ENDOTOXIN AS ADJUVATOR TO THE TRANSPLANTATION OF A MOUSE MAMMARY TUMOR

By JAMES STUART HENDERSON, M.B., Ch.B.

(From The Rockefeller University, New York 10021)

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Tumor cells, if they are to survive transplantation, depend for some time solely on the nutritional and other favorable influences of the tissue fluid surrounding them. The tissue fluid is enriched meanwhile by products of the transplanted cells themselves. Such mutual aid becomes attenuated as the transplanted cells are made to lie farther from each other. Other adjuvators to their successful transplantation are then uncovered (1).

In the work already reported, tumor cells from the breast of a Balb C mouse were transplanted in degrees of isolation by spraying them in single cell suspensions onto the broad subcutaneous expanses of mice of the same inbred stock. With the intrinsic adjuvators thus reduced, extrinsic adjuvators could be assayed. One strong adjuvator, derived from liver, was thought to act by enhancing the general nutritiousness of the fluid supplying the tumor cells as they awaited the restoration of vascular support. Further analysis of the liver adjuvator showed that it was not ether soluble, that it was heat stable, and that it resisted mild hydrolysis with acids and alkalis. Since some properties of endotoxin exhibit similar resistance (2–4), and since the liver stores bacterial endotoxin (5), it seemed worthwhile to test the effects of lipopolysaccharide from *Salmonella typhosa* on descendants of the same cells.

The present paper is to report the adjuvator effects of this endotoxin, and some experiments to illustrate the nature of endotoxin's adjuvator effect.

Materials and Methods

Endotoxin.—A lipopolysaccharide,[1] prepared commercially from *S. typhosa* by Boivin's aceto-acetic acid extraction method, was suspended by shaking it in one or other of the balanced salt solutions appropriate to the experiments.

Mice.—All were young adult females from the notably homogeneous C strain colony maintained for many years in this laboratory. The colony was bedded in autoclaved wood shavings and sustained with tap water and mouse breeder pellets.[2] The weanlings were fed a daily ration of bread and milk mash and kept in equable conditions of temperature and humidity. The mice were prepared, when the experiment called for it, by the splitting of their dorsal subcutaneous tissues with 5 cc of air and 1 cc of modified Earle's solution (E soln) with or without

[1] Lipopolysaccharide B, *S. typhosa* 0901, Difco Laboratories Inc., Detroit, Mich.

[2] Old Guilford Laboratory Animal Diets, The Emory Morse Co., Guilford, Conn.

endotoxin. The procedure was exactly as described before (1), except that the air was withdrawn at once.

Tumor.—The mouse mammary cancer MT 296 (1) was used, from its 42nd to its 48th plated generations, in this series of experiments. It remained a complex of several neoplastic components persisting in apparent equilibrium with one another (see Fig. 4).

Plating.—Tumor cell suspensions were prepared by alternately stirring and sieving the tumor fragments in 0.25% trypsin[3] in E soln, centrifuging and resuspending the loosened cells and cell clumps in 0.04% DNase[4] in spinner salt solution (SS soln) and by finally filtering this through the 5 μ pores of porous steel sieves (see Fig. 2). Such suspensions contained exclusively cells which were both viable and individual. They were suitably diluted in SS soln, with or without endotoxin. Then the cells were plated by injecting the suspensions, 1 cc with 5 cc of air, into the dorsal subcutaneous tissues of the mice. The procedure was as described in detail before (1), except for immediate withdrawal of the air.

Records.—The tumor outlines, seen through close-clipped albino skin, 2 or 3 wk after plating, were first traced on transparent plastic and then transferred to permanent cards.

Histology.—Tumors were fixed in Zenker's solution and stained with hematoxylin and phloxin. The connective tissue expanses, dissected from dermis and body wall, were dropped into Zenker's solution for fixing, embedding, sectioning, and staining in the usual way.

RESULTS

Effects of Endotoxin on the Tumor Cells

Experiment 1. Testing the Effect on Plated Cells of Endotoxin Injected with them.—

Endotoxin, in various strengths, was added, just before plating, to the single cell suspensions. The single cell suspensions had themselves been prepared, in various dilutions, from the same tumor mass. The clear adjuvator effect produced by endotoxin is shown in Fig. 1.

In all these experiments the tumor cells were plated over connective tissue expanses of equal area; so 10,000 of them were more thinly dispersed than 100,000. In all these experiments study of individual tumors also showed, that, once they were visible, their growth rate was the same. A higher tumor yield always resulted from more "takes" and never from visible acceleration of growth. In this experiment an increased yield of tumors was seen, at both degrees of dispersal, where a higher concentration of endotoxin accompanied the tumor cells at the time of plating. The harvest from 100,000 individual tumor cells was similar to that obtained from one-tenth of their number, when 50 μg of endotoxin had been added to them. This meant that nine-tenths of the potential takes were lost, in the control plating, that should have contributed to the next tumor generation had 50 μg of endotoxin been added.

The mice in the experiment depicted were adult females, weighing 15–16 g, about two-thirds grown. Those receiving 50 μg of endotoxin lost about 1 g of their weight within 24 hr and were still 2 g behind the control gain at the end of 10 days. The group of mice receiving the smaller amounts of endotoxin

[3] Trypsin 1:250, Difco.

[4] Deoxyribonuclease 1 (beef pancreas) 1 × crystallized, Worthington Biochemical Corp., Freehold, N. J.

deviated in weight only slightly below the control groups. These losses related purely to endotoxin and were no greater with the larger number of tumor cells plated.

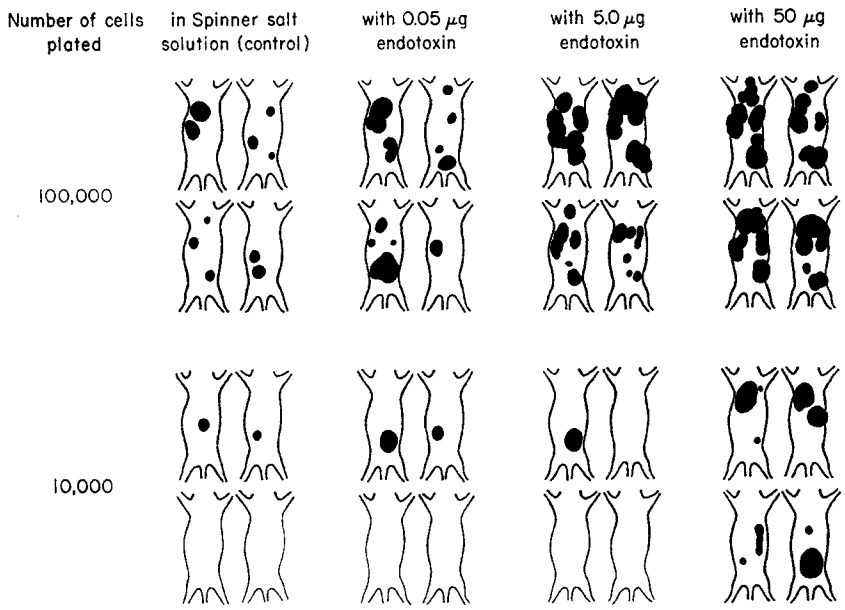

FIG. 1. Experiment 1: Tumor growth from 1 cc of cell suspension after 25 days. Tumor cells were plated in suspensions of different concentration, with different amounts of lipopolysaccharide (endotoxin) from *S. typhosa*. Increase in tumor yield is most obvious where the sparser scattering of cells coincided with the greatest accompaniment of this endotoxin.

Experiment 2. Testing the Effect of Prolonged Association between Endotoxin and the Tumor Cells.—

Endotoxin was added, 50 μg per cc, to one of two tumor aliquots during their 4 hr trypsinization: as the cells were periodically resuspended in fresh trypsin the endotoxin was also restocked. Free endotoxin was discarded, with the trypsinous supernate, when the cells were centrifuged and resuspended in DNase for 5 μ filtering. Then to half of each of these two preparations endotoxin was added, 50 μg per cc, and all four aliquots were plated.

The form and results of this experiment are made clear in Fig. 2. An equally potent adjuvator influence was shown in the two groups which were plated with endotoxin, whether or not endotoxin had also been present during their trypsinization. On the other hand, the tumor cells which had been trypsinized in the presence of endotoxin, but plated in its absence, did no better than those of the control group which had no contact with endotoxin at any time. It is not

likely that this effect of endotoxin would be subverted by such a degree of trypsinization (6). It may be concluded that prolonged contact between endotoxin and the tumor cells had no net adjuvator effect.

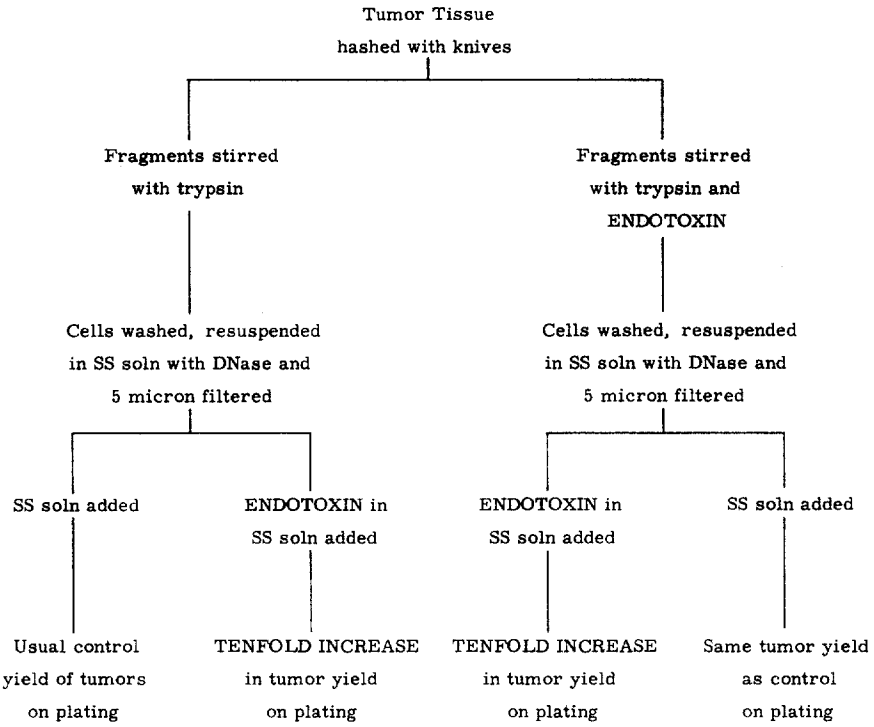

FIG. 2. Experiment 2: The extreme left flow in this figure sets forth the usual scheme by which the tumor cell suspensions are prepared. The others show how tumor growth from 1 cc of cell suspension was increased tenfold, by the addition of 50 μg of endotoxin to the tumor cell suspension in the short, final stages of its preparation. Prolonged association between endotoxin and the tumor cells throughout the much longer period of trypsinization had no adjuvator effect. It is known that the toxic and the antigenic properties of endotoxin resist the effects of trypsin (6). So it seems most likely that the adjuvator effect is on the host.

Experiment 3. Testing the Effect of Disassociated Injection of Endotoxin at the Time of Plating.—

Endotoxin was added, 50 μg per cc, to one of two aliquots of a tumor cell suspension just before plating. The other aliquot was also plated, but into mice which had been injected 30 sec before, in their ventral subcutaneous tissue, with either 50 μg of endotoxin or the appropriate salt solution for a control.

The expected adjuvator effect was seen on cells accompanied by endotoxin. A slightly greater adjuvator effect was seen where the tumor cells were not accompanied but the mice had been injected with the same endotoxin at the other site. Early weight loss was the same in these two groups.

Effects of Endotoxin on the Host

Experiment 4. Splitting the Connective Tissue with Endotoxin before and after Plating.—

Identical groups of mice were prepared, by splitting their subcutaneous tissue with or without endotoxin (50 μg per cc per mouse), on a different day for each pair of groups. The mice were also plated, all at one time, with 1 cc of a tumor cell suspension. Thus preparation and plating were separated by various intervals.

Fig. 3 shows the marked ajuvator effect on tumor cells plated on connective tissue expanses which had been split with endotoxin one day before. This pronounced, 1-day, endotoxin effect contrasts, in timing and duration, with the weak effect obtained by splitting simply with air and E soln alone. The simple splitting effect has been described before (1) and is now seen again in the left column of the Fig. 3. It operates in mice whose tissues were split 1–4 days before plating. It exhibits a poorly defined maximum about the 2nd or 3rd day.

The experiment itself was more extensive than shown in the figure. Mice which were prepared 6 and 7 days before plating (with and without endotoxin), or injected again 1 day after it (with and without endotoxin), had tumor growths comparable to the unprepared control on the left.

Both groups at the top of Fig. 3 were unprepared. Endotoxin was added to the cell suspension for the one on the right replicating the conditions of Experiment 1. Figs. 1 and 3 can be compared, however, only if allowance is made for the former representing tumor growth 25 days after plating and the latter 14 days after plating. The tumors in the mice of the top left and right groups of Fig. 3 had, in fact, grown by 25 days to a size comparable with that of their respective counterparts on the left and right of the lower line in Fig. 1. By that time the other tumors had grown apace towards a confluent maximum and the depiction from them of the 1-day endotoxin effect would not have been so clear.

Experiment 5. Diminishing the Effect of Endogenous Endotoxin.—

Experiment 4 was repeated using mice which had had oxytetracycline[5] in their drinking water for over a month.

The adjuvator effect from simple splitting of the connective tissues with air and E soln had been eliminated in these mice. Indeed the yield of tumors when splitting with air and E soln had been done 1, 2, and 3 days before plating was slightly depressed. The adjuvator effect was still obtained, however, where the tissues had been split with air and E soln containing endotoxin. Its maximum was again in the group prepared 24 hr before plating.

Effects of Endotoxin on the Subcutaneous Expanses

Anatomical Experiments.—Mice, which had their dorsal subcutaneous expanses split as described under the subheading *Mice* in Material and Methods,

[5] Terramycin soluble powder, animal formula, Chas. Pfizer and Co., Inc., New York.

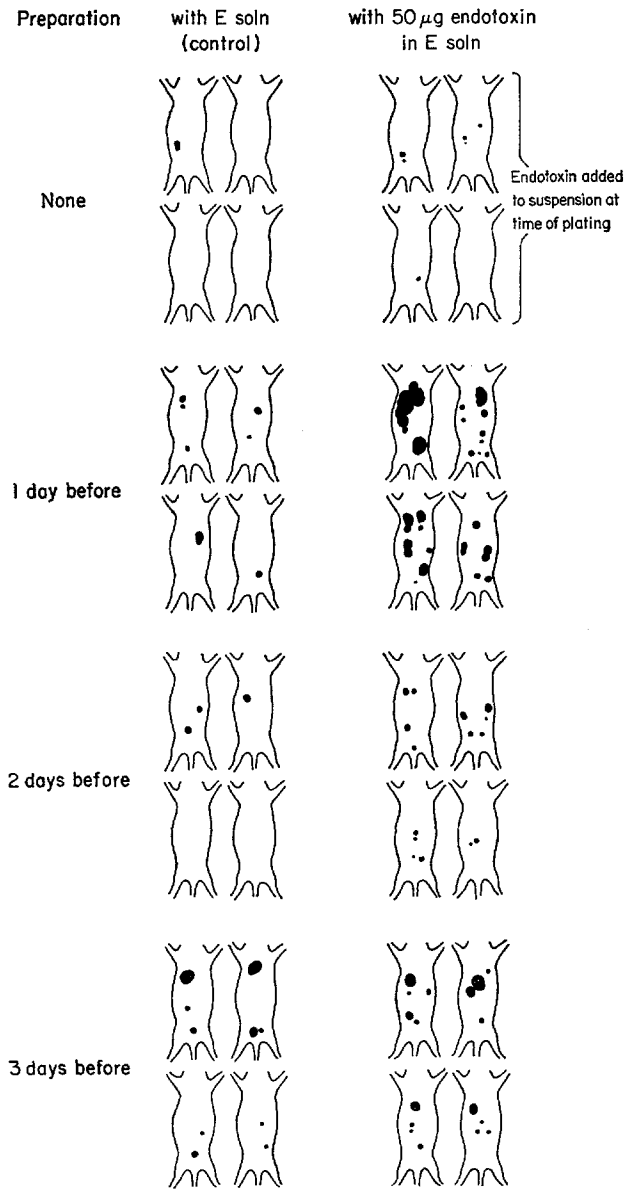

FIG. 3. Experiment 4: Tumor growth from 1 cc of suspension containing 10,000 single cells, after 14 days. The mice on the left had had their dorsal subcutaneous expanses split, by the injection of 1 cc E soln and 5 cc of air, at the noted intervals before these expanses were to receive the plated cells. The mice on the right had had their expanses prepared in the same way except that endotoxin was included in the E soln. At the top of the figure, where there was no such preparation, endotoxin was added to the suspension of tumor cells for plating into the group of mice on the right. Thus it is clearly shown that endotoxin exerts a far greater adjuvator effect when applied to the host 24 hr before the tumor cells. The endotoxin acts entirely upon the host.

were killed and autopsied 24 hr later. These tissues split with air, E soln, and endotoxin (see Figs. 5, 7, 10, 12, and 13) must therefore have been like those receiving the tumor cells most successfully in Experiment 4 (see Fig. 3). Examples of other expanses split with air and E soln are shown in Figs. 5, 8, and 11. An example of the same tissue not previously split is shown in Fig. 9.

Anatomical Remarks.—When an incision was made along the dorsal midline of a mouse, the pelt could be drawn aside with ease. This uncovered a delicate layer of connective tissue under the skin flaps and over the body wall (see Fig. 5). These connective tissue expanses, the same as are created for and by in vivo plating, apparently separate with no damage to vessels or nerves. If the incision was made and the flaps were drawn aside 24 hr after such expanses had been separated by the injection of air and E soln, the connective tissue layers were found thickened and slightly cloudy. If endotoxin had been included in the injection of air and E soln 24 hr before, the split connective tissue was not only thickened and very cloudy but notably hyperemic as well (see Fig. 5).

Histological Remarks.—The loose fascial membrane of the exposed expanses under the pelt is shown in Figs. 6 and 7 and of the corresponding expanses over the trunk in Figs. 8, 9, and 10. These tissue layers, normally quite thin (see Fig. 9), were edematous where split with air and E soln (see Figs. 6 and 8). They were the principal site of a considerable proliferation and congregation of cells where split with air, E soln, and endotoxin (see Figs. 7 and 10).

Very little connective tissue could be teased from pelt or body wall in unprepared mice. But from mice split 24 hr before an abundance was obtained by blunt dissection for fixation and microscopic study. Fig. 11 is from a section of such subcutaneous tissue, split with air and E soln 24 hr before dissection and fixation. Cell nuclei proclaim a population of fibroblasts and macrophages not far removed from the resting state. Fig. 12, in contrast, shows more swollen fibroblast nuclei, more immature macrophages or polyblasts, and many polymorphonuclear leukocytes of the neutrophil type. Most of these last were degenerating. The tissues having this appearance had been split 24 hr before with air and E soln, to which had been added 50 μg of endotoxin. As counterpart to the visible hyperemia (see Fig. 5), the section chosen for Fig. 13 shows capillary dilatation and a plethora of erythrocytes with many of these actually in the tissue spaces. The vein in the center of the picture contained thrombus, and leukocytes had infiltrated its wall.

DISCUSSION

The tumor used in all these experiments was complex. A typical growth is shown in Fig. 4. It contained some, but not all, of the morphologically distinct neoplastic components in the mouse mammary cancer MT 296 of previously reported work (1). MT 296 arose in the breast of a 15 months old breeding female which had borne and suckled at least 38 mice in eight litters. Originally it was typically "pleomorphic." Plating showed that its pleomorphism was in

fact heterogeneity. Repeated platings of entire tumor harvests carried its different components through many transplant generations. These components showed remarkable stability. At the 34th generation, for reasons not yet understood, the only plating made failed to transfer one well-defined component, the easily recognized, broad-banded type. The persisting components have since shown their usual stability. There has been no other occasion of dramatic loss and no new component has ever arisen. Changes in the dominance of one type over others have had the characteristics of slow drift. Each experimental plating now reported was done with a suspension of tumor cells, prepared so far as was possible, with the entire tumor product of one mouse. All components were thus maintained through the plating procedure in the ratio of their occurence. During growth they maintained themselves in about the same relationship despite the experimental manipulations described. The most likely hypothesis to account for this constancy still seems to be as stated before: that this tumor's heterogeneity comes not from repeated cellular mutation late in its development but from diverse potentiality to give rise to cells of specific and differing character, which potentially is inherent in its cells at, or close to, the time of its multicellular origin.

The main aim of this work is to grow tumors, by transplantation in vivo, each tumor from a single cell. It is hoped that a sufficiently large selection of the single cells in any given tumor can be grown thus for a complete cellular analysis of that tumor's potential to be obtained. An adjuvator was found in liver which furthered the impantation of tumor cells plated from single cell suspension (1). The previously reported experiments with this showed that liver was effective only when added to the tumor suspension and plated along with it (1). Experiment 1, testing endotoxin (see Fig. 1), is an exact replica of an experiment testing liver. But here the comparability ends. Liver had no adjuvator effect when it was injected into the subcutaneous tissues of the belly about the time of plating. Experiment 3 of the present series shows that endotoxin was just as effective, even a little more so, when injected there. Liver injected ahead of the plated cells into the dorsal subcutaneous tissue of mice which was to receive these had no net effect on their implantation. Experiments 4 and 5 (see Fig. 3) showed that endotoxin's effect was greater when it preceded the tumor cells by 1 day. When liver and endotoxin were added together to the tumor cell suspensions and injected with them, a slightly cumulative adjuvator effect was obtained. These and other experiments, to be reported at another time, suggest that if endotoxin in liver is a factor in the latter's adjuvator capacity, it is not the only one.

There is some analogy between my crude liver experiments in the living mouse and those carried out in tissue culture with great precision in Eagle's laboratory. Plating efficiency in vitro was modified by the concentration of individual amino acids in the medium (7): the yield of transplanted tumors in vivo was

increased when tumor cell suspensions included liver products (1). It seems most likely that the liver adjuvator included a local nutritional effect. It seems equally unlikely that lipopolysaccharide has any local nutritional value to the newly plated cells. Furthermore, lipopolysaccharide's general nutritional effect must be quite adverse. All the mice in the present report lost substantial weight when given, as most were, 50 μg of endotoxin. The growth of all of them was held back for the duration of the experiments. This emaciating effect of endotoxin is a well-known one, compounded by adipsia (8) and aphagia (9) and resulting in a severely negative carbohydrate balance (10). Such would certainly be inimical to the successful transplantation of this and of the generality of tumors (11). The specific adjuvator effect of endotoxin must be considerable to do so much more than offset the effects of this weight loss.

The broad conclusion from the first three experiments and from the preceding discussion must be that the effect of endotoxin on the tumor cells is insignificant. What then of its effect on the host?

Endotoxin, in the first 4 hr after its injection, increases susceptibility to infection with various microorganisms (12). Pretreatment of mice with endotoxin enables greater multiplication of staphylococci in their peritoneal cavities (13). Impairment of leukocyte migration into the field of increased susceptibility figures large in that phenomenon. Not so with the presently described susceptibility to tumor cell implantation (see Figs. 7, 10, and 12). It reaches its peak at 24 hr, by which time susceptibility to bacterial infection is markedly diminished. Furthermore, the adjuvator effect on MT 296 is shown in mice which, by their breeding and nurture, should have immune tolerance both to the transplanted tumor cells and to the virus connected with their tumorous growth. The adjuvator effect with these tumor cells seems different from the increased susceptibility to infection.

Tumor cell "takes" and the subsequent growth of a new transplant generation require something more than mere survival of the cells transplanted. Tumor cells, to "take," must call forth also from their new host a stromal response supplying new blood vessels. A larger number of individual tumor cells would be surviving to benefit from it if this vascular investment could be accelerated. An adjuvator effect, inherent in the host connective tissue and probably related to such acceleration, has been uncovered by the plating of mouse mammary tumor in single cell suspension (1). It was evoked by splitting the prospective host's connective tissues 1–4 days before these were to receive the plated cells. Although such splitting was followed by no visible inflammation, nor by any detectable repair, the adjuvator effect was thought, on account of the well-known localization of secondary tumors at points of injury (14), to result from stromal reaction already begun. Might the adjuvator effect of endotoxin relate more easily to the accelerated stromal effect than to the nutritional one?

The adjuvator effect inherent in the connective tissue itself was evoked by splitting it with air and E soln. The same effect was found to be evoked when the connective tissues were split by injecting air alone. All seemed to show that this adjuvator effect was an intrinsic one with its endogenous factors released by the slight trauma of

splitting. In the control of Experiment 4 (see the left hand column of Fig. 3) this intrinsic adjuvator effect on tumor takes is seen again in the mice split with air and E soln 1, 2 and 3 days before the tumor cells were plated. In the right hand column of the same Fig. 3 a very striking exaggeration of this adjuvator effect is seen in the mice where endotoxin was included with the E soln used for splitting and the splitting was done 1 day before plating.

The susceptibility of mice to certain effects of endotoxin stems from earlier contact between the mice and Gram-negative bacilli (15). The hemorrhagic necrosis of the skin described by Schwartzman results from intradermal injection of endotoxin followed 24 hr later by intravenous injection of the same substance (16). Appropriately infected mice not infrequently have enough circulating endotoxin for the evocation of a local Schwartzman reaction by the intradermal injection alone (17). This condition of susceptibility is eliminated by their treatment for 1 month with a suitable antibiotic (17). A high proportion of the mice from the colony kept here give a similar "one-shot" Schwartzman reaction, which is not surprising since they are not rigorously shielded from intestinal bacteria. Account must be taken of this in the interpretation of the results in Experiment 5. Elimination of the adjuvator effect from simply splitting the subcutaneous expanses, in these mice which had for a month drunk water containing oxytetracycline, may be the result of having eliminated circulating endotoxin. In confirmation of this, the adjuvator effect of splitting with endotoxin was just as strongly obtained in these mice as it had been in the mice of Experiment 4 (see Fig. 3). Thus the adjuvator effect released by splitting them, and previously thought inherent in the connective tissues, depends on the presence of endogenous or exogenous endotoxin.

Clearly the adjuvator effect of endotoxin is on the host and information about it should be sought first in the connective tissue expanses themselves.

Connective tissue expanses split with fluids containing endotoxin are made redder and more succulent than those split with the fluids alone. This is easily seen when the overlying skin is parted down the midline 24 hr later (see Fig. 5). These gross appearances suggest directly a more favorable nidus for each transplanted tumor cell. But microscopic examination shows, in more detail, the edema and increased cellularity composing the changes and thus raises other questions. Comparison of the sections in Figs 6 and 7, and in Figs. 8 and 10, shows that the subcutaneous tissue subtending the muscular layer of the pelt is thicker, looser, and presumably more edematous where simple splitting occurred without added endotoxin. Whether or not this tissue fluid was helpful to the tumor cells would depend on the balance of nutritive, hormonal, immune, and other factors in it. Whether or not the helpfulness was sustained would depend on the continued restoration of useful factors by a fairly free flow of the fluid. Experiment 4 (see Fig. 3) implies that subcutaneous tissue having the appearance of that in Figs. 6 and 8 is less conducive to tumor implantation than that having the appearance seen in Figs. 7 and 10. While perhaps less edematous, the tissue treated with endotoxin is very much more cellular (see Figs. 7

and 10). Comparison of specimens dissected from the subcutaneous expanses shows that the mononuclear cells already rendered slightly hypertrophic by the simple injection of air and E soln 24 hr before (see Fig. 11) are increased in size and greatly increased in number in tissues where the E soln had 50 μg of endotoxin with it (see Fig. 12). They have been joined in this latter case by many polymorphonuclear cells, all neutrophil and mostly mature if not degenerate. It is not thought that these cells, whose usual function is defensive, would be contributing to the establishment of tumor grafts. But the experiments show that the single tumor cells produce more tumors when scattered on a connective tissue which looks like that of Fig. 12. At the end of the first 24 hr period following the injection of endotoxin there is little generation of new blood vessels. The tissue must be reddened by dilatation of the preexisting blood vessels and by minute hemorrhages as are seen in Fig. 13. These hemorrhages may result from diapedesis, a feature of inflammation, or may result from dysfunction associated with the thrombosis seen in the venule of Fig. 13. Polymorphonuclear leukocytes in the wall of that vessel attest to considerable vascular damage. Such changes are never seen when the connective tissue has been split with air and E soln alone.

Active hyperemia possibly helps tumor engraftment. The other microscopic features together constitute a Schwartzman reaction (16). While clearly of the local type, it is attenuated and spread over a broad expanse of subcutaneous tissue instead of, as in the classical instance, proceeding to necrosis in a confined disc of compact dermis. No role can readily be assigned to any of these last phenomena by which they might contribute to the successful transplantation of tumor cells.

Endotoxin affects established tumors adversely. It has long been known that certain infections might be accompanied by the disappearance of neoplasms. Many experimenters have shown that extracts of various bacteria cause regression of autochthonous and of transplanted tumors when these extracts are injected into the tumor or even into the animal bearing it. The endotoxins of Gram-negative bacteria do this best, causing a hemorrhagic necrosis of the tumor. But any portion of the neoplasm which escapes such destruction continues to grow progressively until the death of the host.

The adjuvator effect ascribed to endotoxin in the present paper contrasts with but does not contradict the results summarized above. For the adjuvator outcome does not stem from the effect of endotoxin on tumor tissue which has an established blood supply. It is the effect of endotoxin on tumor cells and a hitherto tumor-free stroma when these first come together. What endotoxin does in the experiments here described is clear. It acts, when present at the site of slight tissue damage, to promote the success of tumor cell "takes" there, when mammary tumor cells are scattered on the subcutaneous expanses of syngeneic mice. It must not be thought that it could thus cause cancer. But it

might, if this phenomenon is found to have a wide occurrence in nature, be a promoter of metastases and of nascent tumors. As such it may furnish one of the links, long suspected, between chronic infection and the incidence of clinical neoplasia.

SUMMARY

The mouse mammary tumor MT 296 was used in a further series of experiments on the implantation of tumor, plated out in vivo, from suspensions of individual cells.

Lipopolysaccharide from *S. typhosa* was shown to exert an adjuvator influence. But this adjuvator, an endotoxin, had no direct effect on the suspended tumor cells, unlike the liver preparations previously reported.

Lipopolysaccharide from *S. typhosa* was shown to act on the host. It made the host's connective tissue expanses more susceptible to successful implantation by the tumor cells. It did this only if present at the time these connective tissue expanses were split. The increased susceptibility, caused by splitting the connective tissue expanses in the presence of lipopolysaccharide, declined quickly after 24 hr.

The structural changes wrought upon the connective tissues by splitting them in the presence of lipopolysaccharide are described. They show kinship to a Schwartzman reaction of the local type. Their possible role in the adjuvator effect on the plating of single cell suspensions of this tumor is discussed.

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FIG. 4. Tumor, grown from a plating of 10,000 cells, typical of that employed in the present series of experiments. The upper third of this small portion of MT 296 in its 44th generation is solid, alveolar carcinoma. A pure sample of this component was shown in an earlier publication (1). It is combined here with other types. Among them a central wedge of an irregular, thick-walled, cystic tissue is distinct. × 15.5.

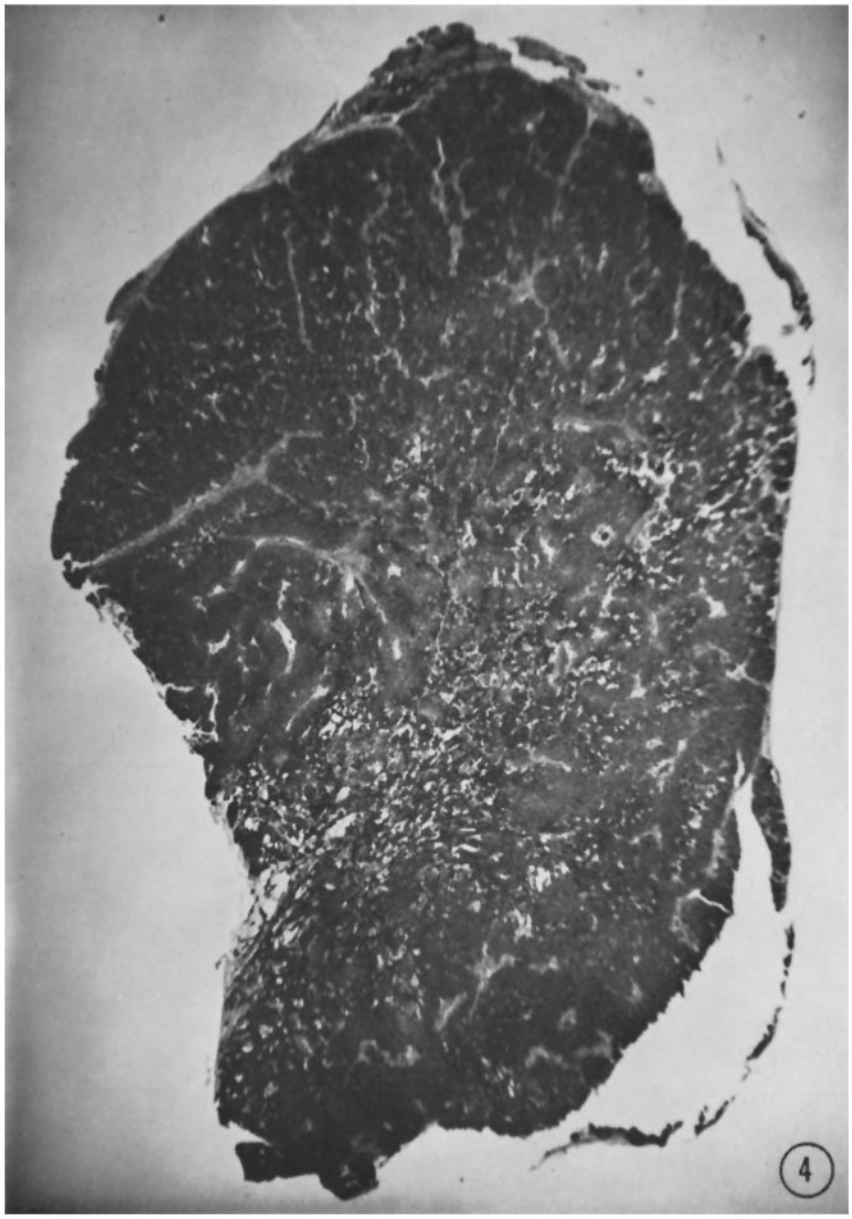
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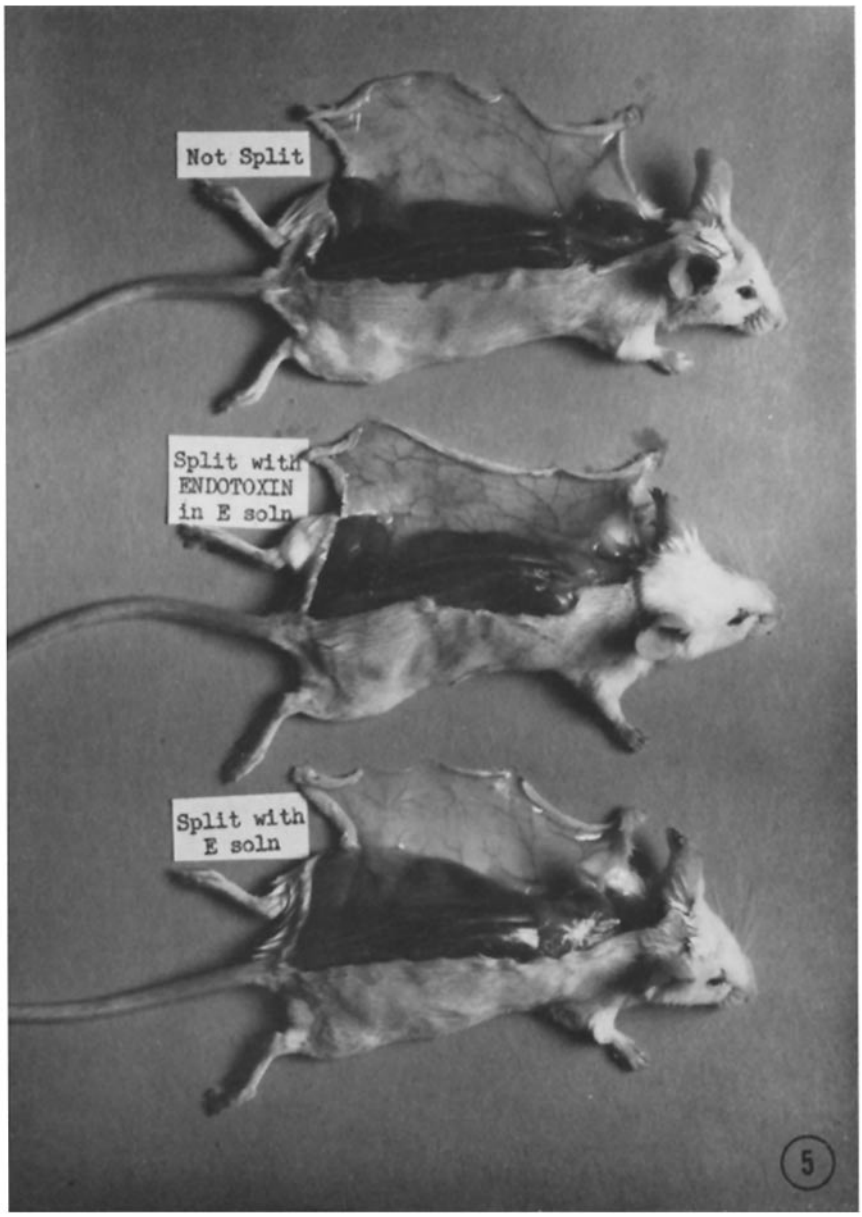
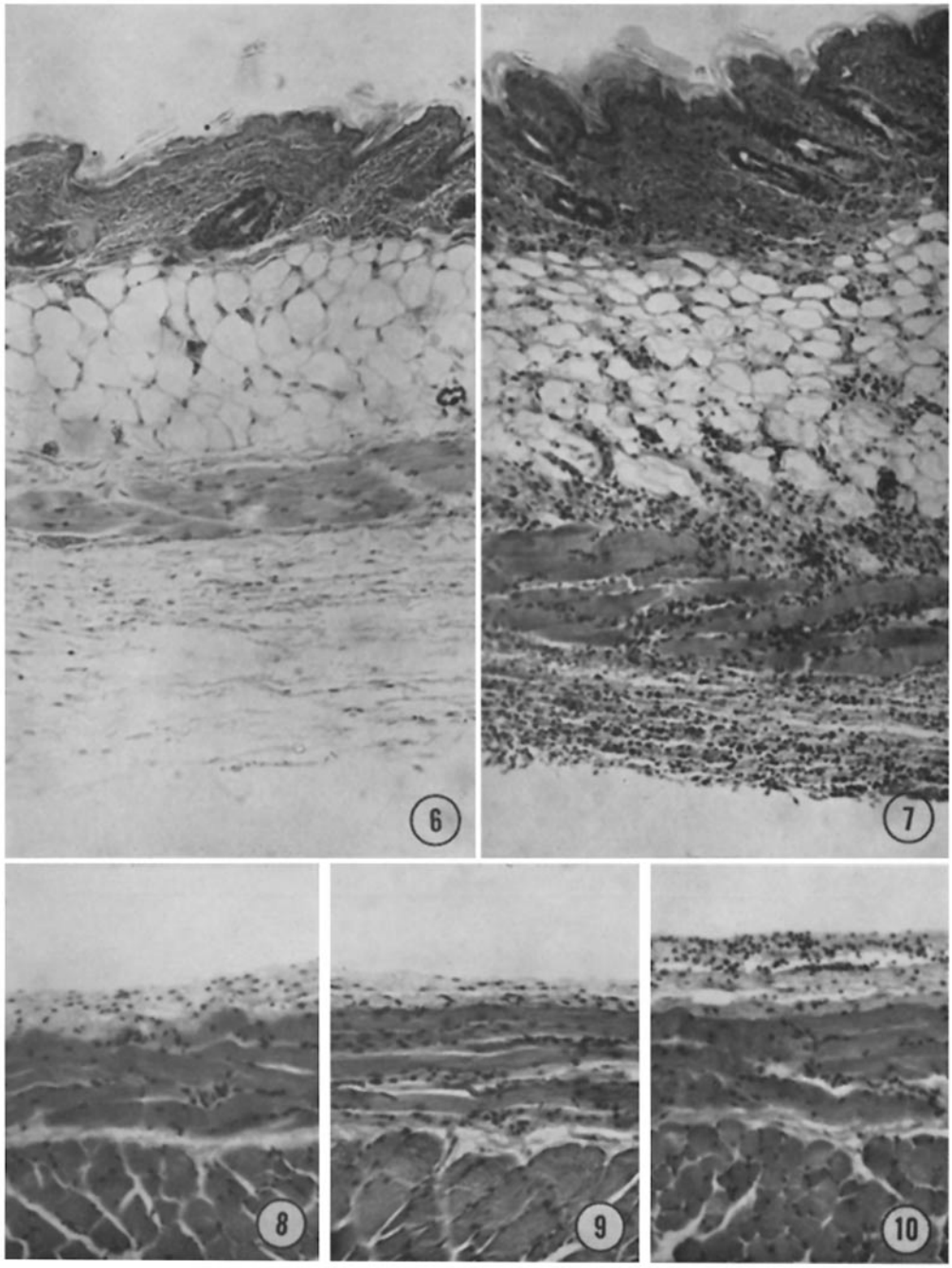
FIG. 5. Mice with part of their subcutaneous expanses laid open to show the vascular effects of having split them 24 hr before. For this photograph, they had been anesthetized with sodium pentobarbital and had received an intracardiac injection of 0.3 cc India ink. Splitting with air and Earle's solution (E soln) caused no discernible hyperemia. Splitting with air and E soln which included lipopolysaccharide (endotoxin) from *S. typhosa* caused a considerable one. Expanses equivalent to the one shown in the middle mouse gave the greatest number of tumor "takes" in Experiment 4 (see Fig. 3). × 0.8.


Not Split
Split with
ENDOTOXIN
in E soln
Split with
E soln
5

FIGS. 6 and 7. The full thickness of dorsal "roof" created by splitting the connective tissue between the pelt and the body wall, as described in Materials and Methods. The fascia, shown here adhering beneath the panniculus carnosus, is similar to its deeper part, shown in Figs. 8, 9, and 10, which continued to adhere to the muscles of the body wall. These are the connective tissue expanses onto which plated tumor cells would be scattered. Fig. 6 shows their appearance 24 hr after having been separated with air and E soln. They are thickened and loosened by edema but hardly more cellular than usual. Fig. 7 shows their appearance 24 hr. after having been separated with air and E soln containing 50 μg endotoxin. The edema is no greater but the population of cells is. These changes affect the areolar tissue between the dermis and the dermal muscle too, but only incidentally to the present study. × 127.

FIGS. 8, 9 and 10. The "floor" corresponding to the roof depicted above. The connective tissue layer defining the dorsal aspect of the trunk, shown here, and that underlying the pelt, shown in Figs. 6 and 7, were continuous in the intact mouse. These two sets of photographs thus show the two connective tissue expanses prepared by splitting as described in Materials and Methods. The layer in Fig. 8 was separated 24 hr before by splitting with air and E soln. It is thickened in comparison with that in Fig. 9 which was not prepared at all. For Fig. 9 the dorsal skin had been gently lifted off the underlying trunk postmortem to expose the normal investment of connective tissue shown here. The connective tissue layer in Fig. 10 was separated by splitting 24 hr before with air and E soln containing 50 μg endotoxin. It is thickened and contains many more cells than the E soln specimen of Fig. 8. Like its counterpart in Fig. 7 it represents the expanses giving the greatest number of tumor takes in Experiment 4. × 127.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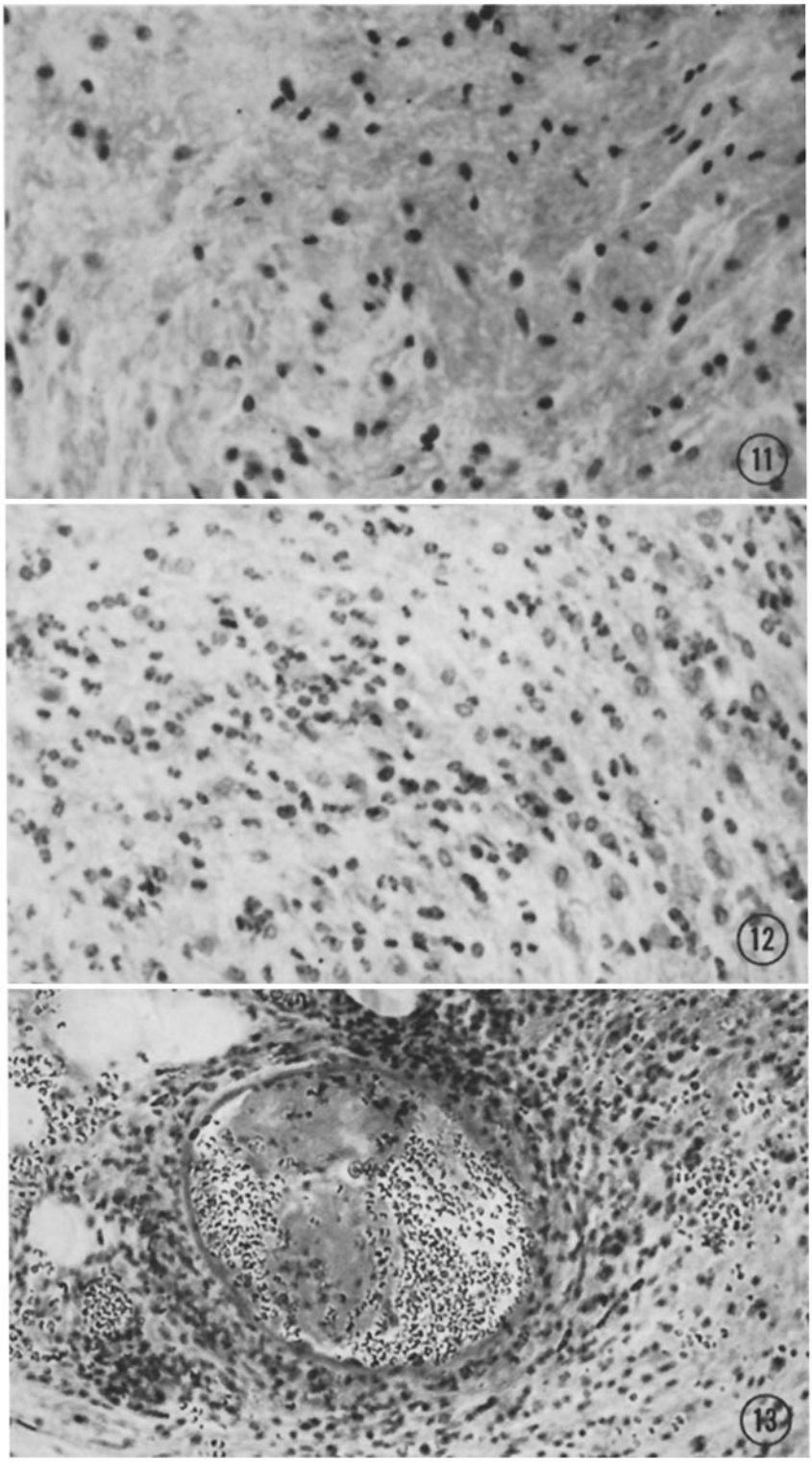
FIGS. 11, 12 and 13. Sections of connective tissue removed, by blunt dissection, off the subcutaneous expanses of mice, 24 hr after their preparation. Fig. 11 shows, in tissue split with air and E soln, the cells a little plumper than those inhabiting the scanty specimens which can be dissected from these parts in intact mice. × 425.

FIG. 12 shows, in tissue split with air and E soln containing 50 μg endotoxin, a considerable increase in the size of this cell population and in the size of its individual members. This polyblastic response is obscured by an immigrant population of neutrophil leukocytes. The totally polymorphonuclear character of the latter indicates that they are all of the same old age. × 425.

FIG. 13 shows, in tissue split with air and E soln containing 50 μg endotoxin, a vein with leukocytes throughout and around its wall. It is partly occluded by recently formed thrombus. Red blood cells have spilled into the tissues. Vascular changes of this sort are not found in tissue split in the absence of endotoxin. × 300.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