A BACTERIOLOGICAL STUDY OF MOUSE TUMORS

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There have been numerous descriptions of the finding of microorganisms in tumors of human, animal, and vegetable origin. A certain divergence exists in the types of bacteria which have been found, and in the interpretation of the significance of their presence. Many have sustained the idea of an etiological relationship between bacteria or microparasites and tumor proliferation. More often the organisms have been regarded as coincidental, or secondary to changes in the tumor or in its overlying surface, or as casual invaders, which, in the case of transplantable tumors, were most probably introduced during one or more of the operations of transplantation, and, as the case might be, were individual to one group of animals or propagated from one generation of tumors to another. As such they might be pathogenic for the animals or carried as harmless saprophytes possibly pathogenic for other species.

The older literature has been amply reviewed by Lewin. Kauffmann, in a series of reports, has described his success in isolating from the Ehrlich mouse carcinoma various strains of a T-bacterium, which he believes to be related to the \textit{B. tumejaciens} of Smith. This organism he discovered in 50 per cent of the tumors; 30 per cent of the tumors showed a banal flora, and 20 per cent were sterile. Kauffmann has studied the biological characteristics of the various T-bacteria with which he was able to produce growths, considered by him to be infectious granulomas, in sunflower and sugar-beet plants, but which in general were non-pathogenic for animals. Other studies have demonstrated that \textit{B. tumejaciens} apparently has no etiological relationship to malignant growth in animals (Borghi and Luzzatto).

Ebert, Jolkiewitsch and Ssobolewa have found large numbers of various types of cocci and bacilli in growths produced by tar in white mice. These authors would attach a certain importance to differences in the flora in different types of proliferation, but it seems difficult to ascribe to such bacteria more than casual importance depending on the specific type of treatment and the accompanying infection. Ikeda, studying the Ehrlich mouse carcinoma and a mouse sarcoma, has found in all but 2 per cent of the tumors bacteria of the coli, paracoli, cloacae, and aerogenes groups; 1 to 2 per cent of the bacteria found showed characteristics similar to \textit{B. tumejaciens}. None of the bacteria examined caused growths in either plants or animals. Arima, working with the Fujinawa sarcoma of the rat and a rat carcinoma, described the isolation of the typhoid bacillus of the mouse from all of the sarcomata and from 3 to 43 carcinomata. The organism was pathogenic for the animals. Sarcoma caused by \textit{Cysticercus fasciolaris} and the Tsunoda sarcoma did not show this bacillus.
Centanni, however, working with an adenocarcinoma of the mouse, has maintained that the tumors are sterile in all but 2 to 6 per cent of the cases, the higher figure corresponding to more advanced states of tumor growth. He has stressed the importance of examining only those tumors with an intact overlying skin, for with subsequent ulceration there is an obvious secondary invasion by many types of bacteria. Shwartzman incidentally has noted that in series of mice with sarcoma 180, despite the occurrence of a spontaneous infection of the animals with \textit{B. enteritidis}, a moderate number of the tumors were sterile, although many showed this microorganism and, rarely, various other bacteria in the tumors, in the body fluids, and in the liver.

**Bacteriological Examination of the Ehrlich Mouse Carcinoma**

As a preliminary to other experiments it has been deemed necessary to examine bacteriologically the tumor used, in this case the Ehrlich adenocarcinoma of the mouse. To obtain results which permit of a certain evaluation, two factors are of extreme importance: the selection of the tumors and the technic. Only those mice were chosen which, apart from the tumor, were in healthy condition, and in which, unless otherwise indicated, the skin covering the tumor was apparently intact and normal. Those tumors with infection of the transplant wound have been avoided, as well as those with excessive distention of the overlying skin caused by expansive growth of the tumor, for in such cases microscopic breaks in the skin may be present through which bacteria can penetrate with ease. Also, when possible, tumors in contact with the peritoneum have been excluded, for excessive expansion in this case could result in secondary infection from the intestinal tract. Transplants have usually been made in the subcutaneous tissue of the subaxillary or lateral thoracic region.

**Technic:** In the beginning the choice of a proper technic caused some difficulty. The first method used was as follows. After rapid washing of the entire mouse in 90 per cent alcohol and dissecting away of the overlying skin, the entire tumor was removed under aseptic precautions, placed in a mortar, and ground with powdered glass; 10 c.c. of sterile physiological saline were added and the resulting suspension was inoculated into nutrient broth. Seventeen tumors were examined in this way, but there arose a suspicion as to the validity of the results when in the cultures from all of the tumors examined there were found after twenty-four-hour incubation large numbers of staphylococci and at times large Gram-positive bacilli of the \textit{B. subtilis} group. The technic was controlled by similar cultivation of normal organs and tissues, liver, kidney, heart, muscle, and fat, and the findings were similar to those obtained in the examination of the tumors. Obviously the error lay in the technic, for with the manipulation incidental to this method contamination may easily occur despite the most careful aseptic precautions. This method was therefore discarded.

It was then attempted to mince or cut the tumor or tissue after removal from the body in a sterile Petri dish with the cover opened only as much as was necessary. The pieces were then directly implanted in nutrient broth. The results were similar to those obtained with the first technic.
Finally, it was decided to transfer small pieces of the tissues and of the
tumor with the aid of a platinum loop directly into the culture medium, with
no further manipulation than cutting the required piece \textit{in situ}. Control
cultures of normal tissues thus obtained have been sterile, and the method
has therefore been used in the examination of the series of tumors. The
mouse is killed by a blow on the head, then washed rapidly in 90 per cent
alcohol. The following steps may be carried out under a glass hood to insure
against contamination from the air. The skin overlying the tumor is dissected
away by means of an incision made at a considerable distance from the mass.
The instruments used in this operation are discarded. The external surface
of the tumor is touched with a heated metal plate to kill bacteria which may
possibly have penetrated superficially through the very thin skin or through
invisible breaks. With a scalpel an incision is made into the tumor, and with
the aid of scissors and forceps pieces of tumor tissue, approximately 0.5 to
1.0 cm. square, are cut from different portions of the growth. With the
platinum loop these pieces are transferred immediately to the culture media.
The fluid medium penetrates rapidly into the piece of tumor, which becomes
partially disintegrated. This is shown by the clouding of the liquid due to
the numerous small particles of tumor tissue which have become suspended in
the broth.

Cultures have been made in nutrient broth and in other media as stated,
and incubated at 37^\circ\ C. for forty-eight to seventy-two and in some cases
ninety-six hours. From two to ten pieces of tumor have been incubated in as
many tubes of broth, depending upon the size of the tumor and the ability to
remove the necessary amount with the least possible manipulation. In all, 52
tumors varying from ten to twenty-one days in development and from 28 dif­
ferent lots have been examined.

\textit{Results of the Bacteriological Examination of the Tumors}

1. Thirty-six tumors were sterile; from 5 of these, anaerobic cultures were
likewise sterile.

2. Two tumors showed \textit{Staphylococcus albus}.

3. One tumor showed \textit{Staphylococcus albus} and in addition a large number
of Gram-negative motile bacilli (flora of intestinal origin?). There was a
possibility, in this instance, of contamination through a hole inadvertently
made in the peritoneum when the culture from the tumor was made.

4. Three tumors, as well as one control inoculated with 0.2 c.c. of sterile
physiological saline, showed a Gram-negative, delicate looking spirillum with
two to three spirals and with a variable motility. The growth in broth was
slow, but in forty-eight hours there was a moderate turbidity. Colonies on
agar were white, raised, oval or round. The microorganism after successive
transplants ceased to grow on artificial media. The use of serum-agar did
not permit the strain to be maintained.

5. Two ulcerated tumors, one in direct smear examination and the other
in culture, showed a large number of Gram-negative bacilli which in broth
were actively motile (flora of intestinal origin?).

6. One tumor showed a slight growth in seventy-two hours of a small
Gram-positive streptobacillus.
7. One tumor showed Gram-negative non-motile bacilli.
8. During the course of the work an obvious infection of five successive lots of tumors occurred. The same phenomenon with a similar course has been noted on another occasion. The cause is unknown; possibly it lies in the use of an occasional tumor containing bacteria as the source of material for the transplant, or to an error in the technic at the time of transplantation, or to the use of inadequately adapted mice—those of a different strain or animals that are too small and in which susceptibility to infection of the transplant is most probably greater.

It is quite probable that during the operation of transplantation, despite the usual aseptic precautions, a varying number of microorganisms is introduced, but that ordinarily with the natural tissue immunity these are destroyed. In such cases the tumor develops free from bacteria. If the contrary is true, infected tumors may result. If the infection, especially with staphylococci, is pronounced, there may be accompanying signs. Such tumors are too large for their age, are inclined to be rounded in a regular fashion; the overlying skin is tense and reddened. The transplant wound is or has been infected; small abscesses may be present in the tissue adjacent to the tumor or, as was observed in a few cases, there is a tendency to the formation of a small abscess within the tumor itself. The tumor usually is gray-white, granular, and extremely friable.

Six of such tumors have been examined, at least one from each of the five infected lots. All were positive for *Staphylococcus albus*. In addition two tumors in direct smear showed a large Gram-positive bifid bacillus, which, however, did not grow in broth, glucose broth, or glycerine broth.

In each lot of these infected transplants the tumor failed to take in a certain number of mice, perhaps 10 to 15 per cent. Three to five days after the transplantation a small abscess formed in the area of the transplant. This opened spontaneously, discharged its contents, and with the healing of the wound the animal remained in normal condition. It is possible that in these cases the infection completely destroyed the viable tumor cells. In the majority of cases, however, the tumor continued to grow well from one generation to another despite the type of infection which it harbored.

It is interesting to note that with each successive transplant the infection tended to subside of its own accord, and that at the sixth transfer the tumor returned to its original sterile state, to continue so in further transplants.

**Pathogenicity of Several of the Isolated Bacteria for Normal Mice**

*Spirillum of Group 4*: A forty-eight-hour sub-culture of the original culture from the tumor was centrifuged until the broth became clear. The sediment was resuspended in 2 c.c. of physiological saline and 0.4 c.c. of this suspension was injected into each of 5 normal mice, in 4 subcutaneously and in 1 intraperitoneally. The latter mouse died on the third day, but autopsy revealed no signs of an infection. Possibly the death was independent of the injection, for the latter was without effect in the other animals.

*Staphylococci from Tumors in Group 8*: Subcutaneous injection of 0.1 c.c. of the twenty-four-hour broth culture from each of the 6 tumors produced small abscesses, except in the case of one culture, which was without effect.
These abscesses opened spontaneously and subsequent healing was complete. The same staphylococci, however, were non-pathogenic for mice injected with the same amount intraperitoneally, although one of these mice died on the seventh day after the injection. Autopsy of this mouse revealed no evidence of infection. One has again an indication that the pathogenicity of a microorganism depends, at least in part, on a variable susceptibility of different tissues.

**Effect of an Induced Bacterial Infection on the Ehrlich Carcinoma**

The study of the effect on malignant tumors of microorganisms and their products, or of an aseptically produced inflammation, is not a new one. It is unnecessary to review the older literature dealing in great part with isolated cases of human cancer treated apparently successfully with induced bacterial infection or with bacterial toxins. These various reports, as well as the subsequent studies of experimental tumors in animals, give the idea of a search for the possibility of injuring selectively the malignant cells, thus causing regression and disappearance of the tumor or at least an inhibition in the rate of growth. An important aspect of the problem is the attempt to find an agent which will act so as to produce regressive changes in the tumor, but will spare absolutely or at least relatively the normal tissues. From a different standpoint there arises the entire question of the relation of various types of inflammatory reaction to neoplastic proliferation.

One is inclined to surmise that the effect on tumors of bacteria or of their products is a direct one, on the malignant cells and the tumor stroma. With this in view, various bacteria or their toxins have been injected directly into tumors or into the general circulation. There are indications pointing to an increased sensitivity of tumor cells to extraneous influences; other factors indicate a greater lability of the newly formed blood vessels of the tumor stroma, so that with rupture of these vessels secondary degenerative changes in the tumor cells may be produced.

Centanni and Rezzesi noted an inhibitory effect of *B. tuberculosis* on the growth of an adenocarcinoma of the mouse. Torrey and Kahn attempted treatment of the Flexner-Jobling rat carcinoma with cultures and filtrates of the proteolytic anaerobes, *B. sporogenes* and *B. histolyticus*. Injections of large doses of *B. sporogenes* were without effect. Cultures and filtrates of *B. histolyticus* injected directly into the tumors apparently caused regressive changes with ultimate disappearance of the tumors in a moderate number of rats.

Lazzarini found that injections of *Sporotrichum bcurmanni* did not affect the development of tar cancer in mice. Comsia noted an inhibitory effect of the spirochete of recurrent fever on the growth of the Ehrlich mouse carcinoma. Karczag, Csaba and Nemeth described a similar effect of *Trypanosoma equiperdum*. Roskin and Romanowa reported retardation of growth of a carcinoma of the mouse following injection of *Schizotrypanum cruzi* or of its endotoxin. The spirochete of recurrent fever and the endotoxin of *B. coli* were without effect. *Trypanosoma equiperdum* was inactive against a guinea-pig tumor.
Carminati obtained inhibition of growth of the Ehrlich mouse carcinoma following intraperitoneal injections of *Thermobacterium bulgaricum*.

Gratia and Linz in their studies of the Schwartzman phenomenon found that they could elicit hemorrhage and necrosis in a liposarcoma of the guinea-pig by simple intravenous injection of a filtrate of *B. coli*.

Shwartzman and Michailovsky described hemorrhages and regressive changes of sarcoma 180 of the mouse after intravenous injection of meningococcus filtrate. Shwartzman reported regression and disappearance of the same tumor in animals infected spontaneously with *B. enteritidis*, as well as after intravenous injections of cultures and filtrates of this bacterium. Cultures and filtrates of *Staphylococcus aureus* had no effect.

Duran-Reynals, studying mouse sarcomata, mouse carcinomata, and the Walker rat sarcoma, found hemorrhage and regression only in the large, more rapidly growing tumors after intravenous and intraperitoneal injection of filtrates of *B. coli*. Later studies of the effect of filtrates of *B. typhosus* and of mouse typhoid on spontaneous carcinoma of mice showed an inhibition rather of smaller tumors, although the original reaction was more intense in the larger ones.

Apitz produced hemorrhages in the Ehrlich mouse carcinoma with intravenous injection of filtrates of *B. coli*. Snake venom caused a fatal hemorrhagic diathesis but no change in the tumor. Tumor hemorrhages were also obtained after injections of agar, antigen-antibody mixtures, and anti-mouse serum.

Shear also observed hemorrhage and recession of mouse sarcoma 180 following intravenous injection of meningococcus filtrates, although injections had no effect on spontaneous mammary carcinoma. *B. coli* filtrate affected a large number of cases of sarcoma 180.

Jacobi used filtrates of *B. typhosus* in mouse sarcoma 180, the Flexner Jobling rat carcinoma, the Walker rat carcinosarcoma 256, and the Rous chicken sarcoma. Injections were first made into the tumors, followed in twenty-four hours by intraperitoneal injection in the mice and rats and intravenous injection in the chickens. Hemorrhage in the tumors with ultimate disappearance of the growths resulted. Simple intratumoral or intraperitoneal injection was without effect.

Pommerenke found that injection of filtrates of *B. histolyticus* grown in a suspension of the Brown-Pearce tumor had no effect upon the course of the tumor in other rabbits.

As to the effect of aseptically produced inflammation upon tumors, it has been demonstrated that oil of turpentine and diatomaceous earth have but little influence upon their rate of growth (Kubo, Lattes).

From these diverse experiments one can note definite results which indicate a deleterious action exerted by various microorganisms or their products upon tumor growth. These effects have been observed especially in transplantable neoplasms, of the carcinomatous as well as of the sarcomatous type.

*Experiments with B. danysz*: In a series of experiments with mice bearing the Ehrlich carcinoma further studies of this problem have been made by the author of this paper, using one of the Salmonellae, *B. danysz*. This organism is extremely pathogenic for mice; without exception, subcutaneous or intra-
peritoneal injection of 0.05 or 0.1 c.c. of the suspension made from a twenty-
four-hour agar-slant culture with 5 c.c. of physiological saline or the same
quantity of a twenty-four-hour broth culture proves fatal in eighteen to forty-
eight hours. There results a septicemia, with the presence constantly of \textit{B. danysz}
in the culture from the heart blood. The bacillus conserves its patho-
genicity, although this is slightly diminished, when cultivated for long periods
on artificial media. In addition to the septicemia, it tends at times, especially
when its virulence is decreased, to cause an acute purulent peritonitis when
injected intraperitoneally, and a small local area of acute inflammatory re-
action with necrosis when injected subcutaneously.

Injections of \textit{B. danysz} were made directly into the tumors at various
stages of their development, from the 12th to the 23rd day. Without excep-
tion, these animals died with the presence of a positive blood culture, tumor-
bearing animals usually succumbing sooner than controls injected with a
similar amount of bacteria. In addition to the study of the effect of \textit{B. danysz}
on the tumor, studies have also been made of the behavior of the bacillus as well as of the tumor, after successive inoculation into the tumor, cultivation of the bacillus from the tumor, reinoculation into another tumor, cultivation, etc., for seven passages. In this way, the bacillus is obliged to
live in contact with tumor tissue for many generations, either directly when
in the tumor, or indirectly when cultivated in broth for twenty-four hours in
the presence of a piece of tumor tissue. The suspension of bacteria was in-
jected into each of three tumors. After twenty-four hours one animal was
sacrificed, culture from the tumor was made in broth, incubated for twenty-
four hours; with this culture a new series of tumors was injected, then a new
culture from a tumor was made, etc. Controls were injected subcutaneously
and intraperitoneally with a similar amount at various steps, and with the
culture obtained at the seventh passage. Naturally, in such an experiment
there are variable factors, differences in susceptibility of different animals,
differences in the size of the tumors, and variation in the amount of tumor
tissue taken for the culture.

In all cases the animals were killed when very sick, the entire tumor was
removed, sectioned completely, and fixed in Duboscq-Brasil fluid. The effects
of the injection were studied histologically in the entire cross-section of the
tumor through its greatest diameter. A small number of animals, unfortunately, died during the night, but the tissues were removed and fixed at the
earliest possible moment. In those animals from which cultures from the
tumor were made, one half of the tumor was used for the culture and the other
half retained for further study. In all, there were 57 tumors in which injec-
tions with bacteria were made.

The injection into an Ehrlich mouse carcinoma of 0.1 c.c. of a suspension
of \textit{B. danysz} made as previously stated, and for convenience of technic diluted
with 0.1 to 0.4 c.c. of physiological saline, produces definite changes in the
structure of the tumor. Grossly, the cross-section of such a tumor usually
shows a reddish or reddish-gray hue, although at times it may present the
gray-white color of untreated cases. Microscopically there are marked
changes. There is a striking hyperemia due to dilatation of the peripheral
as well as the central capillaries. This is in contrast with control tumors, in
which the blood vessels are not especially evident. There is no further discernible reaction of the sparse tumor stroma. However, with this hyperemia there are pronounced regressive changes. In untreated Ehrlich carcinoma regressive change is limited strictly to a greater or lesser area of the central portion, which is well demarcated from the normal wide peripheral band of tumor tissue in active growth. The areas of necrosis and degeneration tend to be well demarcated from the healthy portions. In those tumors treated with \textit{B. danysz} there is a decided increase in the amount of regressive changes, both internally and peripherally, as well as in the areas about the blood vessels, which are usually well conserved in untreated tumors. This process may be almost total, in some cases with practically complete absence of normal tumor tissue. In many tumors large areas are converted into masses of isolated cells with pyknotic nuclei in various phases of degeneration. There may exist portions of apparently spared tumor tissue at the periphery, but upon closer examination these are shown to consist of tumor acini or of smaller groups of cells irregularly mixed with other cells in various stages of degeneration, as shown by the small pyknotic nuclei. In such areas mitotic figures, as well as in the apparently unaffected tumor cells, are either absent or are rare. There is no tendency to demarcation of the necrotic from the healthy tumor tissue, but rather a continuation of the areas of regression or of necrosis to the peripheral portions, where they become insinuated between groups of normal-looking acini. These areas are perhaps to be interpreted as a partial or initial regression. In some tumors there may exist peripheral areas which are of normal appearance and in active proliferation. A number of tumors show little or no effect. As serial sections of entire tumors have not been made, no opinion can be expressed as to the condition of all portions of the tumors. However, the sections as made give a good general impression of the state of the tumor tissue, especially on comparison of the findings with those in untreated controls or in the four controls injected with a similar quantity of sterile physiological saline instead of the bacterial suspension. One of the latter controls did show a small hemorrhagic area in the center of the tumor, probably caused mechanically, but otherwise the condition of this tumor was unchanged.

It is worthy of note that a number of tumors, unfortunately, showed in microscopic sections small groups of staphylococci, as they came from lots of tumors spontaneously infected with that microorganism. But the regressive changes in those tumors treated in addition with \textit{B. danysz} cannot be attributed to the presence of these staphylococci, for four controls spontaneously infected with staphylococci, but not injected with \textit{B. danysz}, or injected with staphylococci alone, showed no greater degree of retrogressive change than was seen in non-treated sterile controls.

The effect of the injection of \textit{B. danysz} in this group of tumors may be summarized as follows:

Marked effect in ........................................ 12
Moderate effect in .................................. 8
Slight effect in ....................................... 6
No effect in ........................................... 9
As has been stated, studies have also been made of the possible variations in pathogenicity of *B. danysz* when it has been in contact with the tumor tissue for a prolonged time. Tumor proliferation, although it depends to some extent upon general conditions of the body, does show characteristics which have caused it to be styled as autonomous. A transplantable tumor is perhaps something still more extraneous, developing as it does in the body of a host from homologous cells which have been introduced from the tumor of another animal. As is already known, however, bacteria grow excellently in tumor tissue. In addition, the tumor, being poor in stroma and always containing necrotic portions, would seem to offer little possibility of defense to pathogenic bacteria, so that it is reasonable to expect an increased virulence of a microorganism after repeated passages in tumor tissue. There is this source of error; as *B. danysz* also invades the blood stream when injected into a tumor, the culture which one makes from a tumor may represent, in part at least, bacilli which come from the blood vessels of the tumor or which have reentered the tumor from the blood stream.

For the successive steps of this experiment, performed as previously described, one-half of the former dose was employed, of the original saline suspension of the bacteria as well as of the broth culture obtained by cultivation from the inoculated tumors. With this smaller dose the animals lived somewhat longer, from a minimum of twenty-four hours up to sixty or seventy-two hours. However, with the successive passages in the tumor there is no definite change in the virulence of the microorganism, for the animals continue to die within approximately the same range of time. Controls inoculated subcutaneously with the original saline suspension died in sixty-five and one hundred and fifteen hours and with the culture obtained after the last passage in the tumor in eighty and ninety hours.

The effect on the tumors has been studied as previously. With the smaller dose this effect was less pronounced. The hyperemia is less intense and the regressive changes are not so extensive. The results may be summarized as follows:

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<th>Count</th>
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<tr>
<td>Marked effect in</td>
<td>5</td>
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<tr>
<td>Moderate effect in</td>
<td>6</td>
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<tr>
<td>Slight effect in</td>
<td>7</td>
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<tr>
<td>No effect in</td>
<td>4</td>
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As to the effect of *B. danysz* on the organs of the body, this has been studied in the liver, spleen, and kidneys of those animals in which injection had been made into the tumor, as well as in the controls. There were observed the general changes of an acute infection, parenchymatous degeneration of the liver cells and of the cells of the renal tubules, rarely with slight proliferation of the cells of the glomerular tufts. A moderate hyperemia occurs in the liver and kidney and usually a more marked hyperemia of the splenic pulp, but none of the organs show areas of necrosis. In addition, in those controls injected subcutaneously and in which there is a local abscess formation, there is demonstrable microscopically an intense acute inflammatory reaction with small areas of necrosis.

Experiments are being conducted by the author which demonstrate that a sterile filtrate of cultures of *B. danysz* has the capacity to produce local
hemorrhagic lesions, of the type described by Shwartzman, in the skin of rabbits. This work as it concerns tumors will be reported later.

**SUMMARY**

1. The Ehrlich mouse carcinoma in a large proportion of cases is sterile. Excluding 6 obviously infected tumors and 2 ulcerated ones, 36 (81.8 per cent) of 44 tumors showed no bacterial growth. The positive cultures from the 8 apparently normal tumors, as well as from one injected with sterile saline, showed no constant types of microorganisms, but instead diverse types, so that further investigation of such casual invaders was not considered to be of importance.

2. The injection into the Ehrlich mouse carcinoma of a microorganism, *B. danysz*, which is highly pathogenic for mice, produced hyperemia and regressive changes in the tumor. There was no change in the virulence of the bacillus after successive injection into the tumors, cultivation from the tumors, reinoculation, etc., for seven passages.

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