Degenerative Changes Induced in Tumor Cells by *Serratia marcescens* Polysaccharide*

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Preliminary reports of these investigations by Diller and Shear (9) and Diller (8) indicated in a general way the degenerative responses of tumor cells to a polysaccharide derived by Shear and his associates (18) from *Serratia marcescens* culture filtrate. The present paper comprises the detailed account of these cellular changes.

The use of bacterial toxins in tumor therapy was reviewed by Shear (15, 17) and more recently the application of bacterial agents in the form of Coley's "mixed toxin" to human neoplasms was surveyed by Nauts, Swift and Coley (13). However, the literature reveals no more than superficial notice of the effects of such agents on tumor cells. Apitz (4) in 1933 noted that there is an edematous appearance of tumor cells following toxin treatment, which may be separate from the anoxia caused by hemorrhage; and Andervont (3) in 1936 reported that treatment with meningococcus and *B. coli* filtrates causes tumor cells to swell prior to hemorrhage "indicating the bacterial products may have some direct effect on tumor cells."

This hypothesis is supported by a microscopic study, made over a 2 year period in our laboratory of the effects produced by Shear's polysaccharide on the tumors of rats, hundreds of mice, and some hospital patients.

MATERIAL AND METHODS

Most of the studies were carried out on transplantable mouse sarcoma 37, supplemented by spontaneously occurring rat and mouse carcinomas and by human biopsy material from 16 sarcoma patients.

Small pieces of each tumor were routinely smeared for rapid examination in acetic-orcein, while the remainder was preserved in a modified Bouin's fixative (1.5 gm. of urea added to the standard formula) so that when supplementary data were needed, paraffin sections could be prepared for orientation studies and pathologic diagnosis. For demonstration of cytoplasmic components and archoplasmic structures, the coverslip smear technic described by Diller (7) was used.

The hosts for transplanted tumors were Carworth Farms albino mice (usually males) approximately 3 months of age. No difference could be noted between males and females with regard to polysaccharide response. Animals with well-established 6 to 10 day tumors which had not perforated the skin were chosen for treatment. Tumors open to the surface tend to be polysaccharide resistant, as noted by Shear and his associates (18), owing probably to the presence of infectious organisms that enhance immunity. Actively proliferating 7 day tumors arising from fragments implanted with a No. 12 trochar in a dorsal position are flattened ovals at least 7 to 10 mm. in their longest dimension, and tumors that had not attained approximately this size were discarded.

The standard mouse dose of polysaccharide employed for the experiments was 0.01 mgm. in a volume of 0.1 cc. of sterile saline, injected intraperitoneally. Other amounts employed are mentioned specifically. Intravenous injection in mice did not alter the course of cytologic response, and the simpler intraperitoneal method was therefore used. The material is highly toxic, and the amount stated usually caused death within 24 hours for 2 to 3 out of every 10 animals treated. Frequently death occurred at 4 to 5 hours, before the maximum cellular response could be realized. At higher dose levels, animals sometimes continued to suc-
cumb as late as 48 hours after administration of the polysaccharide, but when death followed a 0.01 mgm. injection, it occurred most frequently during the first night after treatment.

RESULTS

Effects on Tumors in Animals

Hemorrhage production in sarcoma 37 following polysaccharide administration has been described by Shear (17) and within the limits of individual variation, similar phenomena appeared during our experiments. Cytologically, individual tumors are not equally responsive, a fact which may be dependent upon the number of dividing cells present and the growth pattern of the tumor, as will be discussed subsequently. Microscopic observations indicate that cellular damage is produced in tumors showing little or no hemorrhage, as well as in those in which hemorrhage is extensive, but many tumors are only partially affected. However, no tumor thus far examined failed to respond to a greater or lesser degree by cellular degeneration, whether or not there was gross hemorrhage. In general, the areas of the tumor which were in closest proximity to the blood supply were first and most drastically affected.

For our cell studies, tumors were examined post-injection at hourly intervals, beginning with ½ hour after treatment. At 1½ to 2 hours the first cellular responses appeared in sarcoma 37, whether or not there was macroscopic hemorrhage. Prophase nuclei were primarily affected and reacted by forming surface blisters, or blebs, which were sometimes minute, at other times were more extensive, and in extreme cases there were multiple blebs. (Figs. 2, 3, and 27). In the last case, the entire nucleus appeared to have swollen, and at times pseudopodial processes (Fig. 3) were thrust out.

From 2 to 2½ hours, fewer nuclei with blistered membranes were encountered. Instead, many nuclei were shrunken, as though by withdrawal of fluid. As revealed in paraffin preparations, there was a corresponding shrinkage of the cytosome, which resulted in the disruption of the tumor tissue through separation of the cells into discrete entities. Resting nuclei were in most instances morphologically unchanged; occasionally, however, they were enlarged through apparent imbition of fluid, but no blistering of their membranes was observed.

Correspondingly, those nuclei which were in metaphase at 2 hours after treatment showed considerable damage (Fig. 9). A series of changes appeared to have taken place between 2 and 3 hours which involved various aberrations in nuclear structures. Some were slight, e.g., the displacement of individual chromosomes with respect to the metaphase plate; others showed drastic disorganization such as that in Fig. 10. Furthermore, there was a loss of stainability, which caused the center of the chromosome to appear hollow and vesiculate, although the outer rim was heavily stained. Sometimes these transparent bodies had become confluent and produced enlarged hyaline structures like those of Fig. 5. At other times, coagulation involved all the chromatin, as in Fig. 7.

Nuclei that arrived at the anaphase before this period were apparently successful in completing division, for there was no evidence of anaphase or telophase degeneration; therefore, polysaccharide did not appear to suppress division through any action on the spindle, as is the case with colchicine. At 2 to 3 hours after treatment, cell division was still uninhibited; though a considerable number of prophase nuclei showed surface modifications, and numerous metaphases were disrupted.

Preparations made between 3 and 3½ hours postinjection indicated a more advanced stage of degeneration involving pycnosis of large numbers of nuclei and considerable shrinkage of many others. These nuclei appeared more concentrated with decreasing size and assumed a bean or kidney form.

DESCRIPTION OF FIGURES 1 TO 14

Camera lucida drawings from acetic orcin smears (unless otherwise indicated) of sarcoma 37 treated with 0.01 mgm. of S. marcescens polysaccharide. Mag. X 2,250 (approx.).

Fig. 1.—Untreated prophase nucleus.

Fig. 2.—Prophase nucleus of tetraploid cell (4 nucleoli) 2 hours after treatment. Note blistering of nuclear membrane.

Fig. 3.—Prophase nucleus, 2 hours after treatment; note shrinkage of nucleus and production of pseudopodial processes.

Fig. 4.—Prophase nucleus, 3 hours after treatment.

Figs. 5, 6, and 7.—Metaphase nuclei, 2 hours after treatment.

Fig. 8.—Shrunken nucleus, 3 to 4 hours after treatment.

Figs. 9 and 10.—Metaphases, 3 to 4 hours after treatment.

Fig. 11.—Extrusion of nuclear filaments, 4 hours after treatment.

Fig. 12.—Extrusion of a single longitudinally divided chromosome from metaphase clump 4 hours after treatment (paraffin preparation, Feulgen stain).

Figs. 13 and 14.—Distorted nuclei with extruded filaments, 4 to 5 hours after treatment (paraffin preparation, hemalum stain).
Figs. 15-22
shape (Figs. 4 and 8). Whether these always arose through collapse of previously swollen nuclei, it was not possible to judge. Stages indicating progressive shrinkage of nucleoli were likewise demonstrable, and these bodies ultimately came to be represented only by dark granules that took orcein stain, but not fast green. The cell body also decreased in size and was more heavily stained. Blebbed nuclei could also be found. Metaphases were practically all degenerative, e.g., Fig. 6 (see also Figs. 30 and 31), and there were no anaphase or telophase figures. Sister cells which were in the process of separation at 1½ hours apparently succeeded in completing division, but thereafter the division process usually did not go beyond the metaphase. In many tumors studied the only normal cells visible at 3 hours post treatment were resting ones. Some of the tissues showed large areas in which the orcein, staining the nucleo-proteins, was dispersed throughout the cell instead of being limited to chromatic structures. Furthermore, there appeared at about this time nuclear disruptions so drastic that they must certainly be irreversible. They were the most characteristic feature of polysaccharide response.

The essential feature of this change was the extrusion from the nucleus of filamentous orcein-positive (chromatin) bodies, sometimes involving many threads and sometimes consisting of a single chromosome pair (Fig. 12). Here the chromatin material of what is apparently a degenerating metaphase figure is clumped into a formless mass for which the longitudinally double chromonemata of a single pair of chromosomes have been extruded far beyond the confines of the nucleus, and even of the cell body, to attain an extended dimension approximately 4 times that of the clumped nucleus. This figure (shown also in Fig. 32) is from a paraffin preparation subjected to the Feulgen reaction. The greatly extended thread is Feulgen-positive and still exhibits some faint traces of residual coiling. A dimly discernible body attached to the pycnotic distal granules appears to be the remnant of the nucleolus.

A similar attenuation involving remnants of prophase nuclei appears in Figs. 11 and 13. Even more distorted nuclei filled with fairly individualized chromatin threads drawn out to a considerable length are also present (Fig. 14). These figures are better demonstrated in smears than in paraffin sections, where they are so long that they can seldom be found in one section but appear in cross section as chopped-up fragments. It is barely possible that the pressure exerted in making the smear preparation may tend to exaggerate the effect; but the same phenomenon is apparent to a lesser degree in paraffin preparations, where the tendency is in the other direction, toward shrinkage and a consequent masking of the early effects of the polysaccharide.

At 4 hours practically all metaphase nuclei were clumped (Fig. 8) whether or not there were chromatin extrusions from the central mass. By this time some of the metaphases were nothing but pycnotic spheres, sometimes perforated by vacuoles (Fig. 6). No normal metaphases, and no telophases at all, persisted. Such intact cells as survived were in resting stage, but they often appeared greatly swollen and contained huge, correspondingly swollen nucleoli. Blebbed and reniform nuclei were also present.

A different kind of degeneration of the tumor sometimes occurred about 4 hours postinjection. This involved a complete coagulation of both nucleus and cytosome (Fig. 24).

Between 4 and 5 hours when tumors grossly exhibited considerable hemorrhage, degeneration of initially affected cells neared its peak. Swollen resting nuclei, blebbed prophase nuclei, bean-shaped, shrunken nuclei, clumped metaphases and extruded filaments were all present. The shrunken prostates without extruded filaments had no semblance of ordered form and became twisted "ghosts" from which cell boundaries were lost, while the pale, muddily stained nuclear remnants lay upon an amorphous mass of degenerating cytoplasm. Individually these bodies rounded into spheres of descending sizes or faintly stained bodies of irregular contour, from which all trace of structure had disappeared. The spherical bodies apparently arose from metaphase degenerates, while twisted, folded, and flattened figures were

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DESCRIPTION OF FIGURES 15 TO 22

Figs. 15 and 16.—Degenerating telophases, newly dividing cells, 3 days after polysaccharide treatment (acetic orcein stain).

Fig. 17.—Undamaged resting nucleus, surviving polysaccharide treatment (acetic orcein).

Figs. 18 and 19.—Nuclei, enormously enlarged in resting cells, during 3 day period following polysaccharide treatment (acetic orcein).

Fig. 20.—Resting nucleus during 3 day period of mitotic inhibition following polysaccharide administration (smear, Flemming fixation, Flemming tricolor). Note "fatty degeneration" of cytoplasmic substance.

Figs. 21 and 22.—Degenerating nuclei from cells treated with polysaccharide and x-ray, 24 hours after irradiation.
remnants of nuclei in pre-metaphase stages. Degenerating blood cells were also present (Fig. 36).

The amount of destruction varied considerably, but in some tumors deterioration was so extensive that by 6 hours there was practically complete necrosis; and nothing remained but amorphous cytoplasmic debris with some nuclear fragments (Fig. 26). Recognizable cells were in an advanced stage of degeneration and seemed for the most part to be little more than distorted naked nuclei (Fig. 33).

Beyond this time (6 hours) the stages undergone by as yet undamaged cells were identical with those just described, and the result was merely an extension of the response to include greater areas of the tumor. The length of time during which degeneration can continue, and before any resistant cells are again able to resume division, is discussed in the following section.

Duration of effect.—As mentioned previously, the maximum hemorrhagic effect was reached at approximately 6 hours after injection of the polysaccharide. The tumors were usually encapsulated; and when extreme hemorrhage was induced, the neoplasm appeared on gross examination as a soft, bloody sac. It is not uncommon at this period to find such marked deterioration that at times no solid tissue is available, and little more than a bloody smear can be secured for microscopic examination. During this period the mice show lowered temperatures, accept no food or water, and remain huddled and immobile in their cages. Some casualties may occur from 4 hours onward, but survivors of the 0.01 mgm. dose usually completely recover within 24 hours. They were eager for food and could run and swing on their cages in a normal way, indicating that recovery from the toxic effects was quickly accomplished. In order to determine whether the cellular response of the tumors is correspondingly brief, any persisting residue of undestroyed tissue was studied at 24 hour intervals, up to 5 days postinjection.

For this series of studies tumors were chosen, which, because of extensive hemorrhage, showed that they had reacted. As Andervont (3) demonstrated in connection with tumor response to meningococcus and B. coli filtrates, tumors which are totally hemorrhagic usually form a hard mass of dried blood beneath the skin within 24 hours. In our 24 hour material, the entire tissue was sometimes involved in the hardened mass, while at other times a certain amount of translucent tissue could be secured for examination. Invariably, in such material, the only seemingly normal cells were “resting” ones (Fig. 35). In the transition between interkinesis and the stage of active division the extreme diffusion of the chromatin substance made the distinction between “resting” and dividing nucleus difficult, if not impossible; but nuclei were arbitrarily judged to be in prophase when definite polarization of the heterochromatic proximal ends of the chromatin threads with respect to the nuclear membrane, together with resolution of definitive chromosomes, first became visible.

After 24 hours the only morphologically intact cells were those not visibly preparing for mitosis. There is some evidence that these cells, too, may respond in some way, through changes in the cytoplasmic constituents. An instance of this appears in Fig. 38. This photograph shows a single resting cell remaining undistorted amidst the debris of degenerating cells. The nuclear structures and cell membrane are intact, but the osmiophilic substance has undergone “fatty degeneration” (see also Fig. 20) and is massed in the cytoplasm as blackened spheres. Disrupted cells have extruded osmiophilic substance in the form of spherical masses, and these clumps, large and small, are scattered through the tumor 24 hours after polysaccharide administration.

Degeneration of all types described in the previous section could be discovered at 24 hours, except in cases where all the material had reached the highly attenuated condition. Blood cells were present in large quantities. Erythrocytes by this time began to disintegrate and to confuse further the already chaotic picture by disrupting into amorphous masses and fragments (Fig. 37). Alternately, their substance, which is enucleate, was extruded in filamentous conglomerates resembling in the extremity of their attenuation the drastically elongated chromatic threads already described.

At 48 hours there still were no dividing cells. Resting nuclei appeared greatly enlarged, and judging by the number of nucleoli, which were tremendously swollen and frequently coalesced (Fig. 19), many of these were heteroploid. The necrotic areas now presented an appearance of complete chaos, in which all semblance of nuclear contour had disappeared, and only pyknotic bodies and fragments remained.

The first dividing cells were noted 72 hours after injection. Some of the divisions apparently succeeded, but many were abortive. Frequently, division was halted in metaphase with resultant pyknotic malformations and condensations, but the bulk of the cells degenerated at telophase. This did not involve spindle suppression or destruction but rather the failure of sister nuclei to
Photomicrographs from acetic orcein preparations of sarcoma 37, treated with 0.01 mgm. of polysaccharide.

Fig. 23.—Untreated control tumor. Mag. × 1,000.

Figs. 24 and 25.—Tumor 4 to 5 hours after treatment. (Fig. 4, mag. × 720; Fig. 5, mag. × 1,000).

Fig. 26.—Same, 6 hours after treatment. Mag. × 1,000.
Photomicrographs from acetic orcein preparations of sarcoma 37, treated with 0.01 mgm. of polysaccharide. Mag. X 1,000.

Fig. 27.—Blebbing of nuclear membrane, 1½ hours after treatment.

Fig. 28.—Vacuolization of individual nuclei, 2 hours.

Fig. 29.—Metaphase degeneration in giant cell, 2 hours after polysaccharide.

Fig. 30.—One degenerating, and 1 normal metaphase, 2 hours after treatment.

Fig. 31.—Degenerating metaphase, 2 hours after treatment.

Fig. 32.—Same, 4 hours after treatment, showing extrusion of individual chromonemes. See also Fig. 12.
reconstitute resting cells (Fig. 16). The degenerates were frequently dumbbell-shaped figures comprised of persisting interzonal fibers or a connecting strand of nucleoplasm separating two pyknotic sister nuclei that tended to degenerate asynchronously.

If no resting cells persisted over the 3-day period the tumor was no longer viable, so far as was detected microscopically. No mitotic figures known to arise from the enormously swollen, obviously hyperploid nuclei were positively identified, but polyploid cells in every stage of division were numerous, as were also aberrant metaphases which were attempting regulation, e.g., by throwing out supernumerary chromosomes or by formation of multipolar spindles. Quite possibly these are the products of the giant nuclei. Moreover, at this time it was not uncommon to find "nests" of dividing figures which might involve 6 or 8 metaphases superimposed upon one another in a single microscopic field, in a juxtaposition suggesting that they might be the products of a single giant cell.

By 5 or 6 days after treatment, tumors were again growing actively next to the body wall to an extent discernible even to the naked eye. Alternately, they were being sloughed off at the surface or shrinking down by resorption. The inhibitory effect of the bacterial substance on division processes in sarcoma 37 was, therefore, of no more than 3 days' duration. The first attempts at division were usually unsuccessful; but between 72 and 96 hours, morphologically unaltered resting nuclei were able to resume division and rapid proliferation of the tumor began.

The experiments thus far described were made on 7 to 10 day old tumors. Andervont (3) in discussing reactivity of mouse tumors to bacterial products states that the introduction of such substances "does not produce hemorrhage within skin tumors until the fourth or fifth day after inoculation, although growing tumor tissue is present as early as the third day." In order to discover whether there would be an accompanying failure of nuclear breakdown before the sixth day postimplantation, we studied a series of tumor implants treated with the usual amount of polysaccharide, at daily intervals, i.e., 24, 48, 72, and 96 hours, and at 5 days after implantation. As we shall discuss later, onset of cell division in the tumor implant is probably correlated with availability of blood supply. It is evident upon gross examination that chance decides whether the implanted fragment comes to rest in close proximity to a vascular branch, or whether it must remain quiescent until capillary branching can be elicited, a 3 day process according to Algire's studies (2). This agrees with our own cruder observations and with Andervont's statement (3) that dividing tissue is present as early as 3 days after implantation.

In our own experience, proliferation of sarcoma 37 implants is correlated with establishment of contact with the blood supply. Tumor fragments fortunately placed in juxtaposition to capillaries begin to divide at the outer edges of the implant as early as 28 hours after deposition. On the other hand, fragments not in contact with existing blood capillaries may remain as long as 3 days without blood supply until capillary branches are established. Beyond this period they are apparently incapable of survival. In almost every case, only the part of the implant directly in contact with vascular branches enters into division; the residue degenerates.

Careful examination of many tumors reveals that when cells are dividing they will react typically to polysaccharide, regardless of the age of the implant. The same types of degeneration hitherto described (disrupted metaphases, shrunk crescents and filamentous extrusions) together with structurally normal resting nuclei, are found. Since nearly all the living tissue surrounding the young implant is in rapid division, it is not uncommon to find that 6 hours after administration of the agent to an animal bearing a 2 to 3 day implant, nothing remains except the opaque necrotic mass of implanted tissue which has not yet been absorbed, and a faint trail of blood where the capillary connections have been severed. In a few cases where the total implant had presumably become involved in division within 3 days (as indicated by the absence of any central necrotic plaque of tissue) no tumor tissue remained at the implantation site 6 hours after injection of 0.01 mgm. of polysaccharide.

Older tumors also reacted with the same type of cytologic change. Tumors were studied up to 16 days postimplantation, and although the conditions are more difficult to interpret because of the presence of large amounts of spontaneous necrosis, the expected types of damage were also encountered. Apparently a good many resting cells were dormant in these older growths, since they persisted in large numbers following polysaccharide treatment, and we could never succeed in breaking down a whole tumor but caused only a portion of it to react, as indicated by our microscopic findings and by partial sloughing macroscopically observable.

Effect of multiple or continuous treatments.— In about a fourth of the tumors examined, there was no resumption of division on the third day after
polysaccharide treatment; in the remainder, mitotic figures were plentiful. The obvious practical procedure would appear to be to reinject the host at 3 day intervals in the hope of suppressing each new mitotic wave. This experiment was tried, using the same amount and concentration of polysaccharide in each successive treatment until sloughing was induced or the animal succumbed to the tumor. Much to our disappointment, this induced no appreciable additional cell destruction; and growth, once re-established, was not noticeably impeded. Tumors treated over long periods (about 20 days) tended to become heavily encapsulated, and in only a few cases did we detect metastases. The failure of repeated doses of polysaccharide to suppress growth indicates that either the tumor or the host becomes resistant to the bacterial agent.

Since the amount of the original single injection could not be increased without causing the death of a still greater number of mice, we attempted to use polysaccharide of lower concentration in multiple doses of increasing magnitude. An initial injection of 0.001 mgm. in 0.1 cc. of fluid was administered, and thereafter the injections were increased by 0.001 mgm. daily until a total of 0.02 mgm. had been injected over a period of a week. Two series of tumors were treated simultaneously. One of these was examined microscopically on the seventh day after initial treatment, and the other series was allowed to run until the hosts either succumbed or were able to slough off or resorb the tumor. In the latter series, only 1 animal in 10 was able to rid itself of the growth. The others died within 20 to 24 days.

The tumors examined on the seventh day frequently were scabbed next to the skin and microscopically showed areas of degeneration in which no dividing tissue was present and which did not appear to arise from spontaneous necrosis. In these areas, the cytoplasm tended to be extremely viscous and coagulated. Even tumors exhibiting no gross hemorrhage but filled with clear, translucent tissue, had degenerative areas. In acetic orcein smears the affected portions were stained abnormally, and the cytoplasmic substance, which should have been stained with fast green, was pinkish as though by diffusion of the chromatic substance from the nucleus. Where areas of dividing tissue impinged on degenerating ones, the dividing cells showed a high proportion of polyploid nuclei.

Also, we injected mice with single large doses (0.005 mgm. in 0.5 cc. of fluid) of the low concentration. At 5 to 6 hours there followed marked degeneration of all but resting nuclei, and there was total coagulation in the hemorrhagic areas. In the microscopic preparations, the tumor tissue showed clotting at the center, pycnotic rounded fragments, and heavily stained resting nuclei. At 24 hours, cell division had not been resumed in the affected areas. Nevertheless, tumors similarly treated and not removed for microscopic examination continued to grow and eventually caused the death of the host.

These experiments indicated that multiple doses of low concentration were not as effective in tumor-cell destruction as a single dose of a magnitude approximately 1/4 lethal to the mouse hosts. Furthermore, multiple injections of larger magnitude did not offer much improvement over a single application in breaking down or inhibiting growth in tumor tissue.

Injection directly into the tumor.—Since our studies showed beyond much doubt that polysaccharide has a direct effect on cells apart from that following hemorrhage, it occurred to us to try to obtain cell breakdown by injecting directly into the tumor with the hope that the toxic systemic effects produced in the host could thus be obviated. Injection of 0.005 mgm. of polysaccharide into a 7 day mouse tumor resulted in the disruption and bursting of some of the cells and, in one case, in a severe coagulation of the cytoplasm. Except for this instance there was not much difference in the appearance of cells from tumors so treated and control tumors injected with the same amount of salt solution or distilled water. In no case was division suppressed for more than a few hours and growth was at best only temporarily inhibited. This agrees with observations made in another department of this Institute on tissue cultures treated directly with polysaccharide (Royle, unpublished data), wherein the response was negative.

Effect of polysaccharide on normal body tissues.—Since the hemorrhage-producing effect of S. marcescens polysaccharide appeared to be confined to the region of the tumor, a study was made of normal tissues of treated mice to determine whether the tissue-destructive action of the bacterial product was likewise limited to tumor cells. So far as we were able to determine, this is not the case, although tumor cells did appear to be much more sensitive than nontumor cells, and response was elicited only in those tissues undergoing division or rapid nucleoprotein synthesis. Intestinal epithelium, which appeared to be the most actively dividing tissue in the adult mouse, was also the most reactive to polysaccharide.

Following administration of polysaccharide, extensive filamentous degeneration was produced in the villi of the mouse intestine, which normally
Photomicrographs from smear preparations of sarcoma 37 treated with 0.01 mgm. of polysaccharide. Mag. × 1,000.

Fig. 33.—Tumor, 6 hours after treatment (hemalum-eosin stain).
Fig. 34.—Same (acetic orcein stain).
Fig. 35.—Resting nucleus, morphologically unchanged, 24 hours after treatment. (Flemming tricolor).

Fig. 36.—Tumor 24 hours after polysaccharide treatment, showing degenerating blood cells (paraffin preparation).
Fig. 37.—Tumor 48 hours after treatment; resting nucleus and degenerating blood cells.
Fig. 38.—Three days after treatment; cytosome of resting nucleus filled with osmiophilic bodies which appear degenerative. See also Fig. 20.
FIGS. 39 to 44 Mag. X 1,000.

FIG. 39.—Spontaneous breast carcinoma of mouse, untreated.

FIG. 40.—Same, 6 hours after polysaccharide treatment.

FIG. 41.—Same, 24 hours after treatment.

FIG. 42.—Mammary carcinoma of rat, 48 hours after treatment.

FIG. 43.—Mouse sarcoma 37, 3 days after polysaccharide treatment (just before resumption of division).

FIG. 44.—Same, specimen from a totally nonviable tumor.
contained large numbers of cells in mitosis at all times. No difference was detected in the response of intestinal epithelium of tumor-bearing mice and those having no tumors. Fortunately, the amount of polysaccharide required to produce destruction of nontumorous tissue was greater than that required for degeneration for tumor cells. For instance, the usual dose of 0.01 mgm. produced only slight breakdown in intestinal epithelial cells, and in order to secure an effect comparable to that which would occur in a tumor following administration of the same amount of polysaccharide, a concentration 10 times as great (0.1 mgm.) was required. The reaction of intestinal epithelium to this amount of polysaccharide, 6 hours after intraperitoneal administration, appears in Figs. 46 and 48 (see Figs. 45 and 47).

Other tissues studied were spleen, bone marrow, kidney, liver, endocrine glands, and gonadal tissue. Of these, the damage was greatest in developing blood cells of the bone marrow and in the liver. The amount required to produce extensive damage was also 10 times that used for the destruction of tumor tissue. Kidney, which has few dividing cells, was almost completely resistant. A more puzzling phenomenon was the failure of response of gonadal cells, either spermatogonia or oogonia, to polysaccharide, even when an amount fatal to half the mice was employed. (Fig. 49). When tumor-bearing males and females, treated with polysaccharide in an amount sufficient to cause the sloughing of the entire tumor, were maintained for 2 or 3 months thereafter and bred inter se, normal litters of offspring were produced.

The effect of polysaccharide on the adrenal and other glands will be discussed in a later paper. A decided shrinkage of cells and nuclei of the adrenal, particularly in the medullary region, was produced within 6 hours after injection of 0.01 mgm., but the effect was usually reversible at this dose level within 24 hours.

Treatment of spontaneous neoplasms.—A series of studies of the reactions of mice with primary subcutaneous tumors to hemorrhage-producing polysaccharide was made by Shear (16). From his paper the following is quoted: “Sufficient evidence . . . has been accumulated to show that the production of hemorrhage and necrosis by such agents is by no means restricted to transplanted sarcomas. While it has been found by most workers that, in general, carcinomas are more refractory than sarcomas, nevertheless, several strains of transplantable carcinomas have been reported to be responsive to the action of these bacterial products. . . . Furthermore, this hemorrhagic and necrotic effect is not confined to transplanted tumors.”

The results of Shear’s studies of 750 mice bearing chemically induced tumors show that, while administration of *S. marcescens* polysaccharide regularly produced hemorrhage and necrosis, some portion of the neoplasm usually escaped destruction and continued to grow progressively until the death of the host.

Spontaneous growths in rats and mice.—Cytological studies were made by us of the effect of polysaccharide on spontaneously occurring tumors that appeared in our stock colony of rats and mice, and also of a controlled series of mammary adenocarcinomas in C3H and dba mice. C3H mice with large mammary carcinomas, some of which were multiple, were treated in the same way as mice with transplanted tumors, i.e., 0.01 mgm. of polysaccharide was injected intraperitoneally and the animals were killed at 6 hours. These mammary tumors were highly vascularized; and although treated tumors were scarcely more than sacs of blood at 6 hours posttreatment, we found the control tumors also to be highly hemorrhagic. Therefore the presence of a great deal of blood in the treated tumors could not in this case be taken as a criterion of polysaccharide reaction. However, microscopic comparison of control and treated materials revealed a definite cellular response which, though by no means as pronounced at 6 hours as in sarcomatous growths, was nevertheless striking (see Figs. 39 and 40). The extreme filamentous degeneration in sarcomas at 6 hours was lacking, but there was a rounding and pycnosis of nuclei.

A second series of mammary carcinomas in dba stock was treated with a larger amount of polysaccharide (0.02 mgm.) and the survivors were killed 24 hours after treatment. Considerable areas of the tumors were then found to be responding as in sarcoma 37, by extrusion of nuclear components (Fig. 41).

Spontaneous mammary carcinomas arising in our colony stocks of heterozygous white mice were also studied with similar results. Mammary carcinomas arising in aged albino rats maintained on butter yellow diets were also studied. Pretreatment biopsies were taken and injections of 1 cc. each of polysaccharide of low concentration (i.e. 0.01 mgm. of polysaccharide) were made into the rats. The animals were killed the following day and the tumors prepared by the paraffin method. Definite and typical, though not extensive, nuclear response was detectable, but the most interesting finding was that, although the capillaries penetrating into the neoplasm were still intact, the tissue immediately adjacent was highly degenerative.
Effects on Tumors in Human Beings

The human material available for study was obtained from biopsies taken of 16 sarcoma patients before and after treatment at the Lankenau Hospital during the winter of 1944-45. The clinical responses of these patients and the pathological findings were reported by Holloman (10) and by Oakey (14). Four patients had previously been treated with S. marcescens polysaccharide at another hospital, as described by Brues and Shear (6). The biopsy material available for our studies included specimens of chondrosarcoma, fibrosarcoma, lymphosarcoma, recurrent melanoma, chronic granuloma, secondary spindle-cell sarcoma, and nodes from the neck region of a patient suffering from Hodgkin's disease. The previous finding that carcinomas do not respond as readily as do sarcomas restricted the choice of cases for study to those with sarcomatous growths.

The course of cytological response followed the pattern already exposed by our studies of animal tumors. It differed only in the relatively small amounts of tissue affected. This was to be expected because the amounts of polysaccharide administered to human beings were relatively small in proportion to body weight compared with the doses employed in treatment of mice. As the previous clinical reports describe (10,14) human beings responded with much greater intensity than did mice, as far as toxic effects were concerned, and for this reason in the preliminary trials the amounts used were minute, i.e., 0.01 mgm. in a volume of 1 cc. administered intravenously. Our studies with mice had shown that in sarcoma 37 the ultimate cytological response was the same regardless of the route of injection. The cellular conditions were so similar in all the sarcomas studied that it is not necessary to present examples of every case, but the findings on 4 different types of human neoplasm are presented. Figures 51 to 54 are photographs from paraffin preparations used in pathological diagnosis and are typical of the general histological picture following polysaccharide treatment. The remainder of the figures are from acetic orcein smears prepared especially for nuclear study.

Fibrosarcoma.—This was a very large growth of long standing that involved the entire right arm of a young man. The cell conditions before treatment, as revealed by biopsy, are shown in Fig. 57. Fig. 58 was made from biopsy material excised 4 days after administration of a single dose of 0.01 mgm. of polysaccharide. Externally, the sarcoma was black and blue because of hemorrhage and the gross appearance of the interior, once hard, white and glistening, was soft and blood-filled.

Cells from tissue adjacent to these areas showed degeneration similar to that in mouse sarcomas.

Chondrosarcoma.—The patient had several metastatic tumors growing in the nose and orbital fossa and was beyond further surgical aid. She was given a total of 0.033 mgm. of polysaccharide in 3 separate applications. Two biopsies were taken, at 4 days and 6 weeks, respectively, after treatment. The first posttreatment biopsy showed a part of the tumor to be extremely hemorrhagic. Cells from this region were in the anticipated stages of degeneration (Fig. 56).

Hodgkin's disease.—Another example of polysaccharide response appears in Fig. 60 taken from a neoplasm originally diagnosed as lymphosarcoma of the axillary nodes, but which was later determined to be Hodgkin's disease. The patient was given a total of 9 cc. of 0.09 mgm. of toxin, in three equal doses over a 2 day period; 3 days after cessation of treatment the sarcomatous nodes in the supraclavicular space were dissected out. In contrast to those removed before treatment (which had been firm and of the usual color) these were cyanotic, soft and necrotic. A comparison of the two figures taken from this material (Figs. 59 and 60) shows the extreme extrusion of chromatin threads after treatment, and degeneration of cell nuclei.

Lymphosarcoma.—This neoplasm was imbedded in the groin and was so indefinitely delimited that it was inadvisable to secure biopsy material for pretreatment examination. Therefore, no control figures are presented. The patient was given intravenously 4 cc. (0.05 mgm.) of polysaccharide, followed by the administration of 3 cc. (0.03 mgm.) by continuous intravenous drip; 6 days later, the tumor had become sufficiently shrunken and defined to enable the removal of what appeared to be the entire growth. Photographs of 2 different regions are presented in Fig. 61 and 62. Again there is noticeable filamentous extrusion of the chromatin substance from the nuclei, which are almost totally degenerative in the affected areas.

Polysaccharide Combined with X-Rays

Because of a clinical finding that the administration of polysaccharide apparently assisted in breaking down in a few cases the resistance of human tumors to irradiation, we made a few exploratory experiments concerning the effect on mouse sarcomas of combining polysaccharide injection with x-radiation. Mice with 7 day tumors were treated with 0.01 mgm. of polysaccharide, and 4½ hours later the tumors received a single irradiation of 2,500 r. Dual controls were maintained: (a) Animals with tumors treated with
Photomicrographs of acetic orcein smears from normal body tissues of mice without tumors, treated with 0.1 mgm. of *S. marcescens* polysaccharide.

Figs. 45 and 47.—Low and high power studies of intestinal epithelium of mouse, untreated.

Figs. 46, 48, and 50.—Low and high power studies of intestinal epithelium of mouse, 6 hours after treatment. Fig. 49.—Germ cells from mouse testis, undamaged, following administration of 0.1 mgm. of polysaccharide.
Photomicrographs from paraffin preparations of tumors in human patients, treated and untreated. Mag × 360.

Fig. 51.—Human chondrosarcoma, untreated.

Fig. 52.—Same, 4 days after treatment.

Fig. 53.—Fibrosarcoma, untreated.

Fig. 54.—Same, 4 days after treatment.
0.01 mgm. of polysaccharide; (b) animals with x-ray-treated tumors only (2,500 r in a single application). All x-rayed mice were placed beneath specially prepared lead shields, in which small openings were cut to allow the tumor to project beyond the shield, and the tumors were treated without removing the hair. The amount of x-ray administered was calculated to be approximately equivalent to 500 r over bare skin.

Some animals were killed for microscopic examination of the tumors, but the remainder were maintained until the tumors were shed, or until their continued growth produced the death of the host. Grossly, about 25 per cent of the tumors responded to polysaccharide alone, as was expected. An almost identical number of degenerates was obtained in the group receiving only x-rays. The combined treatment, however, yielded as high as 75 per cent response, as indicated by total sloughing of the tumors, which showed that the effect was not merely an addition but an enhancement of their combined effects. Microscopic examination revealed that in animals killed at 24 and 72 hours after combined treatment, cellular response, too, was something more than the addition of the two types of damage.

It is well known that cells are most sensitive to x-rays at some stage of mitosis, probably metaphase. Ludford (11) reported that in tumors the mitotic effects of irradiation are greatest on those that are fastest growing. In sarcoma 37 no great change was produced microscopically at 6 hours when x-rays alone were employed; but at 24 hours there was a large amount of degenerating tissue, which apparently arose from chromatin fragmentation. Nevertheless, in only 25 per cent of the animals so treated were the tumors completely destroyed. When the two agents acted together only the expected polysaccharide effects were evident microscopically at 6 hours. However, at 24 hours a combined reaction (polysaccharide degenerates and filaments, plus fragmented chromatin strands) could be detected. The resulting masses of debris were often reduced to fine particles and fragments, as though the filamentous degenerates produced by polysaccharide had also been fragmented by the x-rays. A new phenomenon, moreover, appeared in resting cells. Nuclei of many of these were broken into rather large pieces, which still remained sufficiently in juxtaposition to render it possible to identify them as interkinetic nuclei. Such nuclei are shown in Figs. 21 and 22. The oval contours are lost, the nucleus is diminished in size, and cross-fragmentation of the entire nucleus has taken place. These changes did not appear in either of the control series. Since both agents act destructively on cells undergoing mitosis, we should not have been surprised had a combined single treatment produced no greater effect than either alone. The observations reported here are of a preliminary nature only, and much more experimentation must be done in order to determine how x-rays and polysaccharide in combination act to overcome the comparative resistance of the resting cell and also in what way minimum amounts of these two drastic agents can be combined for optimum effectiveness in destruction of tumor tissue.

DISCUSSION

The assumption that tumor cells respond directly to the toxic effect of S. marcescens polysaccharide and that the resultant degeneration may be independent of or supplementary to the anoxia following breakdown of blood supply appears to be substantiated by the evidence presented. In the first place, it does not seem necessary that hemorrhage occur in order to obtain cellular degeneration, since at the time the initial swelling of the cell begins in response to polysaccharide there may be as yet no trace of hemorrhage within the tumor. Although sarcomas that are completely filled with blood at 6 hours post-treatment usually show a greater amount of necrotic tissue than do those that are only moderately hemorrhagic, nevertheless when non-hemorrhagic areas of tumors are selected for study, the cells are found to be reacting in a fashion identical with that observed in cells from blood-filled areas, except that the coagulation phenomenon is not present.

Care was taken to secure bits of tissue from both hemorrhagic and clear areas in order that microscopic comparisons could be made. In biopsy specimens of large human tumors, the areas of degenerating cells were usually located somewhere in the region of the hemorrhage. However, the presence of blood was not the exclusive criterion of cellular destruction, as was shown when a patient with Hodgkin's disease with multiple cervical lymph node enlargements was treated. Some of these nodes responded by severe hemorrhage, while others did not. Even so, both hemorrhagic and nonhemorrhagic nodes surgically removed after polysaccharide treatment showed cellular response, although it was most extensive in the hemorrhagic nodes.

Furthermore, following polysaccharide administration cell destruction in normal body tissues of...
the mouse is not heralded by any marked hemorrhage of tissues; and in intestinal epithelium where damage was greatest, we could never detect macroscopic bloody areas marking the responsive regions, although many blood cells could be found among the degenerating tissues. Neither were there any hemorrhagic patches on liver or spleen following administration of an amount of polysaccharide 10 times as great as that required for tumor necrotization, although destruction of dividing cells in these organs was microscopically demonstrable.

Algire's studies (1) of the vascularization of sarcoma 37 and other tumors led him to observe that "an outstanding characteristic of the tumor cell is its capacity to elicit continually the growth of new capillary endothelium from the host," and Ludford (12) asserted that the vascular damage produced by colchicine in rapidly growing tumors is due to the fact that "the endothelial cells of newly formed capillaries are particularly sensitive to mitotic poisons." A study is now under way to determine, if possible, whether mouse tumor capillaries likewise break down because of polysaccharide destruction of rapidly proliferating endothelial cells. Frequently in rapidly growing tumors, cell multiplication proceeds with a speed with which capillary growth does not keep pace, and the blood travels not in formed capillaries but in spaces surrounded by closely aligned tumor cells. If these tumor cells break down under polysaccharide, hemorrhage naturally follows.

Bearing on these points is the example already mentioned of the stability of capillaries in a less rapidly dividing neoplasm (spontaneous rat carcinoma). In this instance it appeared clear that cellular degeneration was not due to the anoxia incident to deprivation of blood supply.

It can hardly be possible, however, that tumor destruction following polysaccharide treatment is entirely dependent on the direct toxicity to tumor cells; for it is obvious that where lack of oxygen and food supply have occurred through breakdown of the vascular system, there must be additional degeneration of the cells arising from these causes. Moreover, Algire (2) demonstrated by means of the mouse chamber that stasis is observed 3 to 4 hours after polysaccharide, and this may be an added factor in cell degeneration in the earlier stages of response. There is also the possibility that damage to cell walls may result in the leaking of plasma into the intercellular spaces several hours before the red cells come through.

Furthermore, even when some of the tumor cells remain relatively healthy, coagulation of blood and formation of a scab may dispose of some of them by purely mechanical means. That is, the hardened mass of tissue breaks away from the skin and sloughs off, carrying with it some still viable cells mingled with the debris.

On the other hand, we have sometimes observed that tumors which were not sufficiently hemorrhagic to form a hardened mass of blood nevertheless were totally resorbed, indicating that the cells must have been destroyed. These were usually small tumors. In no case was the resorption of a large tumor observed, and this is probably correlated with the greater amount of resting and therefore less responsive tissue present in the older, larger, growths.

Although most of our evidence points to a relatively selective action of the polysaccharide as regards dividing cells, there is no way of determining whether some of the interkinetic cells may not also be affected. However, it is certain that after polysaccharide treatment, cells in mitotic division are destroyed, the mitotic process is suppressed, and the only undamaged nuclei are those of non-dividing cells. As shown previously in this report, the first manifestation of polysaccharide effect is a swelling of the prophase nucleus, accompanied by blistering and other changes in the nuclear membrane. This is followed either by collapse of the nuclear membrane or extrusion of the nuclear contents. In the latter case, there is a seeming failure of the swollen nuclei to withstand the pressure of internal fluid, and the nuclear membrane gives way at some point, permitting the solid structures to be extruded. This is the most characteristic feature of nuclear response to polysaccharide and is seldom encountered during spontaneous degeneration. Our colleagues at the National Cancer Institute report that they have noted this type of nuclear degeneration occasionally in untreated tumors, though it is much less extensive than in treated ones, and usually appears in tumors found to be regressing under bacterial contamination.

The factor of spontaneous degeneration of these tumors renders difficult the cytological evaluation of response to chemotherapeutic agents generally; nevertheless in our experimental tumors it was possible to distinguish between induced and spontaneous necrosis with reasonable certainty. For one thing, cells degenerating spontaneously do not exhibit nuclear membrane changes so far as we have been able to observe, and though nuclei become shrunken and pyknotic, no differential degeneration of dividing cells is demonstrable. Nuclei of all stages shrink down into pyknotic spheres which are much smaller than polysaccharide-induced remnants and which show no
High-power studies (Zeiss 1.5 oil imm. lens) of tumors in human patients, control and treated tissue from biopsies taken before and after treatment. Mag. × 1,000 (approx.).

**FIG. 55.**—Human chondrosarcoma, untreated (acetic orcein smear).

**FIG. 56.**—Same, 4 days after treatment (acetic orcein smear).

**FIG. 57.**—Human fibrosarcoma, untreated (acetic orcein smear).

**FIG. 58.**—Same, 4 days after treatment (acetic orcein smear).
Tumors in human patients, continued.

Fig. 59.—Untreated Hodgkin's lesion from cervical lymph node.

Fig. 60.—Same, 4 days after treatment.

Figs. 61 and 62.—Human lymphosarcoma, 6 days after treatment, showing different regions of the neoplasm (hemorrhagic and non hemorrhagic).
trace of structure. Areas of spontaneous degeneration are usually filled with droplet-like globules of structureless, strongly orcein-positive substance or minute fragments that are likewise heavily stained and granular in appearance; that is, quite different in size and conformation from the components of cellular debris produced within a few hours after polysaccharide administration.

Following polysaccharide, no blistering or collapse of the nuclear membrane was observed in resting cells, even when they were inhibited from division.

The fact that at each progressive stage of nuclear degeneration following polysaccharide treatment there were present figures characteristic of each of the early stages, suggests that the process of morphologic change follows a rhythm, probably that of division, and that each nucleus runs a gamut of degenerative changes, depending upon the stage of division in which it existed on impact of the polysaccharide.

The assumption that the dividing cell is most responsive is bolstered by the observation that the cells not affected are those in resting stage and also by the reactions of nontumor tissue. Furthermore, the fact that there are no longer any anaphase or telophase figures present when the first of the affected nuclei reach the ultimate in degeneration indicates that mitosis was halted earlier. It should be borne in mind that in neoplasms with such a high mitotic index a large proportion of the cells are in some stage of mitosis at all times.

The reason carcinomas fail to respond as rapidly and vigorously to polysaccharide as do sarcomas is not clear and may of course reside in fundamental chemical differences between the cells of these two types of neoplasm. However, if our supposition is correct that dividing cells are more susceptible than resting cells, there may be a correlation between the restricted response of carcinomas and the small number of dividing cells present in any area of such tumors, as revealed by our controls.

It is uncertain whether cessation of mitosis during the 3 day period following treatment is due to polysaccharide suppression, or whether failure of cells to divide may result from loss of capillary connections. Algire's observations that capillaries are not re-established until the third day following polysaccharide damage may favor the second alternative. Our studies of young implants also bear on this point, since it was observed that wherever there are dividing cells they respond to polysaccharide, and that onset of division is dependent upon establishment of vascular connections.

Moreover, the same phenomena occur as were observed during the first 3 days following implantation and before capillary branching is elicited. That is, there are produced many enlarged nuclei with multiple nucleoli. This suggests that many of the cells continue to carry on the process of duplication of the chromatin through “endomitotic” activity (internal mitosis without nuclear or cell division) postulated in neoplasms by Biesele, Poyner and Painter (5). According to Biesele and his collaborators, such nuclei might theoretically arise in cells with an unusually high concentration of thymonucleoprotein “which might be explained on the basis . . . of synthesis over a long period of time.” Such an opportunity for synthesis without chromatin repartition conceivably is afforded during the enforced interkinesis imposed either by polysaccharide inhibition or attendant anoxia.

Our studies of the growth pattern of sarcoma 37 have demonstrated that mitotic activity is not always localized at the periphery but that division centers are scattered throughout the tumor, wherever there is contact with vascular branches. The resultant random distribution of “resting” areas probably accounts for the fact that undamaged fragments of clear tissue, obviously capable of renewed growth, are often macroscopically observable at the periphery of the treated tumor, although the remainder of the growth may have become nothing more than a softened necrotic mass. In collaboration with other members of the Institute, studies are being made on the problem of destroying this persisting tissue through protection of animals against polysaccharide toxicity, breakdown of resistance to repeated treatments, and combination of polysaccharide with x-ray.

SUMMARY

S. marcescens polysaccharide produces nuclear damage to transplanted mouse sarcoma cells, separate from that arising through breakdown of the capillary system. Maximum destruction is attained at 6 hours, and only resting cells persist. These cells are inhibited from division for 3 days following treatment. During this period they may persist morphologically unaltered, they may undergo some degeneration of cytoplasmic components, or they may become enormously swollen cells with huge nuclei and supernumerary nucleoli.

In a significant number of cases, no viable cells could be detected by our methods 3 days after treatment with a dose that killed 20 to 30 per cent of the mice. The same proportion of tumors was sloughed by animals maintained for survival studies. When polysaccharide was combined with x-rays this number was increased threefold.
Injection directly into the tumor did not inhibit growth.
Repeated treatments did not produce greater amounts of destruction, indicating that the tumor, or its host, had become resistant to the bacterial agent.

In primary neoplasms, including human sarcomas, the effects were similar to, but much less extensive than, those produced in mouse sarcomas.

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