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SELECTIVE IMMUNO-SUPPRESSION BY RADIATION *

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ABSTRACT

The lymphocytic family of cells is very sensitive to radiation. The basic reasons for employing radiation as an immunosuppressive agent are to destroy existing lymphocytes as well as to suppress production of new cells and thereby interfere with their role in immunological reactions. In view of the difficulties in the clinical application of lethal and sublethal whole body irradiation for immunosuppression, investigators have attempted a variety of techniques to radiate selectively immunocompetent lymphocytes. The observation that many lymphocytes recirculate from blood to lymph and back to blood has provided one basic lead in the development of these techniques. This unique physiological phenomenon of lymphocyte recirculation enables one to deplete many of immunocompetent, recirculating lymphocytes by radiation without producing harmful effects to body at large.

Techniques that have been explored for selective immunosuppression by radiation include the following; (1) Surface radiation applicators for individual lymphoid organs such as the spleen and the appendix; (2) Radiation of individual lymphoid organs such as the thymus and the spleen; (3) Radiation of allografts either in the immediate post-transplant period or during the rejection episodes; (4) Indwelling intravascular radiation sources; (5) Intravascular or intralymphatic injections of radioisotopes and; (6) Extracorporeal irradiation of blood, central lymph and regional efferent lymph.

The historical aspects of selective lymphocyte radiation are reviewed as well as the problems concerned with dosimetry and the radiosensitivity of circulating blood elements other than lymphocytes.

The possibilities of perturbations in steady state lymphocytopoiesis which might be triggered by products of radiation induced cell death are presented, however the parameters investigated thus far, such as the degree of lymphocytopenia, thoracic duct lymphocyte output and cell cycle times of thoracic duct lymphocytes, have failed to reveal any such perturbations. Studies in adrenalectomized calves have failed to confirm the notion that lymphocytopenia following extracorporeal irradiation of blood and lymph might primarily be accounted for by stress induced corticosteroid hormonal activity.

Of the various techniques enumerated above only local graft irradiation and extracorporeal irradiation of blood have found clinical application. The results obtained have been encouraging and indicate a need for additional, well-controlled clinical trials, especially with regard to the role of ECIB as an adjunct to standard immunosuppressive therapy. The experimental results with extracorporeal irradiation of lymph have also established the potential of this technique for clinical application.

There is an urgent need for studying the influence of irradiation on various subpopulations of lymphocytes with regard to their functional capabilities and in particular with regard to their reproductive potential. Possible influence of selective blood irradiation on circulating stem cells in blood needs to be evaluated.

INTRODUCTION

The extreme sensitivity of the lymphocyte to radiation[1] and the intimate involvement of this cell in immunological reactions[2-5] have

been adequately discussed during the previous sessions of this symposium. With whole body radiation (WBR), one is faced with injury to all proliferating cell systems, free circulating cells, as well as the disturbance of the interrelationship between organ systems. Biologic responses of WBR are thus extremely complicated and it is difficult to sort out the various facets of importance[6]. WBR does indeed lead to a state of immunosuppression consequent upon lymphocyte depletion[7], but as noted above, this approach suffers from a lack of selectivity. Furthermore, lymphocyte subpopulations exhibit different degrees of radiosensitivity[8] and radiation induced immunosuppression has been shown to be dependent upon dose rate and radiation quality[9]. Various approaches for radiation-induced selective immunosuppression have previously been reviewed[7]. The present review will be limited primarily to a discussion of the extracorporeal irradiation of blood, central lymph, and regional lymph, as techniques for selective immunosuppression.

It was demonstrated by Gowans that a fraction of lymphocytes recirculate from blood to lymph and back to blood[10]. Gowans and Knight further demonstrated that the post-capillary venules in lymph nodes and other lymphoreticular tissues are the major sites for the exit of lymphocytes from the blood[11]. Lymphocyte recirculation through peripheral lymphoid tissues was directly demonstrated by the studies of Cronkite et al[12]. Ford has described in detail lymphocyte recirculation within the spleen[13]. The underlying principles for extracorporeal irradiation of blood (ECIB) and lymph (ECIL) are to utilize the extreme sensitivity of lymphocytes to radiation and, by virtue of the traffic patterns of blood and lymph lymphocytes, to induce not only lymphocytopenia in the blood and lymph but also to deplete lymphocytes in the lymphoreticular organs.

EXTRACORPOREAL IRRADIATION: HISTORICAL ASPECTS

Before discussing the current status of ECIB and ECIL, it is appropriate to describe briefly the early attempts at physical treatment of circulating blood outside of the body, an experimental procedure first developed by Heymans in 1921[14]. Similar techniques were subsequently used by Dall'Aqua and Zoppellari[15] and by Ducuing et al[16]. Due to technical difficulties, these investigators were unable to deliver an adequate dose of radiation to the circulating blood. In 1958, Arnould et al reported ECIB in the anesthetized rat[17]. They observed the development of anemia and leukopenia. Pellerin et al studied further the development of anemia and leukopenia[18] and found that leukopenia was predominantly due to an initial depression in the blood lymphocytes. There is no indication in any of these early reports of efforts to suppress immunity by extracorporeal irradiation. In this context it is worth pointing out that as early as 1914 Murphy at the Rockefeller Institute had attempted to modify the rejection of a tumor transplant by interference of lymphocyte function by radiation[19]. In 1962, Lajtha et al discussed the therapeutic potential of ECIB in leukemia and also indicated that destruction of a long-lived circulating cell population in the normal blood might influence the immune response[20].

EXTRACORPOREAL IRRADIATION: TECHNICAL, PHYSIOLOGICAL AND RADIOBIOLOGICAL ASPECTS

At Brookhaven, ECIB and ECIL were developed initially to deplete whole body lymphocytes and study the regulation of lymphocyte production analogous to hemorrhage for study of erythropoiesis[12,21,22]. During the

development of ECIB and ECIL knowledge on lymphocytes grew at a rapid pace and there are now numerous areas under investigation. For ECIB, carotid-jugular shunts are employed in large animals (Fig. 1). The arterial blood traverses the radiation field before returning via the vein. In patients, shunts are similar to radial artery-cephalic vein shunts as employed for hemodialysis. For ECIL, thoracic duct-jugular vein shunts are employed. The technical aspects of large animal and human shunts have been published[23,24].

Radiation Sources and Dosimetry

Various types of radiation sources have been described[20,25-29]. In addition, ordinary x-ray sources[21] and conventional cobalt therapy units[30] have also been employed for extracorporeal irradiation. For the calculation of the radiation dose by the blood during ECIB, both "differential equation" and "exact probability" models have been utilized[31-35]. The radiation dose received by a cell during one circuit past the radiation source is referred to as the transit dose. The transit dose can be calculated from the following formula:

$$D = \frac{V}{F} \times R$$

D = transit dose

V = the volume of external shunt in litres

F = the flow rate in the external shunt in litres/min.

R = the dose rate for the irradiator in rads/min.

The range in residence time of cells and molecules within the blood varies from seconds to an average of four months for erythrocytes. One can estimate the dose distribution only to those entities that remain in

the blood for a period longer than the session of ECIB. For practical purposes this applies to erythrocytes and platelets. The granulocyte leaves the blood randomly with an average sojourn of 9.5 hours and does not return to the blood thus the radiation dose will be only a small multiple of the transit dose. Lymphocytes are in part recycling from the blood to the lymphoid tissues. In this case it is exceedingly difficult to ascertain the dose distribution to the cells. Since the statistics of recycling of lymphocytes are unknown it is assumed that the minimum dose is the transit dose. For this reason, and from the work of Trowell[1], a transit dose of 900 rads was selected for the initial studies because it was thought that 90% of the lymphocytes would be killed by one transit. The accumulation of radiation dose by erythrocytes and platelets does not create any great difficulty in planning ECIB schedules since they are relatively radioresistant[36-38]. Schnappauf et al have shown that radiosensitivity of bovine erythrocytes increases with erythrocyte age[39]. For prolonged irradiation or intermittent irradiation extending for weeks or months, one should not only consider the daily replacement of erythrocytes by newly formed, relatively radioresistant cells but also the influence of accumulated dose of radiation upon erythrocyte life span.

Schedules of ECIB/ECIL and Transit Dose

The ideal situation would be a schedule of extracorporeal irradiation during which all circulating lymphocytes received a lethal dose of radiation. It soon became apparent that with a transit dose of 900 rads and prolonged ECIB, severe hemolysis resulted from

cumulative radiation injury to erythrocytes. Accordingly, the influence of varying transit doses was investigated[40]. Transit doses as low as 15 rads produced lymphocytopenia but of lesser degree than attained at higher transit doses. Compared to continuous ECIB, repetitive short sessions would appear to be more efficient in producing lymphocytopenia and much safer, since the probability of inducing hemolysis by radiation injury to the erythrocytes is diminished. Increased efficiency of repetitive ECIB probably results from the establishment of new equilibria between tissue and blood lymphocyte pools between ECIB sessions. When time is allowed to irradiate 5 blood volumes nearly 100% of the lymphocytes in the blood at commencement of the treatment will have received one transit dose. As shown in Figure 2, continuation of ECIB sessions beyond the time required to irradiate the equivalent of 5 blood volumes, increases the radiation damage to erythrocytes without significantly increasing the proportion of lymphocytes irradiated. Because of these considerations, an average transit dose of ~ 500 rads with daily repetitive sessions of ECIB has been used. In Figure 3, the range of transit dose is superimposed on Trowell's data[1] for the in-vivo radiosensitivity of lymphocytes.

Since the LD_{100} for the cells of interest, the average time spent in the blood, the rate of recycling from blood to lymphoreticular tissues and the average time needed to remove fatally injured cells are not known, it is not possible to define the ideal ECIB schedule. The situation is further complicated by the heterogeneity of lymphocytes with regard to their radiosensitivity, traffic patterns and life span in circulation. The difficulty

is compounded by the fact that thymus-derived cells are intimately involved in immune reactivity, constitute a major fraction of the recycling pool, and are also a major fraction of the newly produced cells in the circulating blood. The latter fraction is short lived and not a part of the recycling pool[41]. Little information concerning the effects of ECIB on various subpopulations of blood lymphocytes such as 'B' and 'T' cells is available. In a preliminary study, no change was observed in PHA response during ECIB[42].

With ECIL any transit dose can be given since one is not concerned with cumulative damage to erythrocytes. With continuous ECIL transit doses around 1000 rads have been used (Fig. 3). Figure 4 shows the influence of low and high transit dose on thoracic duct lymphocyte output. It is evident that the high transit dose is more effective in reducing the output of thoracic duct lymphocytes.

Another reason for utilizing a higher transit dose is to reduce the fraction of cells surviving with "mutations". The radiosensitivity of the lymphocytes and the immune response have been reviewed[8]. In summary, the conclusions of this review indicate that: (i) Radiosensitivity of cells cannot be estimated by the measurement of the radioresistant phenomena such as DNA and enzyme synthesis, enzyme function and antibody synthesis. (ii) There seems to be no difference in response of newly-formed ^{3}H-thymidine labeled lymphocytes versus older lymphocytes to a single dose of x-ray. (iii) Proliferating lymphocytes have about the same radiosensitivity as other cell renewal systems as far as reproductive integrity is concerned. (iv) The only meaningful parameter for radiosensitivity of lymphocytes involved in immune reactions

is the suppression of the reproductive capability.

Lymphocyte Depletion by ECIB/ECIL

ECIB and ECIL produce both a marked blood lymphocytopenia as well as a substantial reduction in the output of thoracic duct lymphocytes[7,12,20-22,30,40,43-52]. Lymphocyte depletion of blood and lymph follows a curve with at least two components. The first component has a half time of 1.2 days and corresponds to the elimination of an easily mobilizable pool of lymphocytes. The second component has a half time of approximately 30 days and corresponds to a more sessile mass of lymphocytes. Calculations based on this data, indicate the size of the easily mobilizable pool to be approximately 10 times the number of circulating blood lymphocytes, or 3-5 x 10^9 lymphocytes/Kg body weight[53]. In untreated calves the volume distribution of thoracic duct lymphocytes is a continuum between 120 and 1200 μ^3, however, within this range there are two overlapping populations of cells; a smaller population with a median of 250 μ^3, and a larger population with a median of 648 μ^3 [54]. Lymphocytopenia induced by extracorporeal irradiation markedly alters the size distribution due principally to a loss of cells within the smaller lymphocyte category[55].

Cottier et al and Ruchti et al have reported on the depletion of lymphoreticular tissues by ECIB[56,57]. Briefly, the thymic cortex is diminished in thickness with a decreased corticomedullary ratio. Splenic lymph follicles are markedly reduced in size as a result of loss of small lymphocytes from the cuffs surrounding germinal centers.

There is also a loss of lymphocytes from the less densely populated areas of the white pulp and from the red pulp. There is a striking loss of lymphocytes from the paracortical areas of the lymph nodes. Disintegrating cells are found in intrafollicular zones of the cortex or near the cortico-medullary junction. Germinal centers in the spleen and lymph nodes are intact and appear to have increased mitotic activity. A considerable proportion (~ 85%) of the lymphoid cells of cortical zone of lymph nodes is sessile while the paracortical areas of lymph nodes and the white pulp of the spleen contain a larger fraction of easily mobilizable lymphocytes (~ 50%)[58].

Influence of ECIB/ECIL on Mechanisms Regulating Lymphocytopoiesis!

The notion that the lymphocytes are highly radiosensitive is due to the rapid disappearance of advanced morphological damage in lymphoid organs following irradiation that produces interphase death of the lymphocytes. Extracorporeal irradiation kills circulating radiosensitive lymphocytes which are then cleared by the lymphoreticular system with presumably little harm to the other cell systems[6]. The effect of the products of lymphocyte death on steady-state lymphocytopoiesis is, however, not known. In a series of experiments, two approaches were utilized in an effort to gain some information pertaining to this problem[59]. In the first approach, removal of cells from the thoracic duct lymph were compared to those obtained by ECIL. It has been suggested, that under normal conditions, large numbers of lymphocytes are lost from the body via the intestinal tract[60]. In the second approach the loss of lymphocytes via a thoracic duct-rumen fistulae was compared to ECIL. There were no significant differences in the degree of lymphocytopenia and/or the output of lymphocytes in the

thoracic duct between ECIL, continuous centrifugation of lymph, or thoracic duct-rumen fistulae.

In another series of experiments, cell cycle times of bovine thoracic duct lymphocytes were measured before and following extracorporeal irradiation. The duration of the various phases of cell cycle was measured by methods of mitotic labeling index and the decrease in mean grain count of labeled mitotic figures. The cell cycle phases were not affected by ECIB[61]. Moreover, there was no discernible increase in the mitotic figures with chromosomal abnormalities. From these observations, it was concluded that lymphocytes passing through the irradiation field were injured to such an extent that they were unable to home on the lymph nodes, proliferate and return to thoracic duct.

In another study, cell cycle parameters were measured by the analysis of labeled mitosis and double labeling before and after ECIL[62]. ECIL had no detectible effect on either the duration of cell cycle phases or the fractional production rate of lymphocytes. Studies of Chan and Hayhoe[63] have suggested that in patients with leukemia, ECIB may alter the feedback mechanisms of growth control resulting in increased proliferation of the leukemic blast cell. There is, however, no information available with regard to similar changes in normal steady state hemopoiesis.

Studies in mice[64], guinea pigs[65] and dogs[66] have shown hemopoietic stem cells to be present in the circulating blood. Yoffey has suggested that small lymphocytes enter the peripheral blood via lymphatics and then seed the bone marrow where they function as hemopoietic stem cells[67]. Attempts in mice[68], rats[69] and dogs[70] to test this hypothesis have provided contradictory results. The majority of the evidence, however,

does not support Yoffey's notion. On the other hand, it has been suggested that hemopoietic stem cell may be morphologically similar to small lymphocytes[71]. In view of the presence of circulating stem cells in the blood and the relative radiosensitivity of these cells[6], it is logical to ask the question as to whether ECIB affects these stem cells? No data are available that bear on this subject.

Influence of Corticosteroids on ECIB/ECIL Induced Lymphocytopenia

The cytolytic action of corticosteroid hormones on lymphoid tissues is well recognized[72]. Surgical and experimental manipulations associated with ECIB/ECIL are stressful procedures resulting in increased endogenous corticosteroid production. It is, therefore, important to establish that blood lymphocytopenia, decreased thoracic duct lymphocyte output and lymphoid tissue depletion are primarily the result of radiation injury rather than increased corticosteroid secretion. Corticosteroid hormones primarily affect the germinal centers as compared to other areas of the lymph nodes, and in addition spare the lymphocytes in the marginal zone of follicles[73]. In studies of the lymphoid tissue following ECIB, depletion of germinal centers was not observed[56,57]. In contrast Storb et al observed germinal center atrophy following ECIB in the baboon[47] most probably attributable to stress induced corticosteroid secretion due to prolonged retention of the experimental animals in restraining chairs.

In order to further clarify this issue, additional studies have been carried out in 10 calves. Five calves were bilaterally adrenalectomized at the age of 4 weeks and maintained on hydrocortisone acetate and deoxycorticosteroid. The remaining group of 5 calves was subjected to

sham adrenalectomy and given no corticosteroids. Two to 3 weeks following surgery 4 animals (2 adrenalectomized, 2 sham-adrenalectomized) were subjected to 10 daily sessions of ECIB each (5 blood volumes/session at a transit dose of 800 rads). Two calves (1 adrenalectomized and 1 sham-adrenalectomized) were submitted to 10 days of continuous ECIL. Four animals served as non-treated controls.

The results are shown in Figures 5 and 6. The drop in blood lymphocyte counts was similar in adrenalectomized and sham-adrenalectomized calves. The minor differences observed in adrenalectomized and sham-adrenalectomized calves following ECIB, were slight and within the variation observed in other calves previously subjected to ECIB. The histological examination of lymphoid tissues has not been completed. We conclude from these data that under the experimental conditions employed in our laboratory, ECIB/ECIL-induced lymphocytopenia is primarily the result of radiation injury to circulating lymphocytes and is not mediated via corticosteroid hormones.

SELECTIVE IMMUNO-SUPPRESSION BY RADIATION

Techniques Other Than Whole Body Irradiation

In examining these possibilities, one must consider the relationship of the lymphocytes to immune reactions. Central and peripheral sensitization theories have been advanced with respect to the initial reaction of lymphocytes to foreign antigen. The end result, however, is the same i.e., production of specific responsive cells which are committed either to react against an allograft or to the production of specific antibodies. These specific cells, initially generated in the regional lymph nodes, migrate

to the distant lymphoid organs via the efferent lymphatics and blood thereby amplifying the immune response. There are several approaches by which one can interrupt the sequence of events through modes of radiation other than whole body exposure. Most of these approaches have been investigated and have been reviewed in detail[7]. Briefly, they include: (i) Irradiation of the arterial blood to an allograft. (ii) Irradiation of the recipient bed prior to transplantation. (iii) Irradiation of the reticulo-endothelial system through radioactive colloids or other radioisotopes. (iv) Irradiation of lymphoid organs such as the thymus, spleen and regional lymph nodes. (v) Irradiation of blood by intravascular radioactive sources. (vi) Irradiation of transplanted organs. (vii) Extracorporeal irradiation of blood and lymph.

The last two approaches are the only approaches that have shown any potential for clinical application. Local graft radiation has been reviewed previously[7]. This technique has been used clinically in transplantation of the kidney, liver and heart. It is used sequentially in the early transplant period and during periods of accelerated rejection. This technique has proven to be of some effectiveness as a clinical and experimental immunosuppressive agent[74,75].

With reference to techniques other than extracorporeal irradiation, certain investigations reported subsequent to the previous review[7], merit some discussion. Ford demonstrated that chronic irradiation of the rat spleen by a ^{32}P-impregnated polyethelene strip applied to the antihilar surface produced a decrease in the numbers of circulating small lymphocytes and a considerable reduction in the output of thoracic

duct cells[76]. Yoshida et al investigated the immunosuppressive potential of this technique and demonstrated a significant depression of both cellular and humoral immune responses[77]. Moderate lymphopenia was observed in rabbits when ^{32}P strips were applied to the appendix but the immune response of these rabbits was not detectably impaired[78]. This later study did, however, confirm the existence of lymphocyte recirculation through the appendix, a finding which is inconsistent with the notion of rabbit appendix being a central lymphoid organ[79].

Near-lethal dose of WBR are effective in suppressing cellular and humoral immune responses[80]. Single, relatively massive doses of radiation given to the regional lymph node just prior to antigenic stimulation, however, do not suppress immunological responsiveness[81], although the immuncompetent lymphocytes are highly radiosensitive[8]. This failure has been explained by the repopulation of the lymph node by blood born lymphocytes[82]. In principle, repeated local irradiation of the regional lymph node following antigenic stimulation might be more effective than a single large dose. Eltringham and Weissman have conducted such studies in rats and have demonstrated a marked suppression of the induction of delayed hypersensitivity and humoral antibody formation following antigenic stimulation with sheep erythrocytes[83].

The immunosuppressive potential of various parenterally administered radioisotopes was previously reviewed[7]. Roser and Ford have since published studies concerning intrasplenic or intra nodal isotope injection[84]. Intrasplenic injection of ^{185}W-tungsten trioxide resulted in a deficit of blood and thoracic duct lymphocytes. This lymphocytopenia persisted for weeks. The primary immune responses to sheep erythrocytes and S. adelaid flagella antigens were depressed, however, secondary responses to flagella were unaffected. With regard to allograft rejection, skin graft survival across a weak histocompatibility barrier was prolonged, however, no prolongation of skin graft survival was observed when crossing strong histocompatibility barrier. This difference was attributed to the presence of a much higher proportion of lymphocytes reactive to strong histocompatibility antigens. Similar results had earlier been obtained by McGregor and Gowans[85]. These investigators produced lymphocytopenia by chronic drainage of the rat thoracic duct lymph. Intrasplenic ^{185}W-tungsten trioxide has been shown to be an effective immunosuppressive technique, but as the authors point out, its clinical potential is limited by the risk of inducing neoplasia.

Extracorporeal Irradiation of Blood

Experimental Studies

Cauchi and Field have utilized ECIB to study the kinetics and radiosensitivity of immunologically competent cells in the blood[86]. In these studies, the survival of irradiated circulating cells was assessed in syngeneic rats of a parental strain by splenomegaly induced by cross circulation with F_1 hybrids. ECIB of the donor was carried out either during cross-circulation or at various time intervals prior to cross-circulation. Influence of ECIB on immunocompetent cells was measured by reduction in GVH - inducing capacity as assayed by changes in recipient spleen weight. The results of these studies suggest the presence of two populations of immunocompetent cells in the blood, both capable of inducing GVH reactions. One population is 6 times as radioresistant as cells in lymphoid organs and has a turnover time of 5 hours; the second population shows the usual radiosensitivity of the lymphoid tissues and restores GVH-inducing capacity to the blood within an hour after this capacity has been destroyed by ECIB. These studies clearly demonstrate the ability of ECIB to deplete the blood of cells participating in cellular immune reactions.

The results of studies concerning the influence of ECIB on bovine skin allograft rejection have been described in a series of papers[49,59,87-90]. Various schedules of ECIB and combinations of ECIB, chemotherapy and thymectomy were employed in these studies. Continuous ECIB for 24 hours at transit doses of 15 to 60 rads, five days prior to skin grafting, did not prolong skin allograft

survival. Repetitive pregraft ECIB at transit doses of 50 to 1000 rads for a total irradiation time of 98 to 114 hours, prolonged the graft survival time by 2 to 3 days and suppressed the intensity of the rejection process. Repetitive pre- and postgraft ECIB was the most effective in prolonging skin allograft survival particularly when used in combination with small doses of azathioprine, which by themselves were ineffective in altering the allograft rejection time. These results suggest that ECIB and immunosuppressive drugs may be synergistic and the combined use might permit one to decrease the dose of chemotherapeutic agents without loss of immunosuppressive potential.

Calves were thymectomized at 56 to 205 days of age. The interval between ECIB and thymectomy ranged from 2 to 205 days of age. ECIB schedules consisted of pre- and postgraft repetitive exposures. The results of these studies demonstrated that the combination of thymectomy and ECIB was no more effective in prolonging skin allograft than ECIB alone. These data do not support the notion that thymectomy is a beneficial part of an immunosuppressive regime in organ transplantation.

The effectiveness of ECIB is limited by: (a) ECIB "kills" only that fraction of immunocompetent cells which are diverted thru the irradiator and (b) ECIB can only be carried out with high transit doses for only a few hours each day due to accumulation of radiation damage to erythrocytes[36,39].

Storb et al[47] have studied on the influence of continuous ECIB on skin allograft survival in baboons. Due to excessive dose delivered

to the erythrocytes, the experimental animals developed severe hemolytic anemia. ECIB resulted in prolonged, severe lymphocytopenia and a significant prolongation of skin allograft survival. Despite germinal center atrophy in these animals there was no depression of antibody formation against sheep erythrocytes. The effects of ECIB on primary and secondary responses to tetanus toxoid have been studied. ECIB failed to abolish antitoxin responses to sucutaneous immunization with either fluid or aluminum phosphate-absorbed tetanus toxoid[91,92]. Following repetitive ECIB delivered over a period of 10 to 30 days (accumulated dose 93,000 to 250,000 rads to erythrocytes), near normal primary responses and normal secondary responses were elicited despite the production of a sustained lymphocytopenia. In these studies antigen was injected subcutaneously in areas where regional lymph nodes are the most important sites for antibody responses. In view of the studies of Roser and Ford[84] it would be of interest to study the effects of prolonged ECIB on antitoxin production when tetanus toxoid is injected intravenously where the spleen rather than the regional lymph nodes would be mainly concerned with antibody responses.

ECIB is being used in a number of institutions for clinical renal transplant immunosuppression[45,48,74,93-95], however, no controlled animal experiments have been conducted. Therefore, the influence of ECIB on goat renal allografts was studied[55,96]. The aim of these studies is to evaluate the influence of ECIB alone and in combination with standard immunosuppressive therapy on renal allograft survival. To date three different schedules of ECIB have been studied. The first schedule consisted of repetitive pregraft ECIB at a transit

dose of approximately 325 rads. About 90 blood volumes were irradiated prior to grafting. Due to unusually high susceptibility of goat red blood cells to hemolysis by irradiation, relatively little ECIB could be given at this relatively high transit dose. To overcome this problem, a second schedule of repetitive pregraft ECIB was employed using a lower transit dose of approximately 45 rads. With this schedule, it was possible to deliver repetitive pregraft irradiation to about 200 blood volumes without inducing hemolysis. The third schedule of ECIB was similar to the second with the exception that repetitive postgraft ECIB (40 to 100 blood volumes) was added. Controls consisted of one group which received no treatment and a second group which received sham-ECIB with schedules similar to those employed for ECIB-III. The results of these studies are shown in Figure 7. Schedule III was most effective in prolonging the renal allograft survival. The general conclusions from these studies are; (1) the total number of blood volumes irradiated prior to grafting may be important for ECIB induced immunosuppression and; (2) Pre- and post graft ECIB is superior to pregraft ECIB only. It is worth mentioning that these results were obtained in randomly matched recipient-donor pairs and without the benefit of standard immunosuppressive drugs. ECIB and azathioprine were shown to have a synergistic immunosuppressive effect on skin allografts[90], and one might anticipate a similar effect on renal allografts.

Clinical Studies

Results of trials with ECIB as an immunosuppressive agent in prolonging the survival of renal transplants have been reported from

a number of institutions[45,48,74,93-95,97]. At only two of these centers[48,95,97] has the number of patients subjected to ECIB been adequate to allow critical comment. In their initial series, Weeke et al[48], reported the results in 11 patients who were subjected to pregraft ECIB. In 10 of these recipients, the transplanted kidney was obtained from a close relative. The 11th patient was subjected to two separate sessions of ECIB prior to receiving a cadaver graft. Eight of the recipients were also given variable amounts of postgraft ECIB. Post-transplant drug therapy consisted of azathioprine and prednisone. It is interesting to note that none of these patients received actinomycin C. Pronounced lymphocytopenia was induced in all patients and during post-transplant period the average lymphocyte concentration remained below 50% of the pre-ECIB value. The authors state that the clinical course in all instances was benign, with no irreversible rejection episodes even though the dose of immunosuppressive drugs was small. In a more recent publication, Weeke and Thaysen[97] have described the influence of ECIB on cadaveric kidney transplantation. This series consisted of 91 recipients of which 37 received pre- and immediate postgraft ECIB. The ECIB treated group suffered from a significantly reduced number of acute rejection episodes and had a 25% better graft survival during the first 6 months postgraft period. In addition the clinical rank of the ECIB treated group was better during the first 6 months of postgraft period. There was no increased incidence of severe infections in ECIB treated group as compared to non-ECIB treated group.

Persson et al[95] have published the results of renal transplantation in 145 recipients, of which 27 were subjected to pregraft ECIB at a transit

dose of approximately 600 rads. Recipients treated by ECIB had fewer and less severe acute rejection episodes. Renal function and graft survival was better in the ECIB treated group as compared to the non-ECIB treated group, although both groups received comparable amounts of chemotherapy.

Although lymphocytopenia was sustained in all patients subjected to ECIB[95,97], there was no clear cut correlation between the degree of lymphocytopenia and the immunosuppressive effects of ECIB. ECIB appears to have resulted in some benefit during the immediate postgraft period. Results of long term followup with regard to graft and patient survival are not yet available. In view of the many variables involved, properly controlled, randomized clinical research studies are necessary to evaluate the effectiveness of ECIB.

Influence of ECIB as an immunosuppressive agent in the treatment of active, non-alcoholic cirrhosis with high serum gammaglobulin levels has been studied by Ranek et al[98]. The results from 4 patients were not encouraging.

Extracorporeal Irradiation of Lymph

Experimental Studies: Central and Regional Lymph

Under certain experimental conditions it is possible to consider thoracic duct lymph as if it were regional lymph[52]. It has been demonstrated that skin allograft antigens reach the regional lymph node via the afferent lymphatics[99], and there is ample evidence to suggest that the immunologically committed lymphocytes emerge from stimulated lymph nodes via efferent lymphatics and enter the blood via central lymph[100,101]. It has also been

suggested that metastatic activation of distant lymph nodes and the spleen is via a similar route[101]. It is possible to place skin allografts on posterior part of the body from which all efferent lymph enters the thoracic duct prior to its entry in to the blood circulation. In this situation, all immunologically committed lymphocytes from the regional lymph node can be subjected to irradiation by ECIL. This approach should assure a complete block of the efferent arc of the immune response and survival of the skin allograft ("posterior grafts") for the duration of ECIL, provided that there are no functional lymphatico-venous or lymphatico-lymphatic communications[102]. Under experimental conditions where the skin allograft ("anterior grafts") are so placed that the efferent lymph does not gain access to the blood circulation via the thoracic duct, irradiation of thoracic duct lymphocytes would be expected to only induce some degree of immunosuppression by depletion of recirculating lymphocytes including a fraction of the recirculating specifically stimulated lymphocytes. Both these approaches have been utilized[50,52,59]. Continuous ECIL for 10 to 20 days prior to grafting resulted in a 5-8 day prolongation of the survival time irrespective of the anatomical location of the skin allograft. Combination of pre- and postgraft ECIB resulted in further prolongation of the graft survival, but in this situation "posterior grafts" survived for the duration of ECIL[52]. The observation that postgraft ECIL further extended the survival of "anterior grafts" suggests that the specifically activated cells do recirculate through the thoracic duct lymph.

In addition to the influence of ECIL on skin allograft survival, cytotoxic antibody responses were also measured in recipients of 'anterior' and "posterior" skin allografts subjected to continuous ECIB for up to 28 days[103]. It is of interest that in calves, which maintained "posterior" grafts until ECIL was discontinued, the cytotoxic antibody response was markedly depressed. Following the termination of ECIL, cytotoxicity of the sera increased presumably the result of specifically stimulated lymphocytes entering the circulation via the thoracic duct. Surgical removal of the grafts prior to rejection indicated that the rejection per se was not necessary for the rise in cytotoxic activity.

Clinical Studies

Despite the encouraging results thus far obtained from experimental studies, there has been no adequate trial of ECIL for immunosuppression in man. Both the results of experimental studies as discussed above and the immunosuppressive effects of thoracic duct lymphocyte depletion by techniques other than ECIL[104-106] suggest a useful potential role of ECIL for clinical immunosuppression.

CONCLUDING REMARKS

Of all the techniques investigated for selective immunosuppression by radiation, as discussed in this and a previous review[7] only ECIB, ECIL and graft irradiation have potential for clinical application. ECIB and graft radiation have actually been applied for immunosuppression in man. The experimental exploitations of other techniques have, however, contributed a great deal of information towards a better understanding of the role of the lymphocyte in the immune response.

Despite the proven usefulness of ECIB as an adjunct immunosuppressive regimen, the best ECIB schedule and transit dose remain unsettled in man[107]. Currently available data indicate that repetitive pre- and postgraft sessions of ECIB at a relatively high transit dose (~ 300 to 500 rads) can be expected to yield the best results in prolonging allograft survival. Even with well planned schedules of ECIB, the dose accumulated by erythrocytes would restrict the duration of postgraft ECIB. In addition, repetitive non-continuous sessions would not permit irradiation of all long-lived recirculating and newly formed lymphocytes entering and leaving the blood between ECIB sessions. A majority of the long-lived, recirculating lymphocytes are thymus-derived. In addition, the blood contains many newly-formed thymus-derived, histocompatibility antigen reactive lymphocytes which do not enter the recirculating pool[41]. The blood transit time of these newly-formed lymphocytes is not known. It is conceivable that a majority of these newly formed lymphocytes enter and exit the blood circulation each day. A similar population of newly-formed cells as described for the rat[41], is suggested in the calf by the studies on thymus cell migration[108,109].

Continuous ECIB reduces the blood and thoracic duct lymphocyte counts to about 20% of the control values within 12 hours[21]. This initial depletion of an easily mobilizable pool of lymphocytes is followed by a period of longer duration during which the number of lymphocytes in various compartments is reduced much more slowly. This latter pool is comprised of relatively sessile and some newly produced

lymphocytes. Continuous ECIL results in a two-component decrease in the output of thoracic duct lymphocytes[55]. The first component, corresponds to the elimination of the easily mobilizable pool and has a half time of about 1.2 days. The second component with a half time of approximately 30 days represents the relatively sessile pool. Extracorporeal irradiation alone, for all practical purposes, is an inadequate procedure for the depletion of this relatively sessile pool[57], which contains immunocompetent cells[58]. Agents capable of mobilizing the sessile pool with resultant lymphocytosis should improve the effectiveness of extracorporeal irradiation[110].

The effectiveness of a particular transit dose has only been assessed by the degree and duration of lymphocytopenia induced following ECIB[40,107]. Such a measurement fails to consider the radiosensitivity of various functional subpopulations of lymphocytes in the circulation[8,111,112]. It is thus not surprising that the level of immunosuppression achieved by ECIB has not shown any definite correlation with the degree of lymphocytopenia[90,96]. In future studies, the functional evaluation of subpopulations of circulating lymphocytes during and following extracorporeal irradiation are essential.

The influence of ECIB on circulating stem cells in the blood[64-66] has not been determined. This question does not arise with ECIL as the lymph does not contain stem cells[68-70]. In view of the obvious implications of possible stem cell damage and depletion by ECIB, such studies are necessary. The technique for stem cell determination in the mouse is the spleen colony forming assay[113], unfortunately, this technique cannot be utilized for the human stem cells. Colony formation

in agar assays the granulocyte and macrophage progenitors[114]. It has been claimed, however, that in dog the number of colony forming units in agar in the peripheral blood is an indicator of the presence of pluipotent hemopoietic stem cells as measured by the in vivo bone marrow repopulation of dogs subjected to 1200 rad WBR and infused with 3 to 40 x 10^9 autologous or allogeneic mononuclear blood cells.(T. M. Fliedner; personal communication). Colony formation in agar might therefore be a suitable technique for application during and following ECIB in man.

In view of the paucity of erythrocytes in the thoracic duct lymph, the restriction imposed by ECIB are fortunately not applicable to ECIL. The best ECIL schedule therefore would appear to be continuous pre- and postgraft irradiation at a high transit dose ($\sim$ 1,000 rads). In practice, however, it would be impossible to continue ECIL indefinitely. This may, however, not be necessary. One might be able to discontinue ECIL at some stage following allografting without jeopardizing the allograft. Hall has reported that in efferent lymph from regional lymph nodes, the numbers of basophilic cells decrease markedly by 20th day postgrafting[100]. Moreover non-specific factors such as the graft adaptation might also result in reduced immunological reactivity[115].

These considerations would lead one to believe that the ideal schedule of extracorporeal irradiation for maximum immunosuppressive activity would be repetitive daily ECIB combined with continuous ECIL.

FIGURE LEGENDS

Fig. 1 A schematic representation of extracorporeal irradiation of blood (ECIB) in goats.

Fig. 2 The relationship of the number of blood volumes irradiated during ECIB to the percent of circulating blood elements (present in circulating blood during the course of ECIB) receiving no radiation. This curve was generated from data obtained in patients and calculated according to the formula of Marsaglia and Thomas[33]. (The authors thank Dr. J. S. Robertson for help in preparation of this figure).

Fig. 3 The in-vivo radiosensitivity of lymphocytes as reported by Trowell[1]. The three arrows and bold horizontal lines indicate the expected lymphocyte survival, according to Trowell's data, at different transit doses. The top, middle and lower arrows correspond to doses of ~ 30 rads, ~ 500 rads and ~ 1,000 rads respectively. The middle (~ 500 rads) and lower (~ 1000 rads) arrows represent the usual transit doses employed by us for ECIB and ECIL respectively.

Fig. 4 The influence of low (~ 30 rads) and high transit dose (~ 1000 rads) extracorporeal irradiation of lymph (ECIL) on thoracic duct (TD) lymphocyte output in the calf.

Fig. 5 The influence of repetitive ECIB on blood lymphocyte levels (transit dose ~ 800 rads) in adrenalectomized and sham-adrenalectomized calves. Each calf was given ten sessions of ECIB with an equivalent of 5 blood volumes per session.

Fig. 6 The influence of continuous ECIL in one adrenalectomized and one sham-adrenalectomized calf on the output of thoracic duct (TD) lymphocytes. A transit dose of approximately 1,000 rads was employed.

Fig. 7 Survival of bilaterally nephrectomized goats receiving renal allografts. The mean and range of recipient survival is shown for various treatment groups. The details of ECIL and sham-ECIB schedules are provided in the text.

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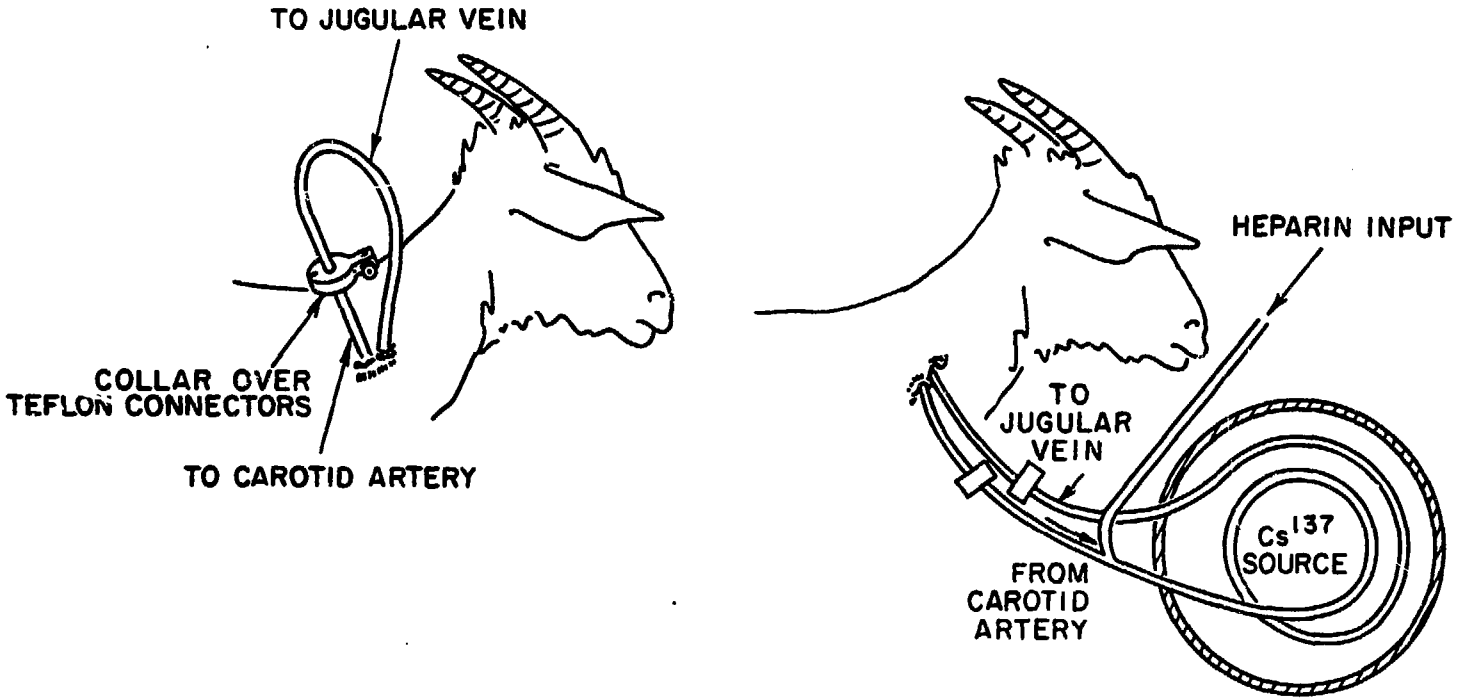

Neg. #1-13-73

FIGURE 1

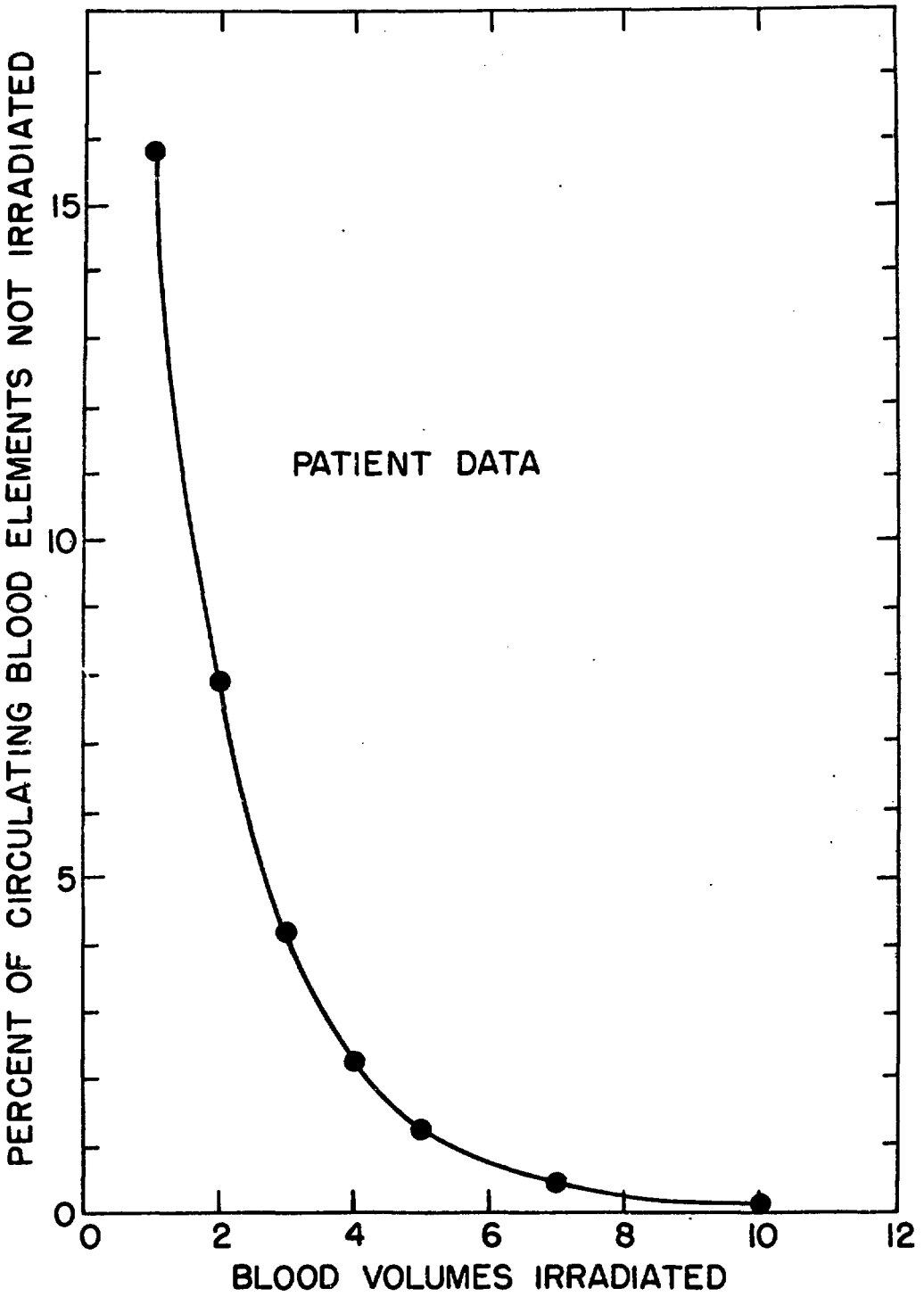

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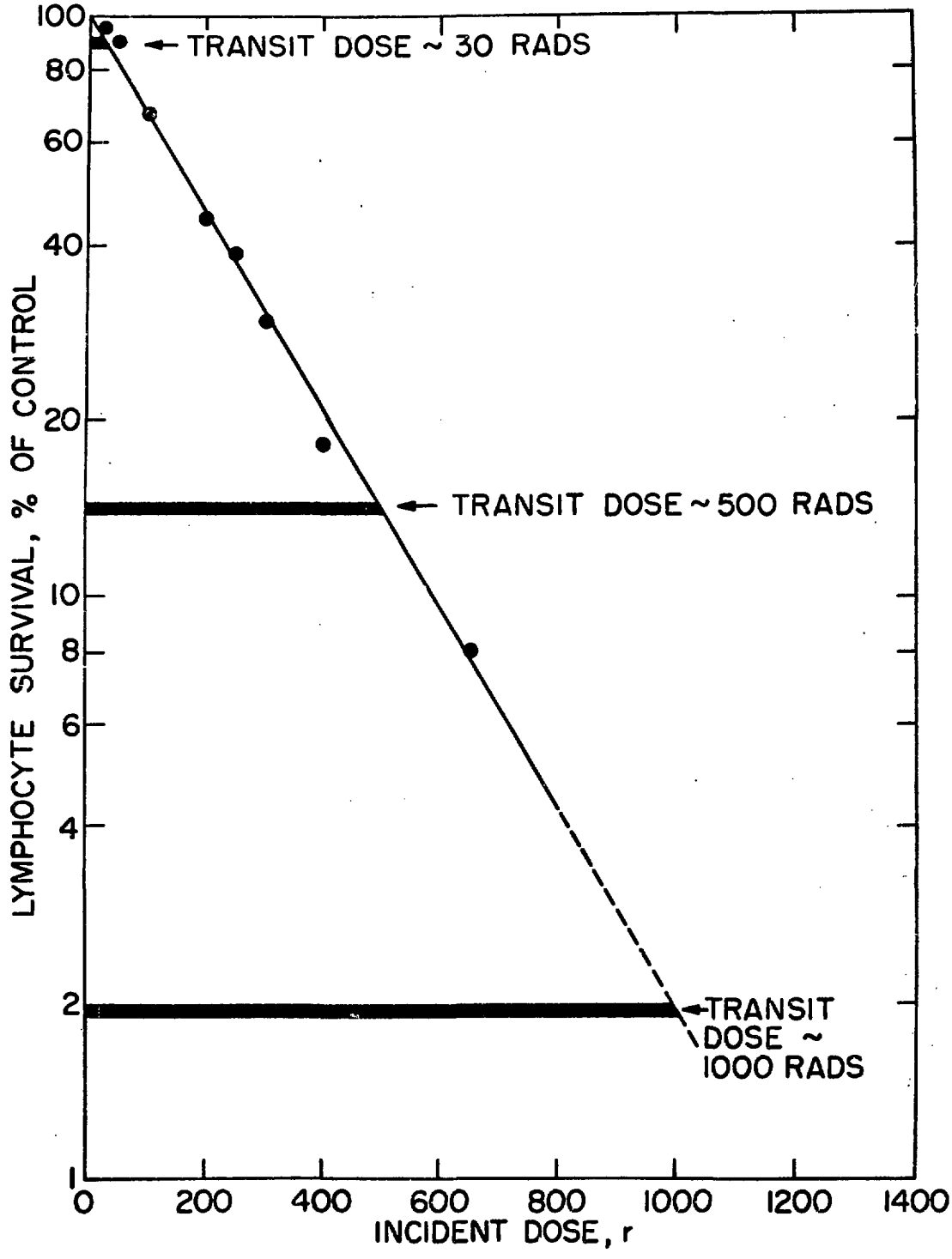
FIGURE 2



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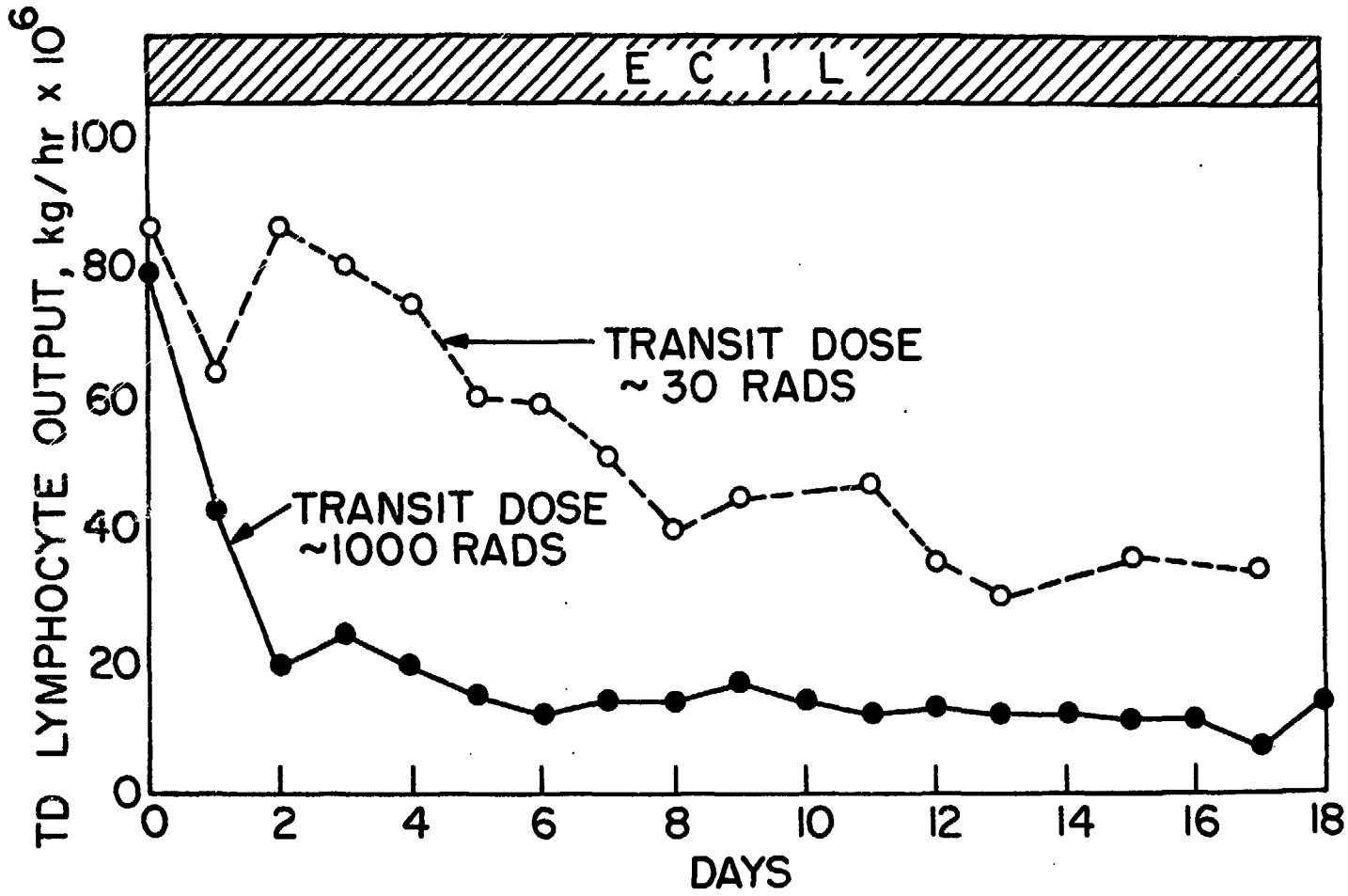

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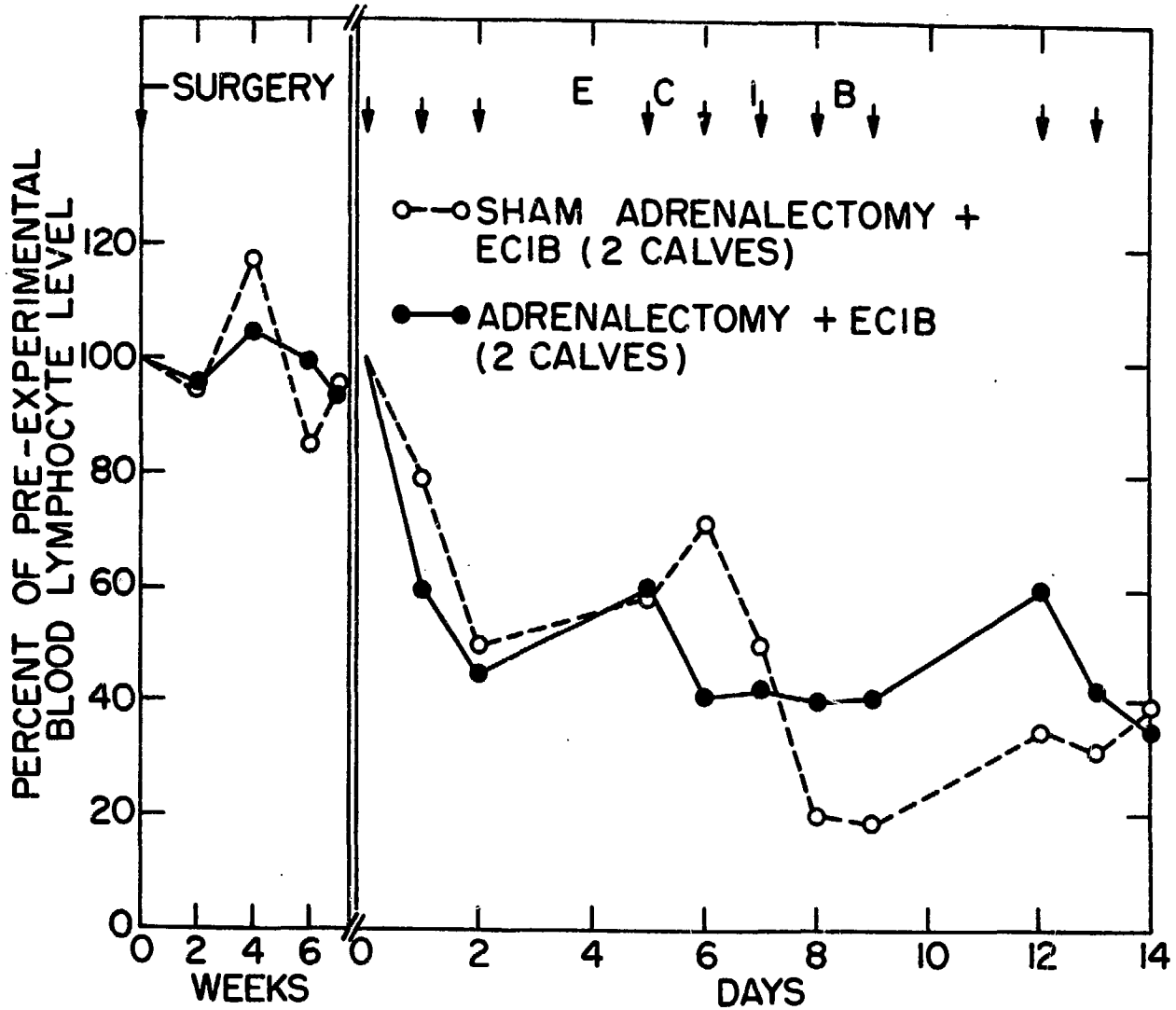
FIGURE 4



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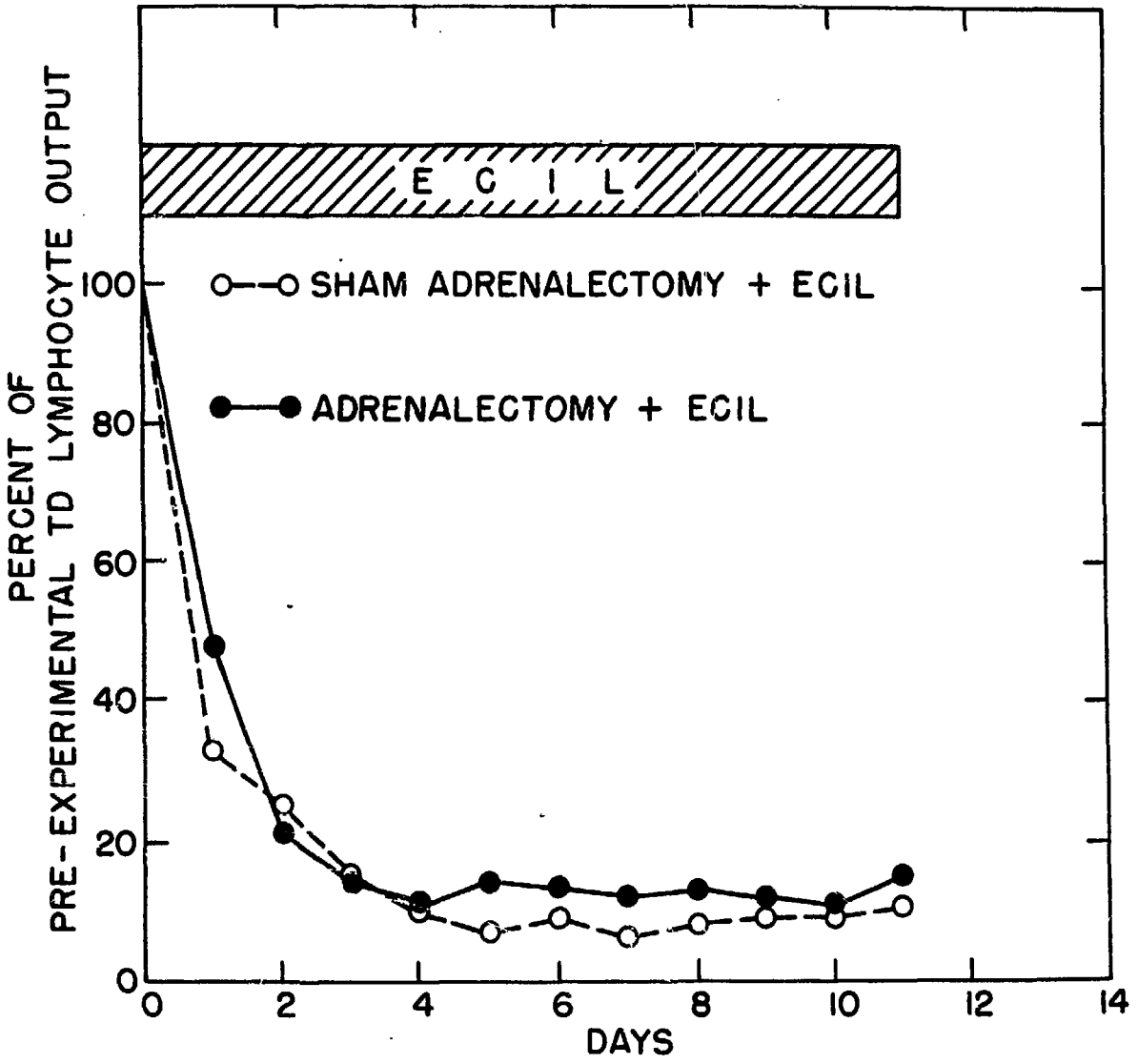

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FIGURE 6

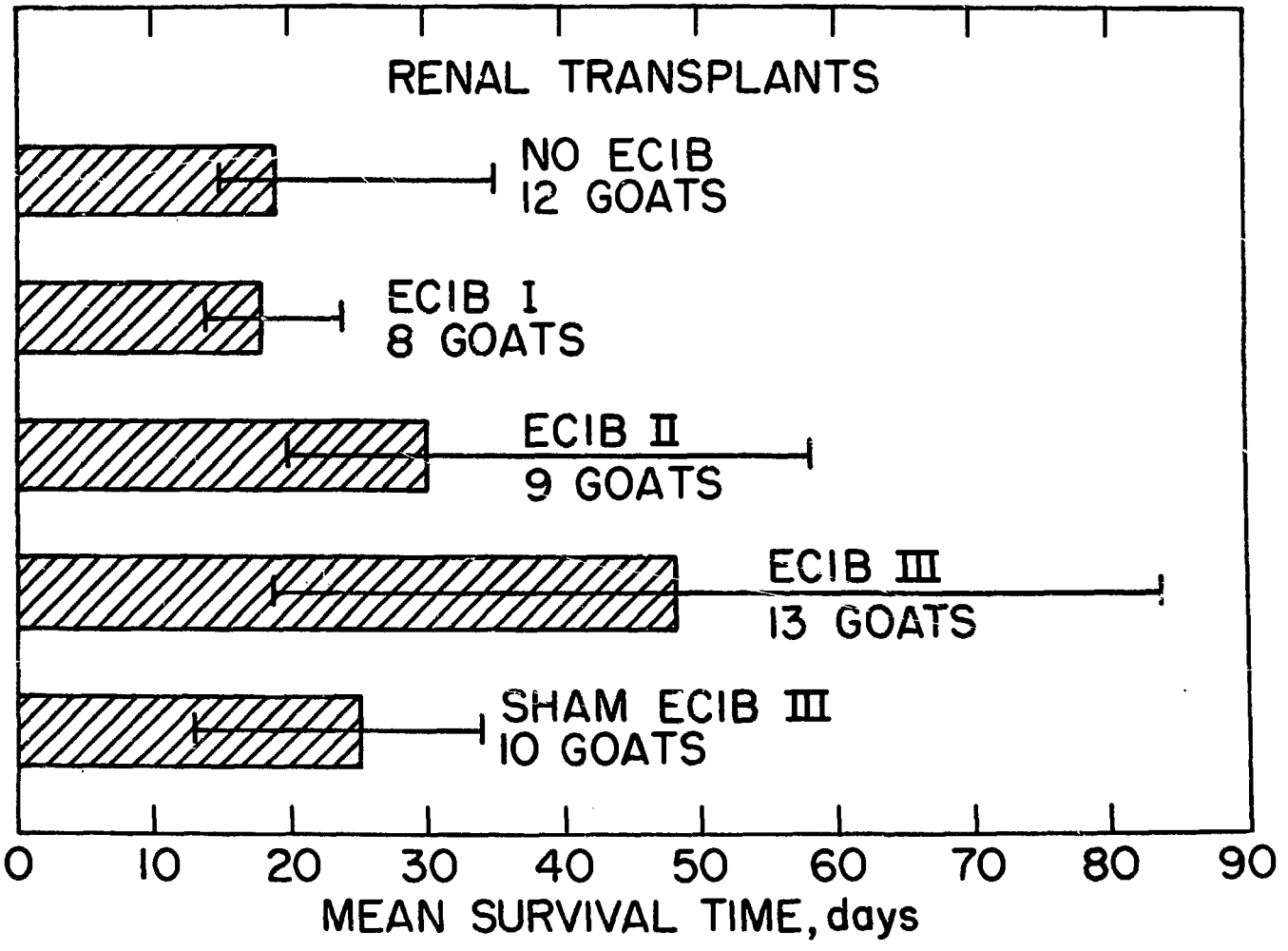

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FIGURE 7

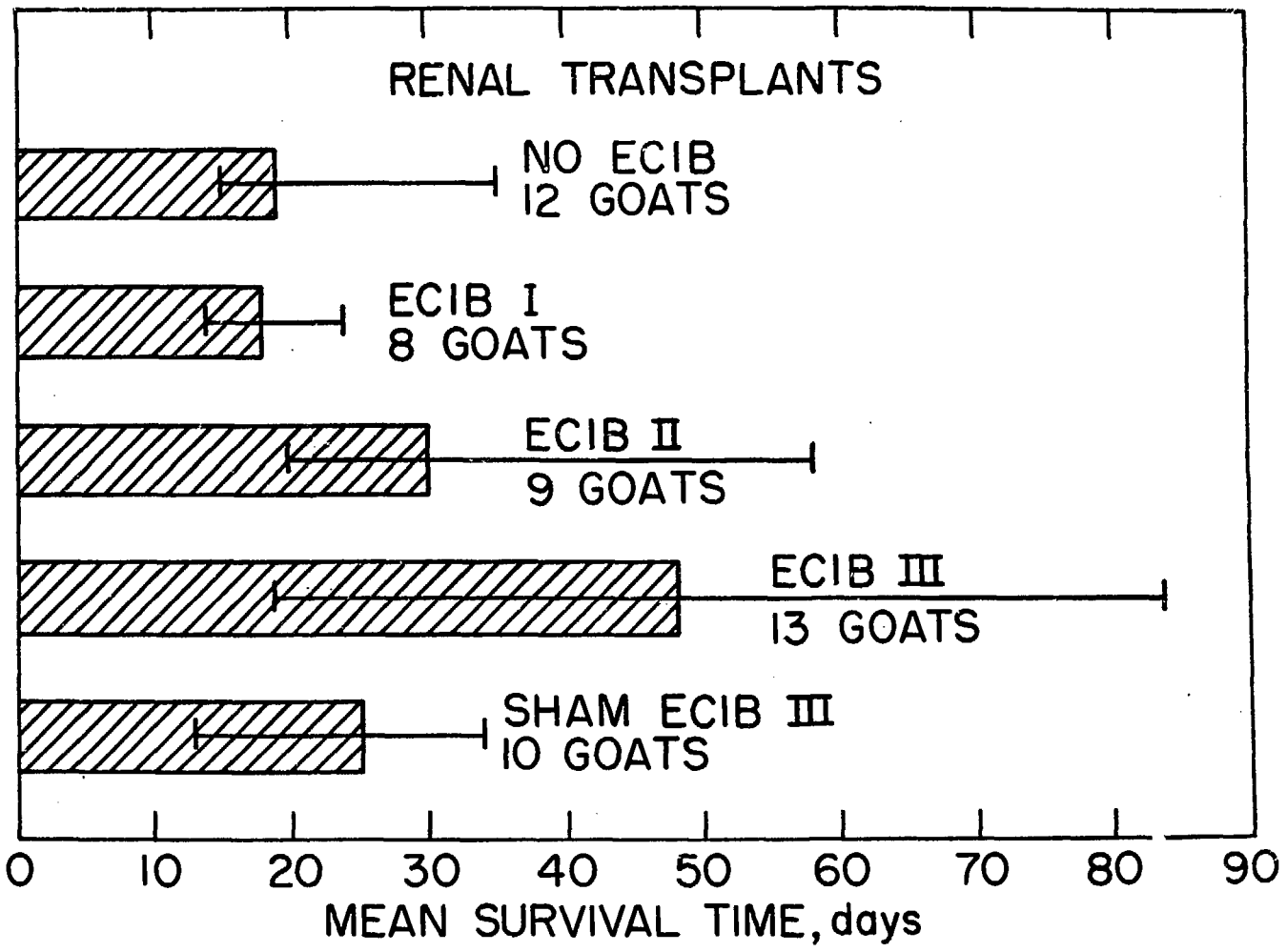

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FIGURE 7