Studies on Lymphocytes. III. Effects of Extracorporeal Irradiation of the Circulating Blood upon the Lymphoreticular Organs in the Calf


Extracorporeal x-irradiation of the circulating blood has been shown to be practical in animals and in man. In applying this procedure in the calf, it is essential to use an extensive amount of heparin as an anticoagulant. Heparin has been shown to produce a blood lymphocytosis and some degree of mobilization of the small lymphocytes from lymphoreticular organs. Accordingly, this fact must be borne in mind in interpreting data on changes in lymphocyte concentration in blood, lymph and tissues. With the technic that has been developed in our laboratory, extracorporeal x-irradiation of the circulating blood in calves, produces a maximal lymphocytopenia within 12 hours. Thereafter, the blood lymphocyte counts remain at a low plateau between 700 and 2,000 cu. mm. while extracorporeal irradiation is maintained. Frank hemolysis with hemoglobinuria has on occasion developed after 28 hours of extracorporeal x-irradiation. However, this has not produced any severe symptomatology in the animals and extracorporeal irradiation has been maintained for as long as 48 hours. The duration of the post irradiation lymphocytopenia in normal calves tends to be correlated with the total number of lymphocytes killed and in some instances it has been many weeks before peripheral blood lymphocytes returned to the pre-irradiation level.

The evaluation of the data in respect to lymphocyte mobilization and/or new production is meaningful only in relation to observed changes in lymphopoietic tissue. In this initial report, the major findings in calf thymus, spleen and lymph nodes after prolonged extracorporeal x-irradiation of the circulating blood will be described and discussed.

Materials and Methods

Initial studies were performed on dogs and swine but the calf was finally selected as the most satisfactory animal for extracorporeal x-irradiation of the circulating blood because of its large blood volume, its temperament and the ease with which surgery can be performed under local anesthesia. The method used and the mathematical considerations relating to the radiation dose delivered to cells and/or molecules remaining within the blood stream have been described and discussed in detail. At present, with a flow rate of approximately 300 ml. per minute, the circulating blood in the shunt receives a transit dose of approximately 900 rads (250 KVP, 30 m.a., HVL of 1.75 mm Cu, at a dose rate of 300 rads per minute).

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Table 1.—Treatment of Calves Used in the Present Study

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<th>Calf #</th>
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<th>Biopsy of Inguinal Lymph Node Prior to Treatment</th>
<th>Heparinized During Experiment*</th>
<th>Duration of Pumping of Blood Through Extracorporeal Circuit (hours)</th>
<th>Duration of Extracorporeal Irradiation of Circulating Blood (hours)</th>
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If not stated otherwise calves were killed for autopsy immediately after treatment.
*(8 mg. of Heparin per Kg. at start and 50 mg. per hour).

The number and types of animals used in this study are tabulated in table 1. Pre-irradiation removal of inguinal or prescapular lymph nodes was performed under 2 per cent xylocaine local anesthesia. For comparative studies identical twin calves were used as experimental and corresponding control animals. Hourly blood samples were obtained prior to and during extracorporeal x-irradiation and hematocrit, total white blood cell counts and differential counts were performed. At the end of the extracorporeal irradiation or pumping of the blood through the extracorporeal shunt without irradiation, the animals were anesthetized with sodium surital, exsanguinated and autopsied for histological study of the organs.

The following methods were used for the evaluation of relative and total lymphocytic cell mass and lymphopoietic activity in the lymphoreticular organs:

(a) organ weights;

(b) planimetric comparison of lymphoreticular tissues densely populated with lymphocytes (e.g. cortex vs. medulla in the thymus and lymph nodes, white vs. the red pulp in the spleen);

(c) planimetric comparison of germinal centers;

(d) mitotic activity in germinal centers and other parts of lymphoreticular tissues;

(e) mean lymphocyte number per argyrophilic fiber along comparable straight lines in lymphoreticular tissue.

**RESULTS**

Characteristic changes in the blood lymphocyte counts prior to and during extracorporeal irradiation are presented in figure 1.
After 30 to 48 hours of extracorporeal irradiation of the circulating blood the following changes were observed in lymphoreticular organs as compared to the appearance of the lymph nodes removed prior to irradiation and/or the control non-irradiated animals:

**Thymus**

The thickness of the thymic cortex has diminished and the planimetrically measured cortical/medullary ratio shows a moderate decrease. The density of
the lymphocyte population in the cortex remains almost unchanged while the medulla contains apparently a greater number of lymphocytes. Pyknotic and karyorrhectic nuclei are unusual. The mitotic activity in the cortex appears to be unchanged or slightly increased. Significant gross weight changes between experiments and controls have not been observed.

**Spleen**

The lymph follicles show a marked decrease in size which is mainly due to a loss of small lymphocytes in the cuffs surrounding the germinal centers. There is also a considerable loss of lymphocytes from the large less densely populated peripheral areas of the white pulp as well as in the red pulp. These latter regions contain numerous neutrophilic granulocytes and some cellular (including nuclear) debris. Disintegrating cells, more often lymphocytes than granulocytes or erythrocytes, are seen throughout the red pulp. The germinal centers appear to be intact. Often they are slightly enlarged and show a marked mitotic activity. Their contours, including the prominent histiocytic mantle are sharply delineated and contact directly the blood-filled neighboring tissue. The latter, after the loss of small lymphocytes, looks similar to the red pulp (fig. 2). However, this area contains numerous neutrophilic granulocytes. The number of tingible bodies within the germinal centers is slightly higher than normal. Hemosiderin containing cells appear in the red pulp as well as within the germinal centers. Numerous macrophages containing red cells are found in the red pulp.

**Lymph Nodes**

In most instances, the planimetrically established portion of densely populated cortical lymphocytic tissue has decreased (fig. 3, 4). Whereas there is no question about the diminution in the thickness of the cortical lymphocyte population it has not as yet been established whether the relative density of the remaining cortical lymphocyte population is reduced in a comparable manner to that of the density in the medulla. By the exclusive use of planimetric measurement such as were used in this study, one very probably underestimates the actual loss of lymphocytes in lymph nodes since no correction has been made for the loss of medullary lymphocytes and for probable changes in the density of cortical lymphocytic tissue. The germinal centers are comparable to those seen in the spleen although the changes are less striking. Some disintegrating cells with karyorrhectic or pyknotic nuclear degeneration are found in the interfollicular zones of the cortex or near the cortico-medullary junction. Increased neutrophilic infiltration in the lymph nodes is not a consistent finding. In all animals studied the loss of small lymphocytes in the lymphoreticular tissues tends to be correlated with the duration of extracorporeal irradiation rather than with the peripheral lymphocyte counts. After 6–12 hours of extracorporeal irradiation, a loss of lymphocytes in the lymphoreticular organs can be detected, but the loss is much greater after 30–48 hours of extracorporeal irradiation as illustrated in figure 4.

Significant changes in the gross weight have not been observed to date.
Fig. 2.—Photographs of splenic lymph follicles after pumping with and without extracorporeal x-irradiation of the circulating blood. (a) Spleen lymph follicle after 46 hours of extracorporeal pumping of the blood without irradiation. Note the well preserved cuff of small lymphocytes (L) around the germinal center (G) and the appreciable lymphocytic infiltration outside of the histiocytic mantle (H) of the follicle. (b) Appearance of spleen lymph follicle after 46 hours of extracorporeal irradiation of the circulating blood: note the well developed germinal center (G) and histiocytic mantle (H) and the almost complete lack of small lymphocytes between the germinal center and the histiocytic mantle as well as outside of the latter. There is a sharp demarkation of the histiocytic mantle against the surrounding red pulp which is filled with blood and contains numerous neutrophils. Both sections are stained with hematoxylin and eosin.
INGUINAL LYMPH NODES AFTER:

- EXTRACORPOREAL IRRADIATION OF CIRCULATING BLOOD
- PUMPING WITHOUT IRRADIATION
- NO TREATMENT
- CORTISONE & HYDROCORTISONE

Fig. 3.—Bar graph showing the relative diminution in the densely populated cortical lymphocytic tissue of inguinal lymph nodes based on planimetric measurements. Neither prolonged pumping without irradiation nor massive treatment with cortisone and hydrocortisone* over a period of 48 hours result in the degree of lymphocyte depletion caused by extracorporeal irradiation of the circulating blood. The percent reduction of the densely populated lymphocytic cortical tissue as measured planimetrically is an underestimate of the actual lymphocyte loss since the decrease in relative lymphocytic cellularity in the cortex and the loss of medullary lymphocytes are not taken into account on this graph. The numbers at the top of each bar refer to the calf numbers.

Other Tissues

After the first few hours of extracorporeal irradiation, disintegrating round cells are observed in the spleen, in the lung capillaries, and to a lesser extent also in the liver sinusoids, some of which have been phagocytized by Kupffer cells. Occasionally, disintegrating cells are seen within the capillaries of other organs. At every time interval studied, after initiation of extracorporeal irradiation, the relative number of damaged cells in the lung, liver or lymph nodes is always less or equal to that in the spleen. During the first few hours of extracorporeal irradiation, the capillaries throughout the examined organs

* Cortisone 1 Gm. intramuscularly followed by continuous intravenous injection of hydrocortisone 1 Gm. over 24 hours and then 250 mgm. of Aristocort (9, α fluoro 16, α hydroxo-prednisotone) intramuscularly every 4 hours for 6 injections. Treatment period 24 hours.
Fig. 4.—Histologic appearance of inguinal lymph nodes of calves prior to and after extracorporeal irradiation of the circulating blood. (a) Histologic picture of inguinal lymph node removed prior to extracorporeal irradiation of the circulating blood. (b) Contralateral inguinal lymph node of the same animal after 44 hours of extracorporeal irradiation of the circulating blood. Note the reduction in thickness of the densely populated lymphocytic tissue in the cortex compared to the pre-irradiation. The smaller size of the node is coincidental. Both photographs from hematoxylin and eosin stained sections.

contain more lymphocytes than normal. The histological appearance of the adrenal glands is not indicative of a marked stress. The bone marrow smears have not shown any obvious changes but further study will be necessary to establish the presence or absence of an effect.
The distribution of dose to any molecule or cell that remains within the blood stream during the period of extracorporeal irradiation is a function of the blood volume, shunt volume, and transit time in the shunt. These factors determine the probability of repetitive trips through the radiation field. In addition some cells such as the lymphocytes may be removed from free circulation as a result of radiation injury after one cycle through the radiation field or the normal average residence time in the blood stream may be significantly less than the period of extracorporeal irradiation. In either case, the dose distribution to the lymphocytes present in the blood can not be estimated because of inadequate information in respect to these two factors. However, the equations for computing the dose distribution to cells or molecules that remain in the blood have been established and programmed on the Brookhaven National Laboratory MERLIN computer for numerical solution. This distribution, at various times after commencement of a 48 hour period of extracorporeal irradiation, is illustrated in figure 5. After 1 hour and 36 minutes of pumping, only 2 per cent of the blood volume had not had a single transit through the radiation field. Ninety-eighth per cent of the blood has received 900 or more rads. Sixty-nine per cent of the blood has received 2700 rads or more. After 6 hours and 24 minutes all of the blood has received more than 400 rads. After 48 hours, the dose distribution ranges from $66 \times 10^3$ to $110 \times 10^3$ rads.

One, of necessity, assumes that the blood returning from the irradiation field mixes completely with the blood volume promptly. After 1 hour and 36 minutes of irradiation 98 per cent of the blood has received a minimal dose of 900 rads. This dose is sufficient to kill most if not all of the exposed lymphocytes. There is cytologic evidence for killing of lymphocytes. Degenerating cells are found, in the order of relative frequency, in the spleen lung capillaries, lymph nodes and capillaries of other organs. If the above assumption is correct, most of the lymphocytes circulating in the peripheral blood 2½ hours after commencement of extracorporeal irradiation must have entered the blood stream after this time interval. In other words, the great majority of these cells must have been released from lymphocyte production sites or stores. A comparison of the lymphocyte counts in the peripheral blood with the planimetrically measured mass of densely populated lymphocytic tissue, as a function of duration of extracorporeal irradiation does not indicate parallel changes of the two. The number of circulating blood lymphocytes drops to a low constant level after 12 hours of extracorporeal irradiation that is maintained up to 48 hours of extracorporeal irradiation. On the other hand, the lymphoreticular tissues continue to lose lymphocytes between the twelfth and forty-eighth hour of extracorporeal irradiation. Therefore the apparent steady-state lymphocyte level in the peripheral blood is maintained by a non-steady-state process in the lymphoreticular organs where the new production of lymphocytes is by far insufficient to replace the cells leaving these tissues. The mechanism governing this release is not as yet
The data does not supply any direct information on the nature of the distribution of transit times.

(1) The blood is primarily feeding the lymphoreticular tissue and the lymph by recycling rather than the lymph nodes feeding the blood by new cell production.

(2) A large fraction of thoracic duct lymphocytes recycle rapidly from blood to lymph or the output of thoracic duct lymphocytes is controlled by the concentration in the blood by some unknown rapid feedback loop. The possibility of an adrenocortical effect upon the lymphocytes has not been excluded. In a pilot study we have shown in the calf that administration of cortisone and hydrocortisone over a period of two days also leads to a loss of some lymphocytes in the lymphoreticular organs. However, the histological changes produced by these hormones are not identical with those obtained by prolonged extracorporeal x-irradiation. One important difference is that the germinal centers are markedly damaged by the administration of cortisone or hydrocortisone within 2 days (increased numbers of tingible

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*The data does not supply any direct information on the nature of the distribution of transit times.*
bodies, decreased mitotic activity, swelling of reticulum cells) but are left intact or even stimulated by extracorporeal x-irradiation. The latter also produces a much more pronounced loss of lymphocytes outside of germinal centers than does the administration of adrenal corticosteroids.

The combination of intact or even more actively proliferating germinal centers than normal with a marked diminution in the concentration of small lymphocytes produced by extracorporeal irradiation within two days is a unique pathologic phenomenon. To the best of our knowledge a similar, yet not identical, picture has only been observed in rats thymectomized shortly after birth,12 or after a period of starvation.13 Prolonged extracorporeal irradiation of the blood thus provides an excellent opportunity to examine the relative importance of germinal centers alone vs. germinal centers and small lymphocytes for immune responses to primary or secondary antigenic stimulation. With the successful development of higher dose rate irradiation facilities and technics for increasing the flow rate of blood, it will be possible to kill even more lymphocytes before hemolysis develops thus improving the efficiency of lymphocyte depletion.

In the first paper of this series, we commented upon the fact that the prolonged lymphopenia might be related to suppression of new cell formation by deposition of large amounts of DNA debris from the destruction of lymphocytes intravascularly. Similarly, the apparent stimulation or preservation of the integrity of the germinal centers in the animals with marked depletion of total body small lymphocytes might be related to the deposition of degradation products of DNA within the lymphoreticular organs. This problem has been under investigation by comparing lymphopenia due to the extracorporeal irradiation of the lymph to lymphocyte depletion by separating lymphocytes from the lymph with re-infusion of cell free lymph in identical twins. The observations of Jaroslow and Taliafferro,14 in which DNA degradation products are said to accelerate the return of antibody producing capabilities in irradiated animals, might be relevant in respect to the apparent increased mitotic activity of the germinal centers. If germinal centers are antibody “factories” it is conceivable but not shown that dumping of large amounts of lymphocytic DNA may have a stimulatory upon mitosis within the germinal centers. Similarly the studies of Feldman et al.15 in which viable spleen cells, splenic DNA and degraded DNA restored antibody production in irradiated animals may have some relevance in our present study. Their studies may even be more pertinent to our present studies on the influence of extracorporeal irradiation upon immunization and homotransplantation. It may well be that the continual destruction of lymphocytes in vivo in an extracorporeal radiation shunt will continue to serve as a stimulus for antibody production rather than suppress it by reducing the number of antibody producing units as we naively supposed. The completion of our current experiments will answer this question.

**Summary and Conclusions**

1. Extracorporeal x-irradiation of the circulating blood of the calf severely depletes the population of small lymphocytes within the body.
2. The number of circulating blood lymphocytes drops to a low constant level after 12 hours of extracorporeal irradiation that is maintained up to 48 hours of extracorporeal irradiation. On the other hand the lymphoreticular tissues continue to lose lymphocytes between the twelfth and forty-eighth hour of extracorporeal irradiation. Therefore, the apparent steady-state lymphocyte level in the peripheral blood is maintained by a non-steady-state process in the lymphoreticular organs where the new production of lymphocytes is far insufficient to replace the cells leaving these tissues.

3. The cellular debris caused by extracorporeal irradiation of the circulating blood is found within the spleen, lung, liver, lymph nodes and elsewhere, in this order of relative concentration.

4. A unique pathologic phenomenon is produced within two days of extracorporeal irradiation of the circulating blood, e.g., a combination of intact or even actively proliferating germinal centers with a marked diminution in the concentration of small lymphocytes. This technic of depletion of small lymphocytes, leaving germinal centers intact, should permit studies for evaluation of the relative importance of germinal centers vs. small lymphocytes in primary and secondary immune responses.

5. The above findings are discussed in relation to lymphocyte production rate, stores, mobilization and recirculation.

Summario in Interlingua

1. Roentgeno-irradiation del sanguine circulante del vitello effectua un sever depletion del population de micre lymphocytos intra le corpore.

2. Le numero del lymphocytos in le sanguine circulante declina a un basse nivello constante post 12 horas de irradiation extracorporee. Iste constante se mantene usque al fin de 48 horas de irradiation extracorporee. Del altere latere, le tissus lymphoreticular continua perder lymphocytos inter le decescunde e le quaranta-octave hora de irradiation extracorporee. Per consequente, le apparente stato stabile del nivello de lymphocytos in le sanguine peripheric es mantenite per un processo de stato non-stabile in le organos lymphoreticular ubi le nove production de lymphocytos es insufficiente per multo a reimplaciar le cellulas que quita iste tissus.

3. Le debris cellular causate per irradiation extracorporee del sanguine circulante es trovate in le splen, le pulmon, le hepate, le nodos lymphatic, e alterubi, in iste ordine de concentration relative.

4. Un unic phenomeno pathologic es producite intra duo dies de irradiation extracorporee del sanguine circulante, i.e., un combination de intacte o mesmo activemente proliferante centros germinal con un marcate diminuition in le concentration de micre lymphocytos. Iste technica de effectuar un depletion del micre lymphocytos in le presentia de intacte centros germinal, deberea permetter studios visante a evalutar le importantia relative del functiones del centros germinal e de illos del micre lymphocytos in primari e secundari responsas immun.

5. Iste constatationes es discutite in relation con le intensitate del production, le thesaurisation, le mobilisation, e le re-circulation de lymphocytos.
REFERENCES


EXTRACORPOREAL IRRADIATION IN THE CALF

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Studies on Lymphocytes. III. Effects of Extracorporeal Irradiation of the Circulating Blood upon the Lymphoreticular Organs in the Calf