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STUDIES ON LYMPHOCYTES.

I. LYMPHOPENIA BY PROLONGED EXTRACORPOREAL IRRADIATION OF CIRCULATING BLOOD.*

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DISCLAIMER

There are numerous conflicting concepts in respect to the function, fate and life span of lymphocytes.[1-5] Radiation of segments of the whole-body or lymphocytes in vitro has long been exploited in the study of lymphocytes.[6-9] However, irradiation of the whole-body or segments injures lymphopoietic tissue and the in vitro environment separates the lymphocyte from homeostatic influences. We reasoned that if it were possible to destroy large numbers of circulating lymphocytes by irradiation of the circulating blood in an extracorporeal shunt under controlled conditions without direct radiation of lymphopoietic tissues that we might be able to study many facets of lymphocyte production, life span and fate not possible or practical by other methods. This is not the initial study on extracorporeal irradiation of circulating blood since O'Brien, with other objectives in mind, studied this in rabbits and dogs.[10-11] However, we believe this is the first presentation of the feasibility of destroying blood lymphocytes in substantial numbers by extracorporeal irradiation of blood flowing at a constant rate with a subsequent prolonged depression of the blood lymphocyte count under conditions where the transit and integral radiation dose to the cells can be computed.[12]

METHODS AND MATERIALS:

ANIMALS: Preliminary studies on dogs and swine under general anesthesia demonstrated the destruction of lymphocytes but these studies were difficult to interpret because of the marked effect of general anesthesia upon the leukocyte picture of these animals[13] and the inability to prolong anesthesia for many hours without deleterious effect on the animals. The calf is an excellent animal for these studies since these animals can be easily restrained and the operative procedures carried out under local

anesthesia. In Fig. 1 is shown a calf with cannulae in the external jugular vein with blood being pumped through the external shunt. Calves from 200-400 lbs in weight were used.

OPERATIVE PROCEDURES: Two per cent local xylocaine anesthesia was used. The external jugular vein was exposed by blunt dissection. After mobilization, one half hour is allowed for hemostasis, before the insertion of cannulae and heparinization. The Tygon cannulae measuring OD 3/8 inch and ID 1/4 inch are inserted towards the head for outflow from the animal and towards the heart for the return to the animal. The cannulae were tied in place by #0 chromic catgut. The wound was closed with sutures for maintaining hemostasis in the skin while heparinized. At the conclusion of the extracorporeal pumping, the wound was reopened, cannulae removed and the vein was either ligated or sutured.

HEPARINIZATION: Six hundred mg. of heparin was given at the time the cannulae were inserted and a continuous intravenous infusion of heparin was maintained at a rate of 100 mg. per hour.

THE EXTRACORPOREAL PUMP: Details of the pump, it's performance, influence on blood counts, and general perfusion utility will be published in detail elsewhere.[14] For all of these studies the pump was operated at 70-80 strokes per minute with stroke volume 3.7-4.2 ml. for a minute volume of approximately 300 ml. per minute. As much of the tubing as possible was surrounded by a water jacket to maintain the temperatures of the blood closely to the body temperature of the calf. In addiditon, an infra red lamp was used to heat the coil.

IRRADIATION: The x-ray machine is a 250 KVP General Electric Maxitron with a 360° head. It was operated at 250 KVP. The filtration is inherent

from the tungsten target. The HVL is 1.75 mm. Cu. The dose rate is about 300 r/min. at the mid-point of the irradiation coil around the x-ray tube. An integrating dosimeter monitors the exit dose. The integral dose has been checked by chemical dosimetry.

The irradiation device (Fig. 2) is a circular frame with a diameter of 50 cm. There are roughly 25 meters of Tygon tubing coiled around the frame with an internal volume of about 1100 ml. The transit time for a unit of fluid to pass through the coil is about 3 minutes. The transit dose is about 1100 r. The integral dose to the blood cells follows a probability function based upon the blood volume, mixing of the irradiated blood in the total blood volume, transit time, and dose rate and the probability of repetitive transits through the irradiation coil. The mathematical method for this computation has been established and will be published separately.[12]

The irradiation coil, pump, and connecting tubing was washed with running water, filled with 30 per cent H_2O_2 for at least 20 minutes to oxidize any pyrogen contaminants, washed with sterile pyrogen-free saline, and then filled with 70 per cent ethanol for final sterilization. Immediately, before cannulation the ethanol was pumped out and two liters of sterile pyrogen free saline pumped through the device to remove the ethanol. The pump, connecting tubing and irradiation coil contained about 1200 ml of sterile saline at the time of cannulation, which is pumped into the animal after the pumping is started thus diluting the blood by saline while the intracorporeal blood volume remains constant.

HEMATOLOGIC PROCEDURES: Microhematocrits were performed with the International centrifuge spinning at 12,000 rpm for 6-1/2 minutes.

Platelet counts were performed by the method of Brecher and Cronkite.[15]

Leukocyte counts were performed by the Coulter Electronic Counter.[16]

Blood smears were made on coverslips and stained by Wright-Giemsa method. Two hundred cell differential counts were made.

Blood samples in the initial studies were taken from the outflow tube from the animal and the inflow to the animal after the circuit through the irradiation coil.

Blood samples were taken at regular intervals after starting the pump and irradiation. Blood counts were performed on these samples immediately and after 6 hours of incubation at 37°C in order to allow irradiation injury to develop in the in vitro environment by the method of Trowell.[7]

Rectal temperature was measured at half-hour intervals by a rectal thermistor as well as the blood, coil and water sheath.

RESULTS

Leukocytes: In figures 3 through 8, the results are plotted. In figure 3 it will be noted that a prompt lymphocytosis and neutrophilic leukocytosis develops following the heparinization operation and during the period of pre-irradiation pumping. This is also observed in Figs. 4,5 and 8. This has become a constant finding and this subject with it's mechanism of action will be reported in detail elsewhere.[17]

Following the commencement of irradiation of the blood there is a prompt diminution in the concentration of the peripheral lymphocytes. In Fig. 3 is shown the influence of two hours of irradiation. The lymphocyte level had decreased at 4 hours after commencement of radiation to 55 per cent of the maximum value observed (5 hours after starting the pump). In Fig. 4 the influence of 6 hours of irradiation is shown. The lymphocyte counts increased for 2 hours after heparinization and pumping and then irradiation of the blood was commenced. After 6 hours of irradiation of blood in the

extracorporeal shunt the lymphocyte count was 40 per cent of the peak value. The normal circulation was reestablished and the following day the lymph level was down to 33 per cent of the peak count. The neutrophilic leukocytosis is apparent during the pumping and irradiation.

Neutrophils have returned to normal levels by the following day. In Fig. 5 the effect of 12 hours of irradiation is observed. The lymphocytes have fallen to 1300 per mm^3 or 17 per cent of the level at commencement of irradiation. In Fig. 6 the influence of the combined operation, heparinization, pumping and irradiation in producing a granulocytosis is illustrated. It is of interest that the granulocytes go through a maximum while the blood is being irradiated. Apparent radiation injury of granulocytes is detected by incubating the irradiated blood for 6 hours in vitro which allows injured cells to disintegrate and results in a lower neutrophil count.

In Fig. 7 the influence of 3 hours irradiation is shown. Lymphocytes fell from 5,400 per mm^3 at commencement of irradiation to 3,200 at completion or 57 per cent of the value at commencement and continued to fall for the next 24 hours to 1600 per mm^3. Thereafter counts commenced to increase but had not attained the pre-irradiation level even 28 days after completion of irradiation.

In Fig. 8 the immediate and long term influence of 6 hours of irradia- is shown. The lymphopenia is similar to previous experiments and the sluggish return is evident still being below the pre-irradiation level 29 days after the irradiation. In this figure the leukocytosis with prompt return to normal values is illustrated. The prolonged time for return of lymphocytes count to normal range will be published in detail elsewhere.[18]

PLATELETS, HEMATOCRIT AND RECTAL TEMPERATURE: There is no effect of this duration of irradiation on the platelet count. The hematocrit is reduced in proportion to dilution of the blood volume by the amount of saline with which the extracorporeal circuit is primed. Upon completion of the extracorporeal shunt and return of all of the blood to the animal, there is a rapid adjustment of blood volume and the hematocrit return to the usual value. The details of the preceding will be published separately.[19]

IN VITRO INCUBATION OF BLOOD: In Figs. 3 and 5 the influence of 6 hours of incubation of the effluent blood from the radiation coil at 37°C is shown. Cells which have passed through the shunt only a single time and which have received about 900 rads are drastically effected showing a dissolution of 87 per cent of the cells in a 6 hour incubation period. Note in both Figs. 3 and 5 that relatively few cells disintegrate during 6 hours incubation prior to commencement of irradiation. It is presumed that this latent radiation injury, not detectible by immediate cytologic observation in vitro but brought out by incubation is "sensed" by the reticuloendothelial system promptly and that the cells or fragments are removed from circulation producing the observed in vivo lymphopenia.

DISCUSSION:

It has been shown that extracorporeal irradiation of the circulating blood will damage lymphocytes. These lymphocytes will disintegrate in vitro. Prolongation of the extracorporeal irradiation produces a progressive lymphopenia. In addition to the lymphopenia a temporary neutrophilic leukocytosis is produced. There is no influence upon the platelet count.

The mechanism of the lymphopenia is not firmly established but is believed due to peripheral radiation injury with subsequent removal of the

lymphocytes presumably by the reticuloendothelial system.

The sluggish return of the lymphocytes to pre-irradiation levels is in marked contrast to the reported rapid return following peripheral depletion by draining lymphocytes from the thoracic duct lymph in the calf.[20] The two methods of production of lymphopenia possess significant differences that may explain the differences in recovery rate. In the extracorporeal radiation a large number of lymphocytes are injured and presumably removed from the circulation by the reticuloendothelial system. Thus the products of the destroyed lymphocytes may influence the capacity of the lymphopoietic organs to produce new lymphocytes. Perhaps the products of destruction of large numbers of lymphocytes over a short period of time acts as a "negative feedback" for new cell production similar to the inhibition of new red cell production by hypertransfusion of red cells[21] or new platelet production by hypertransfusion of platelets.[22] When lymphocytes are drained from the body these cells are lost and can not inhibit new production. Perhaps the loss from the body without feedback by products of destruction removes a brake upon new cell production. This concept is admittedly highly conjectural but for the sake of discussion one can propose the following hypothetical scheme for regulation of lymphocyte production. Normally, lymphocytes in large part presumably recycle from blood to lymph.[23] Thus lymphocyte production may be influenced by the presence of lymphocytes in transit through the lymph nodes. If, in the course of lymphocyte senescence, their products of destruction within nodes acts as an inhibitory influence on new production, the removal of lymphocytes by external drainage should allow an accelerated production rate. If a large surplus of "inhibitory material" were to be dumped into the lymphopoietic tissues by peripheral radiation injury of cells and the subsequent phagocytosis

and destruction within lymphopoietic tissues, one might obtain a depression in new lymphocyte production. On the other hand, if lymphocytes are long lived, of the order of as long as 100 days as the labeling studies in rats would indicate[24,25] and there is a set production rate without any mechanism for increasing this rate significantly one would expect a slow recovery of the lymphocyte mass which would probably be manifested by a prolonged lymphopenia. Admittedly these are hypothetical considerations that do not answer the questions at hand. However, we believe that this method of lymphocyte depletion by extracorporeal irradiation of the circulating blood in combination with enumeration of the outflow of lymphocytes from the lymphocytic ducts, cytology of lymph nodes, DNA labeling techniques will go far in obtaining direct answers on the following important questions:

1. What is the magnitude of lymphocyte recycling from blood to lymph?
2. Mechanisms of regulating lymphocyte production?
3. Life span and production rates of lymphocytes?
4. Stem cell capabilities of lymphocytes?
5. Products of destruction of lymphocytes?

SUMMARY

1. A method for extracorporeal irradiation of the circulating blood has been developed.

2. Extracorporeal irradiation of the blood will produce a lymphopenia promptly which persists for weeks.

3. Heparin in high doses in the calf produces a lymphocytosis and neutrophilic leukocytosis.

Figure 1. Photograph of calf with blood being pumped through an extracorporeal shunt.

A. Lead barrier between animal and x-ray machine.

B. Reciprocating pump.

C. Continuous injection apparatus for heparin.

D. Water bath for heating jacket of extracorporeal shunt.

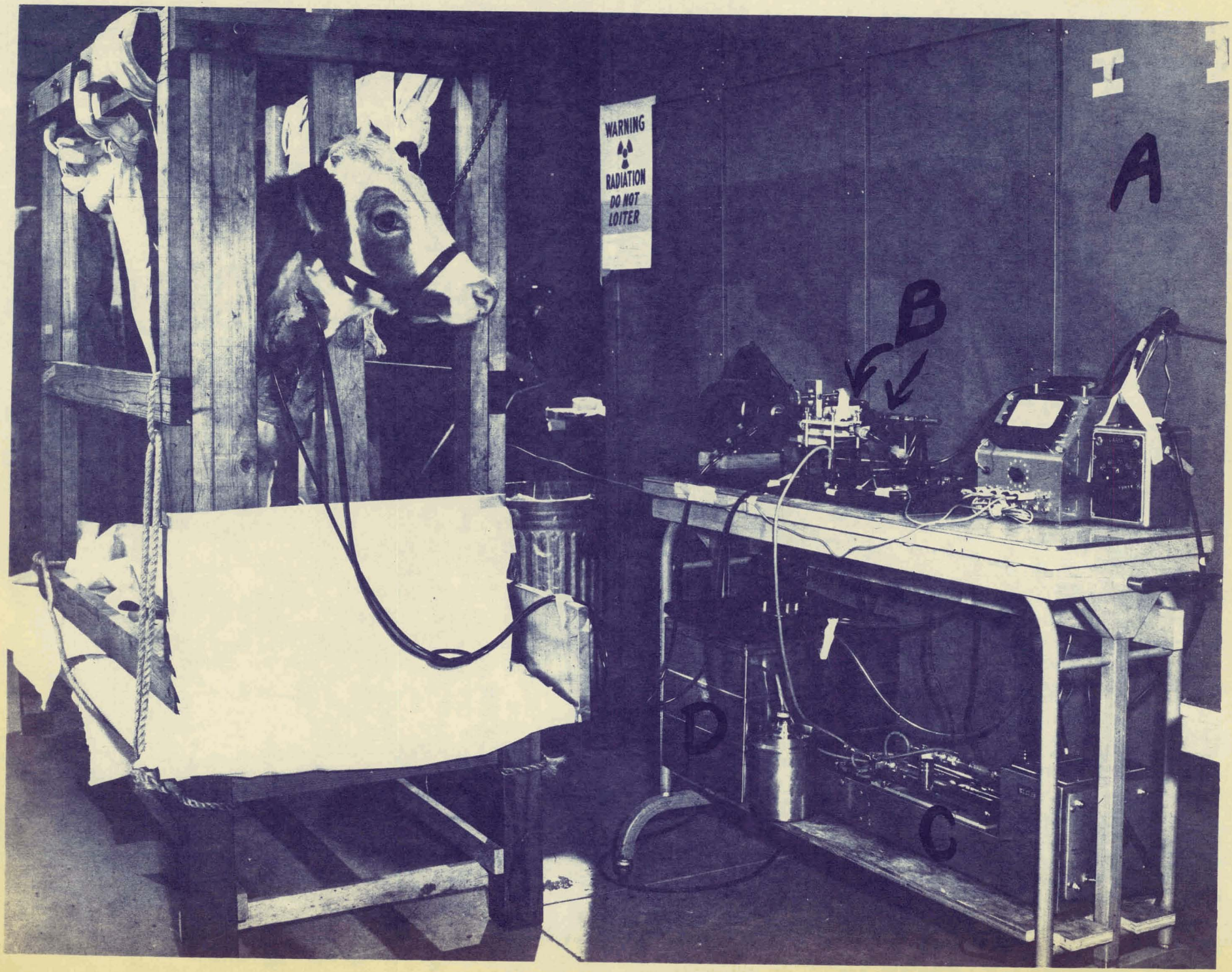

FIG. 1

Figure 2. X-ray machine and irradiation coil.

A. Frame diameter.

B. Tygon coil.

C. Integrating dosimeter.

D. X-ray machine.

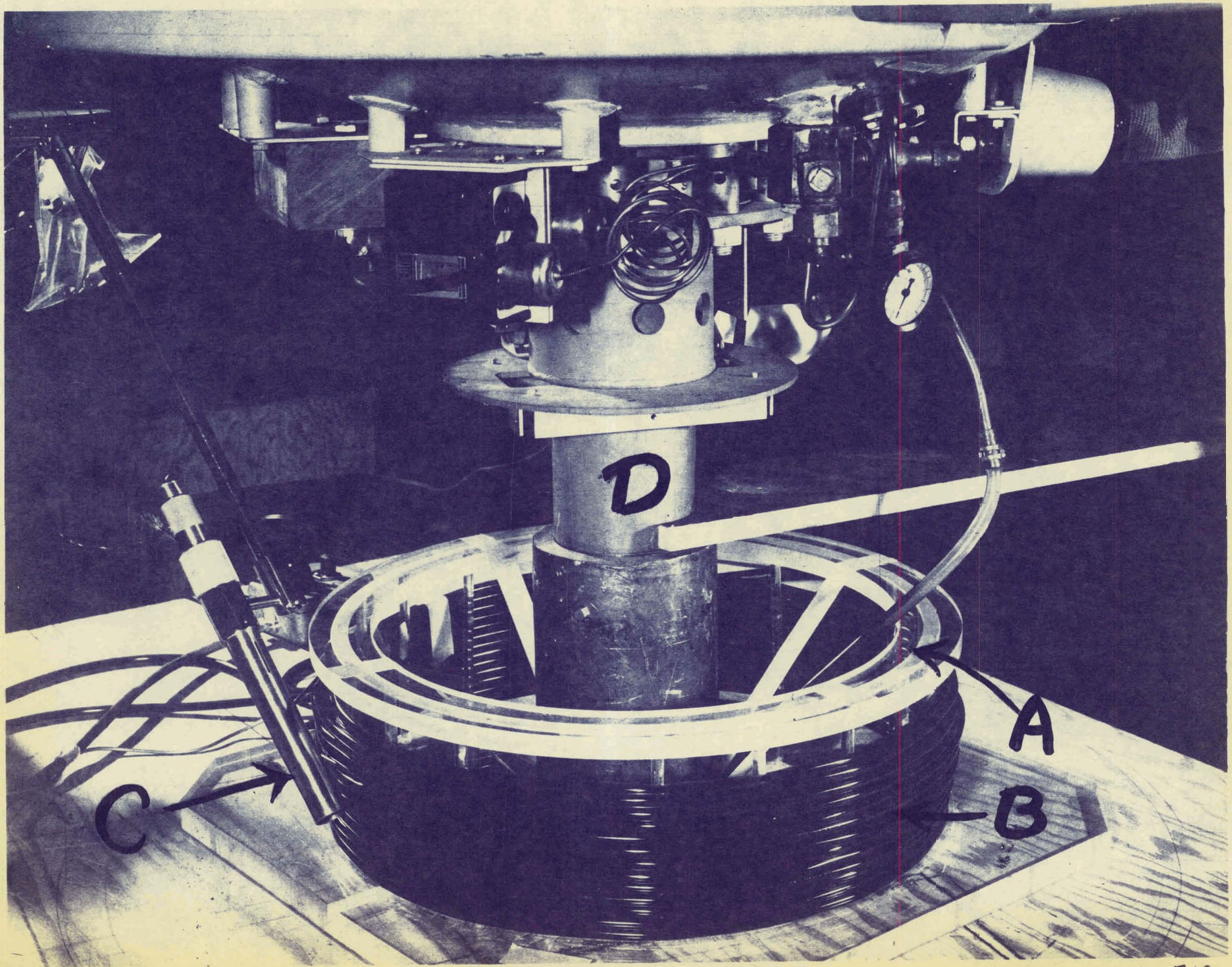

FIG. 2

Figure 3. Effect of heparinization and two hours of extracorporeal irradiation upon the circulating lymphocyte count and the lymphocyte count after six hours of in vitro incubation.

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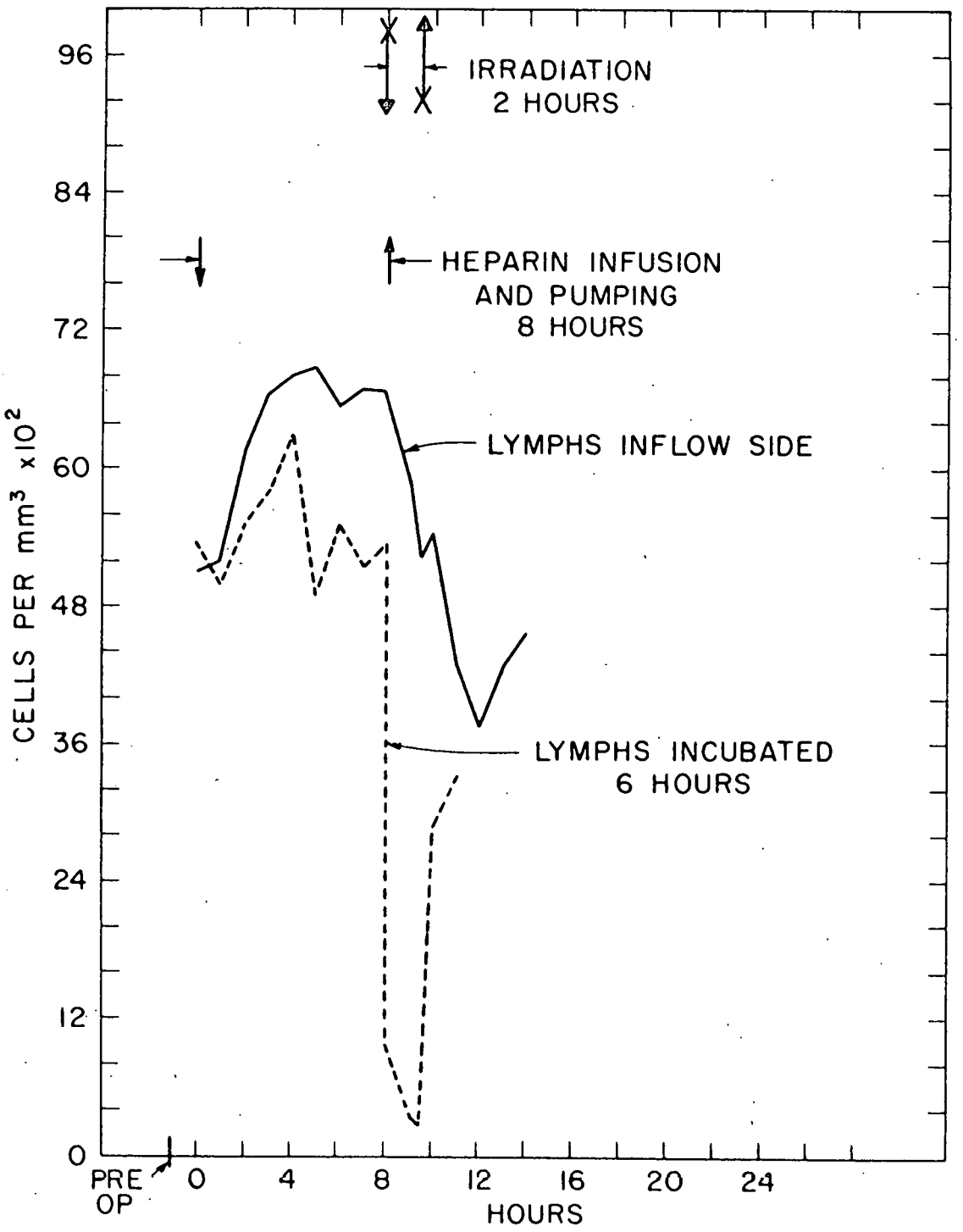
IRRADIATION
2 HOURS
HEPARIN INFUSION
AND PUMPING
8 HOURS
LYMPHS INFLOW SIDE
LYMPHS INCUBATED
6 HOURS
CELLS PER mm³ x10²
96
84
72
60
48
36
24
12
0
PRE
OP
0
4
8
12
16
20
24
HOURS

FIG. 3

Figure 4. Effect of heparinization and six hours of extracorporeal irradiation upon the neutrophil and lymphocyte count.

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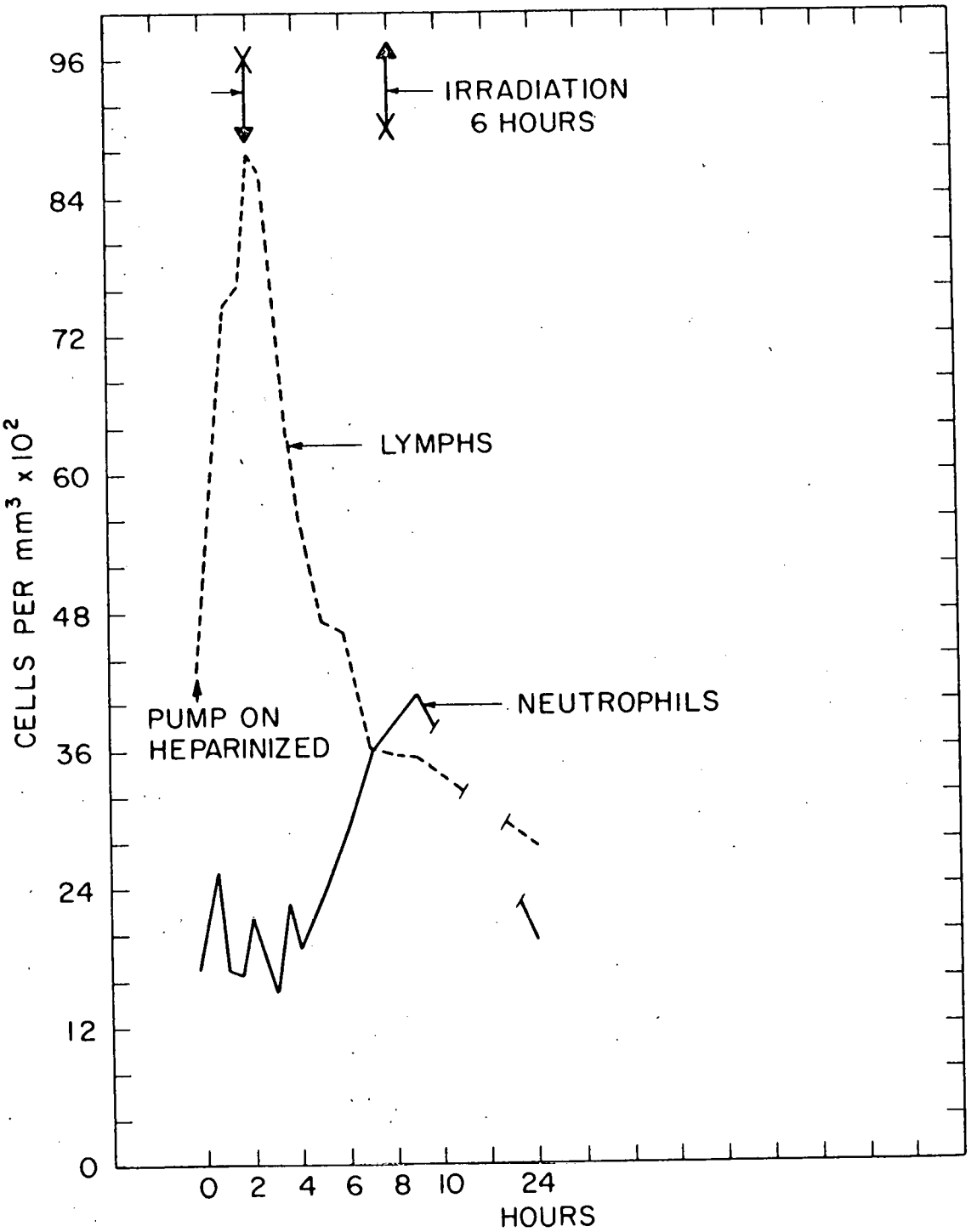

FIG. 4

Figure 5. Effect of heparinization and twelve hours of extracorporeal irradiation upon the circulating lymphocyte count and the count after 6 hours of in vitro incubation.

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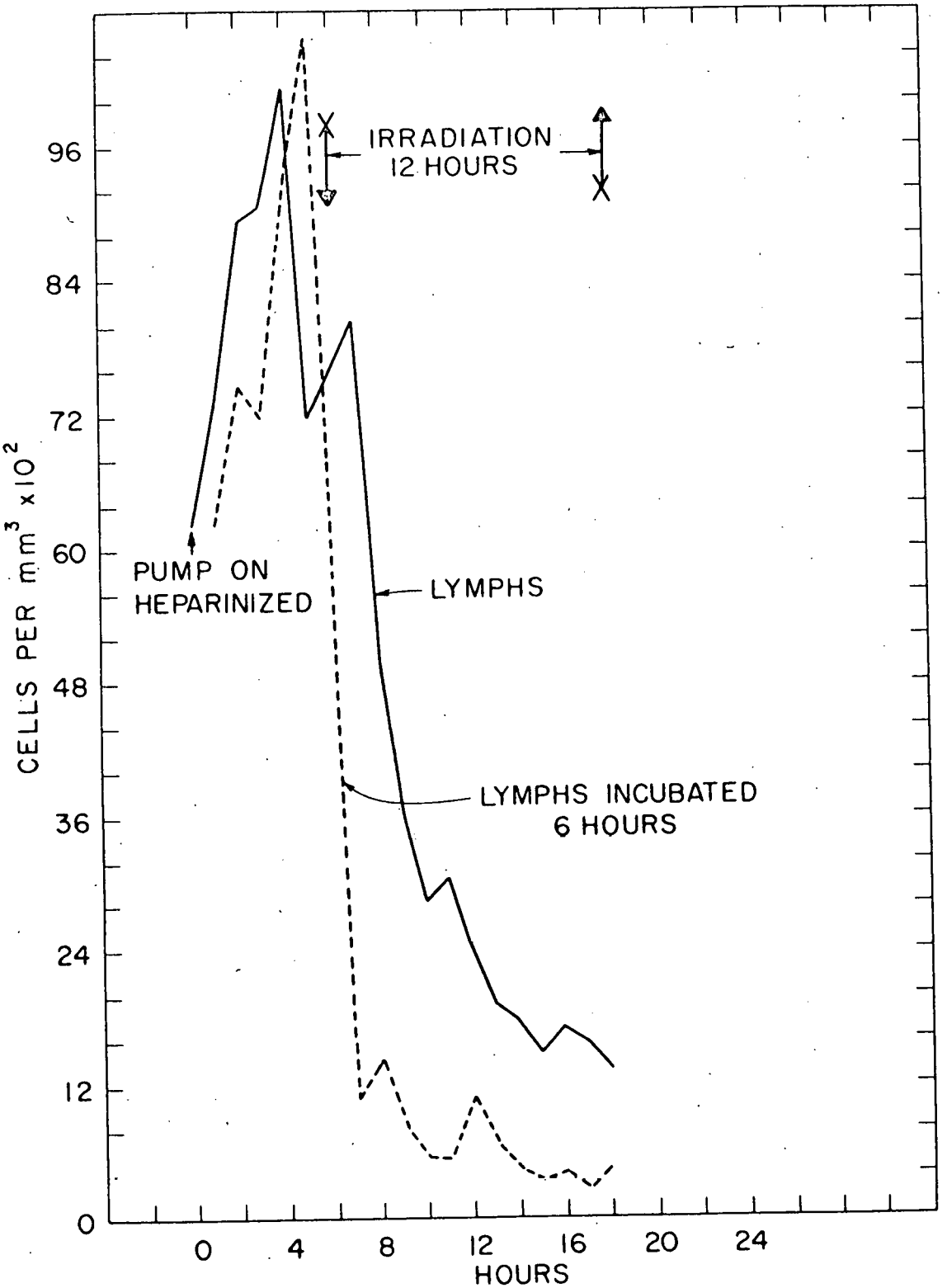
IRRADIATION
12 HOURS
PUMP ON
HEPARINIZED
LYMPHS
LYMPHS INCUBATED
6 HOURS
CELLS PER mm³ x10²
0
12
24
36
48
60
72
84
96
0
4
8
12
16
20
24
HOURS

FIG. 5

Figure 6. Effect of heparinization and hours of extracorporeal irradiation upon the neutrophil count.

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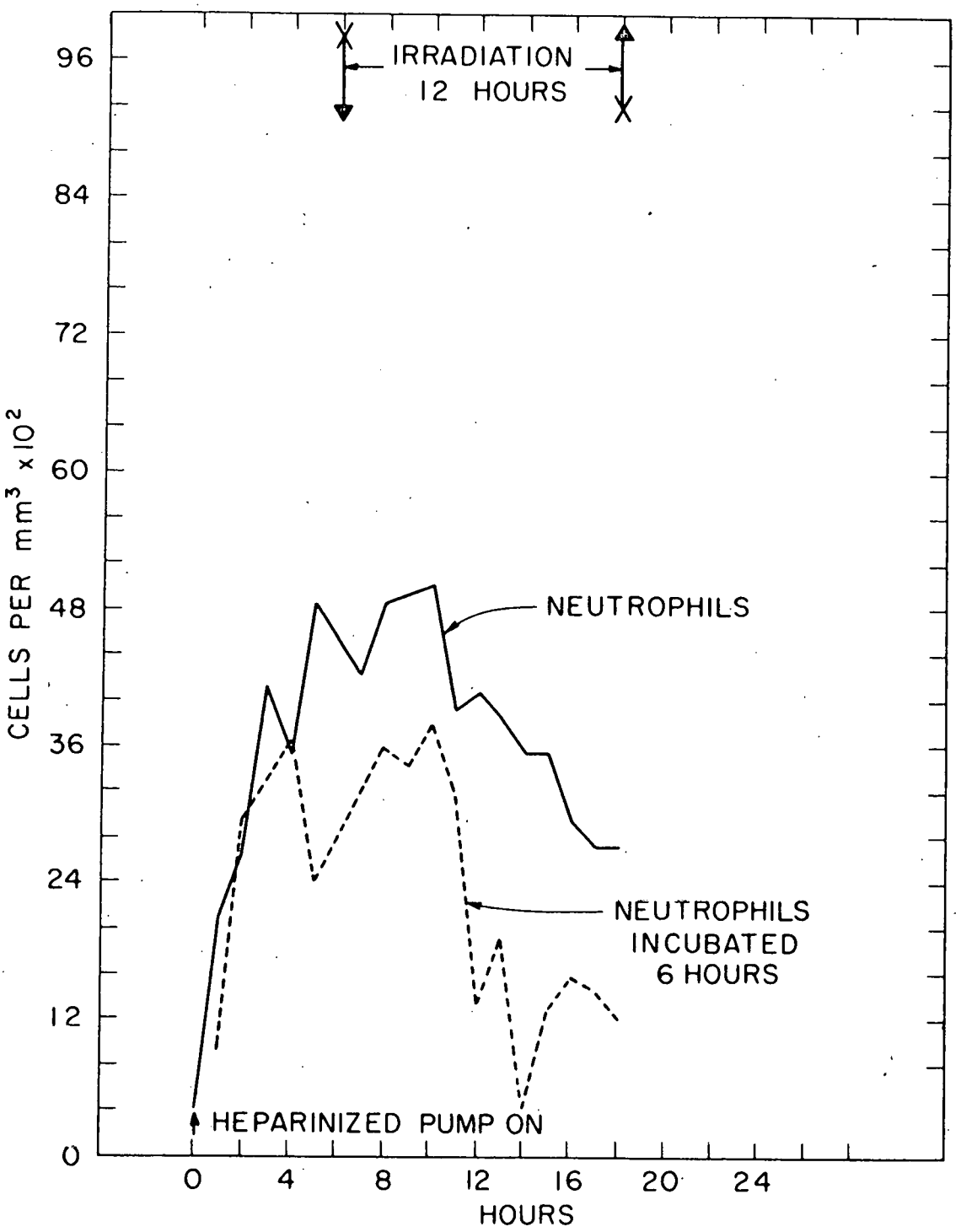
IRRADIATION
12 HOURS
96
84
72
60
48
36
24
12
0
CELLS PER mm³ x 10²
NEUTROPHILS
NEUTROPHILS
INCUBATED
6 HOURS
HEPARINIZED PUMP ON
0
4
8
12
16
20
24
HOURS

FIG. 6

Figure 7. Effect of heparinization and 3 hours of extracorporeal irradiation upon the lymphocyte count promptly followed by the slow rate of recovery.

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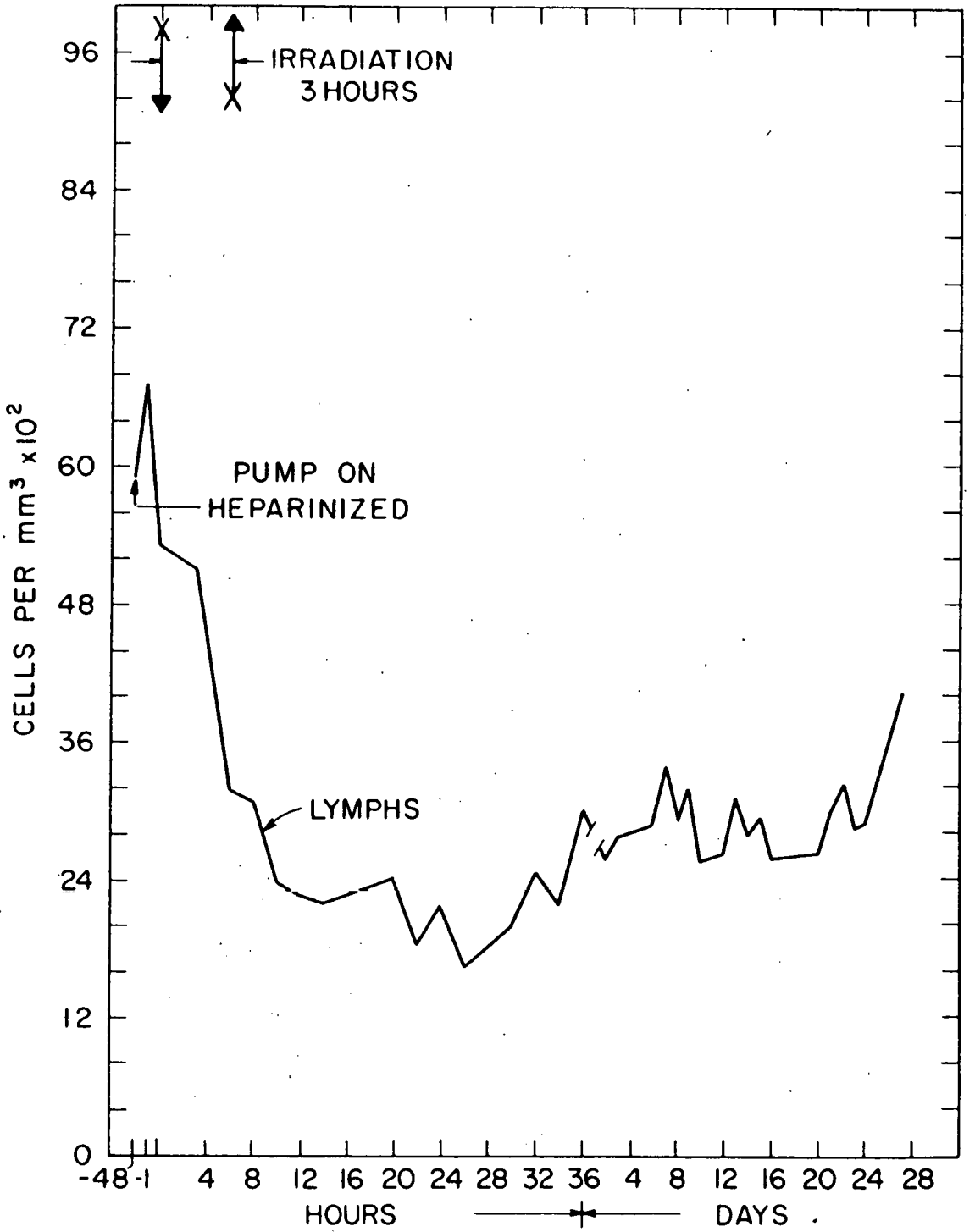
IRRADIATION
3 HOURS
PUMP ON
HEPARINIZED
LYMPHS
CELLS PER mm³ x 10²
96
84
72
60
48
36
24
12
0
-48 -1 4 8 12 16 20 24 28 32 36 4 8 12 16 20 24 28
HOURS
DAYS

FIG. 7

Figure 8. Effect of heparinization and six hours of irradiation upon the lymphocyte and neutrophil count. Note prompt return to normal values with the neutrophils and the slow recovery rate of the lymphocytes.

Bov 9/61

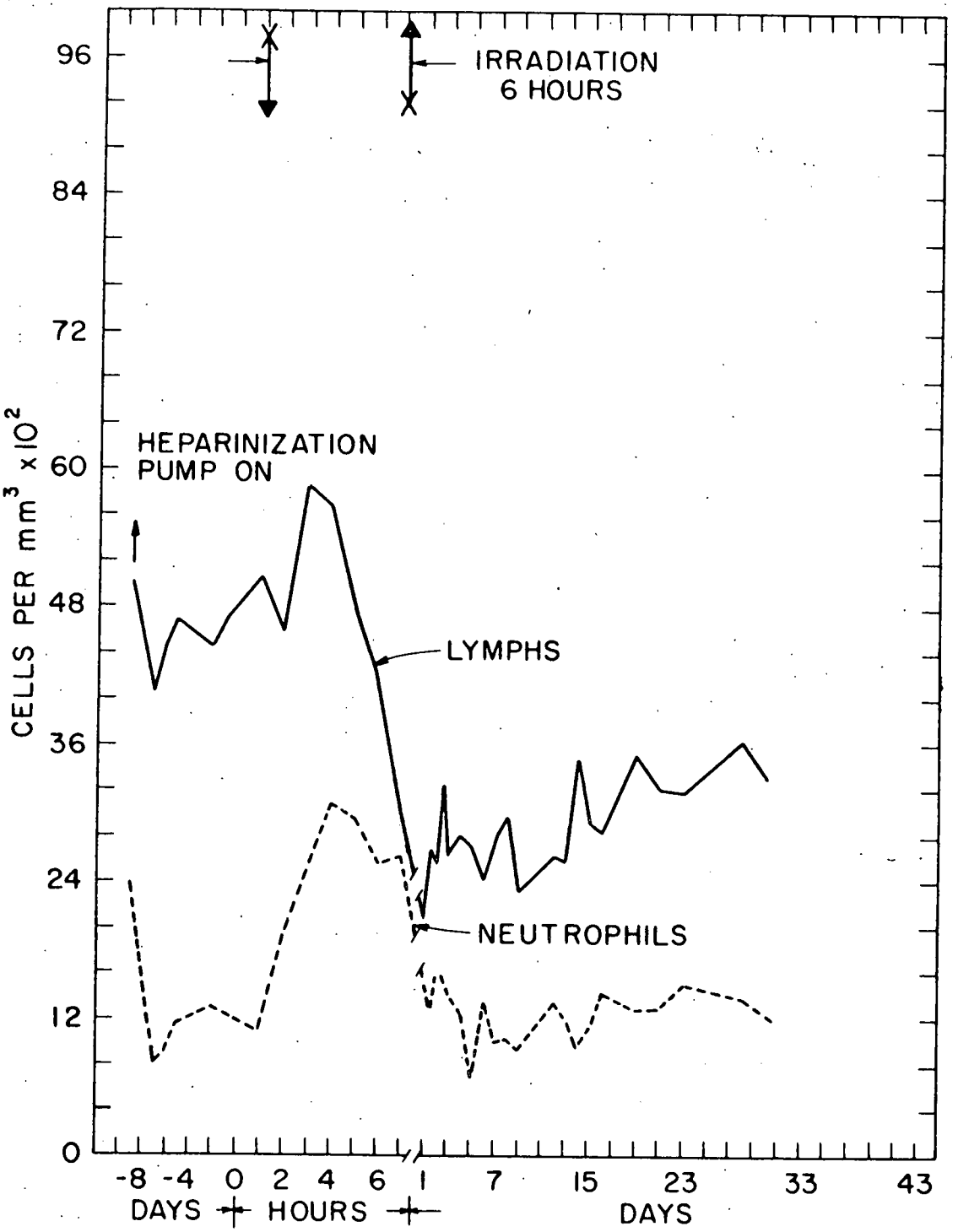
IRRADIATION
6 HOURS
HEPARINIZATION
PUMP ON
LYMPHS
NEUTROPHILS
CELLS PER mm³ x10²
96
84
72
60
48
36
24
12
0
-8 -4 0 2 4 6 1 7 15 23 33 43
DAYS + HOURS + DAYS

FIG. 8

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