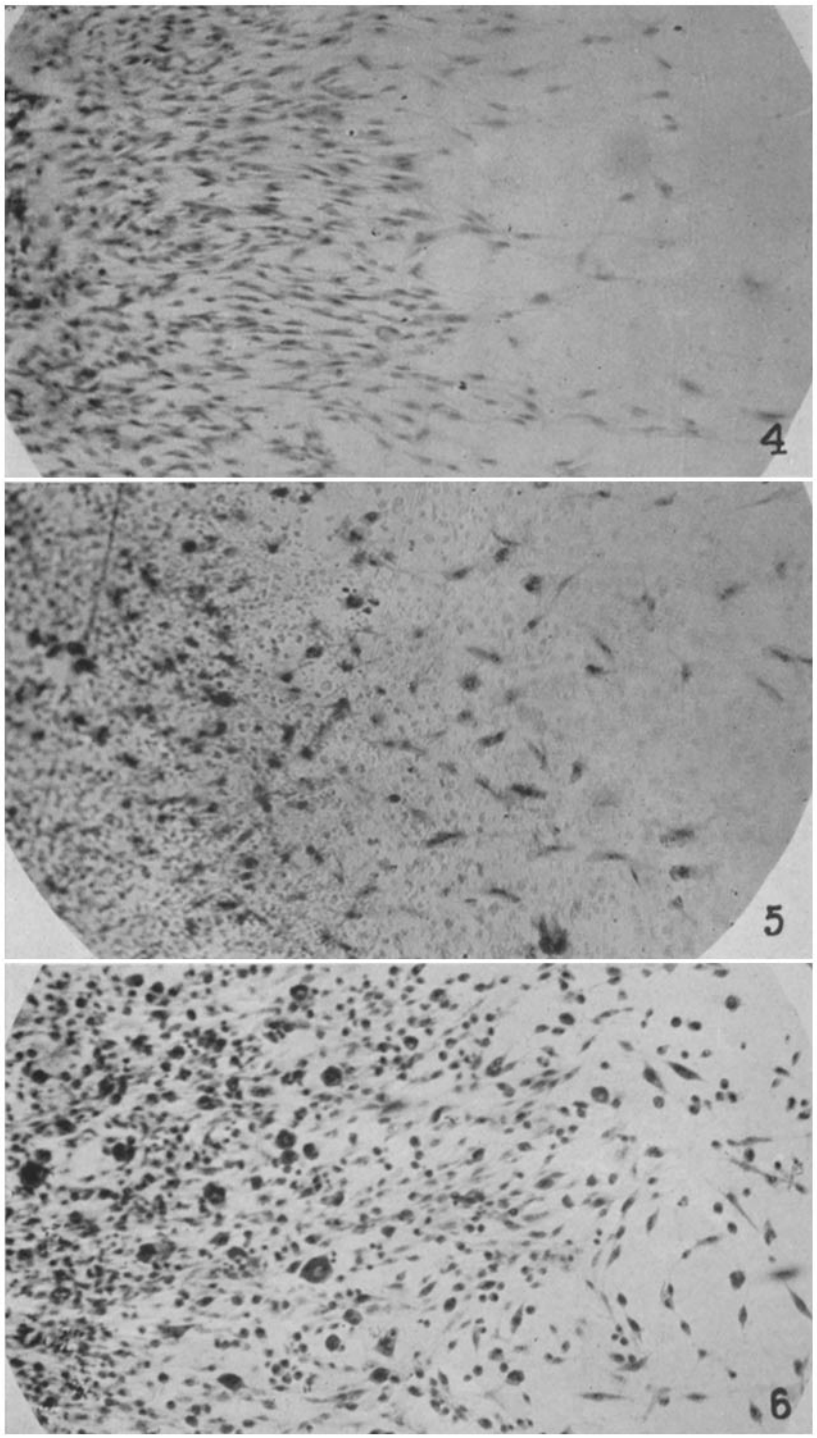
(Cornman: Selective lethal effect of penicillin on sarcoma cells)

PLATE 2

FIGS. 4 and 5. Muscle (Fig. 3) and sarcoma 11 (Fig. 4) growing in the same tube after 6 days' exposure to penicillin followed by 2 days in normal medium. The cells in the muscle migration zone are the normal fibroblastic type whereas the sarcoma migration zone is composed only of deformed cells and debris of disintegrated cells. The effect was graded only as "marked damage," inasmuch as some apparently viable cells remain, but five sister colonies from the same tube, implanted into one rat, failed to produce a tumor. × 100.

FIG. 6. Untreated cells of tumor 132. Same explant as in Fig. 3. × 100.

Fixation in Bouin's; stained with hematoxylin.



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