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The effect of staphylococcus aureus bacteriophage lysates upon malignant growth in hamsters and mice

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THE EFFECT OF STAPHYLOCOCCUS AUREUS BACTERIOPHAGE LYSATES UPON MALIGNANT GROWTH IN HAMSTERS AND MICE

by

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TOPICAL OUTLINE

LIST OF ILLUSTRATIONS

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ABSTRACT

In view of the success of the Danish investigators Christensen and Kjems in inducing regressions of malignant tumors with streptococcus bacteriophage lysates, it was decided to test lysates of hemolytic *Staphylococcus aureus* upon malignancies in hamsters and mice.

Experimental Animals

1. BALB/c albino mice.
2. The golden hamster (*Mesocricetus auratus*).

Neoplasms

1. Lymphoid leukemia induced in the BALB/c mice with the Moloney lymphoid leukemia virus.
2. A spindle-cell sarcoma of the hamster induced by intramuscular injection of methylcholanthrene in 1950, and now in its 89th passage.

Bacteriophage Lysates

Standardized bacterial preparations designated as "Bacterial Antigen Staphage Lysates (*Staphylococcus aureus*, Serologic Types I and III" obtained from Delmont Laboratories, Swarthmore, Pennsylvania.

Experiment I

Two groups of mice were used. One was injected with leukemia virus at birth, and treatment with lysate was not begun until

after leukemic symptoms had developed. The other group was started on injections of lysate before being inoculated with leukemia. Control animals received injections of 0.9% saline. Injections were intraperitoneal; dose begun at 1/8 cc. and increased in increments of 1/8 cc. until the 1/2 cc. level. The type III lysate was used. In neither group was the course of the disease appreciably altered from that of the controls by the lysate therapy.

Experiment II

Sixty hamsters were divided into 3 groups of 20 animals each. Group A was treated with lysate Type I, Group B with lysate Type III, and Group C (control) received sterile beef heart infusion broth. Injections were given subcutaneously between the scapulae 3 times a week and were started the day before the sarcoma was transplanted into the animals. The dose was 1/2 cc. in all cases. The transplants were made into the right cheek pouch with a 15-gauge trochar.

In Group A, 4 tumors (20%) regressed. In Group B, 1 tumor (5%) regressed and 3 showed markedly retarded growth. In Group C, 1 tumor regressed and 1 showed retarded growth. A chi square test of the regressions in Group A against the controls gave a probability of between 10% and 25% that the groups were the same.

Histological study of the regressing tumors showed vascularity to be poor or lacking. Mitoses were rare and the tumors were infiltrated with polymorphonuclear leukocytes.

After excision of the primary transplants, the animals remaining alive in each group were given a second transplant of sarcoma into the other cheek pouch. 36% of the Group B animals proved resistant to the second transplant as compared to 9% in Group A and 14% in Group B. A chi square test of the number of regressions in Group B as compared with the controls gave a probability level of less than 5%.

INTRODUCTION

I. Introduction

A. Historical Background

With the emergence of bacteriology as a science late in the last century, the interest of many workers turned to the possibility that microorganisms might in some way be connected with malignant disease. Some believed that microorganisms were the actual cause of cancer[1,2]; others that microorganisms or their products might be utilized as therapeutic agents in the control of malignancy[3].

Recent advances in antibiotic therapy[4] have renewed hope that a biochemical agent associated with bacteria may be discovered which will exert a destructive effect upon neoplastic growth without seriously interfering with normal cells.

The work relative to microorganisms and tumors has had a largely empirical basis. Nauts[3], in his review of the effects of bacterial products and infections on malignant tumors, points out that for over 200 years reports have been made of the regression of neoplasms following acute infections. If the cancers were not too far advanced and the infections were severe, the tumors disappeared and did not recur; whereas if the infection was mild and the malignancy extensive, only temporary regressions occurred. The infection most commonly involved in these recoveries was erysipelas.

The first attempts at the treatment of malignancy based upon these reports were initiated by Busch[5] in 1868. He endeavored to produce erysipelas in his cancer patients, but was unsuccessful in inducing the infection because he was unaware of its streptococcal origin. Fehliesen[3], after his discovery of the organism responsible for erysipelas in 1882, injected live cultures of the streptococci into cancer patients and obtained "encouraging results". Since then, many microorganisms and their products have been reported to have some therapeutic value in the treatment of malignancy. These have included Serratia marcescens and gonococci, certain spirochaetes, Clostridium histolyticum, Bacillus subtilis, Bacillus coli, Staphylococcus aureus and Klebsiella pneumoniae[6].

Coley[7], following Fehliesen's discovery of the causal agent of erysipelas, began treating cancer patients by direct injection of broth cultures of streptococci into the tumor. His cultures were obtained from erysipelas patients. He induced shrinking of the tumors by this method, but complete regression was obtained only in those patients who developed a systemic erysipelas infection. Sarcoma responded to the treatment more readily than carcinoma in a ratio of about 3:1. However, many patients proved immune to the infection; in some others the infection was fatal. Cultures of erysipelas sterilized by heating or formalin were weak and ineffective.

Coley[8] assumed cancer was caused by a microorganism and felt it "quite easy to understand that the toxic products of the erysipelas streptococci might being about such changes in the blood serum as to destroy the parasite of cancer or inhibit the growth". He believed that the toxic products could be isolated and used without producing erysipelas.

Coley spent the remainder of his life developing a series of preparations for the treatment of cancer. He learned of the work of Roger[9] with S. marcescens (Bacillus prodigiosus) which showed that the presence of this organism or its toxins frequently increases the virulence of associated organisms. Coley tried mixing the toxins of

S. marcescens with those of erysipelas. The resulting product showed some success with certain sarcomas but was not potent enough to cure more resistant types of malignancy.

Coley developed more than 15 formulas for his "Coley's Mixed Toxins", all but 4 of which, according to Nauts (op.cit.) were weak and variable. The toxins were used on a small scale in hospitals and private practice in this country and abroad over a period of about 60 years, but the results are difficult to evaluate. A standard bioassay to insure a definite potency of the products was not used, the techniques of treatment varied widely, and no standard method of recording technique, reactions elicited and results were employed. Nauts, however, after a careful study of 1200 cases treated with the various Coley toxins, concludes that "Toxin therapy (when reasonably potent products are administered with a proper technique) causes destruction of tumor tissue and apparent immunity to further recurrence or metastases". He points out that the preparations were not more widely used because the "existing strong evidence was scattered and had never been presented in such a manner as to allow toxin therapy to compete with its more popular competitors, the X-ray machine, radium, or some of the recently discovered chemotherapeutic agents".

Beebe and Tracy[9] reported "rapid and complete recovery of all tumors" after treatment of lymphosarcoma in dogs with toxins from streptococci and B. prodigiosus. They also tried filtrates of Staphylococcus pyogenes var. aureus, but reported only localized necrosis at the site of injection of the filtrates into the tumor.

Comsia[10] noted arrested development of grafts of Ehrlich tumor in mice which had contracted a recurrent spirochaetal infection. The tumors became granular, encrusted and necrotic. Further study showed a reciprocal relationship: cancerous animals were less susceptible to the fever and infected animals were not as susceptible to the transplants.

In 1928 Shwartzman[11] discovered that a subcutaneous injection of bacterial filtrate into a rabbit or guinea pig, followed in 24 hours by an intravenous injection of the same filtrate, resulted in a severe hemorrhagic necrotic reaction at the site of the original injection.

Work of Gratia and Linz[12] with the Shwartzman phenomenon showed that the hemorrhagic lesions were not restricted to the skin but could also be induced in lymph nodes, thymus, lung, and kidneys. On this basis these authors decided to see if necrosis could be induced in malignant tumors by this method. They used a liposarcoma and were successful in inducing the necrotic reaction in guinea pigs bearing this tumor, without causing hemorrhage in the normal tissues. They later found that the reactions could also be produced without the initial preparatory injection if the guinea pigs had had a bacterial infection such as <u>le charbon</u>, or an involvement with a virus such as vaccinia. This seemed to them to indicate that the infection had caused the necessary "preparation" of the tissues.

Viral etiology of malignant proliferation had been suggested by many workers, and Gratia and Linz felt that if this were a fact the tissues might already be "prepared" by the causative virus and that the intravenous dose alone would suffice to produce necrosis of the tumor. Their further studies with guinea pigs tended to bear this out, although sufficient animals were not employed to draw any definite conclusions.

Shwartzman and Michailovsky(13) also succeeded in producing the Shwartzman reaction without prior preparatory injection, using meningococcus filtrate in mice bearing sarcoma 180.

Duran-Reynals(14) extended the work of Gratia and Linz. He used tumor-bearing experimental animals which are normally resistant to the Shwartzman phenomenon (rats and mice) and injected them intraperitoneally with filtrates of _B. coli._ Slow-growing and non-malignant tumors showed negative results, but rapidly growing malignant tumors such as Walker's sarcoma and M/63 adeno-carcinoma proved nearly 100% susceptible to the hemorrhagic reaction. He, too, found that no previous local injection with the filtrates was necessary to elicit the reaction as would have been necessary with the ordinary Shwartzman reaction in rabbits and guinea pigs. The general resistance of the rats and mice to the ordinary Shwartzman reaction was not changed by the existence of the tumor. Duran-Reynals felt that special conditions must exist in the tumor to cause its blood vessels to react with the blood-carried bacterial toxins.

Further work by Duran-Reynals(15) with both spontaneous and transplanted tumors lead him to the conclusion that the degree of pre-existing necrosis in the tumor is a good index of the degree of tumor susceptibility, necrosis being a special state of vascular fragility which renders the rapidly growing tumor vulnerable to the injected toxin, even if the latter is practically harmless for the normal animal.

The work of Algire[16], described in the following paragraphs, clarifies somewhat the nature of the tissue reactions involved in the studies above. He has observed vascularization in both normal and tumor tissue using the transparent chamber technique. He finds that during vascularization of mammary carcinoma and sarcoma the tumor vessles, though retaining their capillary structure, are sinusoidal in nature and average about 8 times the diameter of adjacent capillaries between the muscle fibers. Vascularity in the surrounding connective tissue is about 25% of the total tissue volume, as compared to about 55% in the tumor tissue. "Vascularity" or "vascular level" as defined by Algire is the percentage of the volume of tissue measured which consists of functional vessels. This is obtained by a method of sampling based on geometric probability which is described in detail in his paper.

Upon injection of the bacterial polysaccharide from B. prodigiosus into the peripheral vascular bed of non-tumor bearing animals Algire noted a rise of vascular level from 25% to 35%, which was caused by a dilatation of the arterioles and venules. Associated with this dilatation was an increasingly sluggish blood flow and "a remarkable increase in leukocytes in the circulating blood". No hemorrhaging was observed. By the next day the vascular level had returned to the usual base line of 25%.

When Algire injected the bacterial polysaccharide into tumor tissue, however, there was no sign of vasodilation. Vascular level dropped steadily and by the end of 4 hours had fallen from 55% to 24%. Numerous petechial hemorrhages developed and gross fluid and red blood cells surrounded the tumor. Functional vascular level had dropped to zero at the end of 4 hours. By the end of the 4th day, the tumor had become entirely necrotic, apparently due to loss of vascular supply, but by the 5th day new capillary sprouts became visible about the periphery and tumor growth again commenced.

The phenomena described by Algire, as he states, "do not rule out the possibility of a direct lethal action of the polysaccharide on the tumor cell, but an adequate explanation of such lethal action would have to explain the vascular phenomena described".

Connell[17] used a slightly different approach. He grew B. histolyticus on scirrhous breast carcinoma suspended in physiological saline in an attempt to stimulate production of enzymes specific for proteolysis of the cancer tissue. He used filtrates of the tumor - bacillus culture in the treatment of human cancers and reported "arrest" and "softening" of the tumors and marked symptomatic relief, but no cures.

Jacobi[18], using a diluted filtrate of B. typhosus, produced the necrotic reaction of Shwartzman in 2 kinds of rat carcinoma and the Rous chicken sarcoma. This was followed by sloughing of the tumors, after which only a few recurrences were noted.

Zahl et al.[19] reported that with few exceptions killed gram negative bacteria induce capillary hemorrhage in implanted mouse tumors. However, he found that this property is almost entirely lacking in gram positive forms. In larger doses their preparations were toxic to normal vascular tissue. The authors feel that the S. marcescens (gram-negative) component of Coley's toxins operates as a vascular poison to the tumor capillary bed. The streptococcal components are nonhemorrhagic and therefore must exert their effect upon the tumors through another mechanism.

Jacobsen[20] points out the lessened frequency of neoplastic disease in patients with active tuberculosis, chronic osteomyelitis, and acute infectious disease in general. He cites the increasing incidence of cancer in the 30-50 age group and concludes that:

1. The reticulo-endothelial system when sufficiently activated by acute infectious processes, may attain the ability to cope with neoplastic disease.

2. The present increase in malignant morbidity is due to the decreased resistive powers of the reticulo-endothelial system resulting from lessened exposure to the infectious diseases which were prevalent before the advent of modern public health.

Jacobsen's conclusions are based largely on statistical surveys, however, and his correlations have since been questioned[21].

Shear [22,23] produced some symptomatic relief by administering S. marcescens polysaccharides to patients with inoperable cancer. Post-mortems showed gross changes in tumor size and consistency as well as microscopic evidence of necrosis and hemorrhage in both animal and human tumors. Shear concludes, however, that immunity reactions, differential tissue resistance and a toxemia resulting from necrosis tend to weaken the rationale for use of hemorrhagic bacterial materials. He refers to the effects caused by the bacterial polysaccharides as "laboratory curiosities", whose mechanism of action is still obscure. Shear agrees with Jacobsen that there is indeed an increase in the incidence of malignant diseases, even when figures are corrected for improved methods of diagnosis and for age distribution, and that there is a concurrent, and possibly related, decline in infectious disease. Shear cites the survey done at Children's Hospital in Boston where it was found that 3/4 of the spontaneous remissions of leukemia occurred following an episode of acute infection.

As in the other forms of malignancy, beneficial effects of acute concurrent infections or fever on leukemia have long been reported in the literature. Pelner *et al.*[24] cites 70 cases in which remission of leukemic symptoms occurred during or following such infections as typhoid, malaria, miliary tuberculosis, staphylococcus pneumonia, influenza and empyema. Remissions have also been reported following administration of Coley's toxins and tuberculin. Pelner feels the observations of such investigators as Southam[25] and Ungar[26] suggest that the reticulo-endothelial system of cancer patients is less active than normal, the ability to invoke the inflammatory reaction being absent in most patients. Such stimuli as toxin or concurrent infections may be able temporarily to stimulate the reticulo-endothelial system.

Wintrobe[27], however, after a critical review of the literature and his own observation of a series of 86 consecutive cases of human leukemia, feels that there is little evidence to support the thesis that a remission of leukemic symptoms frequently occurs in association with an intercurrent infection. He states: "The occurrence of a remission in association with infection in chronic leukemia....cannot be denied. However, a remission is probably a very unusual event rather than a common one."

Jordan *et al.*[28] report massive degeneration and necrosis of tumor tissue when a localized streptococcal adenitis was superimposed upon a localized lymphoma produced by intranasal deposition of ascites-form leukemia cells in AKR mice. The authors feel that the streptococci exert a direct cytopathogenic effect on the tumor cells.

In 1951 a Boston physician gained considerable unofficial publicity by using a hemolytic Staphylococcus aureus phage lysate in the treatment of cancer and allied diseases. The Massachusetts Medical Society published a report[29] on this therapy which concluded that:

> ....no proof has been presented nor was any able to be obtained which has shown that beneficial organic changes have occurred in disease processes treated by that therapy which have been due to that therapy and to that alone.

On this basis the Society did not recommend that any animal experiments be done or any hospital study of the treatment be made. As far as can be discerned by a search of the literature since the Massachusetts Medical Society's Report, no animal experiments have been published involving the use of staphylococcus phage-lysates to treat tumors.

In 1959 Christensen[30] endeavored to confirm the old hypothesis of antagonism between malignant tumors and infectious disease. Accordingly he subjected rabbits with Brown-Pearce carcinoma to repeated massive infections by hemolytic streptococci. Although the primary tumors remained unaffected, there was less occurrence of metastases among the infected animals than among the controls. Similar results were obtained by treating the rabbits with phage-lysed hemolytic streptococci. Experiments with heat-killed or formalin-killed streptococci, endotoxins or exotoxins were negative.

The infections resulted in a high mortality among the rabbits, whereas the lysates proved non-toxic. Since by using lysates the authors found that metastases could be inhibited without the general condition of the animals suffering in any way during treatment, further experimentation along this line was undertaken by Christensen and Kjems[31]. Three different lysates of hemolytic streptococci were used. All three proved capable of inhibiting the growth of tumors in a large number of rabbits. Out of 126 lysate-treated rabbits, 29 had metastases. Out of 113 placebo-treated, 110 had metastases, and all 71 untreated rabbits had metastases. In 44 out of 126 lysate-treated rabbits (35%) no viable tumor tissue was evident macroscopically at autopsy, whereas all placebo-treated and all untreated rabbits had moderate or large quantities of tumor tissue at autopsy.

B. Statement of Purpose

In view of the success of the Danish investigators Christensen and Kjems with streptococcus phage lysates and the renewed interest of many investigators in questions of immunity and cancer, it was thought that an interesting project would be one in which lysates of hemolytic *Staphylococcus aureus* were tested upon malignancies in hamsters and mice.

A pilot study using a small number of hamsters yielded a sufficient number of tumor regressions to make a larger study seem warranted.

It was decided to test the effect of the hemolytic *Staphylococcus aureus* lysates on an induced hamster tumor (available at Boston University) and on a virally caused mouse leukemia.

M A T E R I A L S

A N D

M E T H O D S

II. Materials and Methods

A. Experimental Animals

1. Mice

Dr. J. B. Moloney of the National Cancer Institute, Bethesda, Maryland, kindly supplied both leukemic and normal mice for this experiment. Twenty-six BALB/c albino mice were received from him. Twelve were 42-day-old normal mice. The other 14 were 100 days old and had been inoculated the day after birth with cell-free extracts of lymphocytic* neoplasms.

2. Hamsters

Sixty golden hamsters (Mesocricetus auratus) of both sexes were obtained from the Bioresearch Laboratories, Cambridge, Massachusetts. Their average weight was 80 grams.

The hamster was chosen as an experimental animal because:

a. The richly vascular blind end of the cheek pouch affords a transplantation site which can be viewed as frequently as desired without injury to the animal.

b. The use of aseptic technique has been found by experience not to be of any special benefit.[32]

c. The tumor chosen for these experiments has been shown to yield nearly 100 per cent takes in the hamster[33].

B. Neoplasms

1. Lymphoid leukemia produced by a virus extracted by Moloney[34,35] from sarcoma 37. Inoculation of the virus will induce a generalized

lymphocytic leukemia in essentially 100 per cent of weanling and adult BALB/c mice within 2.5 to 3.0 months. Intraperitoneal inoculation of a homogenate of spleen and lymph nodes of a leukemic mouse will induce a generalized lymphocytic neoplasm in 10 to 14 days[36].

2. Spindle-cell sarcoma induced in the golden hamster by intramuscular injection of methylcholanthrene by Handler[37] in 1950 and now in its 89th passage.

C. Bacteriophage Lysates

Standardized bacterial preparations designated as "Bacterial Antigen Staphage Lysates (*Staphylococcus aureus*, Serologic Types I and III" were obtained from Delmont Laboratories, Swarthmore, Pennsylvania. The lysates are prepared by the commercial laboratory by lysing *Staphylococcus aureus* Serologic Types I and III.

The lysates contain heat-labile and heat-stable antigenic fractions of *Staphylococcus aureus* with its extracellular and intracellular enzymes and the culture media ingredients (sodium chloride, beef heart infusion, Bacto-Tryptose). They are standardized on the basis of bacterial cell content and phage activity. Each m. contains:

2×10^9 lysed *Staphylococcus aureus*

1×10^{10} active *Staphylococcus aureus* bacteriophage particles.

In order to preserve maximum antigenic potency and phage activity no preservative is added. The lysates are tested prior to delivery for safety and sterility according to United States Public Health Service requirements for biologic products.[38]

Experiment I - Viral Leukemia of the Mouse

One of the 14 leukemic mice was killed and an equal quantity of leukemic spleen and lymph node material removed. This was homogenized in an Elvejhem homogenizer and then diluted 1:1 with 0.9% NaCl. This mixture was injected intraperitoneally into 12 healthy mice. Six mice were given lysate Type III intraperitoneally 3 times per week, starting the day before the mice were inoculated with the leukemic homogenate. Dosage of the lysate was started at 1/8 cc and increased by 1/8 cc. until a dosage of 1/2 cc was reached. The dose was then maintained at the 1/2 cc. level. The other 6 mice received intraperitoneal injections of 0.9% NaCl in the same dosage as the lysate.

Of the remaining 13 leukemic mice, 7 received lysate Type III 3 times per week, the other 6 receiving injections of 0.9% NaCl.

Experiment II - Transplanted Sarcoma of the Hamster

The 60 golden hamsters were divided into 3 groups of 20 animals each.

In the pilot study more tumor regressions had been obtained using subcutaneous injection of the lysate than had occurred following direct injection into the tumor. It was therefore decided that the subcutaneous route would be employed. By using this route a standard dose of 0.5 cc. per injection could be maintained, while an intratumor dose would of necessity vary with the size of the tumor. Also the variable introduced by mechanical injection into the tumor would thereby be eliminated.

Subcutaneous injections were made between the scapulae as follows:

Group A - Type I lysate

Group B - Type III lysate

Group C (control) - Sterile heart infusion broth

This was done to insure presence of the lysates in the bodies of the animals before transplantation.

Two days later another 0.5 cc. of the respective materials was given to all the animals and 1 cu. mm.± of the spindle cell sarcoma from a single donor was transplanted to the right cheek pouch.

The right cheek pouch was everted with forceps, pinned out over a lucite block and swabbed with zephiran diluted 1:2000 with mammalian ringer's solution. A 15-gauge trochar was loaded with a piece of tumor tissue 1 cu. mm.± in size and inserted through the stratified squamous epithelium of the cheek pouch. A small pocket was made in the underlying fibrous connective tissue by manipulation of the trochar, the tumor tissue ejected into the pocket, and the trochar removed. The incision closed itself without suturing due to the contractility of the pouch.

The animals were injected with lysate or broth and the tumors observed and measured 3 times per week for 37 days. The first observation was made on the 4th day after transplantation. By this day most of the tumors had "taken" and were beginning to vascularize. One control animal was found dead on this day, so was omitted from the study, making the total number of controls 19. The observations were made under a binocular dissecting microscope until such time as the tumors were large enough to make this unnecessary. The animals were anesthetized with sodium pentabarbital (Nembutal, Abbott) injected intraperitoneally in dosages of 0.15 cc. per 100 grams body weight with fortification of 0.05 cc. when necessary. The majority of the tumors tended to be spherical. Their diameters were measured with a millimeter rule, each measurement being checked twice. It was felt that this method would give as accurate comparative diameters as would the use of calipers.

Mortality in the 3 groups was roughly the same: at the end of the study (37 days after transplantation) there were 16 animals in Group A, 13 in Group B and 15 controls remaining alive.

During the study, tumors which regressed to near the vanishing point were excised, fixed in Zenker's fixative and prepared by the paraffin method for sectioning to determine what cytological changes had occurred. Non-regressing tumors were excised when central necrosis became too large to permit accurate measurement of the tumor mass. The viable sarcoma tissue around the periphery was fixed for sectioning to compare with that of the regressing tumors.

Photographs were taken on days 6, 17 and 32, of a typical control animal and of the experimental animal showing the minimum amount of tumor growth on those days (see Figures 2 through 8).

Retransplantation Study

The animals were permitted a week's rest after excision of their tumors, then lysate injections were resumed at 5-to 6-day intervals to keep the animals immunologically stimulated. Twelve days after completion of the 37-day observation period those animals still living were given a transplant of 1 cu. mm.± of sarcoma to the remaining left cheek pouch. (11 animals in Group A, 11 in Group B and 8 in Group C.)

The purpose of this second transplant was to determine:

1. Whether the first transplantation of tumor followed by regression and/or excision would render the animals resistant to further transplants.

2. Whether those animals who had been immunologically stimulated by the bacterial lysates would reject their transplants more frequently than the control group.

No further doses of lysate were given after the second transplantation was made. The animals were observed at roughly weekly intervals for 30 days.

RESULTS

III. Results

A. Experiment I - Viral Leukemia of the Mouse

1. The mice which were already leukemic when received were all dead within 26 days after arrival. The treated group lived no longer than the controls. At autopsy the spleens and lymph nodes of the treated animals showed as great a degree of enlargement as did the controls.

2. The mice which were started on lysate therapy before the leukemic homogenates were injected were all dead of leukemia within 33 days after injection, with the exception of 2 mice (one control and 1 treated animal) who showed no sign of leukemia upon autopsy.

The treated mice lived no longer than the controls, and the course of their disease appeared unaltered in any way by the lysate therapy.

Since in both A and B above the death rates of treated and control groups were the same, the chi square value for the experiment would be zero.

Figure 1. Mouse with leukemia induced by injection of homogenate of infected spleen and lymph nodes. Note grossly enlarged spleen.

B. Experiment II - Transplanted Sarcoma of the Hamster

1. Comparison of Mean Diameters

A comparison of the mean diameters of the tumors (Table I) in the 3 groups showed the means of both Group A (treated with lysate Type I) and group B (treated with lysate Type III) to be somewhat smaller than those of the controls through the 11th day after transplantation. When standard deviations were calculated[39], however, the mean diameters of the treated groups all fell within one standard deviation of the control group values. Therefore the apparent differences between the means of the 3 groups were not significant.

The mean diameters of the 3 groups were plotted against time following transplantation (see graph Figure 9). The curves for the 3 groups showed an initial period of slow growth through day 7, then rapid growth from day 7 to day 18. By the 21st day the Group B means had stopped their steep rise and the curve began to fluctuate. Groups A and C maintained rapid growth through day 23, then began to taper off. All the groups reached maximum size by day 27, after which the means decreased. The decrease in size was due to the advanced necrosis and sloughing of tissue which altered the spherical shape of the tumors and made accurate measurement impossible. The graph was not continued after the 30th day of the experiment.

Since day 18 was the last day when measurements could be made with any degree of accuracy, and since all regressions which occurred did so within the first 11 days, the graph from day 1 to day 18 was enlarged and points plotted for the standard deviations of Groups A and C. As can be readily seen, the growth curves for all 3 groups fall within one standard deviation of the control group. (Figure 10).

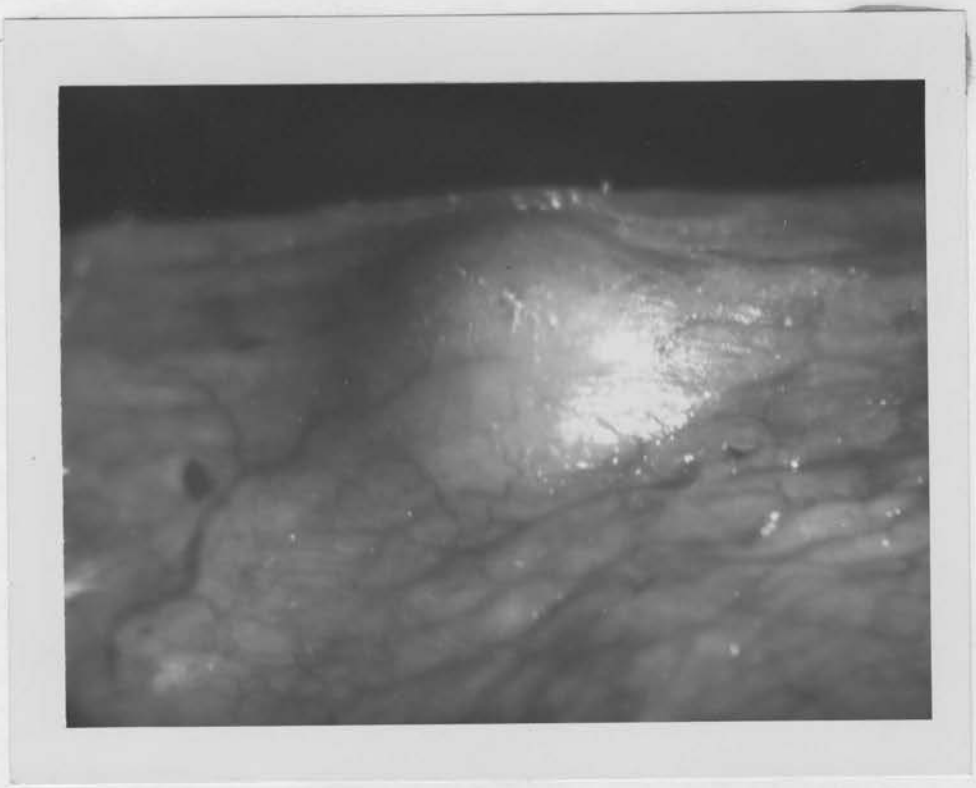


Figure 2. Tumor in cheek pouch of hamster treated with staphylococcus lysate, 6th day after transplantation, diameter 2 mm. Enlargement 30X.

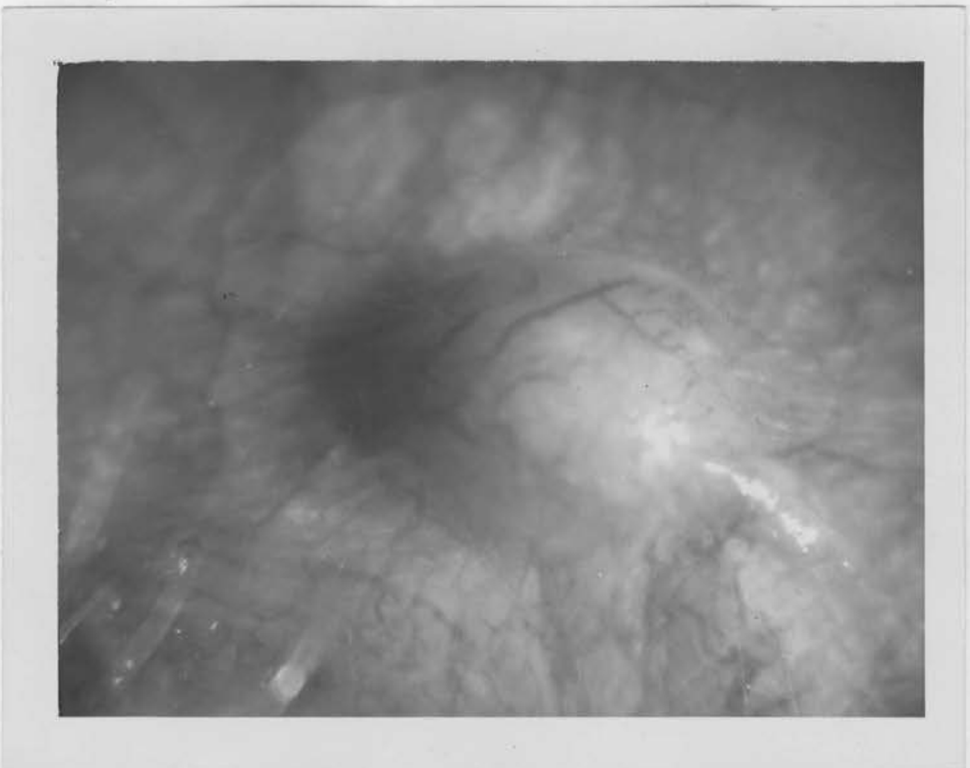


Figure 3. Tumor in cheek pouch of control animal, 6th day after transplantation, $3\frac{1}{2}$ mm. diameter. Enlargement 30X.

Figure 4. Cheek pouch of treated animal 17 days after transplantation. No tumor in evidence. Enlargement 2X

Figure 5. Detail of cheek pouch shown in Figure 3. Arrow indicates site where tumor had been implanted. Enlargement 30X.

Figure 6. Tumor in cheek pouch of control 17 days after transplantation. 13 mm. diam. Enlargement 2X.

Figure 7. Tumor in cheek pouch of treated animal 32 days after transplantation. 10 mm. diameter. Central necrosis just beginning. Enlargement $2\frac{1}{2}$X

Figure 8. Tumor in cheek pouch of control animal 32 days after transplantation. 21 mm. diameter. Necrotic process far advanced. Enlargement $2\frac{1}{2}$X.

TABLE I

Mean Diameters (in mm.) and Standard Deviations

Day	GROUP A		GROUP B		GROUP C	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
4	1.97	0.68	2.17	0.624	2.4	0.77
77	2.47	0.98	2.55	0.72	2.73	0.91
9	3.36	1.46	3.16	0.93	3.32	1.2
11	4.33	1.5	4.0	1.2	4.57	1.4
14	7.48	2.0	6.2	1.46	6.63	1.71
16	9.25	2.15	8.28	2.1	8.8	1.76
18	11.8	2.7	11.0	*	10.8	1.98
21	12.0	4.0	13.6		13.1	1.8
23	13.2	*	17.5		16.0	*
26	14.9		19.1		17.3	
28	13.7		16.3		15.7	
30	14.2		16.3		15.9	
33	14.4		16.0		16.7	
37	15.5		17.3		18.6	

* After this point the variation in the tumors was so great that a negative standard deviation was obtained.

Figure 9 - Mean Tumor Diameter Over Period of 30 Days

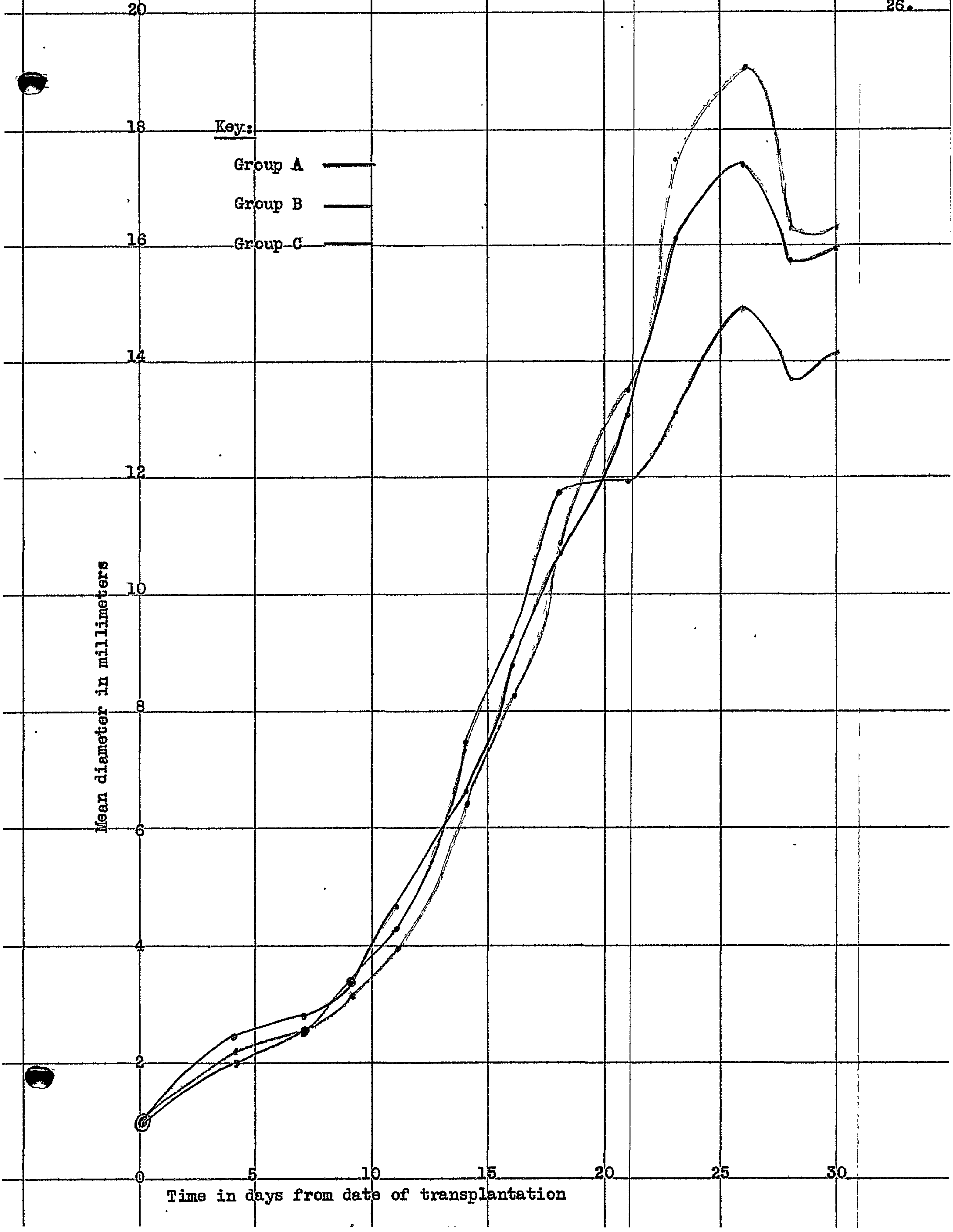
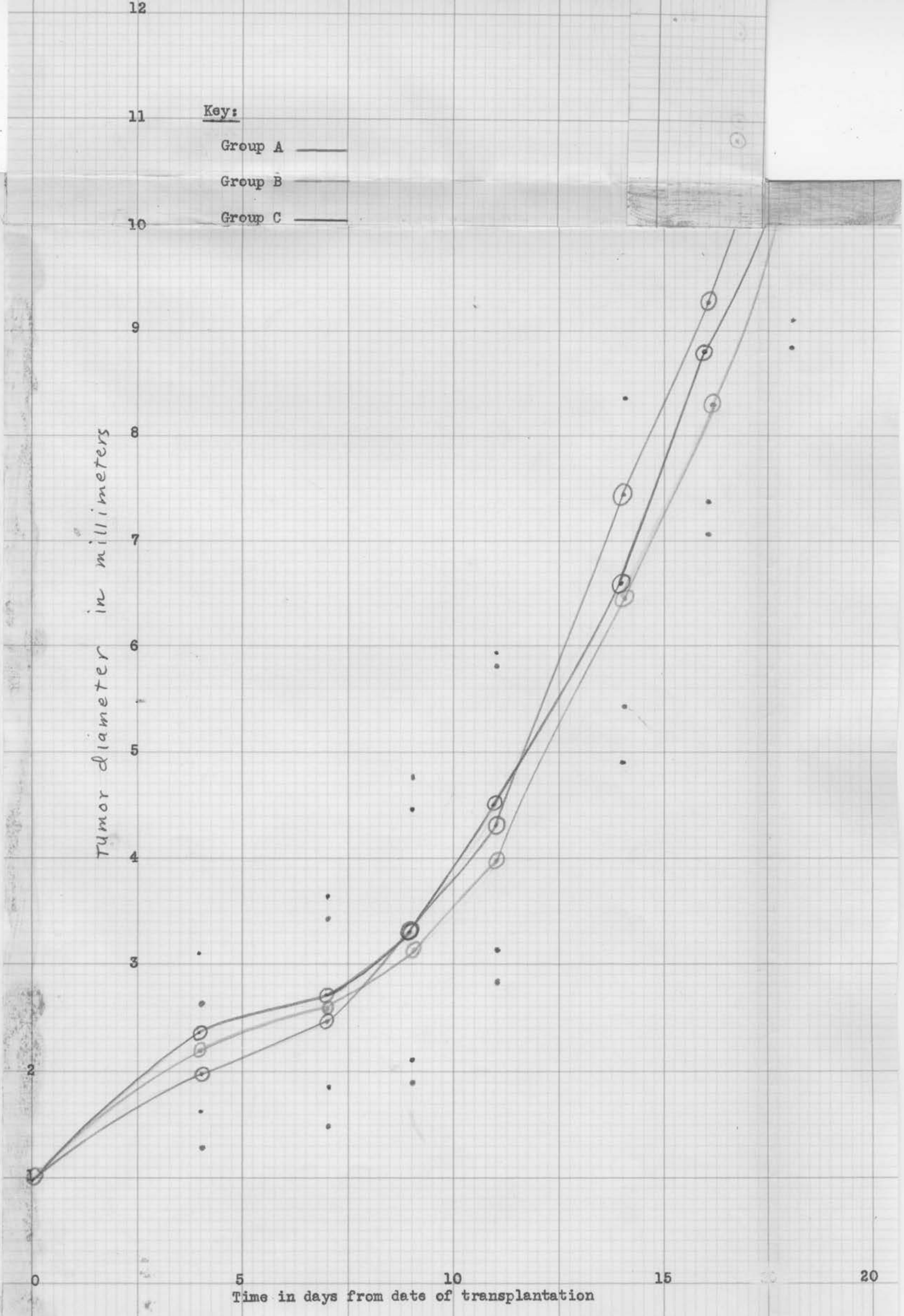


Figure 10 - Mean Tumor Diameter and Standard Deviation



2. Regressions

In Group A, 4 out of 20 tumors (20%) regressed. One tumor (5%) regressed in Group B, and 3 tumors showed retarded growth. One tumor (5%) regressed in Group C, and 1 tumor showed retarded growth.

In order to determine whether 4 regressions had any statistical significance, a chi square test was done of Groups A and C as follows:

	Tumors Growing	Tumors Regressed	Total
Controls	18 (a)	1 (c)	19 (e)
Group A	16 (b)	4 (d)	20 (f)
Totals	34 (g)	5 (h)	39 (k)

Observed	Expected	Δ	Δ^2	Δ^2/Expected
18 (a)	eg/k = (19)(34)/39 = 16.55	1.45	2.1	0.127
16 (b)	fg/k = (20)(34)/39 = 17.42	1.42	2.0	0.115
1 (c)	eh/k = (19)(5)/39 = 2.44	1.44	2.1	0.861
4 (d)	hf/k = (20)(5)/39 = 2.56	1.44	2.1	0.837
Chi square				1.940

A chi square of 1.94 indicates a probability of between 10% and 25% that the difference between the two groups had occurred by chance. According to Snedecor[40], a chi square value must be at least as large as 3.841 (indicating a probability of less than 5%) before the difference between 2 groups can be considered significant.

3. Histological Study

Regressing tumors were excised as they approached the macroscopic vanishing point so that the apparently absorbing tissue could be studied histologically. An equal number of rapidly growing tumors in the same group as the regressing tumors were excised to serve as histological controls. Three tumors in Group B and 1 in Group C which were exhibiting markedly retarded growth curves were also excised and sectioned for study. They were fixed in Zenker's fixative and stained with hematoxylin and eosin.

In general, the healthy control tumors in each group displayed cells with large, pale nuclei bearing one or more dark nucleoli. Mitotic cells were frequently seen, averaging 1.2 mitoses per field. (Oil immersion, 970X enlargement). Ten fields were counted for each tumor. Some polymorphonuclear leukocytes were present, averaging 3.8 per field. Figure 11 is a photomicrograph of a typical section of a control tumor. Group C tumors did not differ appreciably from the treated groups.

The regressing tumors showed shrunken, dark, strongly basophilic nuclei in which nucleoli were not seen. The nuclei were eccentric in shape and were more scattered than in the rapidly growing tumors. More fibrous connective tissue was present within these tumors. Polymorphonuclear leukocytes were numerous, averaging 14.5 per field. Mitoses were rare, averaging 0.4 per field. One tumor displayed many amitotic figures which were not counted as regular mitoses. Vascularization was poor or absent. Figure 13 is a photomicrograph of a typical section of a regressing tumor.

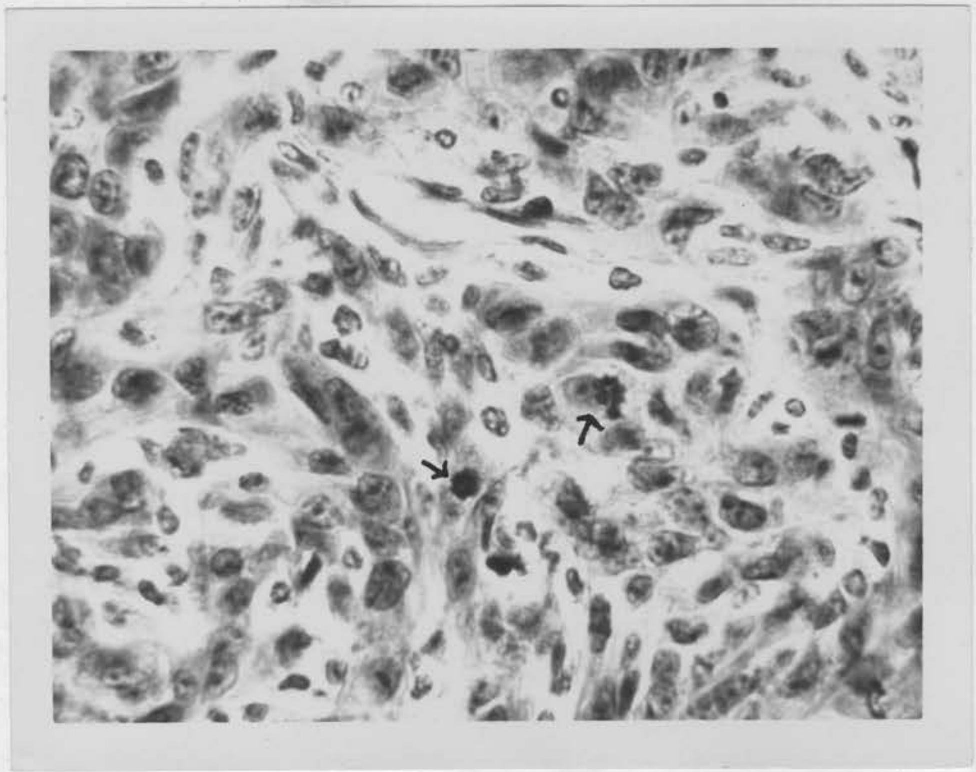


Figure 11. Photomicrograph of typical section of a control tumor. Arrows indicate mitoses. Enlargement 430X.

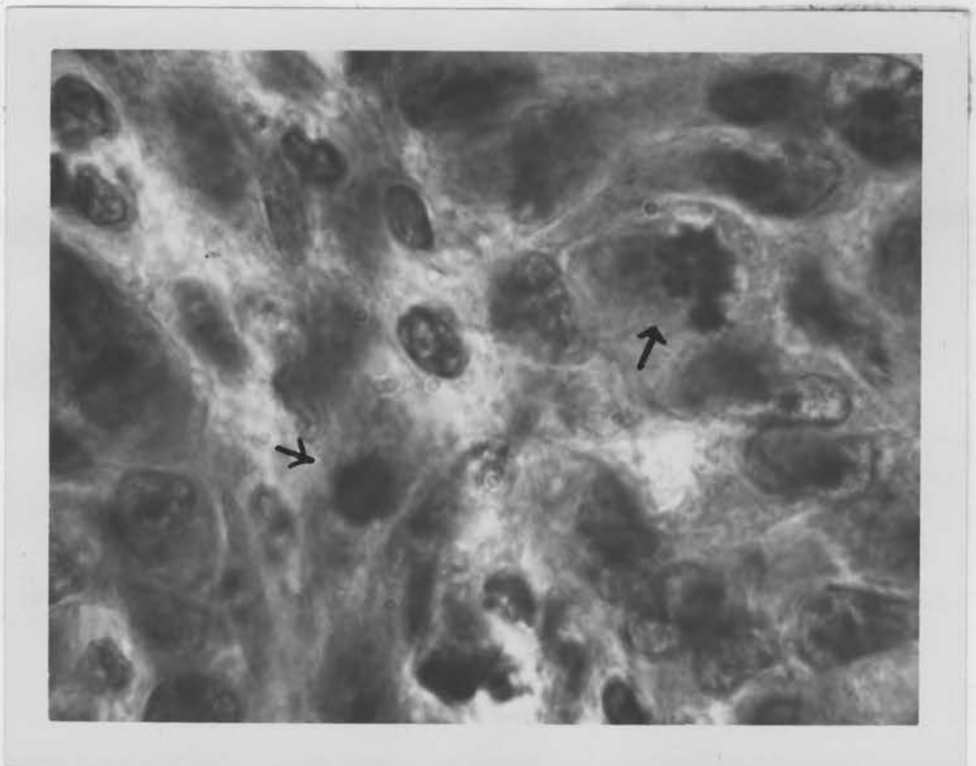


Figure 12. Detail of above, enlargement 900X.

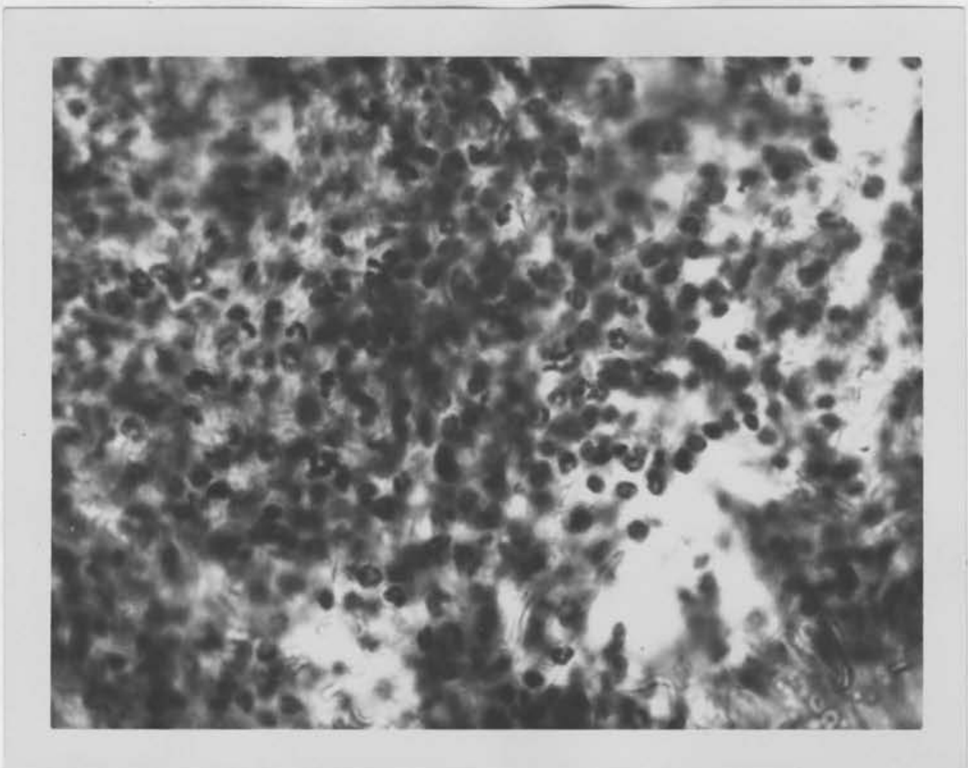


Figure 13. Photomicrograph of a typical section of a regressing tumor. Enlargement 430X.

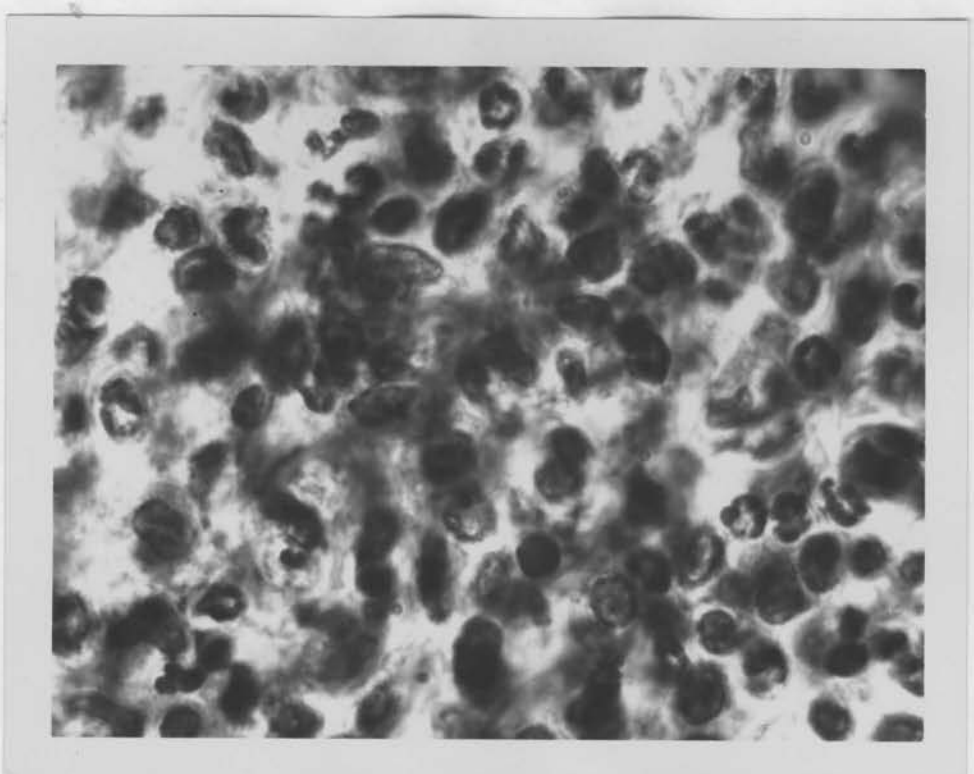


Figure 14. Detail of above, enlargement 900X.

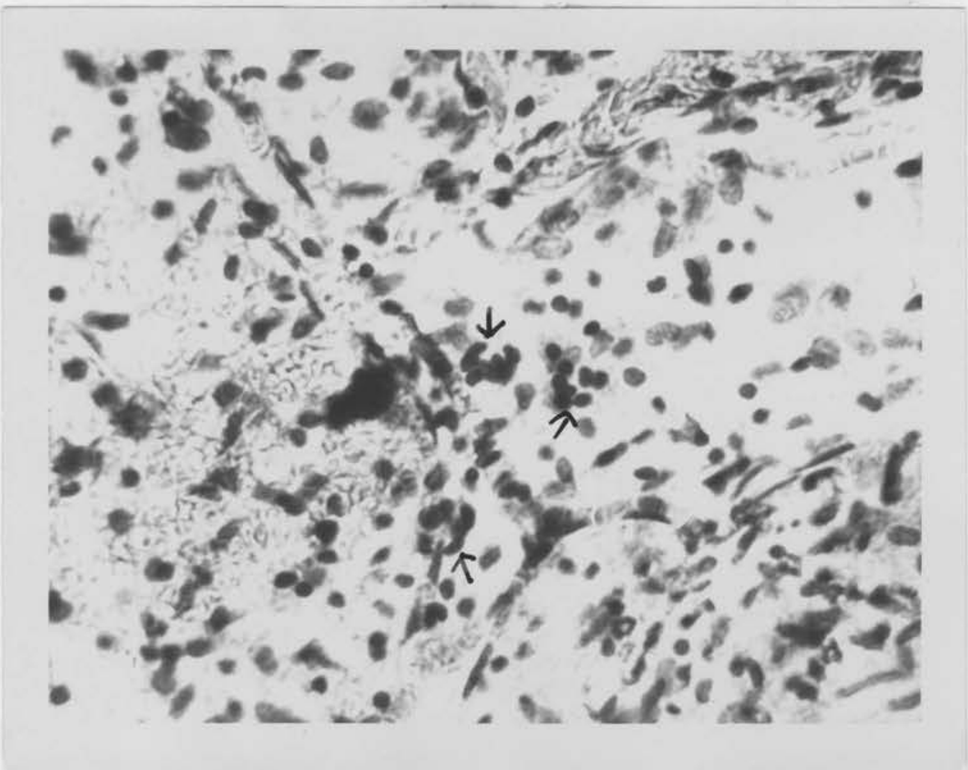


Figure 15. Polynuclear malignant cells in regressed tumor of control. Enlargement 430X.

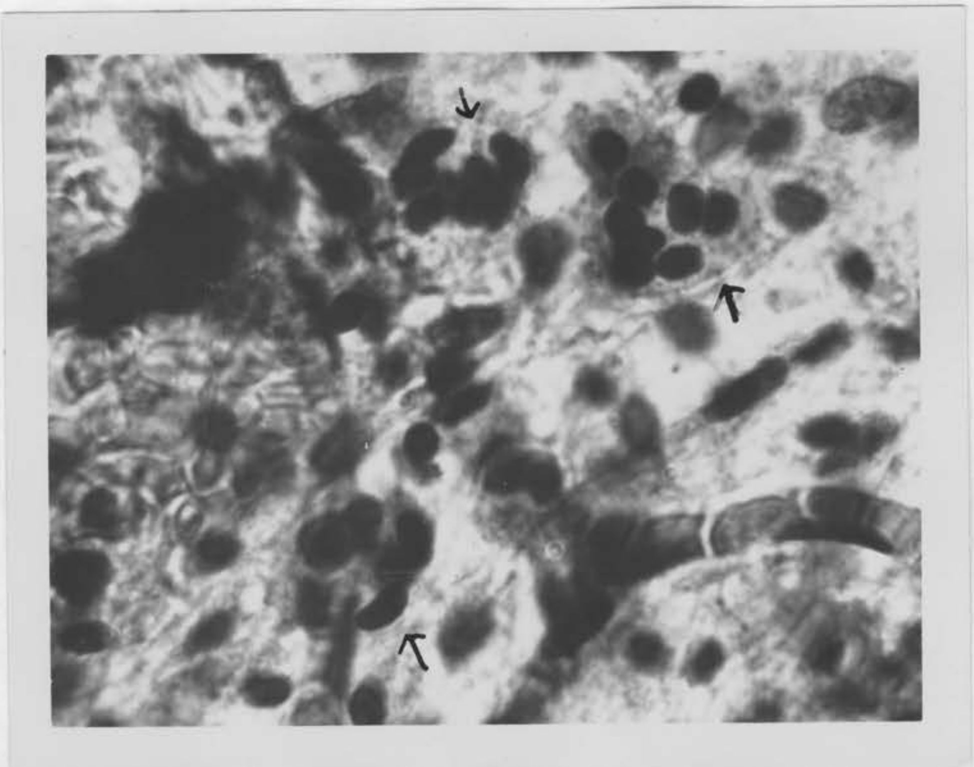


Figure 16. Detail of above, enlargement 900X.

TABLE II - HISTOLOGICAL STUDY: POLYMORPHONUCLEAR LEUKOCYTES AND MITOSES

GROUP A								GROUP B								GROUP C							
Regressed or Stationary				Growing				Regressed or Stationary				Growing				Regressed or Stationary				Growing			
#	Day Excised	Polys per field	Mitoses per field	#	Day Excised	Polys per field	Mitoses per field	#	Day Excised	Polys per field	Mitoses per field	#	Day Excised	Polys per field	Mitoses per field	#	Day Excised	Polys per field	Mitoses per field	#	Day Excised	Polys per field	Mitoses per field
12A	9	0	0	9A	21	8.2	0.9	7B	9	2.4	0	14B*	16	18.9	2.1	1C	9	26.0	0.6	2C*	11	4.0	0
5A	12	10.2	0	13A	21	2.0	0	18B‡	16	21.1	0.8	15B*	16	0.3	0.7					15C	16	0	1.3
16A	12	5.1	0.1	14A	21	0	2.6					3B	21	2.8	2.1					4C	21	3.4	1.3
17A	12	7.9	0.7	7A	37	2.9	1.6					13B	21	2.0	1.0					13C	37	0.5	0.6
Total		23.2	0.8			13.1	5.1			23.5	0.8			24.0	5.9			26.0	0.6			7.9	3.2
Average		5.8	0.2			3.3	1.3			11.7	0.4			6.0	1.5			26.0	0.6			2.0	0.9

‡ Tumor had remained stationary 13 days before excision.

* Tumor had shown markedly retarded growth before excision.

The one regression occurring in the controls differed from those occurring in the treated groups in one respect. There were present in tumor 1C a moderate number of hugh polynuclear malignant cells with up to 6 discrete dark nuclei. These cells were at least 3 times as large as the ordinary cancer cells (see Figure 15).

Table II summarizes the data for mitotic cells and polymorphonuclear leukocytes.

4. <u>Retransplantations</u>

Those animals which had received Type III lysate (Group B) had 4 regressions out of 11 transplants. Group A (Type I lysate) had one "no take" out of 11 transplantations; that is, no sign of tumor tissue could be located 4 days after transplantation. Group C also had 1 "no take" out of 7 transplantations.

It is difficult to decide whether a "no take" should be considered a regression or an error in transplantation, since no histological study was done on this part of the project. Assuming the no takes to be regressions, a chi square test was done as follows:

	Tumors Growing	Tumors Regressed	Total
Controls	6 (a)	1 (c)	7 (e)
Group B	7 (b)	4 (d)	11 (f)
Totals	13 (g)	5 (h)	18 (k)

Observed	Expected		2	2/Expected
7 (a)	eg/k = (7)(13)/18 = 5.05	1.95	3.80	0.75
13 (b)	fg/k = (11)(13)/18= 7.94	5.06	25.60	3.23
1 (c)	eh/k = (7)(5)/18 = 0.67	0.33	0.11	0.16
4 (d)	hf/k = (5)(11)/18 = 3.05	0.95	0.90	0.30
Chi square				4.44

A chi square value of 4.44 corresponds to a probability of less 5% that the 2 groups are the same. Therefore, it may be considered that the number of regressions obtained in Group B is statistically significant.

DISCUSSION AND

CONCLUSIONS

IV. Discussion and Conclusions

A. Discussion

It is well known that microorganisms synthesize an enormous range and variety of products[41]. Many of these products cannot, in fact, be synthesized in any other manner at the present time. As a group, microorganisms produce enzymes which can digest almost any organic substance known[42]. A large portion of the drug industry today is concerned with locating microorganisms which produce substances of antibiotic value[4,42].

It does not seem unreasonable, therefore, in the search for some means of controlling cancer, to investigate the products of microbial metabolism, many of which are potent antigens, as well as the viruses which are parasitic upon these microbes. Since precise knowledge regarding the cause of malignant growth is lacking, this sort of empirical approach seems necessary. Many advances in medical science have been made through such an approach, such as the treatment of pernicious anemia with liver extracts, of decompensated heart failures with digitalis and malaria therapy in general paresis.

Reports of viruses being utilized to effect oncolysis have occurred since 1912[43,44], and this work has received considerable impetus during the last decade. Moore[45,46] lists 11 such viruses which have proved effective in destroying one or more types of tumor, at the expense, however, of also killing the host animal. The majority of these viruses have been neurotropic. Turner and Mulliken[47,48] report prolongation of life in a significant number of leukemic mice treated with vaccinia virus, and Noguchi _et al._[49] report the clearing up of skin cancer lesions with the same agent. Briody[50] also used vaccinia virus to destroy Ehrlich acites carcinoma cells in the mouse. The mechanism for this tumor-destroying effect is unknown, but the investigators cited postulate that viral oncolysis may be brought about through a metabolic competition and/or antagonism.

With regard to the work of Christensen and Kjems, their results seem to indicate that some factor present in bacteriophage lysates of hemolytic streptococci is operating to retard or completely to inhibit malignant growth. (These experiments must, of course, be proved capable of duplication by other investigators before this can be accepted as more than a postulate.) Since there is no known specificity of bacterial viruses for mammalian cells, there is little reason to believe that the antitumor effect is brought about by the same mechanism as that operating with the animal viruses. The authors themselves state, "We do not know whether the presence of the phages is a presupposition for the tumor-inhibitory factor or whether they serve merely to release this factor from the bacteria in a gentle manner". Any conclusion on this problem must, of course, await further data on the metabolism of both viruses and mammalian cells.

More specific possible explanations for the effect of bacterial lysates upon tumors would seem to lie

1. along the line suggested by Algire: that is, that the toxic constituents of the bacterial lysates may become localized in tumors and are capable of acting as selective vascular poisons without

affecting normal tissue; or

2. in the observations of the many investigators cited in the historical section of this thesis, e.g. Shear[22,23] and Jacobsen[20] who feel that stimulation of the specific and/or non-specific immune mechanisms of the animal is involved.

In the experiments involving hamsters treated with the staphylococcus lysates, two of the Group A tumors had regressed to a microscopic level by the fourth day. No blood vessels could be seen microscopically; but whether or not a hemorrhagic vascular reaction had occurred is not known. Another regression showed no sign of hemorrhagic reaction. The fourth regression was hemorrhagic on the fourth day, but so was one tumor in this group which continued to grow rapidly. In Group B the one tumor which regressed and one tumor which had remained stationary were hemorrhagic, but one tumor in this group which attained full size showed early hemorrhage. In control Group C the one regression was not hemorrhagic, but one tumor which grew normally was. It would appear, therefore, that there was no particular correlation between regression and hemorrhagic reaction in these animals.

With a transplanted tumor, of course, it is extremely difficult to determine whether regressions of the tumor is due to a specific host reaction against the tumor cells or merely a generalized reaction against the implantation of foreign tissue. In further studies, control transplants of normal connective tissue from the tumorous hamster used as donor would be instructive.

In Group A (animals treated with lysate Type I) 20% of the tumors regressed as compared with the controls, 5% of which regressed. The statistical analysis of these regressions indicates a rather large probability (between 10 and 25%) that the apparent difference between the 2 groups occurred by chance. Further studies employing a larger number of animals would be necessary in order to determine whether the Type I lysate was actually instrumental in causing regression of the treated tumors.

In any event, in evaluating this study, several points should be held in mind:

1. Different types of malignancy frequently react to a given therapeutic preparation in different ways. Only two types of neoplasm were used in this experiment. The next logical step would be to treat a whole spectrum of tumors, both spontaneous and induced, to determine what range of effects the lysates might have.

2. Experimental animals vary from species to species in their response to certain treatments. Clearly the leukemic mice showed no detected response to lysate therapy. No regressions or apparent remissions occurred, and chi square for the treated vs. the control groups was equal to zero. The hamsters, however, showed a difference in response between treated and control groups which gave a chi square of 1.94 which indicates a probability level of between 10% and 25%. The possibility that other species of animal might respond to lysate therapy should therefore not be ruled out.

3. Only one dosage was used in the hamster, and one route of inoculation. Varied dosages and such routes as the intravenous and

intraperitoneal should also be investigated.

4. There are a very large number of strains of hemolytic staphylococcus, many of which carry a phage which is capable of lysing them. The two strains used in these experiments may not be the strains best suited to the initiation of tumor destruction. The two phages used in these experiments apparently varied between themselves in their effect on the animals. The group treated with Type I lysate had more regressions than the group treated with Type III; yet the animals treated with type III were apparently more able to reject a second transplant.

The small number of animals remaining alive for the retransplantation part of the study prohibits any definite conclusion to be drawn. It is suggestive, however, that the Type III lysate in some manner stimulated the immune mechanism of these animals.

Pelner[24] states "There is considerable evidence to indicate that the ability of an animal or patient to elicit an acute inflammatory reaction is one of the powerful inhibitors to successful transplantation of tumors or to the formation of metastases".

In future work the tumors should be viewed perhaps twice daily following transplantation in order to observe any inflammatory or hemorrhagic reactions which may occur.

The histological study of the regressing tumors was useful in ascertaining whether a pouch in which no tumor was evident on day 4 was a rejection of the tumor by the host or a transplantation error. Apparently no transplantation errors occurred in the main experiment, as regressing tumor cells were located in sections of both pouches which were questionable.

B. Conclusions

1. The treatment of BALB/c leukemic mice with the particular staphylococcus bacteriophage lysates used, at the dosage specified, was totally ineffective.

2. Treatment of hamsters bearing transplanted spindle cell sarcoma with the particular staphylococcus lysates used, at the dosage specified, does not result in a dramatic number of tumor regressions as compared with untreated controls. However, further research along this line does appear justified.

3. There is some evidence that animals treated with lysate Type III during the development of a transplanted sarcoma will, after the sarcoma has been excised, be resistant to a second transplantation of the sarcoma. Untreated controls were not resistant to the second transplantation.