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RELATIONSHIP BETWEEN TUMOR GROWTH AND THE ENVIRONMENTAL CONNECTIVE TISSUE

I. INFLUENCE OF THE CONNECTIVE TISSUE ON THE DEVELOPEMENT OF TUMORS
II. INFLUENCE OF THE CONNECTIVE TISSUE ON THE CHEMOTHERAPEUTIC EFFECTS
III. INFLUECNCE OF THE CONNECTIVE TISSUE ON THE METASTASIS

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By experimental work the connective tissue was studied whether it infiuences on the tumor developement, metastasis and also the effect of anticancer chemotherapy. Ascites Hepatoma 130 and Yoshida Sarcoma was transplanted to the abdominal cavity or subcutaneous space of wistar rats which were treated with chondroitin sulfate, parotin, licopodium, hyaluronidase or α-chymotrypsin. As anticancer agents mitomycin-c and nitromin were used. For the conclusion one hypothesis was considered that the immature type of the connective tissue gave good circumstance to the developement and metastasis of the tumor, whereas it gave good regression of the tumor at administration of anticancer agents. Contrarily the old type of the connective tissue environing tumor cells depressed the developement as well as metastasis of the tumor, whereas the effect of chemotherapy was disturbed.

I. Influence of the Connective Tissue on the Developement of Tumors

The inflammatory response in the stroma lies in all organs of the body being injured by a wide variety of means, whose morphologic manifestations have been well described.[1] However the host defensive activities to neoplasma have not been fully understood. There have been many descriptions that the connective tissue acts as one of biophylaxis to the tumor growth, whereas not few workers denied the defensive reaction of the connective tissue against the tumor developement, and they thought that the connective tissue was introduced only as the secondary reaction of the tumor growth. For example, Vasilief suggested that epithelial invasion, both malignant and nonmalignant, was preceded by the formation of an immature connective tissue bed,[2] which was not agreed by Imai, who enforced the connective tissue as the host defensive reaction to neoplasma.[3] The other thoughts were like that the scar formation of the connective tissue environing the tumor mass depressed the

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tumor developement mechanically,[4] or that the round cell infiltration had antitumor activities.[5] The recent progress in the field of biochemical and histochemical studies about the connective tissue impressed us that it should be considered from many standpoints depending on various sites of the connective tissue to investigate the influence on the tumor developement. This attempt was made, using histological criteria, to evaluate the connective tissue to the transplantable tumor, Ascites Hepatoma 130(AH 130) and Yoshida Sarcoma.

MATERIALS AND METHODS

Gifu Wistar male rats weighing between 130 and 150 g were used in all experiments, and there were ten rats in each group, unless stated otherwise. All rats was kept on a standard pellet diet with drinking water. Ascites Hepatoma 130 (AH 130) and Yoshida Sarcoma which were introduced from the Second Department of Pathology, Nagoya University School of Medicine respectively, was prepared for tumor cell suspension in Ringer's solution.

I.

1) 1×10^7 cells of AH 130 were transplanted into the subcutaneous space of the right side back of rats by using 1/5 gauge needle. The first group of rats was not treated by any drug taking as controls. Four mg per 100 g body weight of chondroitin sulfate produced by Kaken Yakka Kogyo Co. Inc. was started by daily intraperitoneal injection 2 days prior to the tumor cell inoculation for 5 days. All of the following drugs were injected daily into the abdominal cavity for 5 days starting 2 days prior to the tumor cell inoculation. The third group was treated with 0.6 mg per 100 g body weight of parotin which was manufactured by Teikoku Zoki Seiyaku Co. Inc. Tumors of the 4th group were introduced by inoculation of tumor cell suspension mixed together with licopodium powder. The 5th group was treated with hyaluronidase 20 units per 100 g body weight obtained from Mochida Seiyaku Co. Inc. and the last group was injected α-chymotrypsin from Eizai Co. Inc. for the dose of 1 unit per 100 g body weight.

Five days after tumor cell inoculation, tumors became noticeable size at the subcutaneous space on palpation, when started the daily measurement of the tumor size up to the 16th day after the tumor cell inoculation, and the mean size of each group was shown in Fig. 1.

2) 1×10^7 cells of Yoshida Sarcoma were transplanted into the subcutaneous space of the right back of rats. Like the case transplanted AH 130, the first group was taken as controls, and the following each group was treated with chondroitin sulfate, parotin, licopodium, hyaluronidase or α-chymotrypsin, and the dosage was same as described above. The administration of them was started intraperitoneally 2 days prior to the tumor cell inoculation for dura-

tion of 5 days daily, and the tumor size was followed 5 days after the tumor transplantation up to the 16th day.

In cases of both AH 130 and Yoshida Sarcoma, after the 17th day it was stopped to record the tumor size because that the tumors started by that time marked central necrosis with their frequent rupture outside of the skin causing inadequate results for further comparison.

II.

1) Rats were divided into 6 groups, all of which were inoculated 1×10^7 of Ascites Hepatoma 130 cell suspension in Ringer's solution into the abdominal cavity by using 1/5 gauge needle. The first group was taken as controls, and the following groups were treated daily with above noted drugs starting 2 days before tumor cell inoculation for 5 days by subcutaneous injection. The dosage of the drugs was the same as in case of I., that is, chondroitin sulfate (4 mg per 100 g body weight), parotin (0.6 mg per 100 g body weight), hyaluronidase (20 T.R.U. per 100 g body weight), or α-chymotrypsin (1 unit per 100 g body weight). The 4th group of rats was inoculated into the abdominal cavity by tumor cell suspension mixed with licopodium powder. The surviving time of each rat was recorded and shown in Fig. 3 as noted by surviving line of each group.

2) 1×10^7 cells of Yoshida Sarcoma in Ringer's solution was transplanted into the abdominal cavity of rats, which were divided into 6 groups performed the same treatment as noted above. The surviving time of each group was

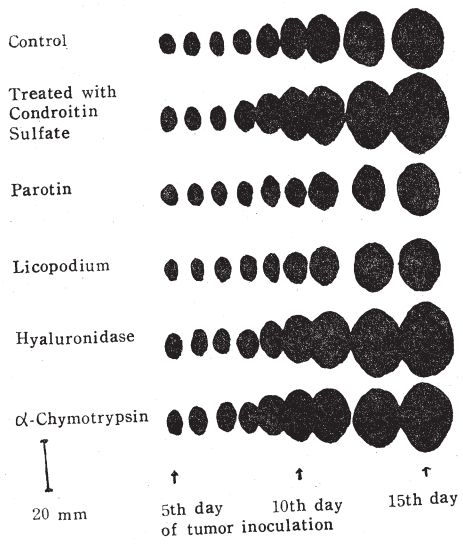


FIG. 1. Mean Size of AH 130 Transplanted into the Subcutaneous Tissue of Rats.

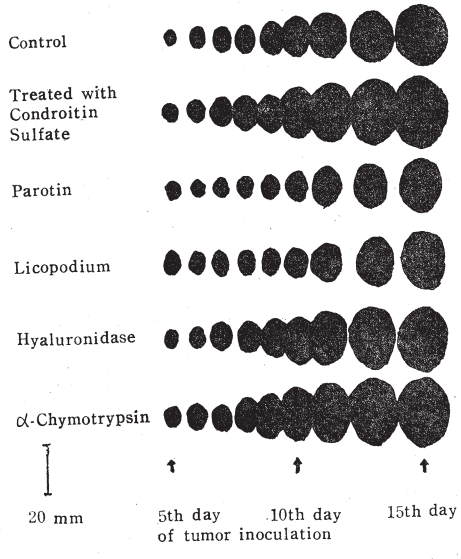


FIG. 2. Mean Size of Yoshida Sarcoma Transplanted into the Subcutaneous Tissue of Rats.

shown in Fig. 4.

III.

1×10^7 of AH 130 or Yoshida Sarcoma cells were transplanted into the subcutaneous tissue of rats, and chondroitin sulfate, parotin, hyaluronidase, or α-chymotrypsin was administered to each group of rats with the same dosage and the same way as noted in case I. One group of rats was inoculated also tumor cell suspension mixed with licopodium powder. Rats of each group were killed on the 5th, 10th and 15th day of the tumor inoculation by ether inhalation, and tumor masses grown in subcutaneous tissue were removed and kept in 10% formalin after recording the size and the weight. Serial sections were made from all these specimens being stained by haematoxylin and eosin stain, silver impregnation method, periodic acid-Schiff (PAS) method and toluidin blue metachromasie stain (TBM). An effort was made to get sections of all areas of tumor mass as possible, because the sites of the tumors showed many variety depending on the location.

RESULTS

I. Size and gross features of transplanted tumors

1) Three days after the tumor transplantation most rats showed palpable tumor masses on the back counted mostly one or rarely two or more which size was rather variable depending on the individual. By the 6th day the size became comparatively constant among each group as shown in Fig. 1. figured by the mean size. Generally solid type tumors of AH 130 tended to show central necrosis from the early days many of which formed like a cyst, and after 2 weeks of tumor inoculation these cyst-like tumor masses started to rupture in the skin followed by spontaneous cure of tumors. Comparing with controls the second group treated with chondroitin sulfate showed rapid growth from the beginning, however the rupture of the tumor by developing central necrosis was less frequent. The 3rd group which was treated with parotin did slow growth, of which consistency was somewhat hard comparing with controls, and the cyst formation or central necrosis was not so marked. Tumors introduced by local inoculation of licopodium mixed together with cell suspension started almost the same size of controls at the beginning, though the developement of the tumor size was slow. On the second week of tumor transplantation was noted frequent developing projection from the original lesion forming a bud like shape which grown rather rapidly and mostly seen in the 3rd and the 4th groups. The 5th group treated with hyaluronidase made a rapid growth with marked central necrosis followed by frequent rupture from the early days. α-Chymotrypsin also made a rapid developement of transplanted tumor growth. From these results the administration of chondroitin sulfate, hyaluronidase as well as α-chymotrypsin introduced an accumulation of AH 130 solid type tumor

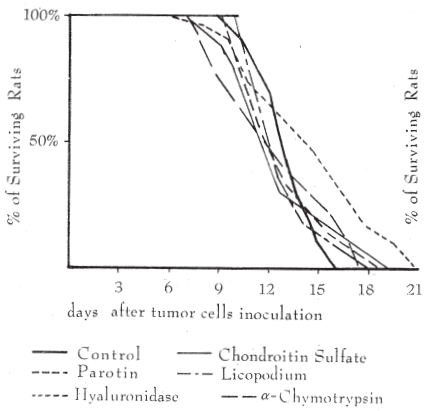


FIG. 3. The Surviving Line of Rats Bearing AH 130 in the Abdominal Cavity.

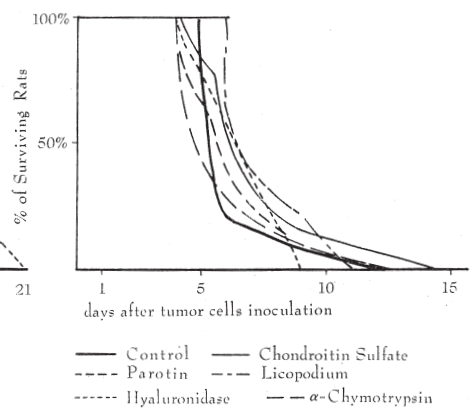


FIG. 4. The Surviving Line of Rats Bearing Yoshida Sarcoma in the Abdominal Cavity.

growth, contrarily parotin and licopodium depressed the growth of tumors.

2) Comparing with AH 130, Yoshida Sarcoma was noted slow developement of central necrosis. The results of these groups were similar to that of (1), that is, in groups administered chondroitin sulfate, hyaluronidase or α-chymotrypsin rapid tumor growth was noted. Parotin and licopodium was seemed to depress the tumor developement.

II. Survival time of rats

1) As shown in Fig. 3, the survival time was observed of rats inoculated AH 130. For some of factors which influence the survival time, noted the season investigated and rats from which tumor cells for inoculation were harvested. Therefore an effort was made to get tumor cells from one host at one time, and to keep constant temperature and moisture of the room as possible. Of the survival time there was not much difference between controls and the groups treated with chondroitin sulfate, parotin, hyaluronidase or α-chymotrypsin. The forth group which was inoculated tumor cells together with licopodium showed also no change on the survival time.

2) Fig. 4 shows the survival time of rats transplanted Yoshida Sarcoma into the abdominal cavity. The results were almost similar to that of (1), that is, chondroitin sulfate, hyaluronidase, parotin, licopodium or α-chymotrypsin did not effect on the survival time. Generally the life of rats inoculated Yoshida Sarcoma was shorter than that having AH 130 in the abdominal cavity.

III. Microscopic findings

1) On the 5th day of tumor transplantation most tumor showed oval shape and somewhat hard in consistency, and its cut surface showed greywhitish in

color with beginning of necrosis of the central area manifested by brownish mottled character. Specimens on the 15th day showed marked developement of central necrosis forming cysts with thin wall consisting external connective tissue involving small amount of tumor cells. Some of them started to rupture outside of the skin after the second week. After 10 days of tumor transplantation the tumor mass started irregular form with projection like a bud which tended to grow rapidly with scanty necrosis. This irregular form was frequent mostly in the 3rd and 4th groups. Microscopic findings showed much variety from the 5th day already. For the conveniences the tumor mass was divided to the three layers, that is, the central portion, the second or intermediate layer and the external layer which is the most external layer of the mass.

At the central portion, tumor cell arrangement was rather simple with scarce mitosis where the connective tissue developement was not marked, and the blood vessel supply was poor. On the 5th day the center of this area started degenerating tumor cells to necrosis with infiltration of round cells and leucocytes. On the 10th day the central necrosis was marked surrounded by comparatively young granulation tissue with cell infiltration where PAS or TBM stain was considerably positive. Excepting this granulation tissue these central portion was noted by poor fibrous proliferation as well as scanty ground substance.

The second layer was the largest part of tumor mass surrounding the central portion. At this area the connective tissue was considerably abundant surrounding each tumor cell, which showed more variety with more increased mitoses indicating high metabolic activity comparing with that seen in the central portion. Irregular reticulin or collagen fibers ran to all directions surrounding tumor cells, and there noted mild positive PAS and TBM substances. Blood vessels including capillaries presented much more than the central portion. This second layer was surrounded by thick connective tissue which fibers ran parallel.

As the external layer or the third layer surrounded the second layer sometimes forming projection like a bud shape, and in this layer the tumor cells showed marked pleomorphism with many mitoses. The blood vessels especially capillaries were abundant and the connective tissue was not developed so much, especially with scanty fibrosis and hyalinization. This layer was surrounded by immature connective tissue with round cell infiltration, and PAS and TBM stain was strongly positive suggesting considerable amount of ground substance, contrarily the reticulin or collagen fiber proliferation was poor. On examining the sections of 10th and 15th day this 3rd layer gradually transformed to the second layer, that is, another new growth developed surrounding the older tumor mass. Late stage of the tumor growth the central

portion was completely necrosed, then gradually the second layer seemed to start the necrosis with forming cyst like structure.

Comparing with the controls the microscopic findings of the second group treated with chondroitin sulfate showed large central necrosis from the earliest day. The connective tissue at the second layer of this group showed somewhat slow developement of fibrous component, and seemed to keep comparatively immature type revealed by silver impregnation, PAS and TBM stain, whereas the third layer was similar to that of controls.

Parotin treated group showed slow developement of tumor mass. The central portion was not different from the controls, but the second layer was noted by the increased fibrosis, where the tumor cell seemed to be shrinked among fibrous connective tissue. As noted previously the third layer projecting rapidly to outside showed marked mitosis and pleomorphism which was similar to that of controls and other groups.

The 4th group treated with licopodium showed also marked developement of fibrosis surrounding the licopodium granules running to the all directions. Tumor cells surrounded by this dense connective tissue showed small population and each cell seemed not to be active nor pleomorphic and found very scarce mitosis. The 3rd layer was noted like above.

The 5th group treated with hyaluronidase which developed rapidly in tumor growth was noted the poor connective tissue proliferation on the second layer. The tumor cells seemed to present more variety with more mitoses, however the quantitative change of ground substance was not demonstrated by PAS or TBM though it was considered enough that hyaluronidase lyzes the intercellular cement substance.

Chymotrypsin made also large central necrosis, and the fibrosis and hyalinization was not so much on the second layer. The central portion and the 3rd layer of the 5th and 6th groups showed also similar findings with controls as described previously.

The groups treated with α-chymotrypsin, hyaluronidase and chondroitin sulfate showed accumulated solid tumor growth, and microscopically tumor cells tended to have more variety with increased number of mitosis in the second layer, where the fibrous component was generally depressed in growth comparing with controls. However the central and external layer of the tumor was not noted much difference. Whereas, the group treated with parotin and licopodium tended to grow slowly, and the microscopic findings showed increased fibrosis and hyalinization, characterizing senile type of the connective tissue environing tumor cells at the second layer, where the tumor cells were generally monotonous and somewhat decreased in mitosis comparing with the controls. On the other hand the ascites form of transplanted tumor was not influenced by the drugs noted above.

DISCUSSION

There is no question about the preventive reaction of the host against the bacterial infection,[1] and also there have been presented many reports insisting host preventive activities to the tumor growth.[3] There was one thought to give one mean of the preventive reactions of host to the lymphoid cells or mast cells against the tumor cells from the standpoint of immunology.[5,6,7,8] By Schwarz[9]; necrosis of tumors was considered to product some preventive substance to tumor growth. Kubo[10] transplanted tumors where chemically or physically induced granulation tissue, and found that the granulation tissue depressed the tumor growth or at least the tumor growth developed to the direction of scanty granulation tissue. According to Nagayo, the capsule formation of the connective tissue surrounding tumor mass was thick when the tumor growth was slow, contrarily the capsule surrounding rapidly growing tumor mass was poor in developement.[11] Oshima[12] by Brown-Pearce tumor of rabbit, and Miyata, Sumida and Okajima[13] by transplantable tumor of rats, reported that the dense connective tissue of granulation surrounding the tumor growth as preventive reaction. For the human carcinoma Hauser[14] described that the immature granulation tissue altered to the mature type of granulation as preventive reaction of hosts on growing of carcinoma, which was agreed by Heidemann[15] who enforcing the connective tissue capsule surrounding the tumor mass to be the preventive reaction against the tumor growth. By Hansemann[16] and Borst[4] cancer cells were assumed to stimulate the developement of the connective tissue. There were several explanations for the connective tissue as mechanical depression to the tumor growth by its constriction.[17,18] While, according to some investigators in the cases of prolonged surviving time of cancer patients, noted the highly differentiated tumor cells, advanced lymphoid cell proliferation, hyalinization or fibrosis of the connective[18,19] tissue. Ewing explained that the connective tissue with abundant blood vessels could be a good bed for the tumor growth, however further developing fibrosis made the tumor cells localizing in small area.[20]

On the other hand not few people denied the antitumor activity of the connective tissue. Borrman[21] also reported neither fibrosis nor hyalinization affected on the cancer developement, and Greenough[22] said that the grade of the connective tissue growth or its fibrosis and hyalinization did not indicate the prognosis of carcinoma. According to Machii[23] the connective tissue growth was induced by the disturbance of lymphatic circulation, and Oota and Tanaka[24] described that fibrosis was induced by bacterial infection copresenting with carcinoma. Kuru[25] reported the cancer cell grew selectively in the scar tissue.

By Ribbert[26] the edematous loose connective tissue with inflammatory reaction was considered to give the better condition for the developement,

contrarily old scar formation of the connective tissue depresses the tumor growth. Recently Vasilief[2] made a description that the immature connective tissue surrounding the transplanted tumor made favourable circumstances for tumor growth. Ozzello and Lasfargeus[27] investigated by human breast cancer that tumor cells grew well in the medium supplemented by acid mucopolysaccarides. Takeuchi[28] observed by his experimental work that tumor growth was rapid in the immature connective tissue. To consider the relationship between the tumor growth and the connectve tissue, the connective tissue itself should be considered biochemically and histochemically through its various sites.[29][30][31][32] The characteristic or quantitative change of fibrin or ground substance forming the connective tissue seemed to alter the influence on the tumor growth. Several considerations were made from these experimental works. By microscopic findings of controls, the central portion of the tumor mass seemed to be the original location where tumor cells were inoculated. The second layer was the place showing many faces of tumor cells related with the host in many means, and the third layer indicated the place showing newly forming tumor cells with relatively low host reaction. At the second layer the tumor cells seemed not to be so violent, and the connective tissue was more mature in character manifested with the increased fibrosis or hyalinization and with rather scanty ground substance. The third layer was not marked in fibrosis and hyalinization, and the ground substance as well as the blood capillary supply was developed, whereas the tumor cells were violent with increased number of mitosis. Considering that the ground substance does as the passway of nutritions from capillary vessels to tumor cells, one hypothesis is thought that the mature connective tissue with decreased ground substance or characteristically altered ground substance accompanied by fibrosis or hyalinization causes nutritional disturbance of tumor cells, while that the immature type of the connective tissue gives good condition to grow tumor cells. Of course though the mechanical depression of the connective tissue with increased fibrosis can be considered. To get further consideration for this hypothesis, several drugs were used which were considered to alter the connective tissue in some way. Chondroitin sulfate, which is known as a component of ground substance, is considered to depress the excessive fibrosis and to keep young type of the connective tissue.[29][30][33][34][35] On giving chondroitin sulfate to rats having transplantable tumor in the abdominal cavity, no definite change in survival time was noted, whereas the solid type tumor mass developed rapidly. By microscopic findings collagenous and reticulin fibers were slow to develope and abundant ground substance was suggested, though it was noted to increase the permeability of the ground substance.

Parotin was used for hormon to accentuate the formation of fibrosis,[35][36] and also noted as ACTH-like activity.[37] By administration of parotin the survival time was not changed among rats having transplantable tumors in

the abdominal cavity, however the solid tumor seemed to be depressed the growth comparing with controls. In fact collagen or reticulin fibers were marked surrounding tumor cells, but there was no evidence of alteration of ground substance.

Licopodium was inoculated together with tumor cells in order to induce granulation tissue. On the early days when PAS and TBM was considerably positive surrounding licopodium granules, tumor cell invasion was rapid, however after certain times the tumor cells seemed to be depressed by scarring granulation tissue.[10] The place outside of this granulation tissue tumor cells showed rapid growth as seen in controls at the third layer.

Next case was administered by hyaluronidase[29)30)] which is known to present in testis and hydrolyze acid mucopolysaccharides. Hyaluronidase was thought to be produced by cancer cells,[38)39)40)] and also reported that hyaluronidase made rapid growth of solid tumor as well as metastasis.[41)] In our experiments also was demonstrated the rapid growth of solid type of AH 130 and Yoshida Sarcoma, though there were some reports that hyaluronidase did not affect on the sarcoma type of tumors.[41)42)] The growth enormously accelerated by hyaluronidase may be considered due not only to increase of the permeability of the ground substance, but also due to scarce fibrous component.

α-Chymotrypsin made rapid growth of solid tumor, and microscopic findings revealed scanty fibrosis, though it was considered the alteration of permeability or quantitative change of ground substance.[40)]

From these results it was suggested that the tumor growth developed rapidly in the immature connective tissue, contrarily the growth was slow in the mature connective tissue with increased fibrosis or hyalinization. For this results many factors were considered like mechanical depression by fibrous contracture, and the alteration of nutritional supply to the tumor cells due to the change of the blood vessel supply as well as the altered ground substance.

SUMMARY

An experimental work was made to investigate the relationship between the connective tissue and the tumor growth, both Ascites Hepatoma 130 and Yoshida Sarcoma were used for this investigation using Wistar rats, and were administered chondroitin sulfate, parotin, licopodium, hyaluronidase or chymotrypsin to obtain various type of the connective tissue surrounding tumor cells.

Solid type of tumor both AH 130 and Yoshida Sarcoma did a rapid growth in groups treated with chondroitin sulfate, hyaluronidase, and chymotrypsin, whereas groups treated with licopodium and parotin revealed slow developement of solid type tumor, However the survival time in ascites form was not influenced so much by these drugs.

For the microscopic findings of solid tumor, tumor showed usually three layers, that is central portion, the second or intermediate layer and the third or external layer. The central portion was noted by early developing central necrosis and poor connective tissue proliferation as well as blood vessel supply, and the second layer was noted by most developed connective tissue with abundant collagen or reticullin fibers. The external layer was noted by rapidly growing violent tumor cells with scanty and immature connective tissue and abundant blood capillaries.

Among groups noted rapid growth of tumor, was investigated the young type connective tissue with rich ground substance and blood capillaries or scanty fibrosis, where the permeability of the connective tissue seemed to increase. While the tumor growth developed slowly when the connective tissue progressed fibrosis or hyalinization altering to mature type, which was mostly like due to the mechanical depression by the connective tissue or the low permeability of ground substance or disturbed blood vessel supply that might decrease the amount of nutritional supply to the tumor cells.

II. Influence of the Connective Tissue on the Chemotherapeutic Effects

The connective tissue has been studied by many workers for the defensive reaction to the tumor growth.[3] As described in Chapter I., the mature type connective tissue with increased fibrosis or hyalinization was thought to depress the tumor growth, whereas the immature connective tissue might give good circumstance for the tumor cell developement.[2] However no description has been obtained whether the connective tissue influences on the effect of anti-cancer chemotherapy. On considering the ground substance of the connective tissue as a passway of nutrition or chemotherapeutic drugs from the capillary vessels to tumor cells, it is considerable that the characteristic or quantitative change of the connective tissue gives influence on the chemotherapeutic effect. From this standpoint one hypothesis can be considered that the immature connective tissue with increased permeability of the ground substance gives good circumstance for the chemotherapy, whereas the scar formation or hyalinization and fibrosis with degenerated ground substance disturbs the effect of chemotherapy. Of course the blood vessels themselves are considered to be obstructed by the scar formation of granulation tissue, while blood circulation may be good enough in the inmmature type.

MATERIALS AND METHODS

Gifu Wistar rats weighing between 130 and 150 g were used in all experi-

ments, which were kept on a standard pellet diet with drinking water. For tumor cell suspensions used AH 130 and Yoshida Sarcoma obtained from the Second Department of Pathology, Nagoya University School of Medicine respectively. Nitromin and mitomycin-c were used as anticancer agents.

I.

1) 1×10^7 cells of AH 130 were transplanted into the subcutaneous space of rats. Rats were divided into 6 groups with each 10 rats, and the first group was taken as the controls. The second group was administered intraperitoneally 4 mg/100 g body weight of chondroitin sulfate, and 0.6 mg/100 g body weight of parotin was administered to the third group, while licopodium was inoculated simultaneously mixed with tumor cells to the subcutaneous space of the forth group. The fifth group was treated with 20 T.R.U./100 g body weight of hyaluronidase, and the sixth group with 1 unit/100 g body weight of α-chymotrypsin. All these drugs excepting licopodium were started daily intraperitoneal administration 2 days before tumor cell inoculation for 5 days. Nine days after tumor cell inoculation when the tumor size developed to be enough to compare, anticancer agents were administered for 2 days intraperitoneally. The dosage of nitromin was 2 mg per 100 g body weight of rats. Tumor size of each rat was measured daily from the 5th day of tumor inoculation to 15th day as shown in Fig. 1.

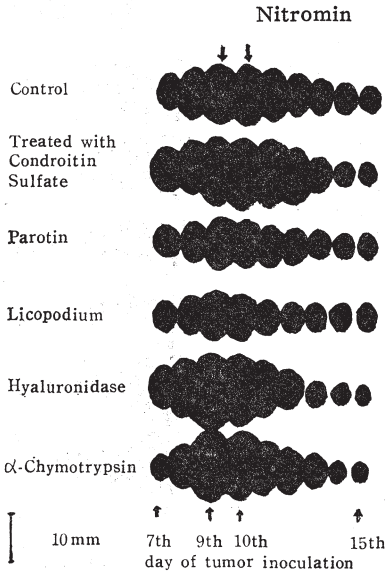


FIG. 1. Tumor Size of AH 130 in the Subcutaneous Tissue on Applying Nitromin.

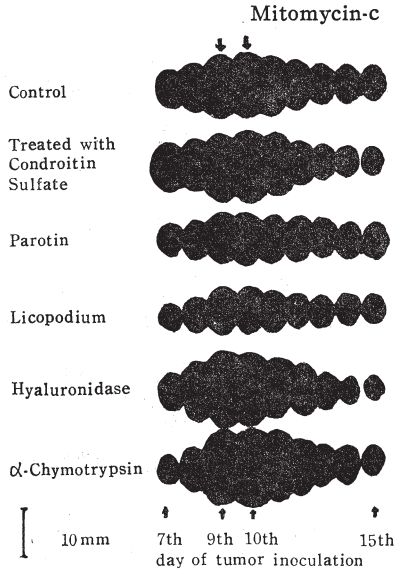


FIG. 2. Tumor Size of AH 130 in the Subcutaneous Tissue on Applying Mitomycin-c.

2) 1×10^7 cells of Yoshida Sarcoma were inoculated to the subcutaneous space of rats which were divided into 6 groups as noted previously. Chondroitin sulfate, parotin, licopodium, hyaluronidase, and α-chymotrypsin were applied to each group for 5 days intraperitoneally starting 2 days before tumor transplantation, which was exactly the same method as noted above. Nitromin or mitomycin-c was also administered 9 and 10 days after tumor cell inoculation. The dosage of above drugs was the same as described in (1). Fig. 2 shows the result of daily measurement of tumor size in each group.

II.

1) Each six group was treated subcutaneously with chondroitin sulfate, parotin, licopodium, hyaluronidase, and α-chymotrypsin. Another one group was taken as a control. Each group was inoculated 1×10^7 cells of AH 130 into the abdominal cavity. Above drugs were administered the same dosage as that of I. 1) and 2). Licopodium was inoculated into the abdominal cavity mixing together with tumor cells in Ringer's solution.

Nitromin or mitomycin-c was administered subcutaneously on the 5th day, whose dosage was the same as noted above. The results were shown on Fig. 3 and 4.

2) Each group was applied in the same way as noted above by chondroitin sulfate, parotin, licopodium, hyaluronidase and α-chymotrypsin, and was inoculated 1×10^7 of Yoshida Sarcoma cells. Nitromin or mitomycin-c was admi-

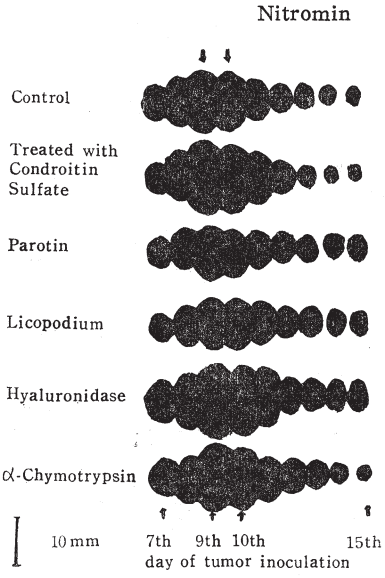


FIG. 3. Tumor Size of Yoshida Sarcoma in the Subcutaneous Tissue on Applying Nitromin.

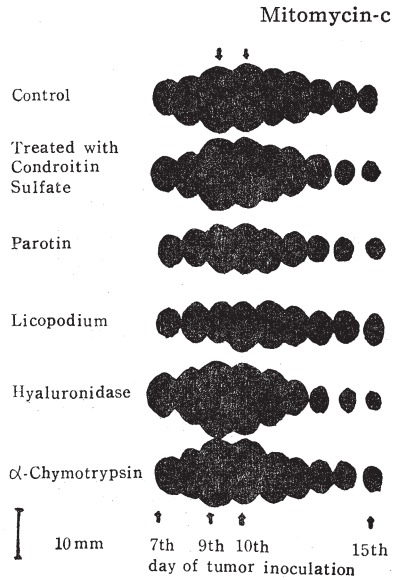


FIG. 4. Tumor Size of Yoshida Sarcoma in the Subcutaneous Tissue on Applying Mitomycin-c.

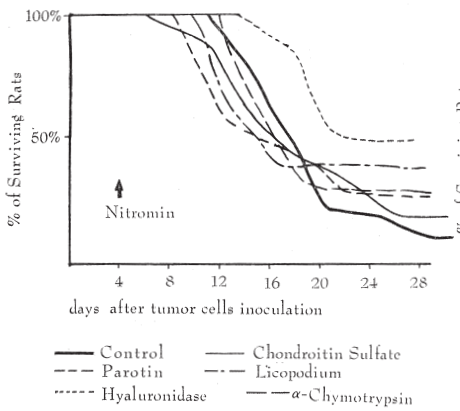


FIG. 5. The Surviving Line of Rats Bearing AH 130 in the Abdominal Cavity on Applying Nitromin by Hypoderm. Inj.

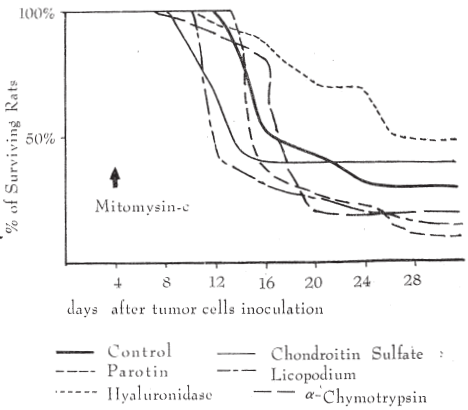


FIG. 6. The Surviving Line of Rats Bearing AH 130 in the Abdominal Cavity on Applying Mitomycin-c Subcutaneously.

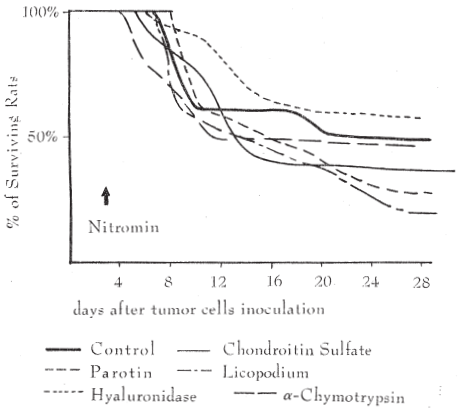


FIG. 7. The Surviving Line of Rats Bearing Yoshida Sarcoma in the Abdominal Cavity on Applying Nitromin Subcutaneously.

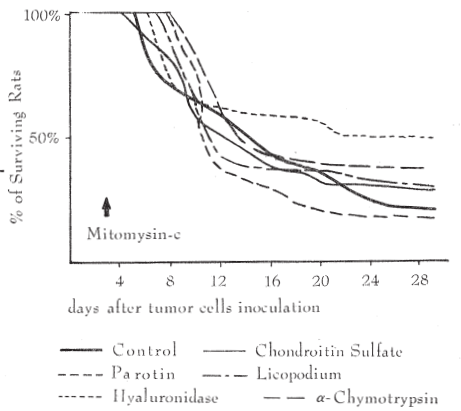


FIG. 8. The Surviving Line of Rats Bearing Yoshida Sarcoma in the Abdominal Cavity on Applying Mitomycin-c Subcutaneously.

nistered subcutaneously on the 4th day, and their dosage was the same with (2). Fig 5 and 6 showed the results.

III.

AH 130 or Yoshida Sarcoma was transplanted into the subcutaneous space of rats which were treated with chondroitin sulfate, parotin, hyaluronidase or licopodium in the same way as noted previously. On the 7th day nitromin or mitomycin-c was administered intraperitoneally, then next day or three days after rats were sacrificed by ether inhalation. Tumor masses were prepared for microscopic examination by serial sectioning.

RESULTS

I. Size and gross features of transplanted tumors

1) The tumor growth was influenced by chondroitin sulfate, parotin, licopodium, hyaluronidase or α-chymotrypsin as noted in chapter 1. By combined therapy with anticancer agents as shown in Fig. 1, tumor regression was marked in groups treated with chondroitin sulfate, hyaluronidase and α-chymotrypsin, whereas the groups treated with parotin and licopodium showed slow regression by anticancer chemotherapy. However there were sometimes noted rapid growth of tumor in the 2nd, 5th and 6th group even though treated by chemotherapeutic agents, which could be considered that the tumor growth was too rapid by these drugs as shown in chapter I. before being influenced by chemotherapy.

2) Fig. 2 shows the results of solid type tumor by Yoshida Sarcoma, and the similar results to 1) were noted, that is, chondroitin sulfate, hyaluronidase or α-chymotrypsin did better effect on combined therapy with anticancer agents than that treated with anticancer agents alone, while parotin and licopodium disturbed the effect of these drugs. Furthermore the 2nd, 5th and 7th groups showed sometimes rapid developement of tumor size as compared with the controls.

II. Survival time of rats

1) The survival time was not influenced by single administration of chondroitin sulfate, parotin, hyaluronidase or α-chymotrypsin or licopodium as shown in chapter I. However the combined therapy with anticancer chemotherapy did delay of survival time in the group treated with hyaluronidase more than single anticancer chemotherapy, whereas the other groups showed similar survival time as compared with the controls. These results were similar between treated by nitromin and mitomycin-c.

2) The case of Yoshida Sarcoma showed also similar results to that of AH 130, namely only hyaluronidase made better effect on combined therapy with anticancer agents. It is noteworthy that chondroitin sulfate, parotin, licopodium or α-chymotrypsin did not influence on the effect of anticancer chemotherapy on the survival time of rats bearing tumors in the abdominal cavity, which changed the effect to the tumor size of solid tumors.

III. Microscopic findings

By microscopic investigation the tumor cell degeneration presented already on the next day after chemotherapy. The earliest change was noted on the external layer of the tumor mass, where was seen most pleomorphism of the cells with scanty and immature connective tissue and abundant blood vessels. Next place easily affected by chemotherapy was central portion, however the location of degeneration was definitely different from the place

of central necrosis occurring spontaneously, that is mostly located near the edge, where rather abundant blood supply was presented. The degeneration of tumor cells by anticancer chemotherapy occurred last at the second layer, where fibrosing or hyalinized granulation tissue was surrounded in marked degree. The second group treated with chondroitin sulfate showed most marked developement of necrosis at the central portion, and even the second layer was found advanced cell degeneration. The external layer was almost the same as the controls. The group treated with parotin was slow in degeneration of tumor cells by chemotherapy at all layers, and similar findings were noted on the group treated with licopodium. Especially the tumor cells near granulation tissue surrounding licopodium granules were found to be affected with most difficulty. The group treated with hyaluronidase or α-chymotrypsin showed similar findings like seen in the second group treated with chondroitin sulfate. The external layer was characteristically similar in all groups where presented similar findings by chemotherapy, contrarily the central portion and the second layer showed different attitude against the chemotherapeutic agents among the 6 groups.

DISCUSSION

The connective tissue was considered to influence on the growth of tumor, though there have been various kinds of thought how it acts on the tumor cells. A trial was made to investigate the influence of the connective tissue on the anticancer chemotherapy if presented. Chondroitin sulfate was administered to rats bearing tumor in their subcutaneous space with anticancer chemotherapy, and made a rapid regression of the tumor size, however the survival time of rats having tumor in the abdominal cavity was not altered by chondroitin sulfate upon chemotherapy. This impressed that chondroitin sulfate did not influence directly on the tumor cells but on the environmental tissue. Chondroitin sulfate depresses the fibrosis and keeps the connective tissue in immature state, and the ground substance seemes to increase quantitatively or at least to increase the permeability,[29) 30) 33) 34) 35)] namely anticancer agents are suggested to reach easily to the tumor cells after coming out through the capillary wall. The chemotherapeutic effect on the survival time of rats bearing tumor in the abdominal cavity was not influenced by parotin or licopodium, however that on the solid tumors was disturbed by parotin and licopodium. Microscopically fibrosis of environmental connective tissue was increased by parotin, and licopodium did contracting scar formation with hyalinization and fibrosis, where degeneration or decrease of the ground substance was naturally considered accompanied with poor capillary vessel supply.[10) 35) 36)] Combined therapy of hyaluronidase and anticancer agents affected on both ascitic form and solid form of transplantable tumors of rats, which

was considered due to increased absorption of anticancer agents at the location by hyaluronidase[43) 44)] as well as due to increased permeability of the vascular wall and the ground substance surrounding tumor cells.[29) 30)] There was a good regression of tumor size with combined therapy of α-chymotrypsin[40) 45)] and anticancer drugs, and microscopically fibrous component of the connective tissue was definitely scanty with marked tumor cell degeneration. As noted above the controls showed rapid regression at the external layer and the central portion where presented low degree of fibrosis, whereas the second layer started last the cell degeneration. Anticancer agents are expected to reach to tumor cells as possible to obtain good result of tumor regression, and for that is mentioned a good vascular supply, increased permeability of the ground substance and of blood vessel wall and/or increased permeaility of tumor cell membrane. In our results the permeability of the tumor cell membrane was not to be considered, since ascites form of tumor cells was not affected by these drugs noted above except hyaluronidase. Fibrosing scar formation seemed to disturb the blood vessel proliferation and also to alter quantitatively or qualitatively the ground substance, contrarily chondroitin sulfate caused increased permeability or quantitative increase of ground substance. Hyaluronidase and chymotrypsin seemed to disturb the fibrosis and to increase the permeability of vascular wall and the ground substance. Between AH 130 and Yoshida Sarcoma similar results were obtained in the present experiment.

SUMMARY

AH 130 and Yoshida Sarcoma were transplanted to Wistar rats intraperitoneally or subcutaneously which were treated with chondroitin sulfate, parotin, licopodium, hyaluronidase and α-chymotrypsin combined with anticancer agents. Upon administration of mitomycin-c and nitromin the group treated with chondroitin sulfate, hyaluronidase and α-chymotrypsin did rapid regression of solid type of tumors, however the surviving time of rats bearing tumors in the abdominal cavity was elongated more than that of the controls only in the group treated with hyaluronidase. Contrarily the group treated with parotin and licopodium minimized the effect of anticancer chemotherapy in solid type of tumors, but no definite influence was observed on the effect of anticancer chemotherapy to ascites form of tumors by these drugs. By microscopic examination of serial sections of these specimens early necrosis or degeneration of tumor cells was noted at the external layer, and then observed at the central portion comparatively locating near the edge. Anticancer chemotherapy affected last on tumor cells of the second layer where dense fibrous connective tissue presented.

The above results suggested that mature type of the connective tissue

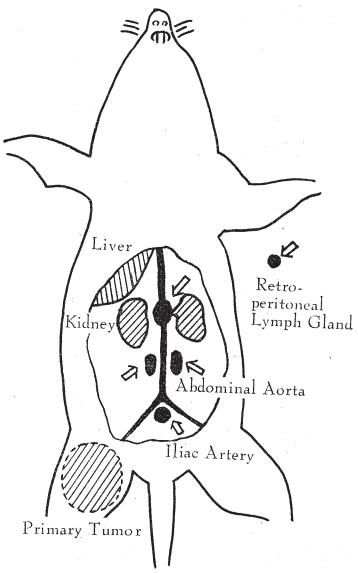
environing tumor cells disturbed the effect of anticancer chemotherapy, contrarily indicated that the immature connective tissue or increased permeability of the ground substance could become a favorable bed for the effect of anticancer chemotherapy.

III. Influence of the Connective Tissue on the Metastasis

For the experimental work many investigators observed the tumor metastasis in the liver or the lung by inoculating tumor cells intravenously,[46)47)] and also studied tumor cells in the circulating blood.[48)49)] Some made the histological studies at the tumor lesions relating to the tumor cell invasions to the blood vessels,[50)] and others to investigate the tumor cell stickness and adhesiveness,[51)52)] etc. etc,. A problem is that one factor cannot be always on one side, that is, it might accumulate the metastasis at one site, but it could be defensive to the metastasis at the other site. Because the site which single cell isolates from primary lesion is different from the site to transport through the lymphatic and the blood vessels and that occurring incidence of new growth at the new place. In this investigation an effort was made to observe whether the connective tissue surrounding tumor cells influence upon metastasis by an experimental work. Rats were inoculated AH 130 and Yoshida Sarcoma into subcutaneous space of the right thigh, and observed the lymphatic metastases at the retroperitoneal lymph nodes.

MATERIALS AND METHODS

Gifu Wistar rats weighing between 100 and 120 g were used which were kept on a standard pellet diet with drinking water, and for the tumors Ascites Hepatoma 130 (AH 130) and Yoshida Sarcoma were used in all experiments. 1×10^7 of tumor cells suspension in Ringer's solution was inoculated into the subcutaneous space of the right thigh of rats. On the 8th day of Yoshida Sarcoma inoculation and 11th day of AH 130 inoculation rats were sacrificed and lymph gland metastases were examined. All rats were divided into 6 groups, the first group was taken as the controls, chondroitin sulfate (4 mg/100 g body weight) was



injected into the abdominal cavity for 5 days starting 2 days prior to tumor cell transplantation to the 2nd group, Parotin (0.6 mg/100 g body weight) to the 3rd group, and to the 4th group licopodium was inoculated together with tumor cells. To the 5th group hyaluronidase (20 units/100 g body weight) was applied, and the 6th group was treated with α-chymotrypsin (1 unit/100 g body weight).

Metastases to the lymph glands were mostly seen at the retroperitoneal area, that is, few at the area where the abdominal aorta divides into the iliac artery, each one on both side of abdominal aorta about 1 cm above of the

TABLE 1. Metastatic Involvement of the Retroperitoneal Lymph Nodes on the 11th Day of AH 130 Inoculation to the Right Thigh of Rats.

Agent	Rat No.	Grade of Metastatic Involvement of Lymph Glands			
		None	No. of Grade 1	No. of Grade 2	No. of Grade 3
Control	1	2	2	0	0
〃	2	2	2	0	0
〃	3	4	0	0	0
〃	4	2	2	0	0
〃	5	1	2	1	0
〃	6	2	2	0	0
〃	7	4	0	0	0
〃	8	0	3	1	0
Chondroitin Sulfate	1	1	2	0	1
〃	2	1	2	1	0
〃	3	0	2	1	1
〃	4	2	2	0	0
〃	5	0	3	1	0
〃	6	1	1	2	0
Parotin	1	2	2	0	0
〃	2	4	0	0	0
〃	3	2	2	0	0
〃	4	3	1	0	0
〃	5	3	1	0	0
〃	6	3	1	0	0
Licopodium	1	2	2	0	0
〃	2	3	1	0	0
〃	3	2	2	0	0
〃	4	3	0	1	0
〃	5	3	1	0	0
〃	6	1	2	1	0
〃	7	4	0	0	0
Hyaluronidase	1	1	1	2	0
〃	2	0	4	0	0
〃	3	2	2	0	0
〃	4	1	2	1	0
〃	5	2	2	0	0
〃	6	1	3	0	0
α-Chymotrypsin	1	0	2	1	1
〃	2	1	3	0	0
〃	3	2	1	1	0
〃	4	4	0	0	0
〃	5	0	2	1	1
〃	6	1	1	2	0

TABLE 2. Metastatic Involvement of the Retroperitoneal Lymph Nodes on the 8th day of Yoshida Sarcoma Inoculation to the Right Thigh of Rats.

Agent	Rat No.	Grade of Metastatic Involvement of Lymph Glands			
		None	No. of Grade 1	No. of Grade 2	No. of Grade 3
Control	1	0	1	2	1
〃	2	2	2	0	0
〃	3	2	0	1	1
〃	4	0	1	2	1
〃	5	1	2	1	0
〃	6	2	1	2	0
〃	7	2	0	2	0
〃	8	2	1	1	0
〃	9	0	2	1	1
〃	10	1	2	1	0
〃	11	0	2	2	0
Chondroitin Sulfate	1	0	2	2	0
〃	2	0	2	1	1
〃	3	1	0	2	1
〃	4	2	0	1	1
〃	5	0	1	1	2
〃	6	1	1	2	0
Parotin	1	2	2	0	0
〃	2	2	1	1	0
〃	3	3	1	0	0
〃	4	2	0	1	1
〃	5	3	0	1	0
〃	6	1	2	1	0
Licopodium	1	3	1	0	0
〃	2	1	1	2	0
〃	3	4	0	0	0
〃	4	2	1	1	0
〃	5	2	0	2	0
〃	6	1	3	0	0
Hyaluronidase	1	2	2	1	0
〃	2	0	1	2	1
〃	3	0	1	3	0
〃	4	2	1	1	0
〃	5	0	1	2	1
〃	6	2	1	1	0
α-Chymotrypsin	1	0	0	1	3
〃	2	0	0	2	2
〃	3	0	0	3	1
〃	4	0	0	2	2
〃	5	0	0	1	3
〃	6	4	0	0	0

1st group, and one or more on the posterior side of the abdominal aorta where the renal arteries divide. Table 1 and 2 show the result of the metastatic lymph nodes involved by taking lymph glands limited as seen in Fig. (arrow sign), which is shown with grade 1, 2 and 3; namely grade 1 indicated the metastases revealed by microscopic examination though the size of the lymph node was not enlarged so much, grade 2 indicated macroscopically definite metastasis; and grade 3 indicated marked enlargement with starting to fuse together between each metastatic lesion. Primary tumors and the

metastatic lesions of all rats were prepared for microscopic examination by serial sectioning.

RESULTS

Table 1 and 2 show the result of lymphatic metastases. In this experiment no tumor metastasis was found. Chondroitin sulfate seemed to stimulate the metastases, and α-chymotrypsin and hyaluronidase also increased metastases comparing with the controls. Contrarily the tumor treated with parotin or licopodium showed decreased number of metastatic involvement as well as small size of metastatic lesions.

By microscopic findings of primary tumor of the controls showed the similar findings to described in chapter I. The lesion at the lymph node started mostly at peripheral area of the lymph node, and at this stage the tumor cells were rather monophoric with less mitoses where the interstitial connective tissue was not proliferated so much. However when the cut surface showed almost metastatic lesion with grey whitisch in color, the microscopic findings were mostly like those of the primary tumors.

On bestowing chondroitin sulfate the primary tumor was just like noted in chapter I, and the developement or secondary metastases in lymph glands was rapid with early starting central necrosis. The primary tumors treated with α-chymotrypsin and hyaluronidase showed also the same as that described in chapter I, and the growth at the lymph nodes was somewhat rapid. The primary lesion of the group treated with parotin or licopodium was also already described in chapter I, but the secondary lesion showed almost similar findings to the controls.

DISCUSSION

Many investigators have been studied about metastasis from various standpoints. For the production of isolated tumor cells from the primary lesions, intercellular adhesiveness[51)52)] or amoeboid[53)] motion of tumor cells has been considered, and Madden explained by his experimental work that a viable single tumor cell production was accumulated under condition of high permeability of intercellular cement substance treated with trypsin and DNase.[54)] Like noted by Rössle it is also interested to observe the tumor cell invasion to the blood vessels.[50)] Moore,[48)] Robert[49)] and many other workers have investigated the circulating tumor cells in the blood. Zeidman,[46)] Fisher[47)] and many others who observed the metastases in the lung and/or the liver following injection of tumor cells through the vessels, explained that anticoagulant substances depressed the metastatic incidence, which was considered contrary to accumulate the production of isolated single cells.[54)] Therefore the factors which influence the metastasis varies depending on the site of metastasis investi-

gated, and this experiment was tried to observe the relationship between the connective tissue environing the tumor cells and the incidence of metastasis.

As noted in Table 1 and 2, α-chymotrypsin increased the number of metastases, which may be explained by that α-chymotrypsin lyzes intercellular cement substance or increase the permeability causing increased number of isolated cell production from the primany[40) 45)] tumor, and the result was against the fact that α-chymotrypsin decreased metastatic incidence in the liver or the lung on inoculating tumor cells from the vessels.[46) 55)] Similar findings were obtained in the groups treated with hyaluronidase, which seemed to be explained by the increased permeability of the intercellular ground[29) 30) 52)] substance. On the other hand chondroitin sulfate did also increase of lymphatic metastases. As noted previously by giving chondroitin sulfate the ground substance seemes to increase and keep the connective tissue in immature[29) 30) 33) 34) 35)] condition with depressed fibrosis and hyalinization, thusfore the immature connective tissue surrounding tumor cells suggests to give increased incidence of metastasis, which is of course necessary to further investigation for the conclusion. By administration of parotin the connective tissue of the primary tumor showed increased[35) 36)] fibrosis, and also noted scar formation with increased fibrosis and hyalinization by licopodium, which seemed to relate to the result that the metastases to retroperitoneal lymphnodes decreased in number and the size. Thus the relationship between metastasis and the environmental connective tissue was suggested, that is, the decrease of intercellular cement substance causing decreased intercellular adhesiveness or increased permeability of the ground substance seemed to accumulate the metastasis. On the other hand the immature type of the granulation tissue is thought to include abundant blood or lymphatic capillaries and intercellular ground substance to cause increased chance of the cell isolation and of transport of tumor cells from the primary tumor, contrarily the mature type of the connective tissue with increased fibrosis and hyalinization was suggested to present poor vessel supply as well as scanty intercellular ground substance with its degeneration disturbing production of isolated tumor cells and their transportation.

SUMMARY

It was invesitaged that the connective tissue environing tumor cells influences on the metastasis by experimental studies. Ascites Hepatoma 130 and Yoshida Sarcoma was transplanted to the subcutaneous space of the right thigh of Gifu Wistar rats, and lymphatic metastases to the retroperitoneal lymph glands were observed. Rats were treated with chondroitin sulfate, parotin, licopodium, hyaluronidase or α-chymotrypsin which were thought to alter

the connective tissue environing tumor cells quantitatively or qualitatively. By chondroitin sulfate, hyaluronidase and α-chymotrypsin, lymphatic metastases were accumulated, on the other hand the metastases decreased in the group treated with parotin or licopodium. From this result one hypothesis was considered that the immature type of the connective tissue at least with increased permeability of the ground substance gave better condition to metastasize, whereas the increased fibrosis or hyalinization namely senile scar formation environing primary tumor depressed the incidence of metastasis.

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EXPLANATION OF PLATE FIGURES

FIG. 1. 2nd layer of AH 130. Control. Hematoxylin and eosin. ×200.
FIG. 2. 2nd layer of AH 130. Control. Silver impregnation. ×400.
FIG. 3. External layer of AH 130. Control. Note pleomorphism of tumor cells with scarce fibrosis and hyalinization. Hematoxylin and eosin. ×200.
FIG. 4. External layer of AH 130. Control. Note the regular arrangement of fibers. Silver impregnation. ×400.
FIG. 5. Central portion of AH 130. Control. Central necrosis is starting. Hematoxylin and eosin. ×200.
FIG. 6. Central portion of AH 130. Control. Fibers are scanty but irregular in shape. Silver impregnation. ×400.
FIG. 7. 2nd layer of AH 130 of the control. Silver impregnation. ×200.
FIG. 8. 2nd layer of AH 130 treated with chondroitin sulfate. Note slow fibrosis comparing to the control. Silver impregnation. ×200.
FIG. 9. 2nd layer of AH 130 treated with parotin. Note increased collagen or reticulin fibers. Silver impregnation. ×200.
FIG. 10. AH 130 inoculated together with licopodium. Increased and irregulary arranged fibers are noted. Silver impregnation. ×200.
FIG. 11. 2nd layer of AH 130 treated with hyaluronidase. Fibrinization is seemed to be depressed. Silver impregnation. ×200.
FIG. 12. 2nd layer of AH 130 treated with chondroitin sulfate. PAS. ×200.
FIG. 13. 2nd layer of Yoshida Sarcoma. Control. Silver impregnation. ×200.
FIG. 14. 2nd layer of Yoshida Sarcoma treated with chondroitin sulfate. Silver impregnation. ×200.
FIG. 15. Yoshida Sarcoma inoculated with licopodium. Note increased and irregulary arranged fibers. ×200.

FIG. 16. 2nd layer of Yoshida Sarcoma treated with chymotrypsin. Note depressed fibrosis. Silver impregnation. ×200.

FIG. 17. Second layer of AH 130 of the control on the next day of nitromin treatment. Note beginning of cell degeneration. Hematoxylin and eosin. ×200.

FIG. 18. External layer of AH 130 of the control on the next day of nitromin treatment. Cell degeneration marked. Hematoxylin and eosin. ×200.

FIG. 19. Central portion of AH 130 of the control on the next day of nitromin treatment. Cell degeneration on the right side differenciated from the left side where is mostly like due to central necrosis occurring sopntaneously. Hematoxylin and eosin. ×200.

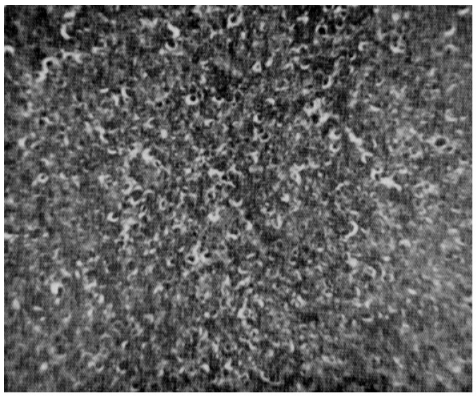
FIG. 20. 2nd layer of AH 130 treated with chondroitin sulfate which was taken on the next day of nitromin treatment. Note advanced cell degeneration. Hematoxylin and eosin. ×200.

FIG. 21. AH 130 inoculated together with licopodium which was taken on the next day of nitromin treatment. Hematoxylin and eosin. ×200.

FIG. 22. Metastatic lesion of AH 130 of the control on the retroperitoneal lymph gland. Hematoxylin and eosin. ×200.

FIG. 23. Yoshida Sarcoma metastsized to the retroperitoneal lymph gland. In the case of the control. Hematoxylin and eosin. ×200.

FIG. 24. Metastatic lesion of AH 130 of the control on the retroperitoneal lymph gland. Hematoxylin and eosin. ×200.



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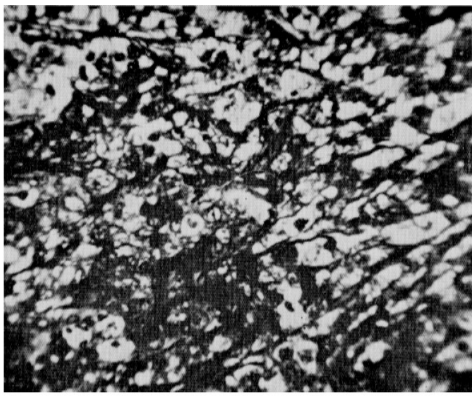

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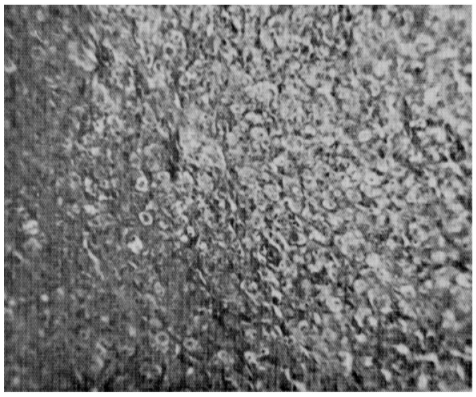

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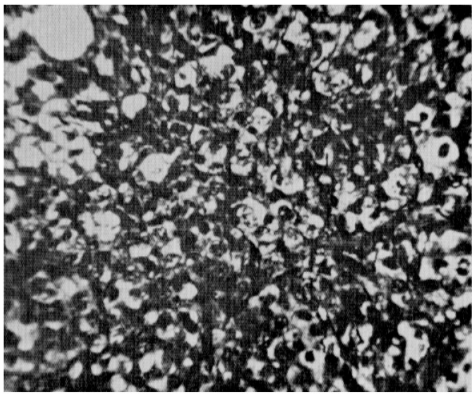

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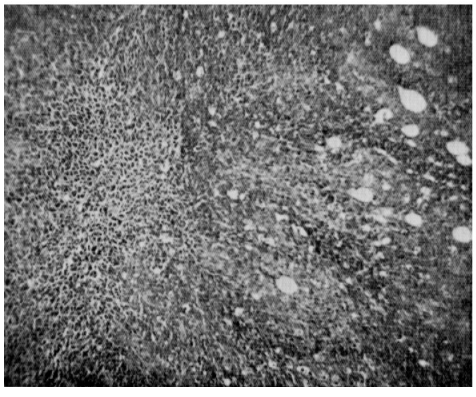

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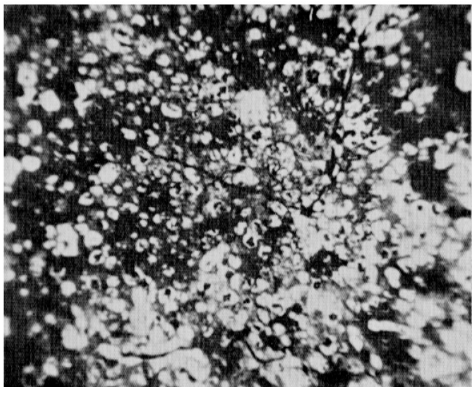

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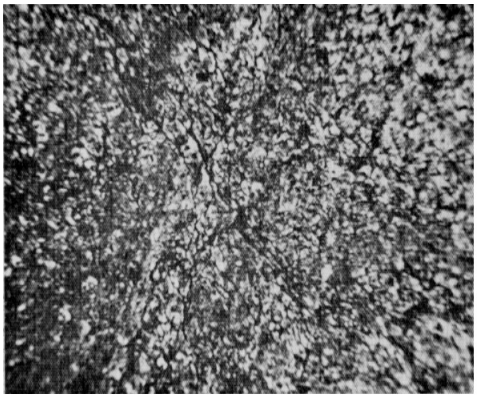

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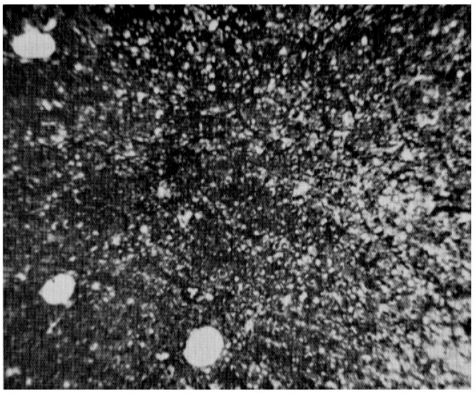

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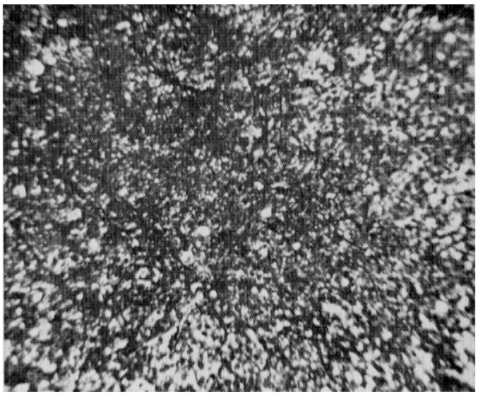

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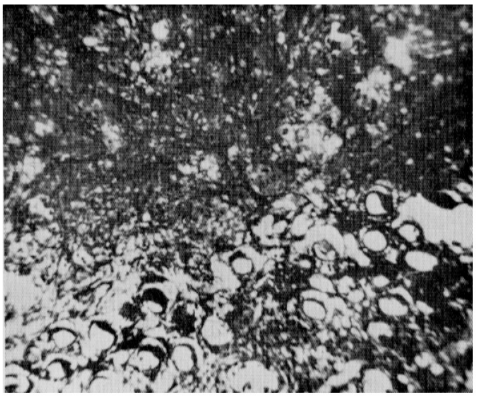

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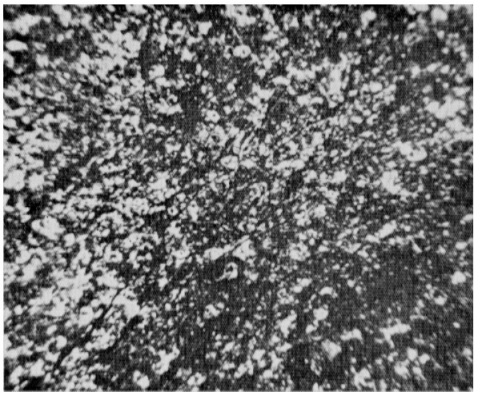

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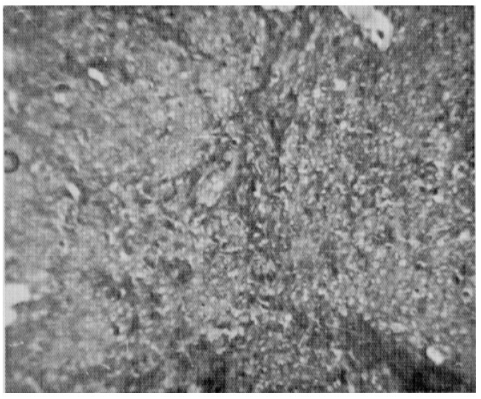

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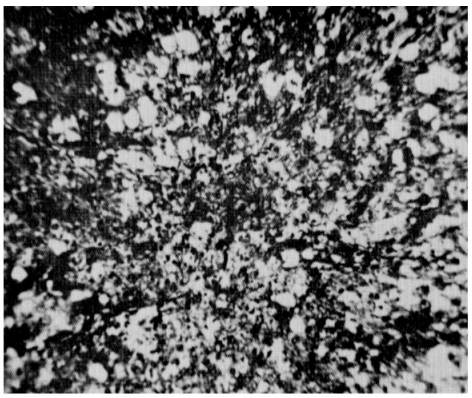

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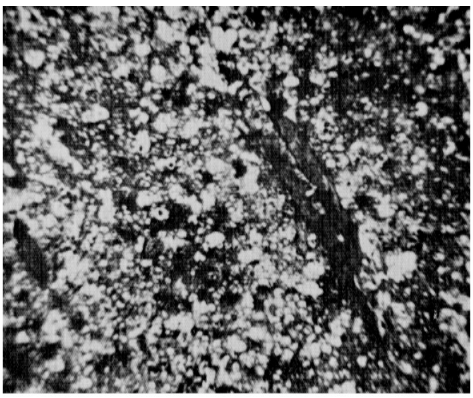

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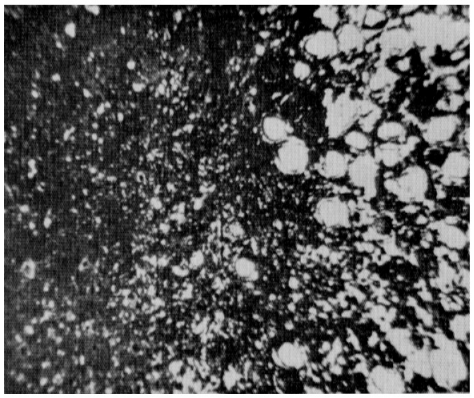

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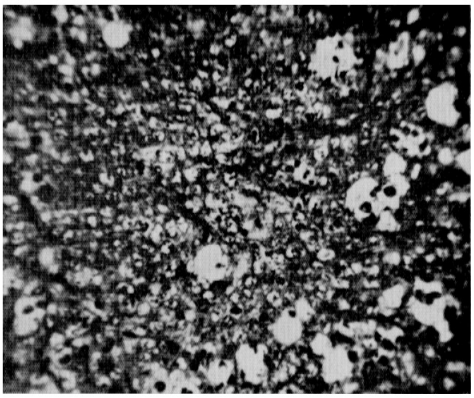

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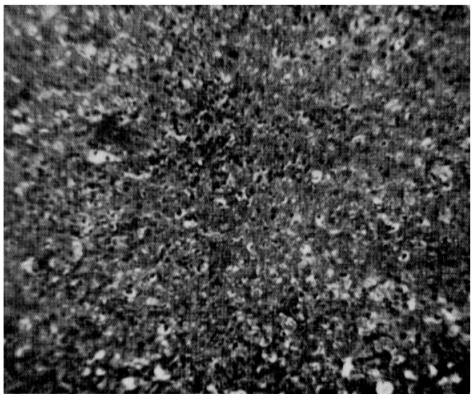

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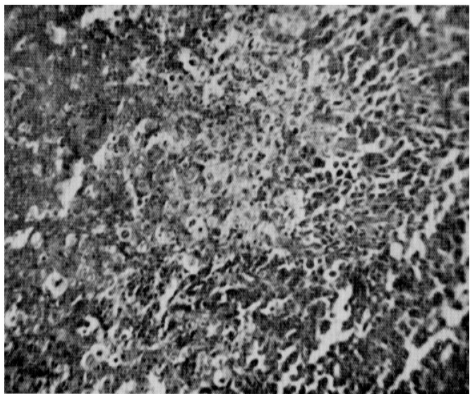

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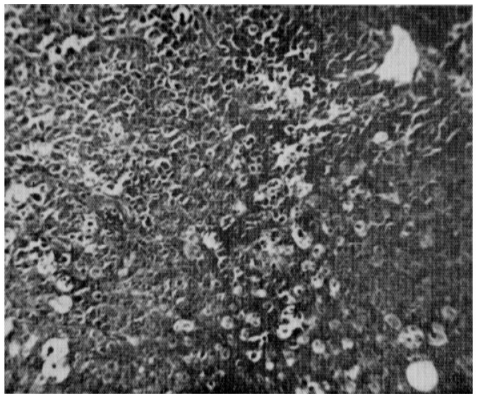

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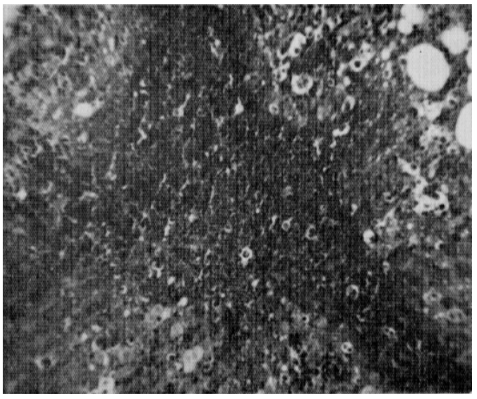

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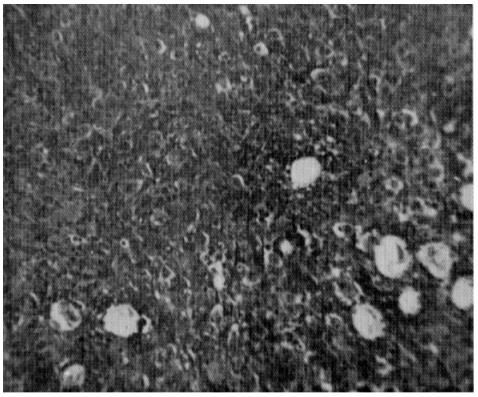

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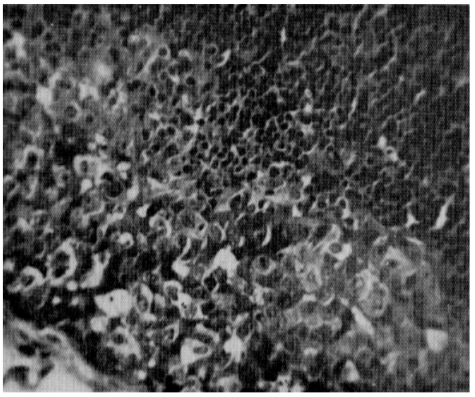

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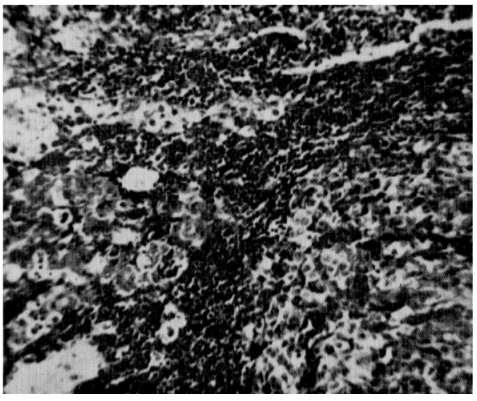

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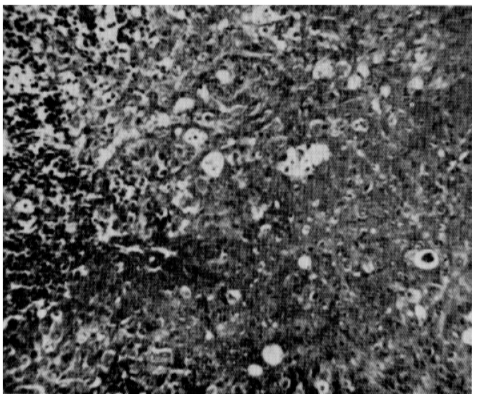

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