VOLUME II JANUARY, 1897 No. 1

THE JOURNAL
OF
EXPERIMENTAL MEDICINE

ON THE PRESENCE OF NERVES IN TUMORS AND OF OTHER STRUCTURES IN THEM AS REVEALED BY A MODIFICATION OF EHRLICH'S METHOD OF "VITAL STAINING" WITH METHYLENE BLUE.

BY HUGH H. YOUNG, M. D.
(From the Anatomical Laboratory of the Johns Hopkins University.)

PLATE I.

Our knowledge of neoplasms, notwithstanding the enormous amount of research to which they have been subjected, is still extremely vague and unsatisfactory. With nearly every advance in histological technique, however, its application to tumors has afforded some additional fact or facts concerning them, and it is only by utilizing for their study the various improvements of older methods or entirely novel procedures, that we may hope for the accumulation of data from which may be deduced a satisfactory explanation of their origin and somatic relations. About a year ago, at the suggestion of Dr. Lewellys F. Barker of the Anatomical Laboratory, I undertook the study of some of the tumors obtainable in the surgical operating room of the Johns Hopkins Hospital, with Golgi's silver-method and with a modification of the method of vital staining by methylene-blue introduced by Ehrlich* of Berlin. I have to thank Dr. Barker for instruction in the use of

* Ehrlich, P., Ueber die Methylenblaureaction der lebenden Nervensubstanz. *Deutsche med. Wochenschr.*, 1886, No. 4.

the methods and for the interest he has manifested in the progress of the research. The silver-method, after repeated trials even with double and triple impregnation, was found to afford such uninteresting results with these tissues that it was discarded. The staining with methylene-blue, however, yielded many striking pictures, and the method as modified may be warmly recommended for revealing certain of the details in fresh pathological tissues as obtained from the living patient. The present studies pretend to no degree of completeness, but since they include a number of observations which would seem to be entirely new, and because for unavoidable reasons the research must for the present be discontinued, I have decided to put the results on record, with the hope that others may be sufficiently interested in them to make further experiments along similar lines.

METHODS AND MATERIAL.

Naturally with human beings, although small doses of the substance appear not to be injurious, the injection *intra vitam* of amounts of methylene-blue sufficient to stain the nerves, as practised by Ehrlich in animals, is out of the question. It has been shown, however, by those who have worked extensively with the methylene-blue staining (Arnstein, Dogiel, Mayer, Smirnow, Apathy and others) that immersion of small pieces of tissue, removed from the animal while living or immediately after death, in dilute solutions of methylene-blue affords results almost, if not equally, as good as those obtained by injection. Accordingly, in my work thin layers of tissue cut with the double knife of Valentine were employed altogether. After acquiring experience in the use of the stain on embryonic tissues in which the nerve fibres and nerve endings are very easily demonstrable, the examination of tissues from adult human beings and of those from the interior of solid tumors was undertaken. Solutions of methylene-blue* of varying strength and consistence were tried with variable results. The suggestions of Lavdowsky† with regard to the use of

* Grübler's "Methylen-blau rectif. nach Ehrlich."

† Lavdowsky, M., Zur Methodik der Methylenblaufärbung und über einige neue Erscheinungen des Chemotropismus. *Ztsch. f. wiss. Mikr.*, Bd. xii (1895), Heft 2, p. 177.

egg albumin in the mixtures were found particularly serviceable. After many trials, the following proportions of materials were selected as affording the most constant results:

Fresh agg albumin,
Physiological salt solution,
0.25 per cent aq. sol. ammon. chlorid.,
1 per cent aq. sol. methylene-blue; of each 10 cc.

These constituents after thorough mixing make a stain ready for immediate use. It must be freshly prepared for each experiment. The sections cut from the tumor are placed upon large glass slides and surrounded on all sides with the stain. The slides are then placed in a warm, moist chamber and examined at intervals under a low power of the microscope. In from 45 minutes to 2 hours, when the acme of differential staining has been reached, the tissues are plunged into the ice-cold fixing fluid of Bethe,* which has the following formula:

Ammon. molybdat.,	1 gramme.
Distilled water,	10 cc.
Hydrogen peroxide,	1 cc.
Acid. hydrochlor.,	1 drop.

The bottle containing the fixing fluid and the specimens is kept in the ice-box over night, after which the bits of tissue are washed carefully in running water for from one to two hours, then quickly dehydrated in cold absolute alcohol, cleared in xylol and embedded as soon as possible in paraffin. Serial sections are then cut and fastened to the slide with Mayer's albumin or by the alcohol method; they may be mounted without further staining in xylol balsam, or if desired—and this has been in many instances of distinct advantage—a differential stain of alum cochineal may be employed after removal of the paraffin. The sections may then be studied, best of all with oil-immersion lenses. The procedure as above outlined, though far more reliable than the early methylene-blue methods recommended, affords by no

* Bethe, A., Angaben über ein neues Verfahren der Methylenblaufixation. *Archiv f. mikr. Anat.*, 1895, Bd. xliv.

means constant results. After some experience with it, however, microscopic pictures, often of very striking beauty, are obtainable

The material used in the investigation, with the exception of the epithelioma of the uterus, which came from the gynæcological service, was drawn entirely from the patients in Prof. Halsted's operating room. I wish to thank Drs. Halsted, Finney and Bloodgood for kindly supplying me with the tumor masses immediately after their excision. Up to the present time I have studied the following growths by this method:

1. Scirrhous carcinoma of the breast.
2. Epithelioma of cervix uteri.
3. Scirrhous carcinoma of breast.
4. Subperiosteal sarcoma of tibia.
5. Scirrhous carcinoma of breast.
6. Myxoma of axilla (very painful).
7. Metastatic carcinoma of glands of neck.
8. Sarcoma of muscles of leg.
9. Mass of mycosis fungoides.
10. Subperiosteal sarcoma of condyle of femur.
11. Intracanalicular myxoma of breast.
12. Amputation neuroma (right arm).
13. Myxoma.

THE PRESENCE OF NERVES IN TUMORS.

The question of the relation of nerves to tumors, aside from true neuromata and tumors of nerves, is practically *terra incognita*. A careful search through the bibliographic references in the catalogue of the Surgeon-General's Library and in the Index Medicus shows no direct reference to the subject. The general text-books on pathology are either entirely silent regarding this topic or dismiss it with an acknowledgment of our ignorance. Thus in Green* we are told that tumors contain no nerves. Senn† says: "But little is known of

* Green, Pathology and Morbid Anatomy, 1889.

† Senn, N., Pathology and Surgical Treatment of Tumors, 1895.

the innervation of tumors. The tenderness and spontaneous pain which belong to certain varieties of tumors would suggest the presence of new nerve fibres and should induce pathologists to make additional researches." No mention is made of nerves in tumors either in the last edition of Ziegler's Pathology* or in Thoma's text-book on the same subject.† The following quotation from Williams well illustrates the poverty of results as yet obtained in this field of investigation: ‡ "Cancers are regarded as nerveless, but vaso-motor filaments probably accompany the stromal blood-vessels, although this is denied by Verneuil and Nefvers. Our knowledge is singularly inadequate."

In their recent work on tumors, Luecke and Zahn§ state that concerning the innervation of tumors but little is known, although from a clinical standpoint no doubt of the presence in them of nerves can be entertained. Thus the possibility of quieting the pain in tumors by injection into them of anodynes speaks for the presence of nerves. It is probable, however, in their opinion, that the nerves were present before the formation of the tumor, and that none are newly formed. They refer to a report of a case by Billroth of nasal polyp, in which he found nerve fibres which he considered to be newly formed. But in a similar tumor examined by Zahn, which likewise contained nerve fibres near its origin, these were thought by him to be without doubt old preformed fibres.

In view of the foregoing statements, the findings in the cases included in the present study can scarcely fail to be of general interest; excluding tumor No. 6, which was very painful and probably represents a myxoma developing in the course of a small cutaneous nerve, the mycosis fungoides (No. 9) and the amputation neuroma (No. 12), we have left ten definite new growths which represent neither tumors developing from nerves nor true neuromata. In no less than five of the ten the presence of nerves was positively demonstrated, and in

* Ziegler, E., Lehrbuch der allgem. u. spec. path. Anat., 1896.

† Thoma, R., Text-book of General Pathology and Pathol. Anatomy. Trans. by Bruce, London, 1896.

‡ Diseases of the Breast, 1894.

§ Chirurgie der Geschwülste, Teil 1, p. 18, 1896.

some of them nerve fibres were present in considerable numbers, even in the minute pieces submitted to examination. Thus in No. 1, a carcinoma of the breast, a bundle of medullated nerves is visible adjacent to a milk duct, both being closely surrounded by carcinomatous tissue. In tumor No. 2, an epithelioma of the cervix, while the progress of the staining was being watched under a low power, a distinct though small bundle of nerve fibres containing four axones took the stain intensely and could be followed directly into the carcinomatous tissue. In tumor No. 3, a carcinoma of the breast in which the skin of the nipple is almost entirely replaced by cancerous tissue, a large bundle of medullated nerves has taken the stain. The possibility, however, that this may be a cutaneous nerve which has been surrounded by the ingrowing neoplasm cannot be excluded.

In tumor No. 4, a small round-celled sarcoma of the tibia, a small bundle of non-medullated nerves is to be seen closely bound to a small artery and vein by a moderate sheath of connective tissue. The section in this instance was taken from the middle of the tumor, and these vessels and nerves are near the centre of it and entirely surrounded by sarcoma cells. (Plate I, Fig. 1.)

In No. 10, a subperiosteal sarcoma of the lower end of the femur containing both spindle cells and round cells, three different bundles of nerves were made out in different sections. The tumor was very soft and showed large areas of œdema. The largest bundle of nerves —composed of about 50 medullated fibres—is accompanied by an artery and two veins, and is surrounded by a zone of œdematous fibrous tissue around which are closely packed sarcoma cells. Another bundle of six or more small axis cylinders accompanied by a vein and artery is surrounded by sarcomatous tissue, which is œdematous for a short distance and then becomes denser. One of these axis cylinders can be seen dividing, the branch accompanying the main fibre for a short distance and then becoming lost to view. The third and smallest bundle, composed of about five very fine axones, could be followed in its course among densely packed sarcoma cells. It was situated in the neighborhood of two minute blood-vessels belonging to the tumor.

The results even in these few cases would seem to justify the conclusion that nerves are of much more frequent occurrence within tumors than has previously been suspected. The examination of such minute particles of the tumors as those involved in these studies having revealed the presence in considerable numbers of nerves in one-half of the specimens looked at, it may certainly be assumed that, were it practicable to examine the whole of a tumor mass for its constituent nerves, (1) the number in some instances would be found to be enormous, and (2) the percentage of cases in which nerves are present would be even larger than this investigation would indicate.

More important, however, than the demonstration of the frequent occurrence of nerves in tumors is the question of the actual relation of the nerves to the tissue making up the tumor. Are these nerves so frequently present in the interior of tumors to be regarded simply as normal structures belonging to the tissues in which the tumor appears, which happen to have been included by the neoplastic tissues in their onward march, or are there grounds for believing that under some circumstances the nerve fibres belong to the tumor proper, forming really an integral part of it? While more extended research is essential for the establishment of the exact relations in all the different forms of tumors, I believe that my specimens justify the statement that in sarcomata, at least, there may be nerves which are as much an integral portion of the tumor substance as are the sarcomatous blood-vessels. The study of tumor No. 4 and of tumor No. 10, both subperiosteal sarcomata, shows the existence of axis cylinders within the central parts of the tumors in places in which they are not accompanied by tissues foreign to the tumors and where they are widely removed from the normal structures at the periphery. (Plate I, Fig. 1.)

As to the function of these nerves we can as yet only speculate. It is not impossible, inasmuch as in the two sarcomata mentioned they accompanied blood-vessels, that a part of them at least may be connected with the vaso-motor system. In this connection the distribution of the nerve terminals would be of help, but unfortunately these were not met with in the sections studied, and if they were present they remained unstained. In view of this deficiency further investigations will be required to clear up this point.

As to the nature of the nerves present in the carcinomata studied, I am unable to state positively that any one of them represents a portion of the tumor *per se*; indeed, for the present I rather favor the view that those found represent nerves of normal structures which have been surrounded by the invading cancer cells. It would not be surprising, however, if subsequent investigations should prove the presence, at least in the stroma of cancers, of nerves which may properly be considered actual parts of the new growth.

In No. 6 of the series, a painful tumor of the left axilla, when the capsule was cut, the tumor, which was smooth and glistening, was found attached to it only at one point by a narrow fibrous band in which no nerves could be definitely made out macroscopically. A microscopic section of the tumor showed, however, that it contained a large number of nerves. The axones stained brightly in the methylene-blue, and varied from large varicose fibres to very fine wavy fibrils. About some of the axones no medullary sheath could be detected. The individual fibres were separated from each other by varying amounts of myxomatous tissue and were not bound together by a common sheath. The total number of nerve fibres seen in this tumor is very great. This tumor must, therefore, be looked upon as a myxoma developing in the course of a small subcutaneous nerve and having its origin probably in the endo- or peri-neurium. The findings in this case account for the fact that clinically this tumor was extremely painful.

The amputation neuroma (No. 12) was studied simply as a matter of interest in order to observe the relations of the nerves to the scar tissue as revealed by the vital method of staining, and the results of course have no bearing upon the special subject here under consideration. Enormous numbers of multiply branched axones were easily demonstrable in the dense cicatricial tissues.

ON CERTAIN CELLS AND CELL INCLUSIONS IN TUMORS WHICH STAIN BY THIS METHOD.

a. Mastzellen and Plasma-cells.—The fact that with the vital staining method Mastzellen have a special affinity for the dye, a fact em-

phasized by Lavdowsky and others, I have frequently confirmed in studying sections of the skin and tongue, but I was surprised to find them in very considerable numbers in many of the tumors examined, both sarcomata and carcinomata. The forms assumed by the Mastzellen are various, but correspond closely to those met with in normal tissues. Two types predominate: (1) a form showing long processes, the protoplasm of the cell being filled with distinct granules which take a deep blue stain; and (2) a round or oval type smaller than the foregoing, the protoplasm tending to stain almost diffusely, though with the high powers extremely fine granules can be made out. Many of these probably correspond to what have been described as plasma cells. The Mastzellen, though most frequent about blood-vessels, may lie apparently anywhere among the tumor cells which do not take the stain.

A review of the bibliography teaches that Mastzellen have been before observed in various tumors by different investigators who used the older methods. Thus they are described by Hansemann* in the stroma of cancers, by Unna† in neuro-fibromata, and in leio-myomata by Jadassohn,‡ Gottschalk § and Reich.¶ In my methylene-blue preparations the Mastzellen form one of the most striking appearances in the microscopic picture, and the method affords perhaps the clearest demonstration of them in the tissues of tumors.

b. Red Blood Corpuscles.—In tumors as well as in other tissues some of the red blood corpuscles stain intensely with methylene-blue soon after its application. No pathological change has been made out in those which stain, and the proportion of red corpuscles taking the stain varies in different cases.

c. Cell Inclusions.—In tumor No. 4, a sarcoma of the tibia, numerous cells are to be seen which contain within their protoplasm certain irregularly shaped bodies or granules which take an intense blue stain.

* Hansemann, Das Krebsstroma und die Grawitz'sche Theorie der Schlummerzellen. Virchow's *Archiv*, Bd. cxxxiii, p. 147.

† Unna, Die Histopathologie der Hautkrankheiten, 1894.

‡ Virchow's *Archiv*, Bd. cxxi, 1890, p. 88.

§ *Arch. f. Gynäk.*, Bd. xliii, 1893, p. 534.

¶ *Arb. aus d. path. Inst. in Göttingen*, 1893, p. 216.

These cells, which differ entirely in appearance from Mastzellen, are generally larger than the other sarcoma cells adjacent. They appear swollen; their protoplasm is pale, but their nuclei are well preserved, showing no degenerative changes and staining well in alum cochineal.

The intracellular blue bodies vary in size from very minute granules, to be made out only with oil-immersion lenses, to bodies one-third the size of the nucleus of the cell. The smallest and some of the larger granules appear to be solid, but the majority of the larger bodies are vacuolated, showing an unstained or faintly stained centre surrounded by a deeply stained irregular granular peripheral ring. In shape they are ordinarily nearly spherical, but some of them are oval and some are even crescentic or horseshoe-shaped. Usually from three to five are found in a single cell, but as many as ten or more may occur. They lie in the protoplasm, have no apparent connection with the nucleus or with each other, being as a rule discrete, though sometimes they are massed together in heaps. Very different sizes and forms of the granules and bodies may be seen in one and the same cell. (Plate I, Fig. 2.) Similar solid extra-cellular bodies as well as vacuolated forms are to be seen lying apparently free among sarcoma cells, but they are not nearly so numerous as those within the cells.

In No. 10, another sarcoma of bone, similar bodies are to be seen, but they are smaller and not nearly so numerous as in the tumor just referred to. They are present in No. 8 (also a sarcoma), but in only a few of the cells. A careful search for them was made in the other tumors, but they were not found except in a carcinomatous skin nodule. Here in an alveolus of cancer cells some distance from the skin two cells containing similar vacuolated blue bodies are to be seen. None of the epidermal cells contain them.

We are ignorant as to the character of these bodies. They do not appear to be identical with Russell's fuchsin bodies,* which are believed by some to be confluent Mastzellen granules, by others to be proteid coagula,† since they are said to occur chiefly in carcinomata and the bodies described above were commonest in sarcomata.‡ They

* *Brit. Med. Jour.*, 1890, vol. ii, p. 1356.

† Touton, Virchow's *Archiv*, Bd. cxxxii, p. 427, 1893.

‡ Lubarsch states that the fuchsin bodies occur occasionally in sarcomata.

are not probably related to eleidin granules or to keratohyalin, as the epithelial cells of the skin treated by the methylene-blue method show none of them. Whether or not they correspond to the granule-like forms which have been described within certain cells of sarcomata after fixing in the fluids of Flemming, Zenker and Altmann I cannot be sure, as the descriptions given in the literature are very meagre. I have studied these sarcomatous tissues before the stain had taken effect, but could make out none of the granular bodies in the cells. It must remain doubtful then whether they represent pre-formed structures within the cell body or are to be looked upon as precipitates, possibly of nucleo-albumins, carried down by the methylene-blue. The same difficulty has been encountered by Held* in his studies of the so-called Nissl bodies within the protoplasm of nerve cells, but he concludes that the appearance of the blue masses in them is due to the precipitating effect of the dye.

FIBRES OTHER THAN NERVES WHICH TAKE THE BLUE STAIN IN TUMORS.

In the sections of sarcomata and carcinomata treated by this method a considerable number of fibrils which have a special affinity for the blue dye have been met with. In carcinomata they are seen here and there in the fibrous stroma, which otherwise, with the exception of its Mastzellen, ordinarily remains entirely unstained. They appear as fine wavy deep blue fibres with smooth contours, and are often of considerable length. In sarcomata similar blue stained fibres can be seen running in among the cells. Here the fibres are often of great length, in one case passing more than 100 sarcoma cells, and as a rule they are very straight, though in some shorter wavy fibres are to be seen. Some of them at least branch and anastomose with others; some are quite coarse, while others are very fine. No one with much experience with the method could confound them with nerve axones. Leredde* has described basophilic fibres occurring in mycosis fungoides; but in the case of this disease (specimen No. 9), which I have

* Held, Beiträge zur Structur der Nervenzellen und ihrer Fortsätze. *Arch. f. Anat. und Physiol. (Anat. Abth.)*, 1895, p. 396.

* Leredde, *Journal d. maladies cutan. et syph.*, August, 1895, p. 502.

examined by the methylene-blue method, none of the blue fibres so common in sarcomata and carcinomata were met with. The relation in which the fibres which stain in these tumors stand—indeed if any such relation exists—to the ordinary connective tissue fibrils can be decided only by the making of control experiments on the same tissues, fixing and staining by the special methods for studying white and elastic fibres and reticulum.

In the myxomata (Nos. 6 and 11) the methylene-blue has stained some of the cells and their processes beautifully. The processes appear as long slender wavy fibres. Most of the cells have two processes; some only one; others three or more. In tumor No. 13 fibres of greater length, often running through several fields of the microscope, stained intensely in the blue, especially at the peripheral parts of the section where the piece of tissue was most exposed to the staining fluid. These fibres run out from the poles of the somewhat spindle-shaped myxoma cells, and as far as can be judged from this method alone are integral portions of them. It would be unsafe to deny, however, the possibility of the fibres being distinct from the cells, as the latter may be simply closely attached to the former.

DESCRIPTION OF PLATE I.

Fig. 1.—A section of a sarcoma of the tibia (tumor No. 4) in which four fine, wavy, slightly varicose axis cylinders are seen stained a blue color by Lavdowsky's modification of Ehrlich's method. Fixed by the method of Bethe; counterstained with alum cochineal. One fibre is seen dividing into two at this point. A small artery and vein are visible adjacent to the nerve fibres, all running in different directions and entirely surrounded by sarcoma cells. The nerve and blood vessels are evidently integral parts of the tumor. Three cells are to be seen which contain within their protoplasm the bodies or substances which stain blue, and show more highly magnified in Fig. 2.

Fig. 2.—Intracellular bodies from the mixed celled sarcoma of the tibia which stained by the blue of the same method. Various forms are shown: the small solid-looking spherical bodies and the larger vacuolated and crescent-like forms. The cells containing them are seen to be larger, swollen, somewhat irregular in shape, and contain feebly staining protoplasm.

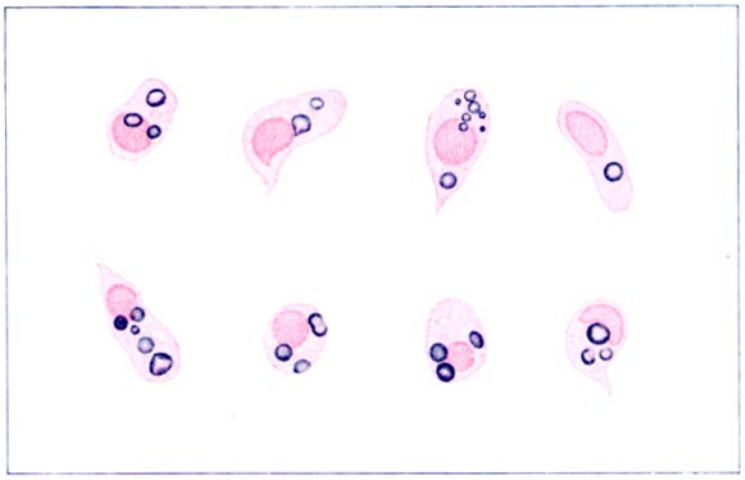

FIG. 2.

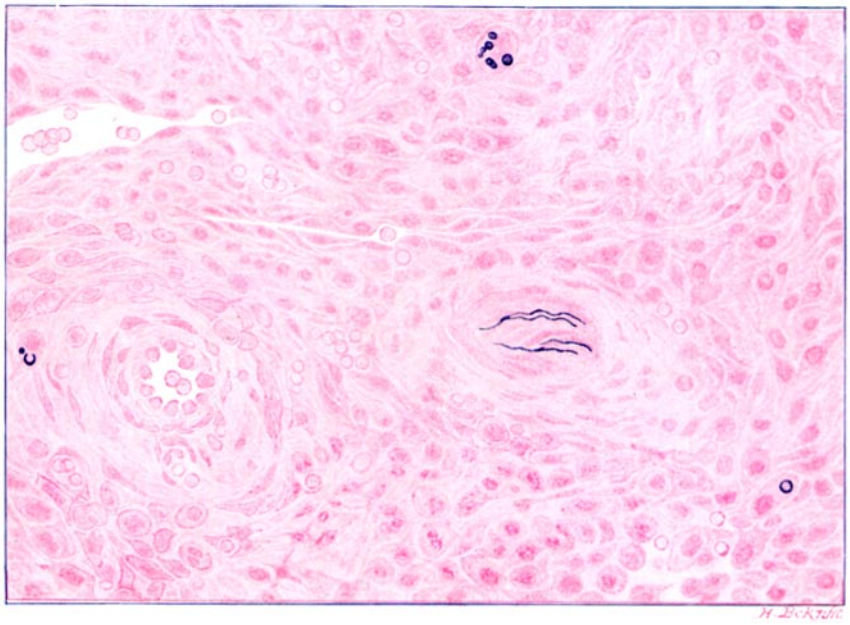

FIG. 1.