THE EFFECT OF COLCHICINE ON TUMOR CELL IMPLANTATION IN MICE

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by

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Single tumor cells were sprayed onto the dorsal subcutaneous connective tissue expanses of mice which had been pouched with air and Earle's solution one day earlier. The oral administration of colchicine just prior to pouching, brought about the implantation of more tumor cells. This enhancement has been called the adjuvator effect. No adjuvator effect of colchicine was seen when mice were not pouched suggesting that pouching affords a site of injury on the tissues where colchicine may influence the adjuvator phenomenon.

The adjuvator influence of colchicine was eliminated in mice pretreated with antibiotic for 2 weeks. It was restored in such mice by feeding them a 2 day culture of bacteria from the small intestines of other mice. It was restored by the culture supernatant fluid too. It appears therefore that endotoxins derived from dead bacteria were responsible for the adjuvator effect and that colchicine by its destructive action on the mucosal lining of the intestine, allowed these endotoxins to be absorbed and to act at the site of injury due to pouching.

The adjuvator effect due to the supernatant fraction was obtained when it was injected intraperitoneally but was eliminated when supernatant and colchicine both were injected intraperitoneally (ip.) into terramycin-fed mice. It was obtained again when the colchicine was given orally. Colchicine was thought to be largely localized at the cells of the gut mucosa when it was given orally, but to reach more effectively the cells of

the connective tissues when it was injected ip. Its direct effect on the connective tissue either offset the adjuvator action of supernatant or failed to do so depending on its route of administration.

Colchicine, whether given by mouth or ip., and the supernatant fraction of enteric bacterial cultures caused a destruction of thymus-dependent lymphocytes in the general lymphoid system of terramycin-fed mice. Since adjuvator activity was observed by colchicine only when fed by mouth it seems that the adjuvator effect of supernatant fraction is not by the lymphoid tissue changes. Rather, it is thought that mediation occurs by changes in the connective tissues upon which the tumor cells come to lie.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

I. INTRODUCTION

1. Review of the Literature

Man has tumors. These have been shown sufficiently similar in their natural history to tumors of other animals to make studies of the latter pertinent (23). Studies of man's environment, backed by experiments with colonies of laboratory animals (29), have defined many carcinogenic influences. Yet its epidemiology in man shows that human cancer is a sporadic disease. Not all men, or even all animals in carefully controlled experiments, respond equally to carcinogens by the production of tumors. It is important therefore to seek co-carcinogenic factors which are not themselves carcinogenic (18,19,20,21).

One such co-carcinogen is endotoxin, which has been shown to increase tumor takes when a mammary tumor was injected in a certain way (3), and to increase the yield of autochthonous tumors evoked by methylcholanthrene, a powerful chemical carcinogen (22).

Endotoxin is no mere laboratory curiosity. We carry a considerable load of it in our gut. Might it be on account of this that oral colchicine also causes an endotoxin-like enhancing effect in tumor transplantation, as has been previously shown (25).

Injury to the gut wall allows endotoxin from the gut to gain access to the systemic circulation. Cuevas and Fine(1) demonstrated that ischemic injury of the gut wall produced

endotoxemia in rabbits within five minutes after occlusion of the superior mesenteric artery. The same authors(2) simulated septic peritonitis by an intraperitoneal injection of cecal contents and also by an 18 hour culture of pathogenic Escherichia coli fortified with hemoglobin. This gave rise to endotoxemia within three hours. Removal by filtration of the bacteria just prior to the injection of culture filtrate delayed the onset of endotoxemia but did not prevent shock and death. The authors also induced chemical peritonitis by injecting bile, gastric juice or pancreatic extract intraperitoneally(2). All these agents evoked endotoxemia at different times followed by shock and death in all animals.

Unlike these agents which cause peritonitis, colchicine's injurious action occurs from within the intestinal wall on the mucosal lining. The normal structure of the small intestine has numerous villus projections which are especially morphologically suited for absorption of soluble materials from the gut lumen. Arterial branches enter the base of each villus and give way to a capillary network which is in close proximity to the epithelial cells. These epithelial cells alone thus separate the vascular system from the gut contents with its proportion of endotoxin.

Colchicine is an alkylating agent which arrests cells in mitosis. Bertalanffy(28) found using colchicine that the cells arrested in mitosis were almost exclusively in the crypts of Lieberkuhn of the small intestine. He further noted that the epithelial cells formed by mitosis in the crypts, reached the

tips of the villi from where they desquamated or were sloughed off. Therefore delay in cell replacement because of mitotic arrest in the crypts leads to denudation at the tips of the villi.

Stemmermann and Hayashi(7) have described how colchicine aptly causes marked villus atrophy of the intestine in humans who had taken lethal doses of colchicine either parenterally or orally. That colchicine's injury to the intestinal mucosa allows parenteral access of native endotoxins is suggested by experiments in which colchicine induced the generalized Shwartzman reaction(6) in pregnant or non-pregnant rats(26) and in the pregnant Golden hamster(27). The purpose of the present thesis is to determine the relationship of the adjuvator effect of colchicine by mouth(25) to the adjuvator effect of endotoxin(3).

2. Defining the Adjuvator Phenomenon

Tumor cells if they are to survive transplantation, must depend upon the favourable circumstances of the surrounding tissue fluid during the several days before they become vascularized (30). When fragments of tumors are scattered upon the subcutaneous connective tissues, the dying cells of the fragments afford aid in the form of nutrition to the surviving ones. If single cells are plated and a finer separation of individual neoplastic components is achieved, these aid one another less and more information is obtained about the susceptibility and/or the resistance of the subcutaneous expanses upon which these cells

come to lie(5).

Extrinsic influences which increase the susceptibility of the connective tissues, with the result of more "takes" from single-celled suspensions, have been called adjuvators(5).

II. MATERIALS AND METHODS

All mice used were from the notably homogeneous Balb C strain colony maintained for many years in this laboratory. In any experiment, the sets of males or females used were always homogeneous in weight, being within a range of no more than 4 grams in any one experiment. The total range of weights of the mice used in the entire study was 11 to 22 grams; that is to say, most mice used were young adults nearly two-thirds grown. The colony was bedded in autoclaved wood shavings and sustained with tap water and mouse breeder pellets.[1] All mice were prepared when the experiment called for it, by the splitting of their dorsal subcutaneous tissues (termed pouching) with a blunted needle and syringe containing 4 cc air and 1 cc Earle's solution (Figure 1A). The modified Earle's solution is a divalent-cation-free solution. The space where the needle enters between the pelt and body wall is quite loose and makes the entry of the needle and the forcing of Earle's solution by air quite simple. Air was then withdrawn immediately.

A single, complex, mammary cancer (MT296) was used throughout for all experiments. This had arisen in an old breeding female. It was always transplanted by plating tumor

[1]Purina Lab Chow.

cells, where the entire tumor growth in the donor mouse was used for each transfer. The plated generations used were 78-79, 84-88, and 91. The tumor maintained a complex of several neoplastic components persisting in apparent equilibrium with one another despite the many serial passages.

The cell suspensions were prepared firstly by excising tumors from mice killed with chloroform and hashing them with sharp scalpels until no fragment remained which exceeded 1 cu. mm. Suspensions were then prepared by alternately stirring and sieving the tumor fragments in 0.25% trypsin[2] in Earle's solution, using fresh solution each time. A Monel metal sieve with 50 micron pores was used. The suspension which passed through the sieve after the third round (nearly four hours after tumor excision) was generally used. The cells and small clumps in it were precipitated by centrifugation (500 g. for 10 min.) while kept at 0°C. The trypsinous supernate was decanted and replaced by another calcium-free modification of Earle's solution which contained the same chemicals but extra $MgCl_2$. It is termed Spinner salt solution (SS solution). The precipitate is a white, slimy clot, and was shaken by hand in the SS solution at 37°C, until the opacity of the new suspension had been brought to a maximum by cells shaken free. The clot was then removed

[2]Trypsin 1:250, Difco Laboratories, Inc., Detroit, Mich.

through a pipette, and the slimy remnants were broken up by adding 0.04% deoxyribonuclease[3] (DNase) in SS solution.

The cell suspension was next passed through a porous steel tube of 20 micron pore size. A small sample of the final suspension was mixed with a 10 fold volume of trypan blue,[4] 0.25% in isotonic saline, and its cells were counted in a hemocytometer. All cells remained viable and no less than 90% were singular, the remaining cells being in small clumps of 2 or 3 cells.

The plating of the cell suspension carried out using a blunted needle and a syringe containing 4 cc air and 1 cc cell suspension. The mouse was held horizontally, and the tip of the needle entered a skin slit made above the back of the knee, through the posterior thigh muscle, into the subcutaneous plane of the dorsal mid-line (Figure 1B). Air was then immediately withdrawn. The area unto which cells are plated is the same area which was separated from the pelt of the mouse by pouching a day before.

The oxytetracycline used was oxytetracycline HCl[5] (commercially called terramycin). It was given by mouth to mice for 2 to 4 weeks. It continues to kill bacteria in the gut and in the blood. Terramycin biscuits were prepared fresh daily by firstly mixing the soluble powdered terramycin (0.2 mg

[3]Deoxyribonuclease 1 (beef pancreas) 1 X Crystallized, Worthington Biochemical Corporation, Freehold, N.J.
[4]Trypanblau, Dr. G. Grubler & Co., Leipzig, Germany.
[5]Oxytetracycline HCl, Chas. Pfizer & Co., Inc., New York, N.Y.

per mouse) and pulverized chow pellets, and then adding tap water till a mashy consistency formed.

Bacterial cultures were prepared by removing a small portion of the small intestine from mice just killed by cervical dislocation, and placing it into a vial containing Trypticase Soy Broth[6]. This was incubated for 48 hours at 37°C. The broth is known to be capable of maintaining most any microorganisms as well as fungi. After 2 days, the murky broth was pipetted off into a new vial. This was centrifuged at 3,000 g. for 30 min. The supernatant fraction was pipetted off from the particulate into another vial. The remaining supernatant was decanted. The particulate left over was then resuspended in fresh broth. Both vials were centrifuged and the supernatant and particulate collected again. The particulate was kept very concentrated by resuspending it in a minimal quantity of broth. No attempts were made to analyze the contents of the supernatant although some bacteria were seen to remain present.

Colchicine U.S.P.[7] ($C_{22}H_{25}O_6N$) is a plant product largely obtained from Colchicum autumnale (autumn crocus or meadow saffron). It was dispersed in an appropriate solution of Earle, or broth depending upon the experiment. A syringe and a blunted needle with a tapered, smoothed end, were used for

[6]Trypticase Soy Broth, Dir Becton, Dickirrson & Co., Cockeysville, Md.
[7]Colchicine, Fisher Scientific Co., New York, N.Y.

the oral administration of colchicine. The hollow bougie was slipped towards the back of the mouse's mouth whereupon it swallowed the suspension, slowly forced out by the syringe.

Endotoxin used was a lipopolysaccharide[8], prepared commercially from Salmonella typhosa by Boivin's aceto-acetic acid extraction method. It was suspended in either Earle's solution or broth, depending upon the experiment.

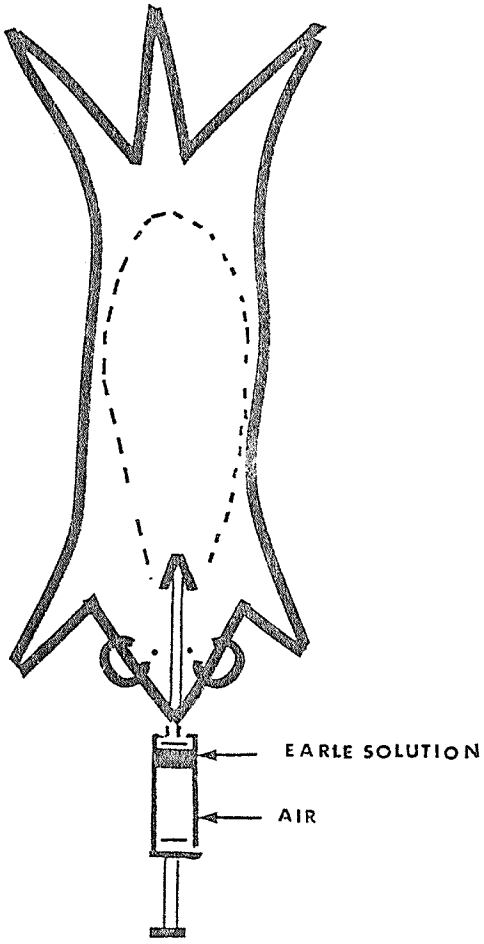
Tiny visible tumors were examined and counted on the connective tissue expanses 10 to 15 days after plating by cutting open the dorsal skin and pinning it aside to expose the connective tissues and their tumors (Figure 1C). All statistical analyses were based on these counts.

Histological sections were prepared from axillary and inguinal lymph nodes, spleens and thymuses. Tumor sections were also prepared from the tumors used for transplanting and maintaining the original tumor. The sections were fixed in buffered formalin and stained with Ehrlich's hematoxylin and eosin stains.

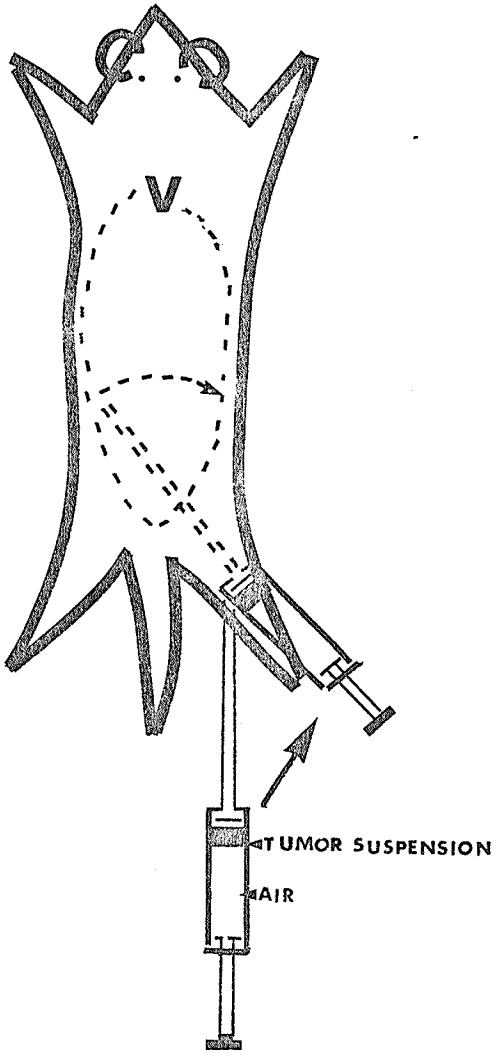
[8]Lipopolysaccharide B, S. typhosa, Difco Laboratories, Inc., Detroit, Mich.

FIGURE 1

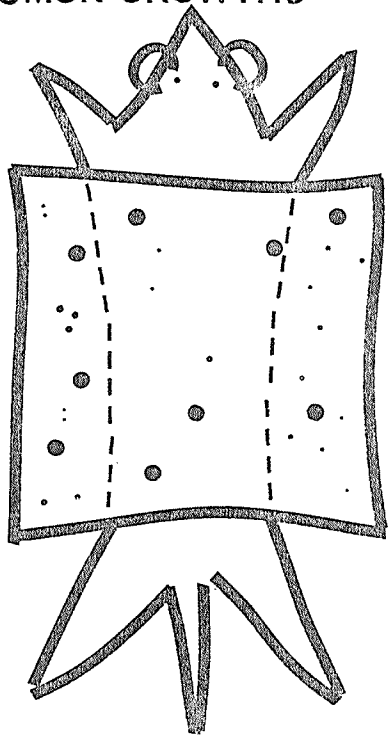
1A POUCHING



1B PLATING



1C TUMOR GROWTHS



III. RESULTS

A. Refinement of the Method

1. Necessity for Timely Pouching

Preliminary experiments in which unpouched mice were given 0.25 mg. colchicine by mouth (higher doses killed the mice), did not demonstrate an adjuvator effect on the connective tissue expanses for the implantation of tumor cells. The numbers of tumors counted in these mice within two weeks after the plating of single tumor cells were not significantly different from the numbers in those mice which were neither pouched nor offered colchicine by mouth. If an adjuvator effect was to be observed by feeding colchicine, it was evident from this experiment that preliminary pouching of the subcutaneous tissues was necessary for evoking the adjuvator phenomenon. Hence all subsequent experiments employed pre-pouched mice.

2. Setting the Dose of Colchicine

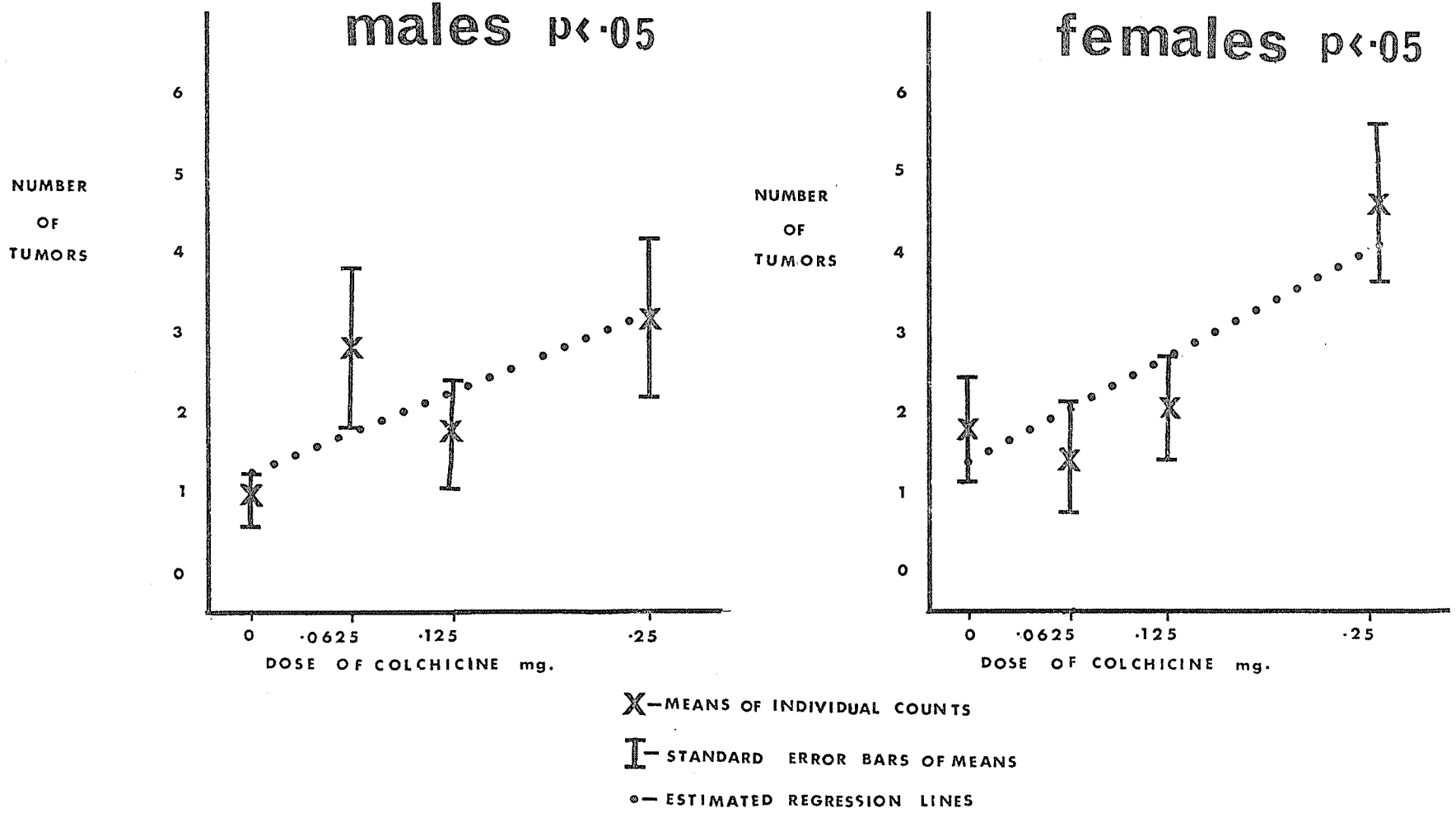
Male and female mice (each 25 in number) were delivered various doses of colchicine by mouth to determine which dose would yield a maximum adjuvator effect on the pre-pouched connective tissue expanses for the implantation of tumor cells. Three groups of 5 mice of each sex were challenged with 0.0625, 0.1250, and 0.2500 mg. respectively. Earle's solution without colchicine added, was administered to 10 mice of each sex. It was found that as the dose of colchicine increased, the number of tumors counted after

a few weeks also increased. This is shown in Figure 2. Crosses in Figure 2 represent the means of individual tumor counts for each dose of colchicine. The bars represent the standard errors of the means. The estimated regression lines as drawn were calculated for both male and female sets of mice. It was found that the positive relationship between the dose of colchicine and the number of tumors on the connective tissue expanses was significant at the 95% confidence levels for both sets of mice. As noted in the figure, the highest tumor counts for both male and female sets of mice were achieved at an oral dose of 0.25 mg. colchicine. Such a dose incurred few deaths. Therefore in all further experiments where colchicine was administered by mouth, a dose of 0.25 mg. was used.

3. Timing the Administration of Colchicine

An oral dose of 0.25 mg. colchicine was given to mice at various days before the day of plating cells as well as on the day of plating. Another group of control mice did not receive any colchicine. Mice received different treatments to determine if there would be any significant variation in the number of tumors formed over the experiment as a whole, and if so, then at which day relative to the day of plating would colchicine exert its maximum adjuvator effect. Mice were given colchicine 1, 2, 3, 4, and 7 days prior to plating.

FIGURE 2
COLCHICINE–TUMOR RELATIONSHIPS



The number of mice used for such treatments were respectively 5, 5, 4, 2, and 3. The group given colchicine on the day of plating and the group not offered any colchicine each consisted of five mice.

Figure 3 describes the variation in the number of tumors due to the different treatments used on the mice, and that this was significant at the 95% confidence level. The figure reveals that those mice which received colchicine one day before plating yielded more tumors than any other group; this was found to be significant at the 98% confidence level when compared to the control group of mice which had not received colchicine. Therefore in all the experiments to follow, mice which were to receive colchicine by mouth, were given 0.25 mg. colchicine one day before plating and up to a few hours before they are pouched.

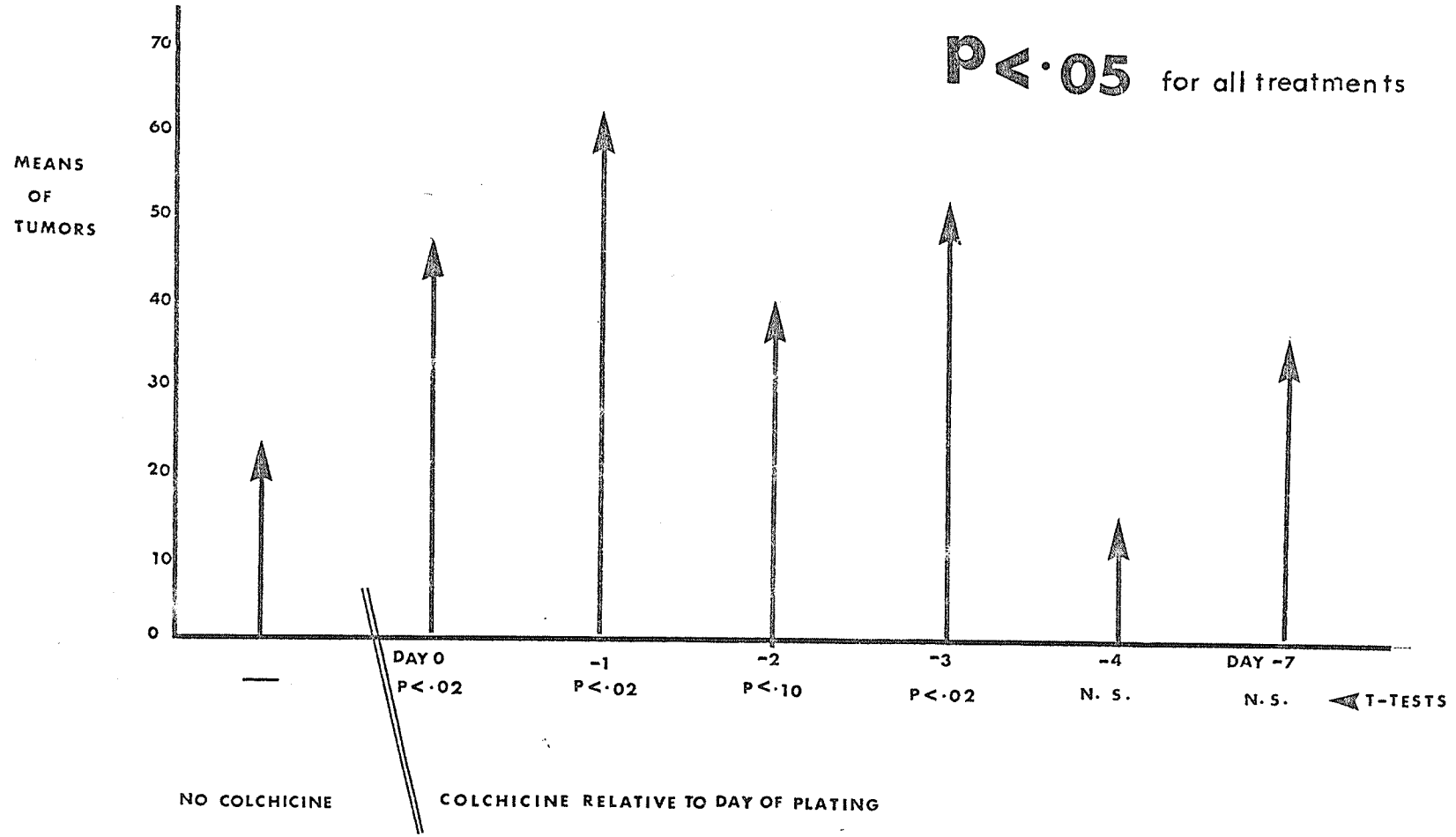
B. Determining the Mode of Action of Colchicine on Tumor Cell Implantation

1. Elimination of the Adjuvator Effect of Colchicine by Reduction of the Gut Flora

To determine if endotoxins play any role in the adjuvator effect shown by colchicine, it was necessary to eliminate the presence of endotoxins and then test the ability of colchicine to cause its own adjuvator effect on tumor implantation. Since endotoxins come from bacteria, then endotoxins in mice can be reduced by "sterilizing" the guts with antibiotic.

FIGURE 3

tumor variation due to different colchicine treatments

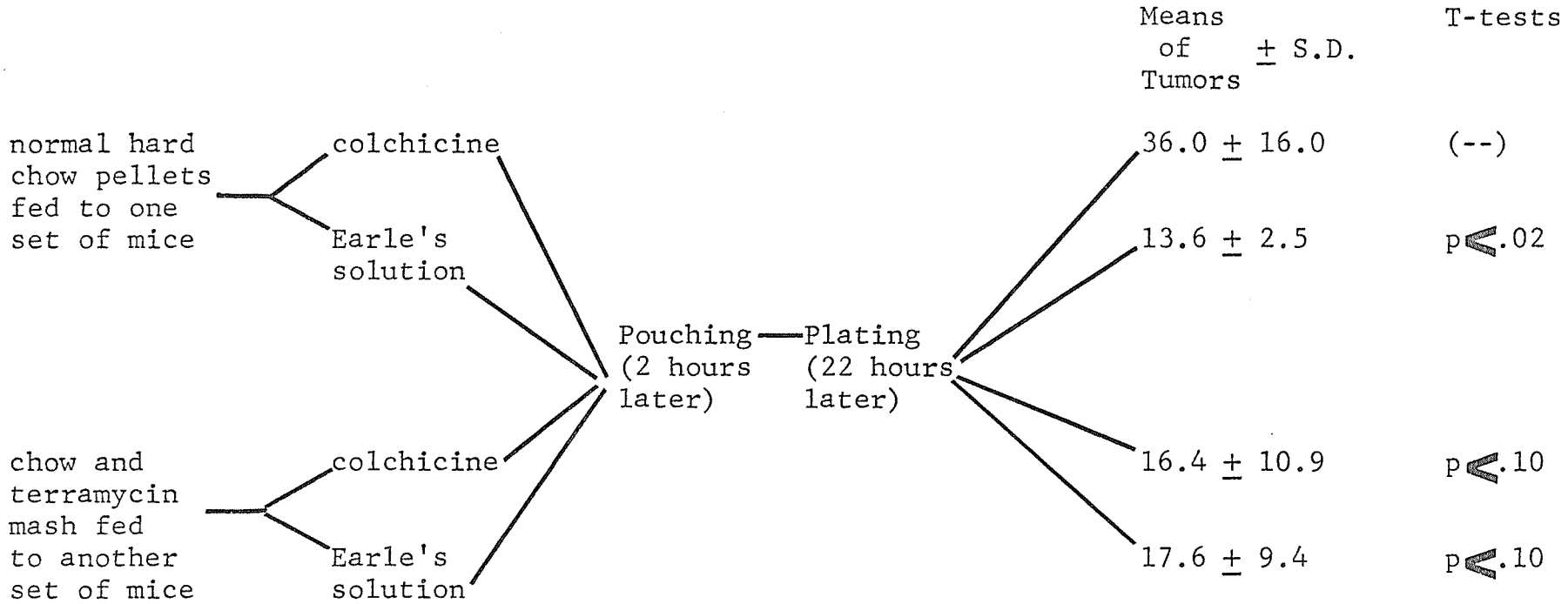


An experiment was designed to see whether the treatment of the guts of mice with antibiotic over a few weeks would eliminate the adjuvator effect due to colchicine. After receiving terramycin biscuits each day for two weeks, mice of one box were fed 0.25 mg. colchicine while mice of the other box were given Earle's solution orally. Control sets of mice, 2 boxes of mice each, were maintained on normal chow pellets. Mice of one box received 0.25 mg. colchicine by mouth and mice of the other box were fed Earle's solution. The feeding of terramycin biscuits and chow pellets was continued in the appropriate mice till the time of their sacrifice two weeks after being plated with cells. The oral administration of either colchicine or Earle's solution was followed two hours later by pouching and one day later by plating cells.

Tumors were counted two weeks after plating. The means of the tumor counts for the mice of each treatment and their standard errors are recorded in Figure 4. It is immediately clear that tumor counts in the mice which had been offered colchicine and chow pellets were more than double the tumor counts in the mice of the other groups which either had received Earle's solution with or without terramycin, or colchicine with terramycin. A t-test was done between the group given colchicine and not fed terramycin, and the group given Earle's solution and not fed terramycin. The difference was significant at the 98% confidence level. However, a t-test performed between the group

FIGURE 4

ADJUVATOR OF COLCHICINE ELIMINATED BY TERRAMYCIN



delivered colchicine and terramycin and the group given Earle's solution and terramycin, showed no significant difference. This demonstrates that the adjuvator effect of colchicine is eliminated if mice are fed terramycin consistently every day until their time of sacrifice.

2. Restoring of the Adjuvator Effect in Mice with Sterile Guts by Materials Given Orally which Contain Bacteria or their Endotoxins

(a) Adjuvator Effects of Bacterial Cultures and Their Supernatant Fractions on Oral Administration of Colchicine

Supernatant and bacterial fractions of a bacterial suspension were derived from mouse small intestine incubated in trypticase soy broth for two days. Both fractions were then tested for their adjuvator effects on cell implantation in mice maintained on terramycin for a few weeks.

The supernatant, its bacterial counterparts, and fresh broth itself were fed in ¼ cc. amounts one day before plating cells, to three different groups of mice all kept on terramycin over a few weeks. Each group contained 10 mice. After 2½ hours, the mice were orally administered 0.25 mg. colchicine. The mice were then pouched one hour later. Terramycin biscuits were removed permanently four hours before the different groups of mice were to receive supernatant, particulate or broth respectively on the day of pouching. The mice remained off food till after

pouching. Then they were fed normal chow pellets till they were killed. The feeding of terramycin was cut short since it would only have killed any living bacteria in the particulate and thus increased any existing amounts of endotoxin in the gut.

The mean of the tumor counts of mice offered broth was much lower than the means of either of the other two groups of mice given bacterial particulate or supernatant (Fig. 5). When t-tests were performed between the one group given the broth and either of the other two groups, the differences were found to be significant at the 95% confidence levels.

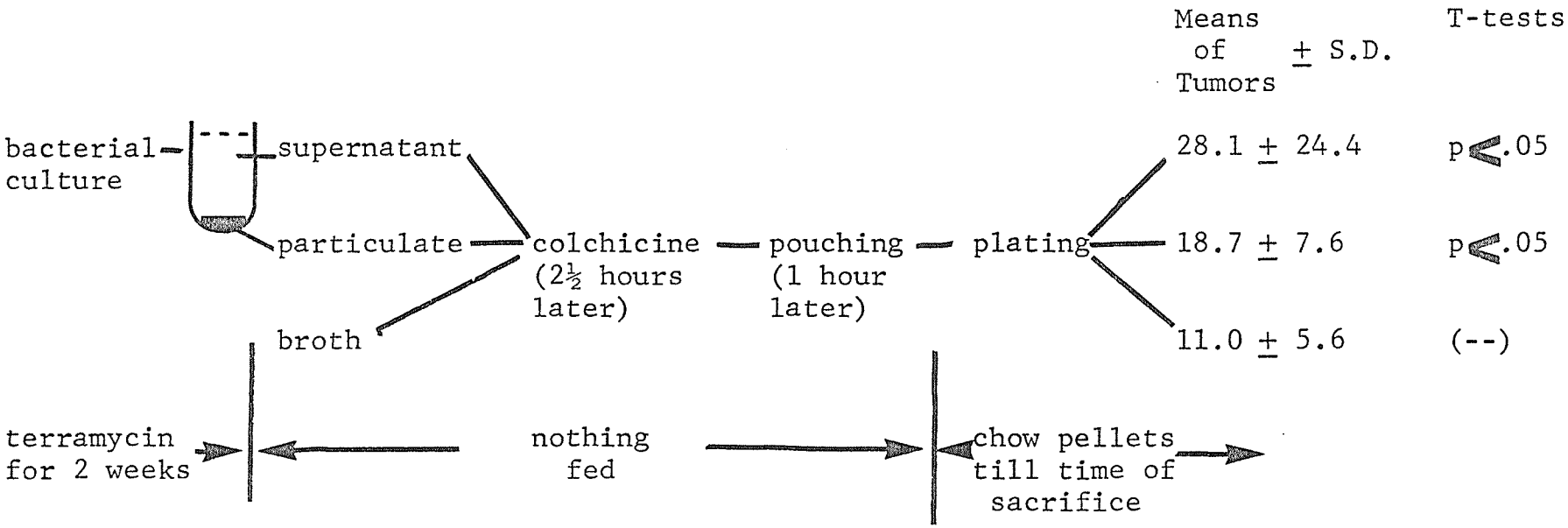
(b) The Adjuvator Effect of Chow Pellets in the Presence of Colchicine

A group of 20 mice were fed on terramycin for 2 weeks. Two days before plating, 10 of them received ¼ cc broth by mouth and were then placed on chow pellets. A day prior to plating, the other group received ¼ cc broth by mouth. On the same day and 2½ hours after the latter group was given broth, all the mice were given 0.25 mg. colchicine orally, followed an hour later by pouching. The latter group was then taken off terramycin and provided chow pellets. All the mice were plated with cells one day later.

When a t-test was performed for the significance of the difference between numbers of tumors resulting in the two treated groups of mice, it was found that the mice fed chow pellets

FIGURE 5

ADJUVATORS OF BACTERIAL PARTICULATE AND SUPERNATANT FRACTIONS



two days before plating had more tumors and that the difference was significant at the 95% confidence level. This is seen in Figure 6. The results of this experiment bring to light the importance of maintaining the exhibition of terramycin up to the time of pouching. The implications of this shall be further discussed.

(c) The Adjuvator Effect of the Supernatant Fraction in the Absence of Colchicine

Supernatant was given orally to twenty mice fed for 2 weeks on terramycin. After 2½ hours, half of the mice received broth medium orally and the other half 0.25 mg. colchicine orally. Ten mice were used as controls to those mice which received supernatant and colchicine orally. These mice (also on terramycin) received broth, followed 2½ hours later by 0.25 mg. colchicine orally. Mice were pouched one hour after receiving their second oral dosages, and were plated with cells the following day.

When t-tests were performed to test for the significance of the differences between those mice which received supernatant, with or without colchicine, and those mice which received broth and colchicine, the differences were significant for both tests at the 95% confidence levels (Fig. 7). Indeed, on the basis of a statistical comparison, whether or not colchicine was used in mice fed on terramycin had little bearing on the enhancement of cell implantation due to the action of supernatant. It is clear from the figure that broth by itself has no adjuvator

FIGURE 6

ADJUVATOR OF CHOW PELLETS GIVEN TWO DAYS BEFORE PLATING

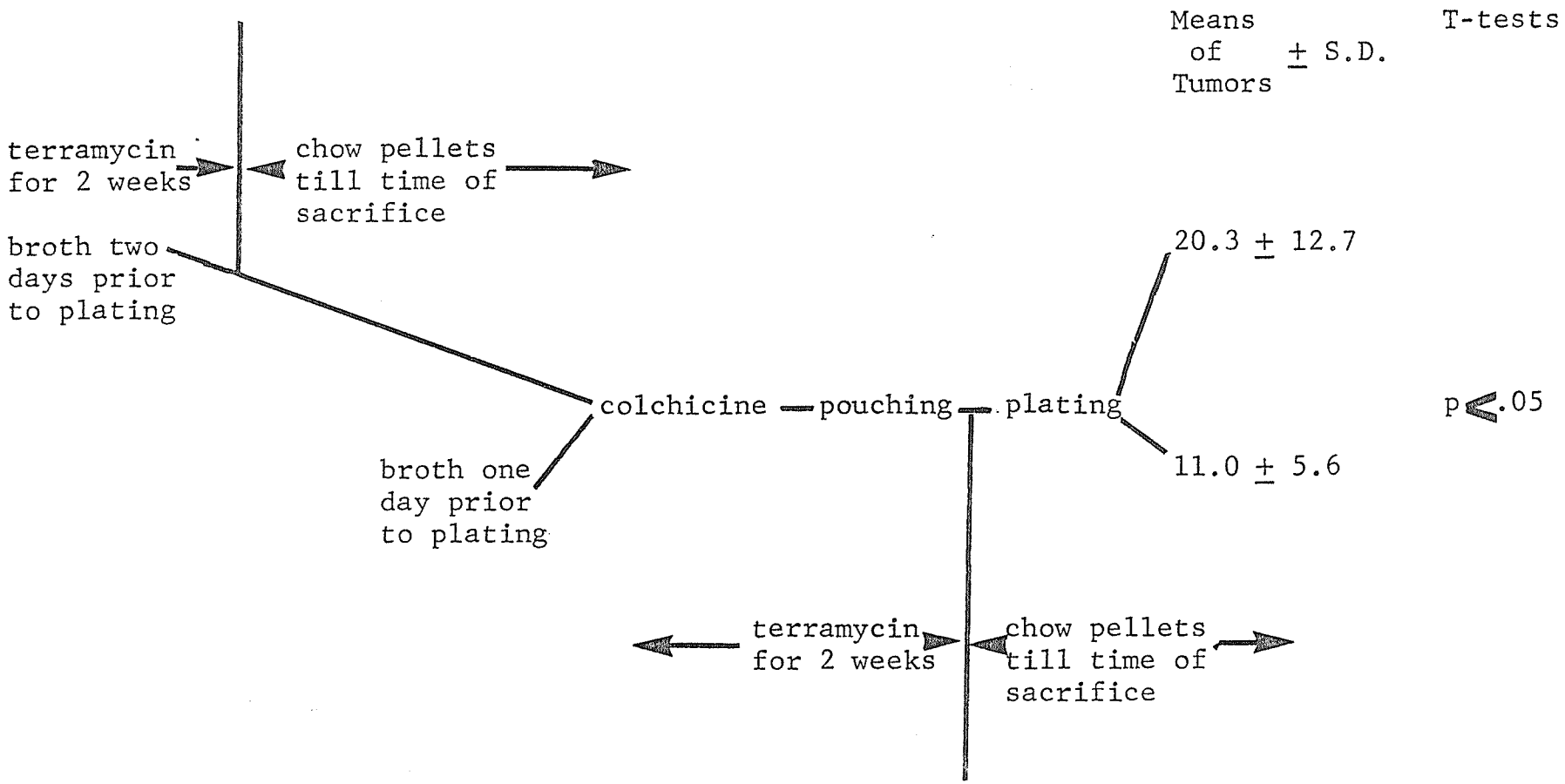
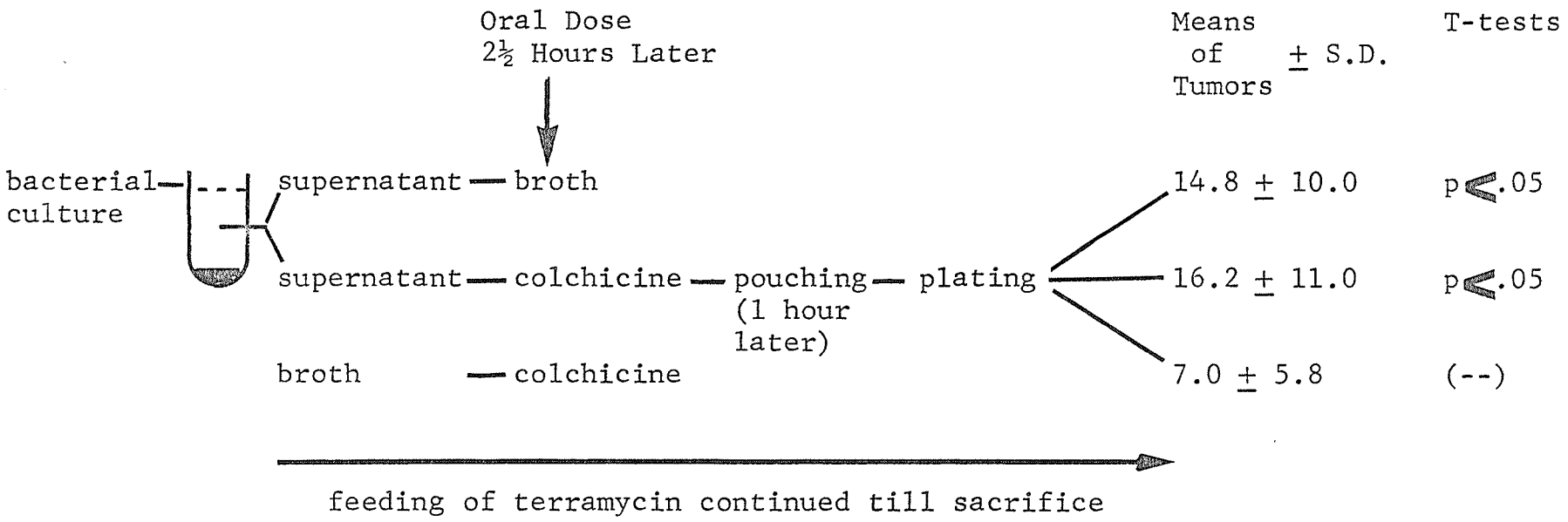


FIGURE 7

ADJUVATOR OF SUPERNATANT IN THE PRESENCE OR ABSENCE OF COLCHICINE



effect. Rather, the supernatant not only restores the principle eliminated by terramycin, but can accomplish the equivalent work by the colchicine on the intestine.

3. Elimination of the Adjuvator Effect of the Supernatant Fraction by the Intraperitoneal Injection of Colchicine

Colchicine when injected ip. into mice already injected with supernatant or endotoxin, was found to be synergistically toxic to the mice. When supernatant and colchicine were injected together the maximum dose of colchicine at which all mice remained alive was 0.01 mg. That dose therefore was chosen for this experiment. The supernatant fraction for the experiment was obtained in the usual manner. It was injected ip. in ¼ cc amounts into 22 mice fed on terramycin. After 2½ hours, broth medium was injected ip. into ten of the mice while 0.01 mg. colchicine (dispersed in broth) was injected ip. into the other twelve mice.

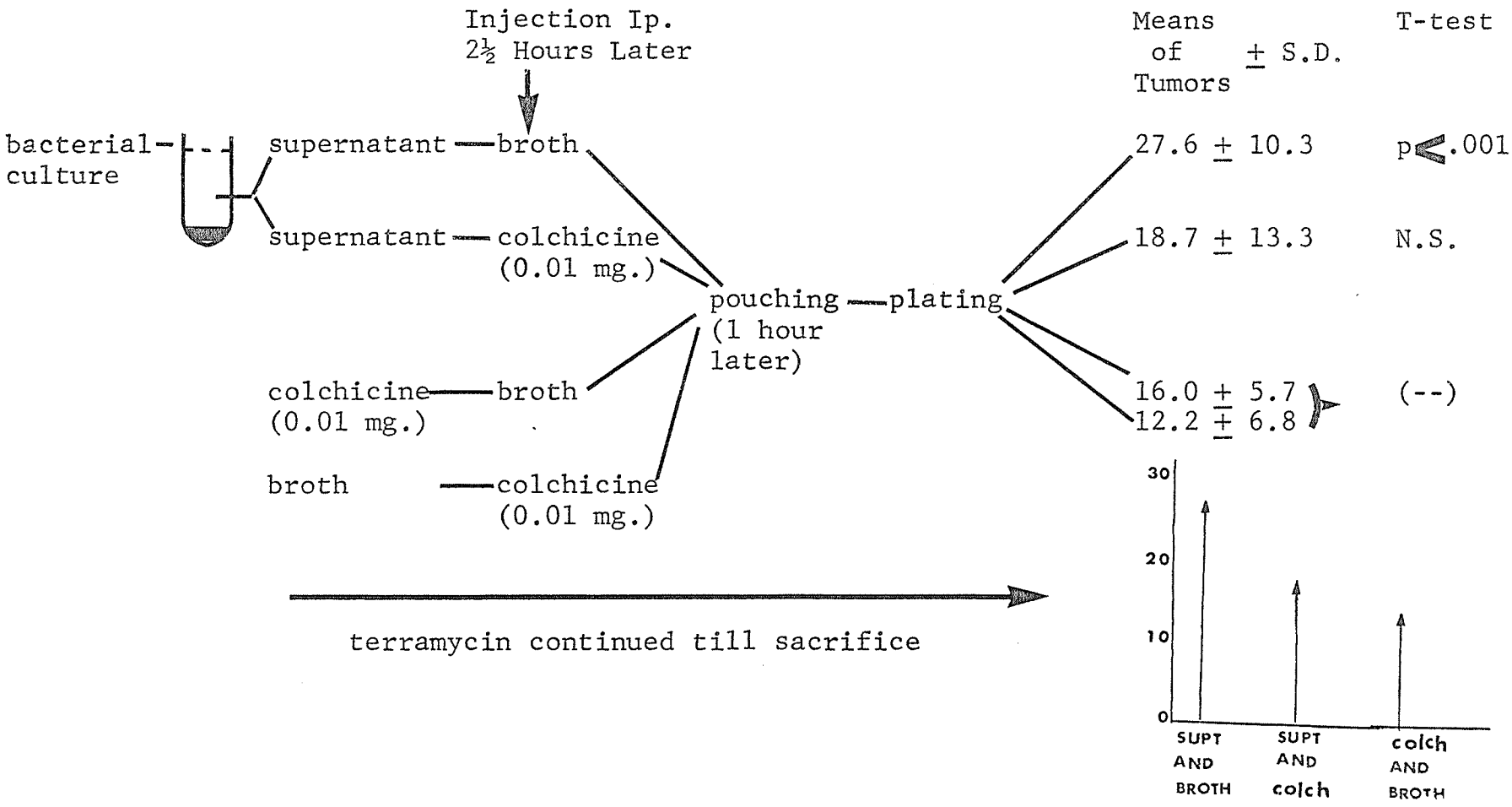
Twenty mice fed on terramycin like those above were used as controls. Half of these control mice were injected firstly with 0.01 mg. colchicine ip. followed 2½ hours later by broth medium ip. The other half of the mice were injected firstly with the broth and then by 0.01 mg. colchicine 2½ hours later. The reversal of dosages in the two halves of the control group of mice was done to balance any influence of the timing of the colchicine injections. The mean and standard deviation of the numbers of tumors occurring in the former half of the control

group were not significantly different from that of the latter half of the control group. This is shown in Figure 8. Hence whether colchicine was given one hour or three hours before pouching had little consequence on the outcome of successful tumor "takes". The individual tumor counts were therefore grouped together for both halves of the control mice and only one mean and one standard deviation were calculated for all 20 control mice.

T-tests were done to test the significance of the difference between the mean of the control group of 20 mice and the means of the groups of mice which received supernatant with or without colchicine. Mice which received ip. injections of supernatant and broth showed many more tumors than control mice. This difference was significant at the 99.9% confidence level, as is shown in Figure 8. Similar results had been realized in the experiment shown in Figure 7 where mice were given oral doses of supernatant and broth. These demonstrate therefore that the supernatant has an adjuvator effect in mice whether it is given by the oral or by the ip. route. In contrast, mice injected with supernatant and colchicine had tumor counts not significantly different from the mean of the tumor counts in the control group. Hence the adjuvator effect due to the injection of supernatant ip. was eliminated where 0.01 mg. colchicine was injected as well. Colchicine's effect, when it was injected ip. was to inhibit tumor cell implantation. The mechanism by which an ip. injection

FIGURE 8

INJECTION INTRAPERITONEALLY OF COLCHICINE ELIMINATED THE ADJUVATOR OF SUPERNATANT



of colchicine brought about a reduction of cell "takes" remains to be discussed. The means of the tumor counts of all the three groups tested are shown graphically at the lower right side of Figure 8.

C. Changes Observed in the Lymphoid Organs When Colchicine, Endotoxin, or Supernatant are Given

1. The Action of Colchicine Fed to Mice with Normal Gut Flora

An experiment was designed to compare the lymphoid changes in two groups of mice with normal gut flora, one group of which had received colchicine orally. All mice were pouched two hours after the one group received colchicine and were plated with cells the following day. Four boxes of mice were used for the experiment. Each box contained six mice. Four of the six mice in each box received 0.25 mg. colchicine orally. The colchicine was dispersed in Earle's solution. The other two mice in each box received only Earle's solution and served as controls. The mice of one box were killed per day, respectively 1, 2, 3, and 9 days after the mice had been plated. The axillary and inguinal lymph nodes, the spleen and the thymus of each mouse were excised immediately, fixed in buffered formalin and prepared for histological examination.

All three lymphoid organs of mice delivered colchicine demonstrated a loss of thymus-dependent lymphocytes. This was especially true in the lymph nodes and the thymuses. The changes in all

the organs became strongly evident on the third day after plating cells.

The entire thymus of each mouse given colchicine and examined three days after plating, showed a loss of lobular differentiation. There was also a loss of structural landmarks: neither the cortex nor the medulla appeared as clearly defined structures. The organ was reduced by 40 to 90% of its usual size. Such changes were not present in the thymus of a control mouse which did not receive colchicine. On examining the thymic cortex of each mouse offered colchicine it was clear that the cortical lymphocytes were almost totally absent. This is illustrated in figure 10(b). Thymic depletion of cortical lymphocytes was already apparent on the second day after plating. Control mice had a normal host of lymphocytes (Figure 10(a)).

The lymph nodes of mice given colchicine resembled their thymuses in that the cortex was markedly atrophied and bore only a small number of lymphocytes. These features were most pronounced in the nodes examined three days after plating. The lymph nodes of control mice maintained their full complement of lymphocytes. In contrast to lymphocytes in the cortices of the thymuses and lymph nodes which are depleted, the bursal-dependent lymphocytes in the lymph nodes remained unaltered by the effect of colchicine or were even rendered hyperplastic. Germinal centres were prominent both in the subcapsular areas of the lymph nodes and in the medullary cords. This was most pronounced

three days after plating. On examining the lymph nodes of control mice, germinal centres were found only in the subcapsular regions. No distinct centres were seen in the medullary cords.

The differences in the changes brought about in the spleens of mice were much less remarkable than the differences seen in either the thymuses or lymph nodes. More pyknotic cells were observed in the red pulp of the spleens of mice given colchicine. Germinal centres were as prominent in the spleen white pulp of mice whether the mice were given colchicine or not.

It is obvious from these three organs that the thymus-dependent lymphocytes were largely depleted where colchicine was delivered to the mice. The mechanism by which colchicine brings about such a depletion of lymphocytes accompanied by fatty changes as seen in the thymus, will be further discussed. The bursal-dependent lymphocytes of the germ centres, otherwise known as pyroninophilic cells, remained unaltered by the effects of colchicine or were even hyperplastic. Germinal centres were prevalent in both the subcapsular areas and in the medullary cords of lymph nodes, and in the white pulp of the spleen. The lymphoid organs of mice from each group killed nine days after plating were indistinguishable from one another. Hence the changes observed in the first three days on the thymus-dependent and bursal-dependent lymphocytes are reversed by the ninth day.

2. The Action of Colchicine Injected Ip. in Mice Fed on Terramycin

The contrasting effects due to the administration of colchicine on the two cell types allowed an opportunity to determine whether the same effects would occur if colchicine were injected into mice fed on terramycin. The changes in these cell types could either be the direct effect of colchicine or the effect of endotoxins from the gut. Feeding mice on terramycin for a few weeks would eliminate bacteria and their endotoxins from the gut and would thereby eliminate the changes if they were due to endotoxins.

A dose of 0.05 mg. colchicine was injected ip. into 15 mice fed on terramycin for 2 weeks. The colchicine was dispersed in broth medium. The injected dose approximately represented the LD50 of colchicine. There were seven deaths within a day of being injected and two more within the next three days. The lymphoid organs of the remaining six mice were used for histological examination. Eight control mice also fed on terramycin were injected with broth. Half of the control group and half of the group injected with colchicine were sacrificed two days after plating. The other halves were sacrificed three days after plating. All mice had been pouched two hours after the injections of colchicine or broth, and were then plated the following day.

The lymphoid organs of mice injected with colchicine showed similar changes to those mice in the previous experiment which had normal gut flora and which were given colchicine orally. These changes in mice injected with colchicine were not seen

in mice injected with broth. The most pronounced changes in mice injected with colchicine occurred on the third day after plating. The same was true in the previous experiment for mice given colchicine orally.

The thymus was largely replaced by fatty tissue. Large reticular-like cells with pale cytoplasm and large pale nuclei occupied almost the entire border of the cortex. Such cells were present in the medulla but were somewhat obscured by a repopulation of younger lymphocytes. Many more lymphocytes occupied the medullary section. Such changes were not seen in the thymuses of mice injected with broth. Cortices had a full complement of lymphocytes and the areas of the medulla showed many reticular cells and only a small number of lymphocytes. These differences are illustrated in figures 11(a) and 11(b).

The cortices of the lymph nodes of mice injected with colchicine were totally depleted of living lymphocytes. Those lymphocytes remaining had darkened clumped nuclei and were thus considered pyknotic. The lymph nodes of control mice injected with broth showed a full population of lymphocytes in their cortices and only a small number were pyknotic. The spleens of mice injected with colchicine showed a remarkable stimulation of bursal cells in both the red and white pulp. Few red blood cells were seen in the red pulp. They were evidently replaced or obscured by the hyperplastic condition of the bursal cells.

Clearly, the histology of the lymphoid organs is similar

in mice with normal gut flora fed colchicine and in terramycin-fed mice injected ip. with colchicine. The implications of this for the understanding of the adjuvator effect of colchicine will be discussed.

It should be noted that a strong bursal stimulation was evident in the spleens of terramycin-fed mice injected with colchicine. Although the dose was very large representing approximately the LD50 for colchicine, the lymphocytotoxic effect of colchicine was borne out only on the thymus-dependent lymphocytes but not on the bursal cells. The stimulation of bursal cells in the spleens is in fact stronger than in the spleens of normal mice given a less toxic dose of colchicine orally. Clearly these are direct effects of colchicine not mediated by release of endotoxin from the gut.

3. <u>The Actions of Endotoxin and of the Supernatant Fraction in Mice Fed on Terramycin</u>

The previous experiment in which two terramycin-fed groups of mice were injected ip. with either colchicine or with broth, also included two more terramycin-fed groups of mice. One group of 10 mice was injected ip. with 0.32 mg. S. typhosa endotoxin. The other group of ten mice was injected ip. with the supernatant fraction (obtained in the usual manner) in ¼ cc parts. The mice of both groups were

pouched two hours later and plated with cells the following day. The changes observed in the lymphoid organs in both the groups of mice were then compared to the changes found in mice treated with colchicine.

The dose of endotoxin injected into mice represented about the LD50 for endotoxin. Half of the ten mice died within a day of the injection. Another mouse died within the next three days. Four mice remained for histological examination. The ten mice injected with supernatant all survived. Half of the four mice injected with endotoxin and half of the ten mice injected with supernatant were sacrificed two days after plating cells. The other halves were sacrificed three days after plating cells.

The changes in the lymphoid organs due to the action of endotoxin were almost identical to the changes which occurred in either the terramycin-fed mice injected ip. with colchicine or in the normal mice given colchicine orally. Fundamental changes in lymphoid organs concerning the two cell types already were pronounced in mice sacrificed two days after plating. Maximum changes occurred in mice killed three days after plating. The white and red pulps of the spleen showed marked bursal stimulation. Bursal cells were also formed in the medullary cords of the lymph nodes. Cortical depletion of lymphocytes in the lymph nodes was not apparent. However, all those lymphocytes remaining were pyknotic. The thymus

showed a loss of cortical lymphocytes. The medulla of the thymus showed a repopulation of younger lymphocytes. Much of the thymus was replaced by fatty tissue. In one particular section of a thymus, the structural landmarks of both cortex and medulla were barely defined.

The histology of the lymphoid organs of mice injected with supernatant ip. very much resembled that of mice injected with colchicine ip. or with endotoxin ip., but to a lesser degree. Bursal stimulation was prominent in the spleen. The thymus showed a loss of cortical thymus-dependent lymphocytes and some loss of structural landmarks (Figure 11(c)). The comparable changes in the histology of the lymphoid organs of mice injected with supernatant or endotoxin are compatible with the hypothesis that endotoxins are the factors present in the supernatant which are responsible for these changes. Such changes did not occur in mice injected with broth. The large dose of endotoxin (LD50) injected into mice more than likely accounts for its more pronounced effect on the lymphoid organs than supernatant.

The lymphoid changes in mice injected with S. typhosa endotoxin were no different from the changes in mice given colchicine by mouth or by ip. injection. The changes of the latter, however, cannot be ascribed to endotoxin and must result from colchicine itself.

4. Gross Morphological Changes in the Lymphoid Organs

Gross morphological features of the lymphoid organs were also noted in mice given different ip. injections of colchicine, endotoxin, supernatant or broth. The spleens of mice given endotoxin or supernatant were decidedly enlarged. The spleens of mice given colchicine or broth had remained nearly normal. In one experiment, 0.05 mg. colchicine and 0.01 mg. endotoxin were injected ip. separately into two different groups of mice fed on terramycin which were then plated with tumor cells and killed 3 days later. A control group also on terramycin was injected with Earle's solution. Spleen weights of mice given endotoxin were double the spleen weights of mice injected with either colchicine or with Earle's solution.

IV. DISCUSSION OF THE RESULTS

A. Resume

The first experiment (Fig. 2) demonstrates that there is an adjuvator effect on the transplantation of the tumors used after colchicine is administered orally. The second experiment (Fig.3) reveals that this adjuvator effect is maximum when the colchicine is fed on the day of pouching. This critical timing is similar to that of endotoxin injection with which the maximum adjuvator effect of that substance was obtained(3). Therefore the hypothesis was formed that colchicine's effect and that of endotoxin were related but not necessarily the same.

The third experiment (Fig.4) demonstrates that the adjuvator effect of colchicine given orally is eliminated in mice fed terramycin over a few weeks. This suggests that colchicine potentiates endotoxin already associated with the host, as it is known that tetracycline diminishes the bacterial flora in the guts of normal mice which contribute to a reservoir of endotoxin. The fourth experiment (Fig.5) shows that the adjuvator effect of colchicine is restored by feeding bacterial matter to tetracycline-fed mice. This supports the notion that tetracycline eliminates the enteric bacteria and thereby the products responsible for the adjuvator phenomenon. It is likely that such products are endotoxins as either the bacterial particulate which would retain endotoxins on the body walls of dead or dying bacteria, or the

supernatant fraction of the bacterial suspension which would contain endotoxins in solubilized form, will restore the adjuvator effect lost in the terramycin-fed mice offered colchicine.

The seventh experiment (Fig.8) describes an inhibitory effect of colchicine on tumor implantation when it is applied more directly to the site of implantation even in the presence of the oral administration of supernatant. The reasons for this are discussed later.

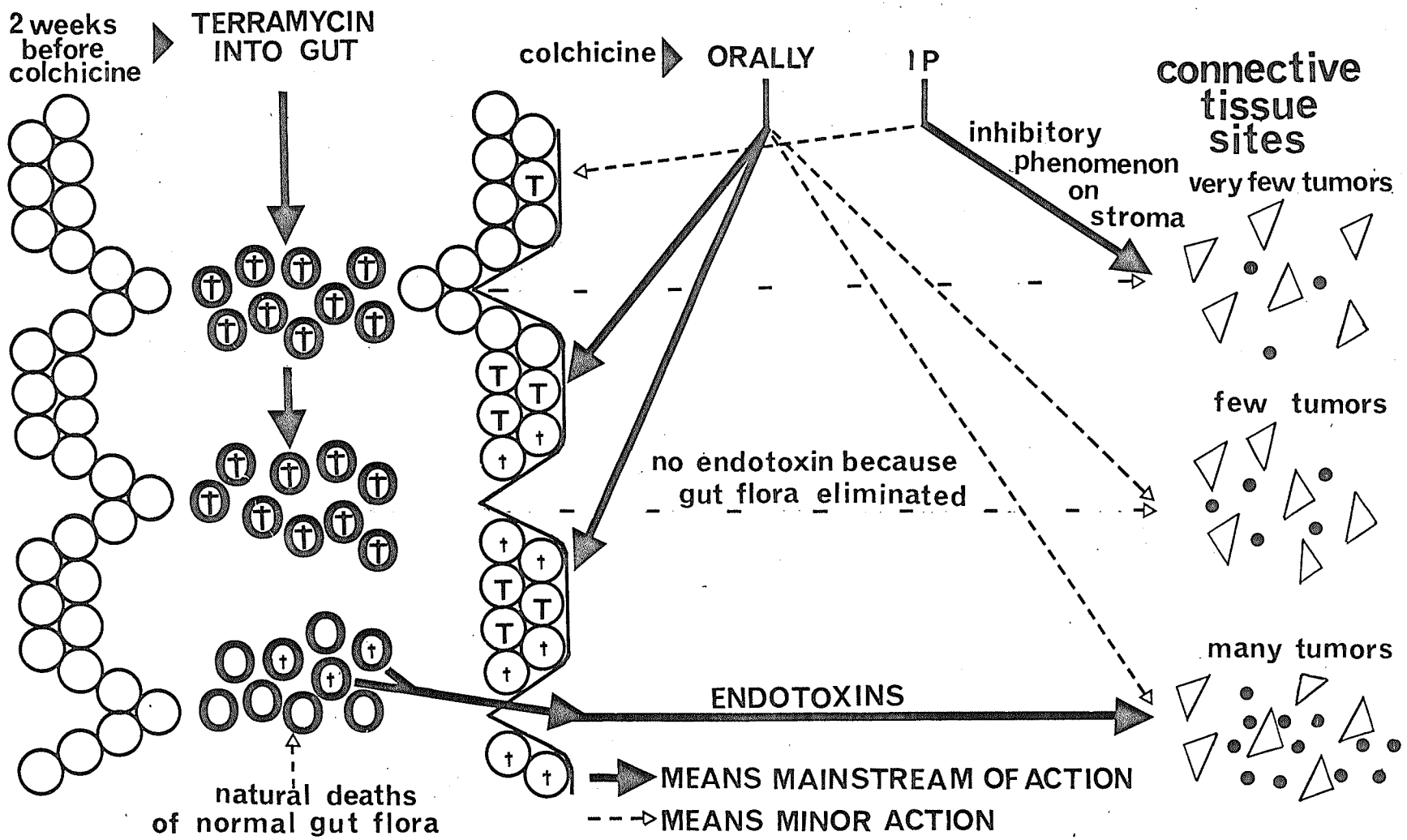
Fig.9 is a diagrammatical review of how colchicine behaves under varying circumstances including that of its administration after the use of terramycin. The figure clarifies how the enhancement of tumor cell implantation in mice fed colchicine without terramycin, is attributed not to its direct action, but to its action on the intestinal wall giving passage then to intestinal endotoxin. An inhibitory effect of colchicine on the stroma is shown when it is injected ip.

B. Action of Endotoxin

Henderson(5) observed that adjuvators to tumor implantation seemed inherent in the subcutaneous tissue itself. They could be evoked by pouching with Earle's solution and air. In the light of new experiments(3) he explained the above phenomenon in terms of the action of endogenous endotoxin on the site of damage evoked by splitting. Mice which had drunk water containing an antibiotic, oxytetracycline, for one month showed no adjuvator

FIGURE 9

DIAGRAMMATIC REVIEW OF ACTION OF COLCHICINE



effect when pouched. In fact, the yield of tumors was slightly depressed. He thought the elimination of the adjuvator effect was the result of having eliminated endogenous circulating endotoxin. In support of this explanation, he found the adjuvator effect was restored by pouching those mice on oxytetracycline with endotoxin dispersed in Earle's solution. The tumor yield was as great as in untreated mice which were never on the antibiotic and had been pouched with an equivalent dose of endotoxin. Thus the adjuvator effect due to pouching is not inherent in the tissue but depends upon the presence of endogenous or exogenous endotoxin which itself acts on the connective tissue at the time of pouching. In the experiments of this thesis, the adjuvator effect by oral colchicine could likewise be restored in antibiotic treated mice by the supernatant fraction of a bacterial culture.

C. The Direct Effects of Colchicine at the Sites of Tumor Implantation on the Connective Tissue Expanses

1. Direct Effect of Colchicine on Tumor Cells

Can the elimination of the adjuvator effect due to supernatant in terramycin-fed mice injected with colchicine and supernatant ip. (Figure 8) be simply due to an arresting of the mitoses of tumor cells by colchicine? If tumor cells are brought into contact in vitro with colchicine over a certain period of time, many cells will become arrested in mitosis. The plating of these cells results in significantly fewer tumors than if tumor

cells which were suspended in Earle's solution for the same length of time were plated in like fashion (unpublished results).

Brues(13,14) has found that a major proportion of an iv. administered dose of colchicine is sequestered to the intestine at least up to several hours. The site of the intestine is far removed anatomically from the connective tissues upon which tumor cells are sprayed. It could be argued that the blood would contain some of the colchicine from the intestine and distribute it to the connective tissue. This is in fact true to some extent, but Walaszek et al(24) discovered that the excretion of unchanged colchicine in the urine represents only 0.3 percent in mice within the second 24 hour period. Thus it is unlikely that any large concentration of colchicine lingers in the body fluids of the connective tissues during that time. It is at this time of course that tumor cells are first introduced there, one day after injection of colchicine. Colchicine would long since have been metabolized or rendered inactive before its contact with the tumor cells. The direct effect of colchicine on tumor cell mitosis cannot therefore explain the inhibition seen in Fig.8. The action of colchicine on the connective tissue must therefore be examined.

2. Direct Effect of Colchicine on the Host Connective Tissue

A more immediate effect of colchicine injected into mice is its anti-mitotic action on the dividing cells of the connective tissues. This action of colchicine may diminish the reactivity

of the connective tissue thus postponing the provision of nutrient stroma for the implantation of tumor cells. Such an influence could suitably explain the inhibition phenomenon of colchicine in the last experiment.

D. Consequences of Colchicine Delivered Intravenously or by Mouth

When colchicine was injected ip. in experiment 7 (Fig.8) into mice just previously injected with supernatant, it was determined that colchicine eliminated the adjuvator effect due to the action of supernatant from a bacterial culture. However, when comparable doses of colchicine were given orally, the adjuvator effect of supernatant persisted. This is noted in both figures 5 and 7. Hence the route of administration of colchicine is important for the outcome of successful tumor cell implantation. The essential problem to the paradoxical outcome of the adjuvator effect of the supernatant in these experiments depends upon the route of administration of colchicine and its distribution within the body. When it is given orally, the colchicine is absorbed from the gastrointestinal tract and passes through the liver where it is largely deacetylated. Colchicine is excreted back into the intestine largely via bile as shown by biliary fistula, and to a lesser extent directly into the isolated loops of the small intestine(13,14). This fact plus the high turnover rate of intestinal epithelium explains its atrophy by colchicine. After oral administration the intestinal epithelium bears the

full burden of the colchicine dose.

When colchicine is injected ip., there is still some shunting of unchanged colchicine to and fro between the intestine and the liver. Wallace et al(8) found that the half life of colchicine in the plasma is 15 minutes after its iv. injection and that its concentration there was near zero within one hour. This demonstrates the rapidity at which colchicine becomes localized in tissue compartments. But during the hour after it is injected ip., colchicine must come into effective contact with the subcutaneous connective tissue expanses which are to receive the tumor cells.

As discussed in section C, colchicine's effect there tends to be against the success of tumor cell implantation. There would still be some intestinal epithelial damage if the colchicine were injected ip., such that the supernatant would flood into the general circulation as determined by experiment 7 (Fig.8). But the colchicine also alters the connective tissue sites in such a way that the adjuvator effect of supernatant becomes nullified.

E. <u>The Effectiveness of Terramycin in the Elimination of Bacteria and Their Endotoxins and of the Adjuvator Effect of Colchicine</u>

Adjuvator effect was seen in mice with normal gut flora fed colchicine. But this effect was eliminated when mice were fed terramycin as in the third experiment (Figure 4). It was thought that terramycin might have inhibited the adjuvator action

of colchicine. However experiments 4, 6, and 7 (Figures 5, 7 and 8) in which supernatant from bacterial cultures was given to mice placed on terramycin revealed that an adjuvator effect could still be demonstrated. This therefore refutes the possibility that terramycin was directly inhibitory.

It was discovered during the course of the present experiments that mice which were fed terramycin were much more susceptible to the toxic effects of endotoxin or supernatant than normal mice. This observation agrees with those of Floersheim and Logara-Kalantzis(15) who have found synergistic toxicity between endotoxin and terramycin. Altura et al(16) have demonstrated that tetracycline depressed the rate of carbon clearance (phagocytic index) in normal rats by 40%, and in respiratory-infected rats, which had a naturally occuring phagocytic capacity of the reticular endothelial system, by 75%. This depressed phagocytic activity of the RES in tetracycline-treated mice might therefore have enhanced the effect of any residual endotoxins in the third experiment. As there was no adjuvator effect, endotoxin could not have been present. Either the endotoxins occuring in the gut must have been effectively eliminated by the action of terramycin on the bacteria, or the toxic effects of endotoxin is not an indicator of whether or not it can demonstrate an adjuvator effect. In any case the adjuvator effect of oral colchicine is eliminated by terramycin and restored when challenged by bacterial products.

F. Comparable Actions of Endotoxins and Colchicine in Relation to Timing

Henderson(3) found that the adjuvator effect of endotoxin could only be demonstrated if it was present at the time the connective tissue expanses were pouched. He ascribed the action of endotoxin as acting at the site of slight tissue damage evoked by splitting of the subcutaneous expanses to promote the successes of tumor "takes" there. Preliminary experiments to those described in this paper showed that there was no increase in tumor counts in mice which had received 0.25 mg. colchicine orally, but were not pouched. Clearly, pouching was first necessary to evoke some tissue damage on the connective tissue expanses before the gut products, or moreover, the endotoxins in the supernatant, were to have a demonstrable adjuvator effect.

By comparing the tumor yields of mice given colchicine orally at various days prior to, and on the day of plating cells (Figure 3), it is seen that the greatest increase of tumors occurred where colchicine was administered one day prior to the plating of cells. Henderson(3) has similarily uncovered a maximum adjuvator action of endotoxin where the increased susceptibility caused by pouching with endotoxin, declined rapidly after 24 hours.

Clearly, even the timing of colchicine administration

compares with previous endotoxin studies thus providing further evidence that it is endotoxin and not colchicine itself which promotes tumor cell implantation.

The results of the fifth experiment (Fig.6) relate the importance of the necessary timing between the administration of another source of endotoxin and of pouching the mice, before an adjuvator effect is shown. Two days before plating, mice which were previously maintained on terramycin, were suddenly placed on a chow pellet diet. Administering germ-containing pellets in the absence of further terramycin plus feeding the mice nutrient broth, allowed bacteria to grow avidly in one day before pouching. In these mice an endotoxin-like adjuvator effect was obtained at the site of damage evoked by pouching(3). The same was not so in a similar group of mice provided chow pellets after being pouched. The amount of endotoxin present in this latter group about the time of pouching must have been negligible. This experiment emphasizes the care needed to keep the subject mice endotoxin free.

G. Histological Remarks and Their Relation to Tumor Implantation

The histological changes in the thymuses of mice fed colchicine, resemble in microscopic detail, the changes described by Landy(11) in the thymuses of rabbits injected iv. with 5 micrograms (mcg.) S. enteritidis somatic polysaccharide, and the changes described by Rowlands et al(12) in the thymuses of young mice injected iv. with 100 mcg. S. typhi endotoxin. Landy noted striking

acellularity with fatty and fibrous remnants. The primary loss was of cortical cells. The medullar elements came to consist of large reticular cells only. Lobular differentiation and structural landmarks were lost. The changes brought about by the experiments described in this paper are identical.

Rowlands et al noted progressive destruction of thymic lymphoid cells and thymic architecture, accompanied by a relative increase in the number of pyroniniphilic cells from the third to the fifth day after the injection of endotoxin. It was assumed that the thymus acquired such bursal cells from other lymphoid organs such as the spleen, which showed an increase in the numbers of plaque forming cells. The lymph nodes and spleens of mice given colchicine orally in the experiments described in this paper similarly reveal a strong bursal stimulation in spite of destruction of the thymus-dependent lymphocytes. Cells which resemble pyroninophils are seen in one particular section of a thymus 4 days after the mouse had received 50 mcg. S. typhosa endotoxin ip. The cells are aggregated as a small focus, cuffing the edge of a sinus.

The striking similarities in the histological changes of thymuses recorded from rabbits and mice after the iv. injection of endotoxin, and from mice given colchicine orally, certainly supports the hypothesis that seepage of endotoxin from the guts of mice offered colchicine might have caused the thymic changes. But Morris(10) has examined the number of metaphases in lymphoid

tissues of mice after colchicine was injected ip. He recorded an increase of metaphase counts in thymic cortex, spleen (red pulp and white pulp), and lymph node, within 2½ to 3½ hours after the injection. The number of dead cells increased steadily after 3½ hours and reached a maximum 6 hours later. Hence, the lymphocytic cell pyknosis seen in mice given colchicine orally, may be attributed to lymphocytoxic actions both of endotoxin and of colchicine. Mice were therefore fed antibiotic to reduce or eliminate the cytotoxic action of endotoxin and to assess the degree of destruction of lymphocytes by colchicine alone.

Colchicine, supernatant, and endotoxin, injected into three different groups of mice fed on terramycin, all behaved similarly in that they led to pyknosis and depletion of the thymus-dependent lymphocytes in all lymphoid tissues examined, but stimulated hyperplasia of pyroninophils. Destruction of lymphoid and thymic cells may follow the metaphase arrest of their mitoses. This action can be related to the specific cytotoxicity of colchicine combining with tubulo-proteins. Destruction of lymphoid and thymic cells may also be related to stress on the body, causing the release of cortisone and other lymphocytolytic hormones from the adrenals.

According to Leblond and Segal(4), colchicine is a powerful alarming stimulus in rats. The involution of hemo-lymphatic organs and the thymus were found to be mediated by the adrenal hormones since adrenalectomy suppressed this effect of colchicine. Similar events were unravelled by Selye(17) using a variety

of alarming stimuli including bacterial toxins. The loss of thymus weight was preceded by nuclear pyknosis there and complete dissolution of the thymocytes. Lymph nodes and spleens were similarly affected but did not involute as readily. Adrenalectomy prevented involution of any lymphoid organs under these circumstances. Corticoid hormones and some non-adrenal steroid hormones produced thymic involution even in adrenalectomized animals. It could be said that either colchicine or endotoxin would be a sufficient alarming stimuli in large doses to stimulate excess cortical hormone secretion and thereby result in thymic involution.

Mitotic arrest of the lymphocyte series with further pyknosis and cell death, would be directed at the lymphoblasts and medium sized lymphocytes (prolymphocytes). Their proliferation occurs in all parts of the lymphoid tissue and not only in the bone marrow. Bursal cells are not similarly affected presumably because they derive from the long-lived lymphocyte series which rarely divide over days(9). Thus the lymphoid tissue changes seen in mice fed colchicine, are attributable to the actions of either colchicine or endotoxin or both together on these tissues.

The adjuvator effect of colchicine is elicited only in mice with normal gut flora and not in terramycin fed mice (figure 4). The lymphoid changes when colchicine is given to mice, however, occur equally whether terramycin has been fed or not. Therefore these lymphoid changes are not the cause of the adjuvator effect. Similar lymphoid changes have been seen when endotoxin is given.

The findings with colchicine suggest that the adjuvator effect of endotoxin too is perhaps not mediated by way of the affected lymphoid tissue.

H. Colchicine in Human Therapy

The present work demonstrates in animals with normal gut flora that colchicine administration presents a hitherto unsuspected hazard. It promotes the successful sowing of tumor cells at the site of an injury. The tumor enhancing effects are reduced or eliminated by the use of an antibiotic.

Colchicine has long been the outstanding drug for the relief of pain in acute gouty attacks. It has also been employed in the treatment of polyarthritis associated with sarcoidosis. It temporarily ameliorates the symptoms of leukemia but since the leukemic process is materially unaltered, its use has been largely curtailed and replaced by more effective drugs.

The significance of the effect of colchicine on the successful implantation of tumor cells becomes relevant where patients are suspected of bearing neoplastic growths or where patients have recently had tumors excised. The injury of excision itself provides a suitable site (like that of pouching the backs of mice), for endotoxins from the gut to promote the establishment of metastatic tumor cells. Colchicine of course, by its injury to the intestine would lead passage to a flood of endotoxins to circulate.

Finally, colchicine delivered by mouth carries more danger with respect to tumor enhancement than colchicine injected parenterally. The neoplastic risk from orally administered colchicine could be diminished by antibiotic treatment beforehand.

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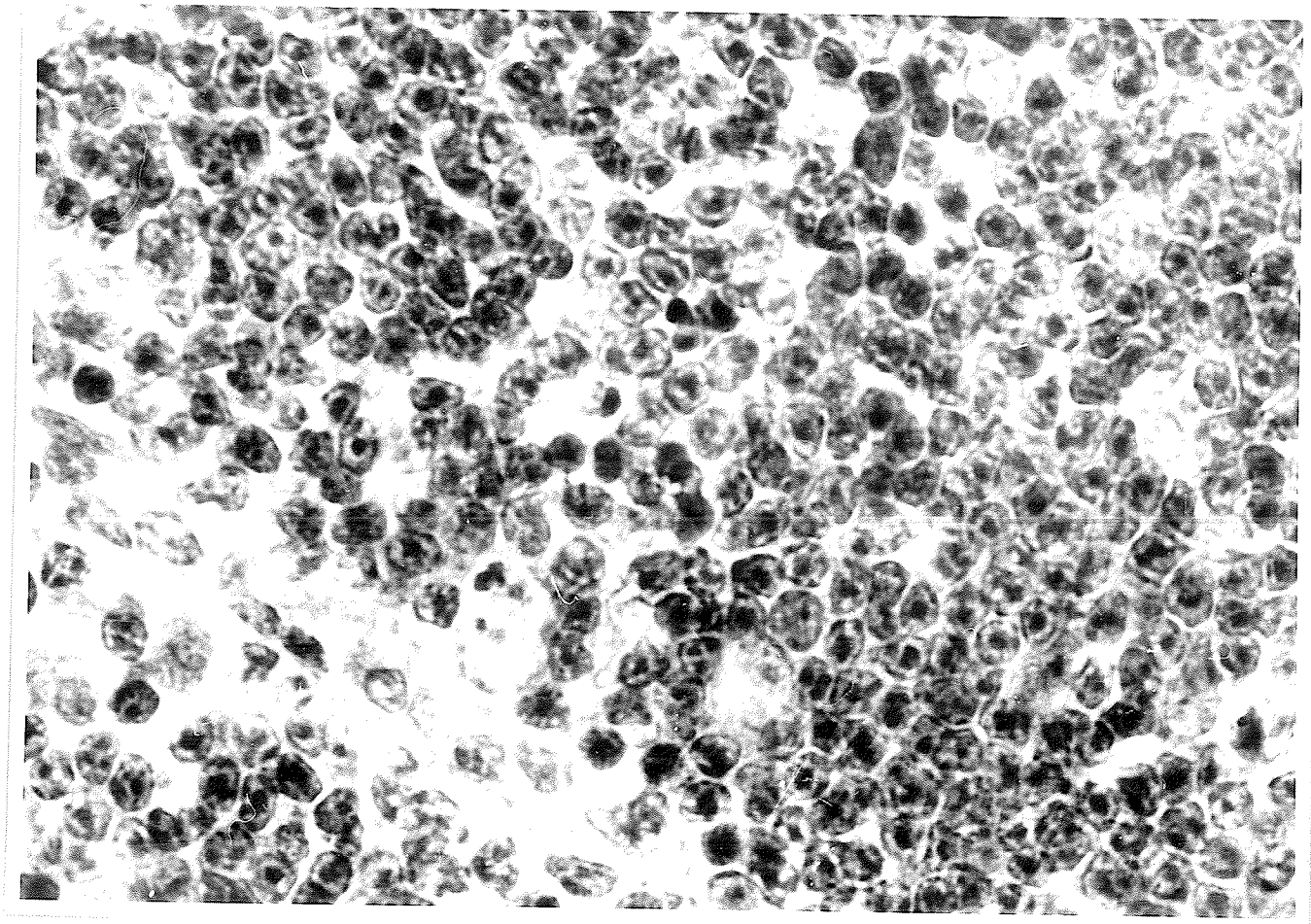

FIG. 10a. ABUNDANT LYMPHOCYTES IN THE THYMIC CORTEX OF A MOUSE WHOSE CONNECTIVE TISSUES HAD BEEN POUCHED AND PLATED 3 DAYS BEFORE. (x50)

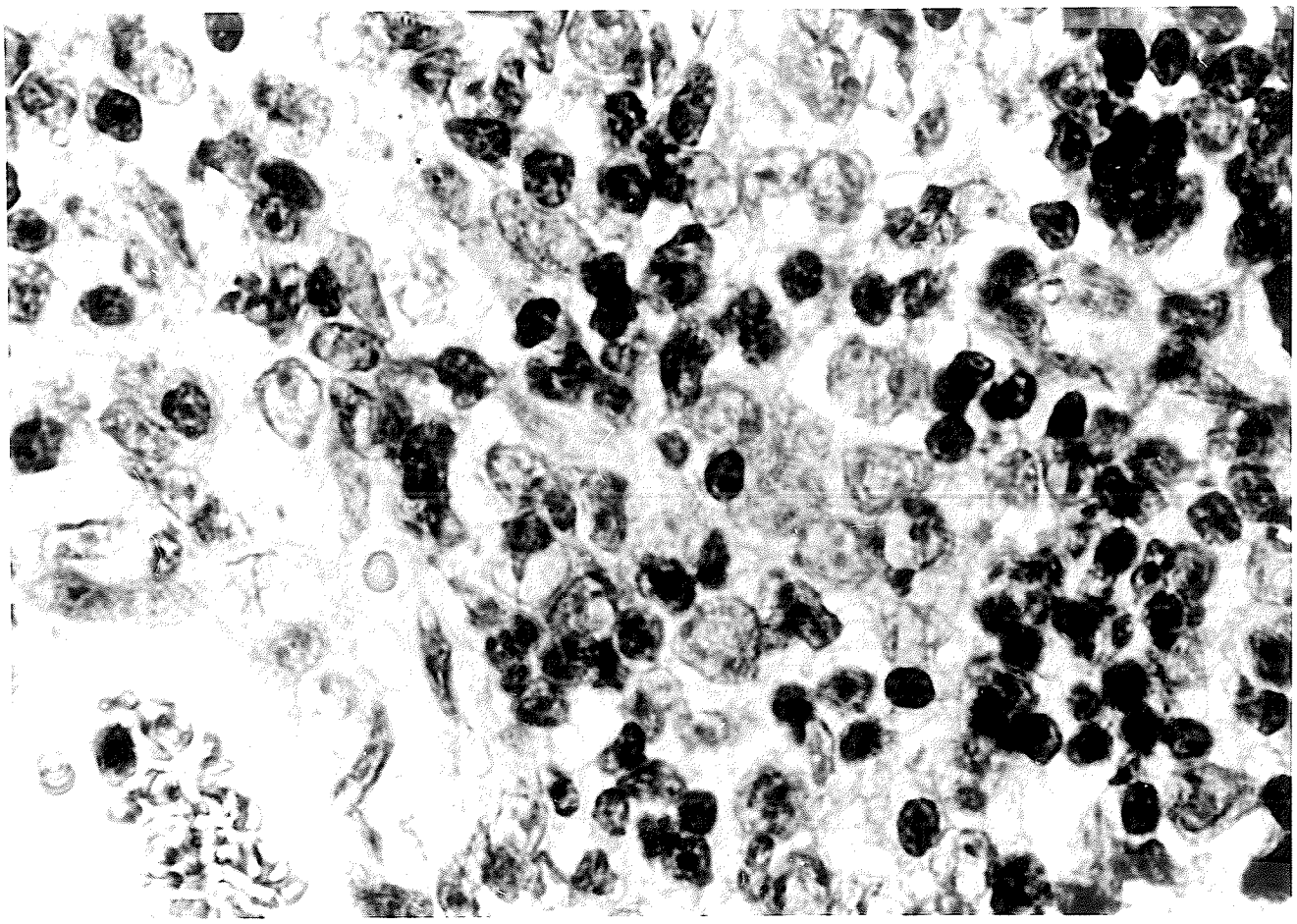

FIG. 10b. LYMPHOCYTES, PYKNOTIC AND REDUCED IN NUMBER, IN THE THYMIC CORTEX OF A POUCHED AND PLATED MOUSE WHICH HAD BEEN FED 0.25 mgm OF COLCHICINE ABOUT THE TIME OF POUCHING. THE LARGE, EPITHELIOID CELLS NOW PROMINENT ARE NORMALLY OBSCURED BY ABUNDANT LYMPHOCYTES AS IN FIG. 10a. (x50)

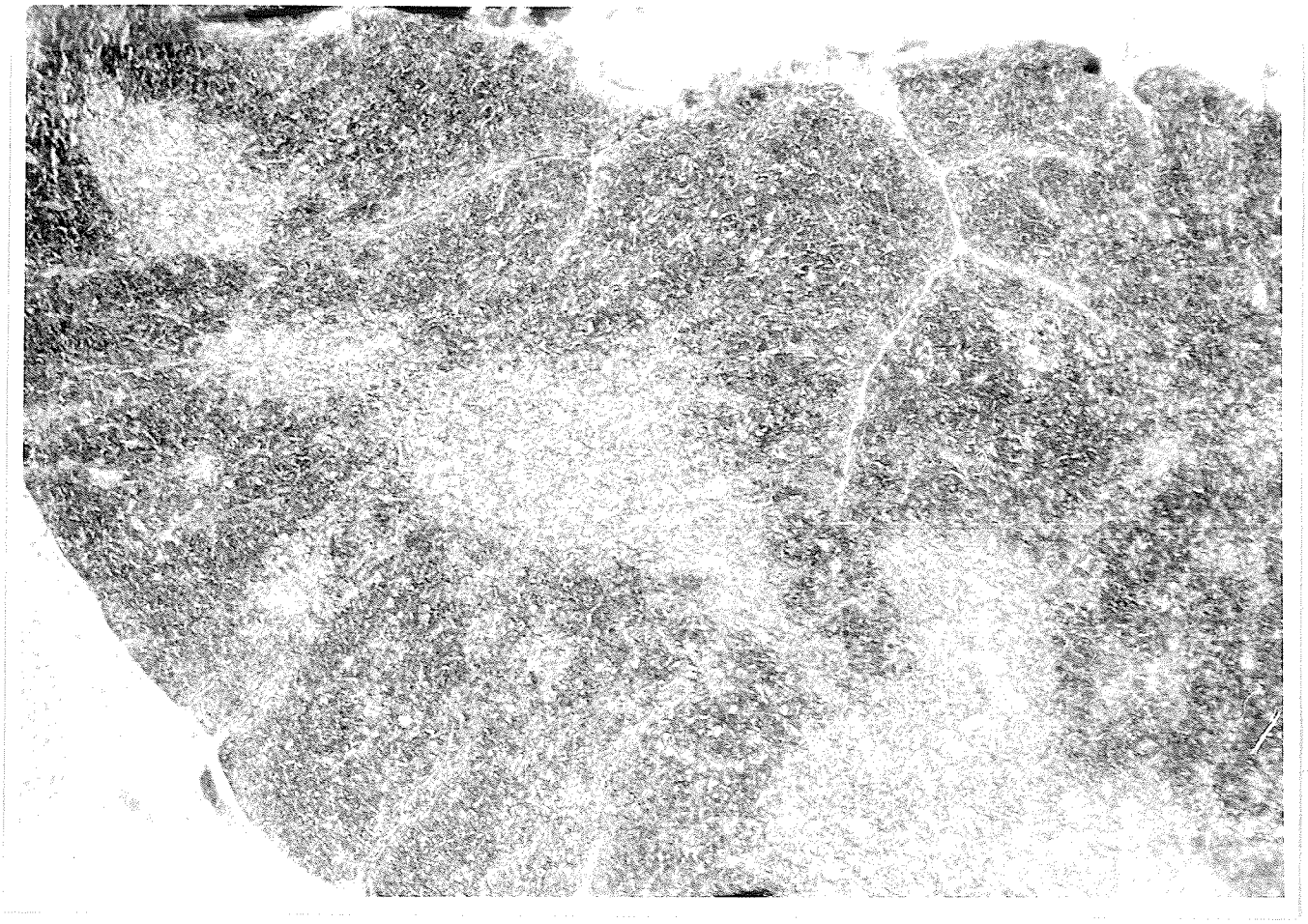

FIG. 11a. THYMUS OF A MOUSE KILLED 3 DAYS AFTER BEING INJECTED INTRAPERITONEALLY WITH TRYPTICASE BROTH THEN POUCHED AND PLATED IN DUE COURSE. CORTEX AND MEDULLA ARE WELL DEFINED. (X800)

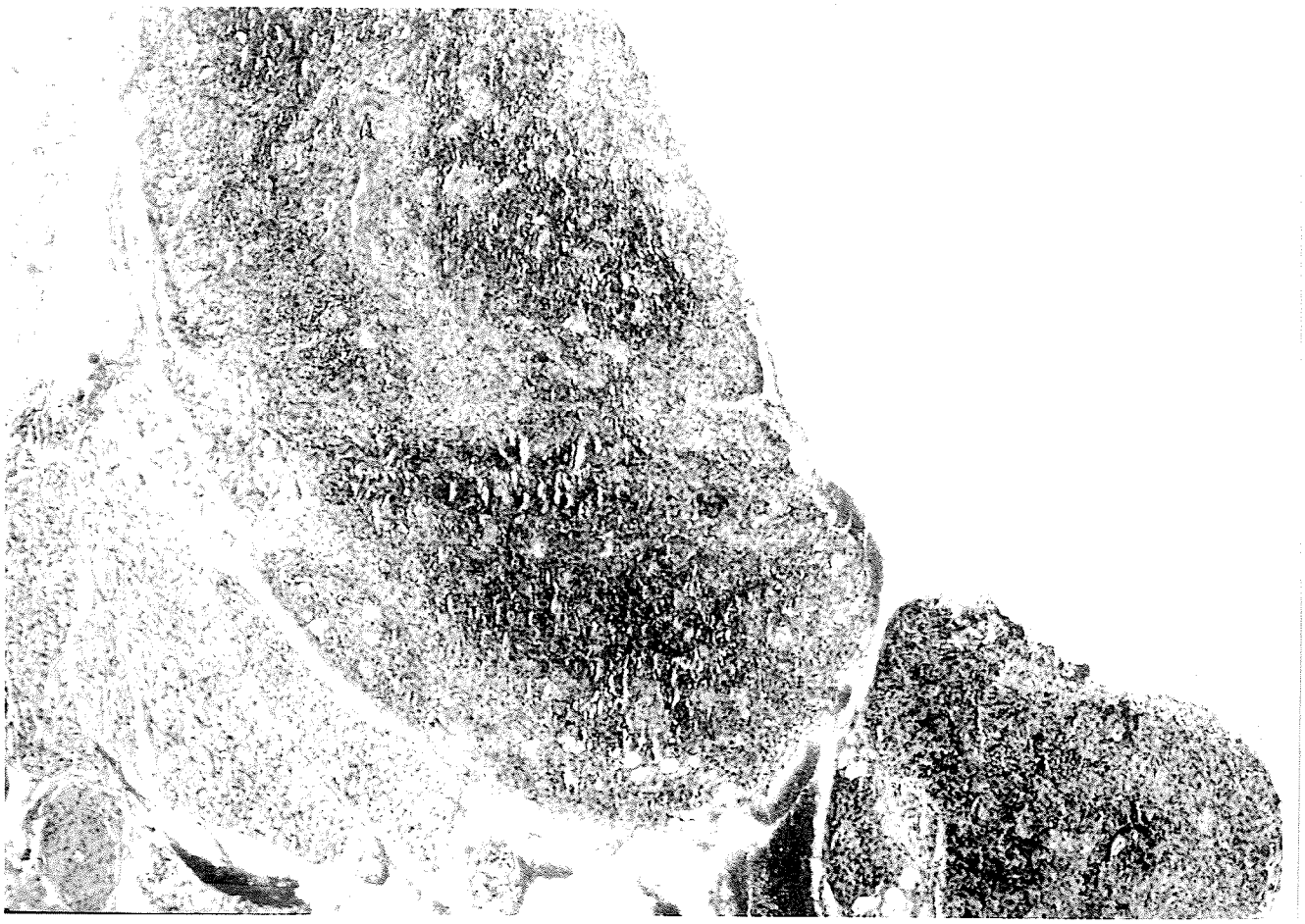

FIG. 11b. THYMUS OF A MOUSE KILLED 3 DAYS AFTER BEING INJECTED INTRAPERITONEALLY WITH COLCHICINE IN TRYPTICASE BROTH THEN POUCHED AND PLATED AS THE MOUSE OF FIG. 11a. HAD BEEN. THE ORGAN IS SHRUNK LARGELY AT THE EXPENSE OF ITS CORTEX WHICH IS NOW DIFFICULT TO DISTINGUISH FROM THE MEDULLA. THE PERIPHERAL PARTS ARE QUITE VOID OF LYMPHOCYTES. (X800)

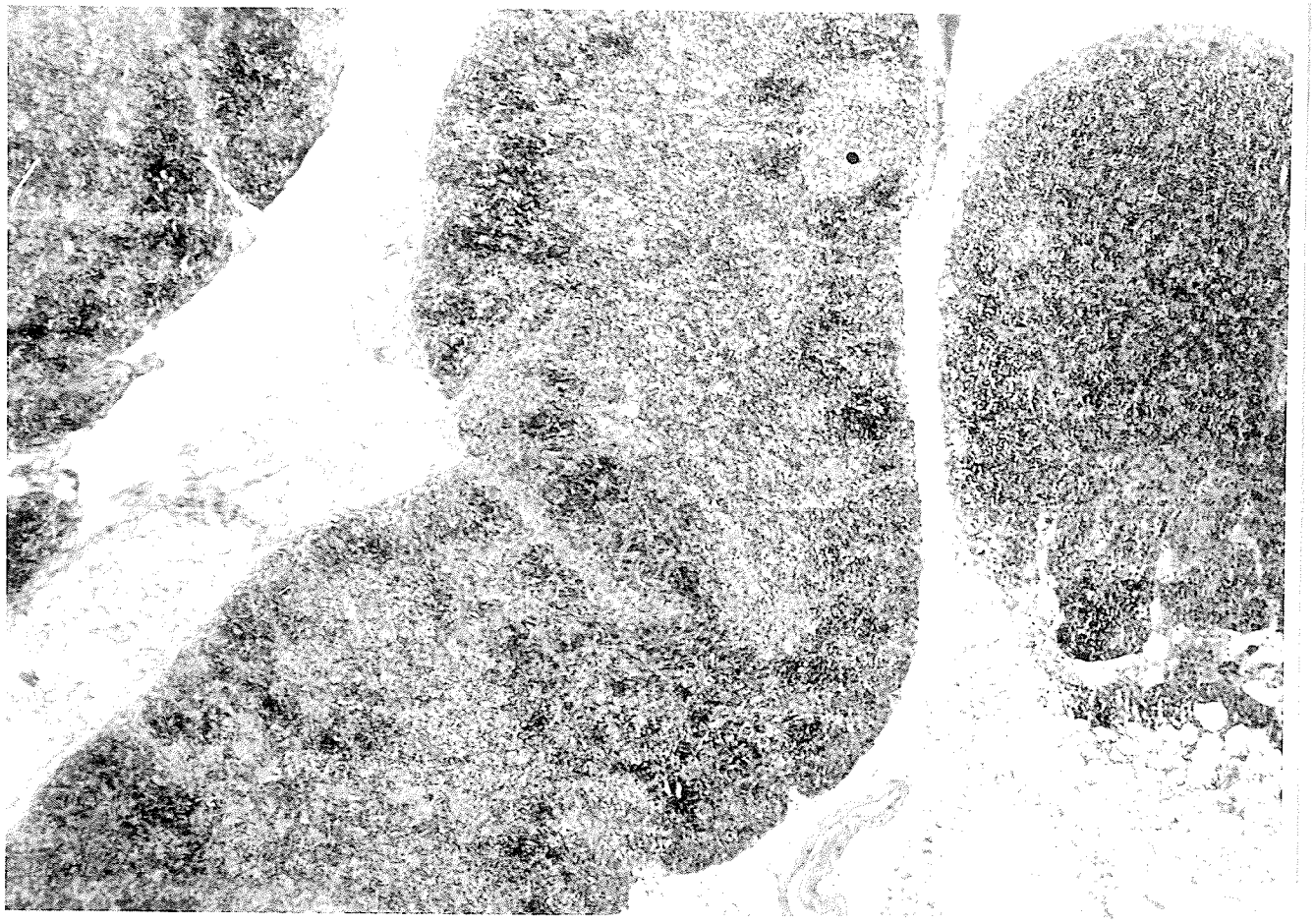

FIG. 11c. THYMUS OF A MOUSE KILLED 3 DAYS AFTER BEING INJECTED INTRAPERITONEALLY WITH THE SUPERNATANT FROM AN ENTERIC BACTERIAL CULTURE IN TRYPTICASE BROTH THEN POUCHED AND PLATED WITH THE SAME TUMOR CELLS AS THE MICE OF FIGS 11a AND 11b HAD BEEN. THE ORGAN IS SHRUNK APPARENTLY AT THE EXPENSE OF THE CORTEX IN WHICH THE FEW LYMPHOCYTES REMAINING ARE PYKNOTIC. (X800)