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THE ANTAGONISM OF PLATELET-ACTIVATING FACTOR
AND HYPERACUTE ORGAN REJECTION

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I. INTRODUCTION

Humorally mediated hyperacute rejection (HAR) of primarily vascularized renal allografts was originally recognized in patients who received kidneys from ABO blood group incompatible donors (1). Isohemagglutinins to ABO antigens resulted in rapid renal allograft rejection with histologic similarity to the generalized Schwartzman reaction (2). It is therefore universally accepted that incompatibility for these blood group antigens is a major barrier to kidney transplantation.

Terasaki and colleagues later provided evidence that HAR was also possible in the presence of ABO red cell group compatibility (3), which was demonstrated to be associated with circulating preformed antidonor antibodies to specific MHC determinants. This state of recipient presensitization or presence of preformed antibodies occurs in those patients who have rejected a previous transplant (4), in those who have received multiple blood transfusions (5), and secondary to pregnancy (6). A positive crossmatch to antibodies directed against HLA -A, B, and in some cases HLA-DR, constitutes a contraindication to kidney transplantation. However, the same events may not be completely applicable to the liver, which has been felt to be resistant to this kind of antibody-mediated injury, although rejection of the human heart within a few minutes to a few hours has also been reported (7).

} Humorally mediated HAR due to preformed antibodies is also the mechanism of rejection in discordant xenografts, i.e., between widely disparate species. The entire process is pathophysiologically very similar, if not identical, to that observed in ABO blood group major incompatibility or in the presence of serum cytotoxic antigraft antibodies. Therefore HAR represent a specific and defined, but incompletely understood, entity which can be elicited in different transplant situations and in different organs. At present, HAR cannot be managed with any significant consistency, and presents the greatest threat to kidney transplants, and is increasingly becoming an important consideration in cardiac and hepatic transplantation.

The implications for a better understanding of the mechanisms involved in and the management of hyperacute allograft rejection are great. Patients with wide ranging antibodies who have become noncandidates for renal transplantation could be helped. For all organs, the possibility of using xenografts could be considered in the future.

II. MECHANISM OF ANTIBODY-MEDIATED HYPERACUTE REJECTION

The hyperacute rejection which occurs when kidney allografts are exposed to a recipient antigraft antibodies in the three different situations already mentioned, fundamentally represents a devascularization in which small vessels become plugged with coagulation products and formed blood elements (8). This immunologically precipitated failure of the microcirculatory environment may well follow many of the same final common pathways of injury and organ function that can be caused by ischemia and toxins as well. Failure of the microcirculatory environment from immune and other causes must be viewed as the product of a complex inflammatory process.

The acute inflammatory mediators responsible for tissue injury are derived either from plasma proteins or from several different types of cells, such as polymorphonuclear leukocytes (PMNs), platelets, monocytes, macrophages, lymphocytes and endothelial cells. The four classical systems responsible for inflammation (i.e., the complement, coagulation, fibrinolysis and the kallikrein-kinin systems) generate the inflammatory plasma proteins responsible for some of the key pathophysiologic processes seen in HAR. These four protein systems interact with one another during the activation process, and furthermore, interact with and activate the cellular elements already alluded to. In fact, it has been the insight recently gained into the

} cell derived mediators which has allowed a fuller explanation and understanding of the interrelationships of the various pathophysiologic processes which are further outlined below.

Preformed mediators such as chemotactic factors, lymphokines, histamines, serotonin, oxygen-derived free radicals, arachidonic acid metabolites, and acetylated alkyl phosphoglycerides (PAF) are released from cells after cell stimulation or injury. Such cell-derived mediators have been implicated in the modulation of many aspects of the acute inflammatory process, and specifically in HAR. Furthermore, the vascular endothelium is now considered an important component of the inflammatory response. The endothelial cell can release various inflammatory mediators including prostaglandins (9), hydroxy fatty acids (10), and PAF (11). Endothelial cells can present Ia antigens to circulating lymphocytes and antibodies (12), and it can release chemotactic factors for neutrophils (13). The endothelial cells that participate in most inflammatory responses are found in the microvasculature (14).

The original description of HAR recognized that platelets and neutrophils contributed to the process. It is well known that various blood elements, specifically platelets and neutrophils, interact with the vascular endothelium during the process of thrombin formation and vascular occlusion (14) in a variety of pathophysiologic processes including antibody mediated organ rejection and organ ischemia. Interactions among these cells can lead to the formation and release of vasoactive

} substances that have the potential to modulate regional blood flow (15), and the importance of localized microcirculatory vessel constriction and underperfusion of the vascular bed should not be underestimated.

The inflammatory reactions involved in HAR are obviously remarkably complex. Furthermore, they are surprisingly resilient and redundant in their organization, so that the same set of functions can be executed through many alternative backup systems. Consequently, even if the potent biologic activities of one specific mediator could be totally blocked by highly specific antagonists, which has yet to be completely achieved, the combined release of other inflammatory and biologically active agents could easily replace the functions of the missing agent and mediate an unwanted process. An understanding of these complexities have very important implications for the therapeutic approach to HAR.

The pathogenesis of hyperacute rejection for the kidney and the heart has been extensively investigated and fairly well established. The causal event of an antibody mediated injury (IgG and IgM) to the vascular endothelium (16), which is propagated by secondary vasoconstriction (17), the recruitment of polymorphonuclear leukocytes (18), and aggregated platelets (19,20), then followed by intravascular coagulation (19-21). The combination of vasoconstriction and the platelet-leukocyte-plugs occlude the small arterioles and capillaries inducing ischemic necrosis of the organ. It is tentatively proposed that the

recruitment of leukocytes and platelets is initially due to immune adherence and specific aggregation and later due to stasis and nonspecific entrapment. This entire process is accompanied by fibrin deposition and rapid total destruction of the vascular system of the organ (2,8). The pathologic description of this entity today, remains exactly the same as the original descriptions (22), and in fact, at that time the similarity of hyperacute rejection of the kidney to the events of the experimental generalized Shwartzman reaction was suggested (2).

After the binding of antigraft antibody to antigens on the endothelium, all of the events that occur thereafter are immunologically non specific, namely, activation of clotting, fibrin deposition, fibrinolysis, capillary leaking and the influx of inflammatory cells with their own release of mediators, and finally injury to the renal parenchyma. The process of hyperacute rejection involves most, if not all, of the components of the typical inflammatory response (23). Obstruction of the small arteries by the platelet-neutrophil-plugs induces an accompanying ischemia in cells which may activate the complement system by the alternate pathway. The release of the vasoactive substances C3a and C5a results in enhanced inflammation with neutrophil margination and migration to the damaged tissue. This results in neutrophil activation, with a burst of oxygen consumption and the production of oxygen derived free radicals. The activation of neutrophils leads to an increase in phospholipase A₂ activity which releases arachidonic acid from membrane phospholipids (23).

) A cascade is thus begun which produces a large number of biologically active lipid products which include the prostaglandins, leukotrienes and thromboxanes. Two of the more important lipid mediators include leukotriene B₁ which is a very potent neutrophil chemoattractant and thromboxane A₂ which is an extremely potent vasoconstrictor, and platelet pro-aggregator. Thus, the many arms of the inflammatory process come together to create a relentless and mutually perpetuating progression resulting in organ destruction.

III. THE POTENTIAL ROLE FOR PAF AND ITS ANTAGONISM IN HAR

Platelet-activating factor (PAF) is a potent mediator of many inflammatory reactions because of its broad range of biological activities as outlined in this test. PAF is released by many cell types including the endothelium, and can also affect different cell types. It can be mediated by immunologic as well as non-immunologic stimuli (24). PAF can evoke almost all the well-known cardinal signs of acute inflammation and probably plays a central role in the induction of tissue injury (25). The biological effects of PAF, which include platelet and neutrophil aggregation and activation, increase in vascular permeability, smooth muscle contraction and the release of numerous vasoactive and potent biological mediators, can influence the microvasculature of all organs and therefore must be considered a key participant in the disruption of microvascular flow. There is already considerable evidence for the candidacy of PAF as a central and key biologic mediator in the pathogenesis of humorally-mediated hyperacute allograft rejection.

In vitro studies have demonstrated that PAF is released when endothelial cells are incubated with antibody to cell surface angiotensin converting enzyme. This has important implications for hyperacute rejection, in which the specific initiating event is a transplantation antigen-antibody interaction on the endothelial surface. In a model of hyperacute renal allograft rejection in sensitized rabbits, PAF was identified in the blood

effluent from the transplanted kidney, following immediate antibody and complement fixation to the endothelium of glomerular and peritubular capillaries (26). No PAF could be detected in rabbits without clinical or histologic signs of hyperacute rejection (i.e., there was no PAF release in syngeneic controls or renal allografts transplanted into unsensitized recipients). The PAF release was detected within 2 to 10 minutes after revascularization and was followed by the recruitment of platelets, endothelial sticking, and obliteration of capillaries by platelet-PMN plugs and evidence of cellular degranulation (26). Whether PAF was released from the endothelial cells of the graft or from the inflammatory cells infiltrating the organ was not determined.

The importance of PAF, even in the absence of inflammatory cells, was demonstrated in a model of ex vivo perfusion of the rabbit heart with transplantation alloantibodies (27). The release of PAF followed bradycardia, conduction arrhythmias, decreased coronary flow, and cessation of heart action. These alterations in cardiac function and, moreover, the release of PAF, were prevented by complement inactivation. PAF itself induced a negative inotropic effect and reduction of coronary flow in isolated guinea pig heart muscle by a direct action on the CA^{++} slow channel. Moreover, the injection of synthetic, purified PAF into the renal artery of rabbits reproduced a pathologic picture identical to that seen in a Schwartzman

} reaction of the kidney. The pathophysiology of hyperacute renal rejection in humans was originally described as a Shwartzman reaction (2).

The nonspecific effector inflammatory cascade that ensues once an antigen-antibody reaction has initiated hyperacute rejection has served as the target for therapeutic intervention by many different approaches. Classically, the platelet-coagulation system has been considered to be the most important and has received the greatest attention. These therapeutic modalities have included heparin, aspirin, dextran, citrate, defibrinating agents, cobra venom factor, induction of thrombocytopenia by antibodies etc., and various prostaglandins (28-30). All these approaches have been either too toxic or the results too inconclusive to be accepted for generalized use.

The difficulty in overcoming the inflammatory process in hyperacute rejection and in explaining the discrepancies in therapeutic results rest not only in the complexity of the biologic reactions, but also in their resiliency and redundancy. That is, there are numerous alternative mediator pathways that can backup the same set of functions. Thus, the inhibition of one arm of an inflammatory reaction can easily be replaced by the release of other inflammatory autocooids with complete mediation of a disease process. PAF may represent somewhat of an exception to this hypothesis, because of its potency and its unique

) potential for mediating almost every aspect of acute inflammatory reactions. Nonetheless, the argument for polypharmaceutical therapy of hyperacute rejection appropriate.

With the availability of specific receptor antagonists of PAF, further insight into the role of PAF in the pathogenesis of HAR is now possible. The potential for a new method of controlling the effector cascade of HAR by pharmacologic intervention can now also be exploited.

A number of PAF acether antagonists have been reported to block PAF binding to its high-affinity receptor and their application has been documented in various pathological conditions. Included in these disease states are the inhibition/or reversal of PAF-induced hypotension (31,32), neutropenia (33), bronchospasm (34,35), endotoxic shock (36), and anaphylaxis (37) in animal models.

There has been little evidence to date that PAF antagonists are capable of preventing or diminishing the phenomenon of antibody-mediated HAR. In the model described previously of the rabbit heart perfused ex vivo with transplantation alloantibodies, PAF was released in the absence of inflammatory cells. The addition of a PAF receptor antagonist, SRI 63-072, (Sandoz Company) prevented the reduction of coronary flow by 70% within 2 to 4 minutes after its addition to the perfusate. In this model, heart action was prolonged after the addition of SRI 63-072, however, eventual hyperacute rejection was not prevented. Implications that PAF may also contribute to cell-mediated

) cardiac allograft rejection have also been reported by Foegh et al. (38). A slight but significant prolongation of rat heterotopic heart allograft was achieved following administration of a PAF-receptor antagonist, BN52021 (Henri Beaufour Institute, Paris, France), in nonsensitized recipients. The current study evaluates two types of invivo models of HAR, namely, in the hypersensitized recipient and in xeno transplantation. These studies not only lend further support to the prominent role of PAF in the regulation of the inflammatory response leading to hyperacute rejection, but also introduce a new and potentially significant therapeutic approach to this entity. The agent used in all of these studies, SRI 63-441, is specific PAF receptor antagonist manufactured by the Sandoz Company (East Hanover, New Jersey). It represents a family of compounds that can be synthesized and modified to meet desired specifications, i.e., an oral compound recently has been produced.

IV. THE EFFECT OF SRI 63-441 ALONE AND IN COMBINATION
WITH IMMUNOSUPPRESSION ON EXPERIMENTAL CARDIAC
HYPERACUTE REJECTION IN PRESENSITIZED RECIPIENTS

A. BACKGROUND

Experimental studies targeted to investigate HAR of heart and renal allografts originally utilized non-inbred models, primarily the dog (20,39). The genetic composition of these combinations were generally unknown and despite calculated donor-specific presensitization, HAR was not consistently achieved (40). Moreover, these experiments could not be adequately controlled with syngeneic pairs without the interference of rejection. Data derived from these investigations must therefore be cautiously transferred to the clinical setting, as separate examiners were inclined to emphasize alternate morphologic features as being characteristic or central to this process (21,41). While the rat would appear to be a logical model of choice for investigating these issues, it was previously thought not to possess an adequate complement system in order to mount such a response. Guttman and colleagues (42) first demonstrated that HAR of heterotopic heart allografts could be regularly produced in specific inbred rat strain combinations without necessitating an exogenous source of complement or direct graft infusion. These donor-recipient combinations were selected on the basis of a strong histocompatibility barrier. The

} establishment of the sensitized rat as a suitable model for experimental HAR is a particularly impressive finding in that controlled investigations designed to elucidate the mechanism(s) of HAR could be undertaken. Further investigations have described the importance of immunization across the MHC in rats as essential for the induction of HAR, with particular reference histocompatibility antigens (Class I) encoded by the RT1.A locus. Complement components were also deemed essential for this response as C₃ depleted recipients failed to reveal functional and pathological changes generally associated with HAR. Moreover, the rat has been well defined genetically and there exists a high degree of homology between the MHC of man and rat.

B. MATERIALS AND METHODS

Animals

Commercially available inbred male Lewis (L) (RT1^l) and ACI (RT1^a) rats weighing 200 to 220 grams were purchased from Harlan Sprague Dawley, Indianapolis, Indiana. This strain combination was selected on the basis of a strong histoincompatibility to provide a reproducible and reliable model of hyperacute rejection (42). Rats were acclimatized in the central animal facility for at least two weeks prior to investigation and received standard rat chow and water ad libitum.

SURGICAL PROCEDURES

Skin Grafting

Presentation of L recipients was carried out under methoxyflurane anesthesia by removing full thickness ventral abdominal skin from ACI donor rats. A 2.5 cm circular skin graft was transplanted to the dorsum of the Lewis recipient and fastened with Clay Adams wound clips. Casting material was then applied for 4 days after the grafting procedure to immobilize the tissue. Grafts were evaluated visually for rejection. A second, third and fourth graft were placed at intervals of 14 days in a similar manner.

Heterotopic Heart Transplantation

Heterotopic heart transplants were performed 17-20 days following the fourth skin graft. Contemporaneous controls (sodium acetate buffer only) were performed for each experimental group.

Animals were anesthetized with 40 mg/kg sodium pentobarbital IP and supplemented with methoxyflurane. Surgical procedures for the primarily vascularized cardiac transplants in rats have been well described previously (43). Briefly, an end-to-side anastomosis is performed between the donor and recipient aorta, as well as the donor pulmonary artery to recipient vena cava.

Impulses of the transplanted hearts were monitored every 30 minutes for the initial 15 hours, and then twice daily thereafter

} by palpation through the recipient abdominal wall. Rejection was considered complete when there were no palpable contractions and confirmed visually at laparotomy and by histological evaluation. All experimental animals were weighed daily.

PHARMACOLOGIC ADMINISTRATION

PAF Antagonist

SRI 63-441: The receptor-specific antagonist of PAF, SRI 63-441, was supplied by Sandoz Research Institute (East Hanover, New Jersey), and its properties and pharmacology have been described elsewhere in this text. The powdered form was reconstituted in 0.68% sodium acetate buffer and 0.9% sodium chloride (pH 5.3) to a final concentration of 10 mg/ml, and placed in a warm water bath (26-28°) for 5 minutes. A single bolus injection was administered via the inferior vena cava at four minutes prior to revascularization. The dosages evaluated are listed in the individual tables.

Immunosuppression

FK 506: FK 506 has strong immunosuppressive activity against mixed lymphocyte reactions (44), suppresses T cell-mediated injury (perhaps by suppressing both interleukins 2 and its receptor expression on T cells) and has been reported to inhibit rejection of heterotopic heart transplants in rats (45). Crystalline powder FK 506 (MW 882) was supplied by Fujisawa Pharmaceutical Company, Ltd, (Osaka, Japan). A final

} concentration of 1.28 mg/ml was prepared in 0.9% sodium chloride. FK 506 was administered at a dosage of 1.28 mg/kg/day for a duration of 14 days (or until rejection) by intramuscular injection commencing three days prior to transplantation.

Cyclosporine (CsA): A commercially available CsA formulation of 100 mg/ml (Sandoz Company) was diluted in olive oil to a final concentration of 10 mg/ml and administered orally at a dosage of 15 mg/kg/day commencing 3 days prior to transplantation and for a duration of 14 days (or until rejection).

Antibody Titers

A 0.5 ml blood sample was obtained under methoxyflurane anesthesia from the inferior vena cava immediately prior to revascularization of the transplanted organ. A complement-dependent-cytolytic assay (CDC) was performed to assess antibody titers.

C. RESULTS

SRI 63-441 Alone

Cardiac allograft survival for control and SRI 63-441 alone treated animals is summarized in Table I. Control Lewis rats that had been previously sensitized by ACI strain skin grafts and then received ACI donor hearts, consistently rejected these in a hyperacute fashion (MST 237 minutes \pm 47.4 n=60). Treatment of sensitized rats with SRI 63-441 alone as a single intravenous

) bolus prior to revascularization resulted in a varied response. Significant prolongation of graft survival was achieved in animals treated with 15 mg/kg of SRI 63-441 (MST 2403 minutes \pm 542, $P < 0.001$ $n=20$) as compared to controls. In fact, 9 of these hearts functioned from 2 to 5 days following transplantation. There was no significant change in weight in these rats.

Histological Evaluation

Tissue sections stained with H+E were reviewed without knowledge of treatment protocols. The morphological features which were considered diagnostic of cardiac hyperacute rejection included extensive interstitial hemorrhage, occlusion of vessels by platelets, endothelial cell damage and myocyte necrosis (Figure 1). Polymorphonuclear cell infiltration was not a constant feature. These histologic features were evident in all control rats.

Heart allografts that functioned long term (i.e., 2-4 days) and then ceased to function exhibited an entirely different histologic picture. Sections from these hearts exhibited an abundant mononuclear cell infiltrate, many of which demonstrated features of blast formation (Figure 2). This finding was interpreted as acute cellular rejection. Typically, there was much less interstitial hemorrhage and little or no platelet plugging of vessels noted. Myocyte degeneration and fibroblast

) proliferation were frequently extensive. No overlap with hyperacute rejection was recognized. The native hearts of the animals in all groups were consistently without morphologic change.

SRI 63-441 and Immunosuppression

Animals pretreated with multiple administration either CsA alone or in combination with SRI 63-441 failed to demonstrate a beneficial effect (Table II). A slight but significant prolongation of allograft survival was evident when a single dose of CsA was administered at 6 hours prior to transplantation (1523 minutes \pm 566 versus 237 minutes \pm 47.4 p < 0.05).

As evident in Table III, treatment with FK 506 alone prior to transplantation resulted in increased graft survival time. Of even greater significance was the response in cardiac allograft survival observed when SRI 63-441 treatment (at 2 dosage levels) was combined with immunosuppression by FK 506. In several cases, graft survival either approached or exceeded one month.

In general, level of circulating antidonor antibodies did not correlate well with organ graft rejection times. This observation is analogous to that experienced clinically at this time. It is now generally accepted that tests for antibodies before and after transplantation must be perfected with further emphasis placed on a distinction between deleterious, beneficial and insipid antibodies (46).

Histologic Evaluation

The extent and quality of the mononuclear infiltrate observed in the grafts of those animals treated with SRI 63-441 alone and SRI 63-441 and FK 506 (Figure 3) was quite different: The grafts of those animals treated with SRI 63-441 alone contained a more abundant mononuclear cell infiltrate, many of which exhibited features of blast formation. Photomicrographs of myocardial sections from animals treated with SRI 63-441 and FK 506 generally exhibited features of mild acute cellular rejection.

D. SUMMARY

Since the original description of hyperacute rejection of the kidney, numerous experimental and clinical studies have attempted to define the precise mechanism(s) of this destructive process. While our interpretation of HAR has advanced, one must recognize this phenomenon as multifactorial and the product of a complex immune/inflammatory reaction. Platelet-activating factor has been proposed as a key biological candidate in the overall evolution of this type of inflammatory process and as a central mediator in the pathogenesis of antibody-mediated hyperacute rejection. In a model of heterotopic cardiac transplantation into sensitized rats, we were able to demonstrate that the phenomenon of hyperacute rejection previously thought to be refractory and uncontrollable, could be converted into a potentially more manageable form of classic rejection by a single

bolus administration of a PAF antagonist, SRI 63-441, prior to revascularization. Indeed, hearts in treated animals that ceased to function at two to four days after transplantation demonstrated a very aggressive histological picture of acute cellular rejection with marked edema and extensive mononuclear cell infiltration. This suggested the prospect of adding classic immunosuppression in an attempt to overcome the cellular rejection.

It was clear that very potent immunosuppression would be required because the cellular rejection of hearts in the SRI 63-441 treated animals was much more aggressive and was occurring much earlier than is usually seen in the same donor-recipient combination without prior sensitization. This probably reflects the aggressive rejection of an organ made vulnerable by antibody attack. The combination of FK 506 and SRI 63-441 treatment in this model of hyperacute cardiac rejection resulted in remarkable prolongation of graft survival in healthy animals. The survival, for the most part, far exceeded the allograft survival in nonsensitized recipients (6.3 ± 0.5 days) and in several cases, graft survival either approached or exceeded one month survival.

These studies provide further insight into the complex immunologic and inflammatory reactions involved in the process of hyperacute rejection. Moreover, we introduce a novel and

) potentially effective therapeutic approach that relies on a combined attack on the very potent and unusually diverse spectrum of biologic activities of a key inflammatory mediator as well as on a very aggressive form of classic cell-mediated rejection.

V. THE EFFECT OF SRI 63-441 ALONE AND IN COMBINATION WITH
PROSTANOIDS ON EXPERIMENTAL XENOTRANSPLANTATION

A. BACKGROUND

Pig-to-dog renal heterotransplantation results in a rapid and violent form of cortical necrosis and ischemic destruction of the organ, which mimics the situation in humans and is due to preformed humoral antibodies in the recipient (19,20). It represents an appropriate severe model in which to evaluate new methods of therapeutic intervention. The studies reported evaluated the effect of a novel and potent synthetic PAF receptor antagonist, SRI 63-441, alone and in combination with other prostanoids, on this renal xenograft model.

B. MATERIALS AND METHODS

Animals

Female, white outbred SPF pigs that weighed 15-20 kg were purchased from Shadyside Farms, Ohio and served as donors. Male beagles that were bred for research and weighed 13-16 kg were purchased from Laboratory Research Enterprise, Kalamazoo, Michigan and served as recipients. All animals were housed in a central animal facility and were allowed to acclimatize for at least 1 week before experimentation. They received standard animal chow and water ad libitum and were fasted for 24 hours before surgery.

SURGICAL PROCEDURES

Orthotopic Renal Transplantation

All animals for both the donor and recipient procedures were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Recipient dogs were maintained with 30% oxygen through an endotracheal tube. Additional anesthetic (pentobarbital, 3 mg/kg IV) was given as required.

Donor pigs received intravenous Ringer's lactate (1000 mL), mannitol (12.5 g), and Thorazine^R (50 mg). After systemic heparinization (5000 units) and in situ cooling with cold (4 C) Ringer's lactate through an aortic cannula, the kidneys were removed en bloc. The kidneys were then separated and transplanted simultaneously into two recipient dogs, always with less than 1 hour of cold ischemia time. A paired design was therefore performed for each experiment, by assigning one kidney as the control and the other as the test (treated) kidney.

All pig-to-dog kidney heterotransplantation were performed by a standard technique. The porcine renal artery was anastomosed end-to-end to the recipient right common iliac artery, and the renal vein was anastomosed end-to-side to the right common iliac vein. The anastomosis time was always less than 30 minutes. The ureter was cannulated with polyethylene tubing and urine was collected throughout the transplant period. For intra-arterial administration of prostaglandins, an 18-gauge polyethylene catheter was inserted through the middle sacral artery and threaded towards the orifice of the right common iliac

) artery. To ensure that solution infused into the terminal aorta would exclusively run into the graft renal artery, the left common iliac, middle sacral, and other smaller arteries originating at or near the aortic bifurcation were ligated near their origin. For those experiments where renal vein and artery platelet counts were measured, 18-gauge polyethylene catheters were introduced into the right femoral vein and its tip positioned at the venous anastomosis, and into the left common iliac artery and its tip positioned at the orifice of the right common iliac artery.

Each dog underwent native nephrectomy after the porcine kidney had been revascularized. A change in color or mottling of the transplanted pig kidney was considered evidence of the onset of rejection. However, the end point of the experiment was signaled by a cessation of urine flow or by the onset of frank hematuria.

PHARMACOLOGIC ADMINISTRATION

Prostaglandins: Naturally occurring prostacyclin (PGI₂) and prostaglandin E₁ (PGE₁) or Prostin-VR were supplied by the Upjohn Company, Kalamazoo, Michigan (M.J. Ruwart). PGI₂ was reconstituted and infused in a 0.1 M glycine buffer, adjusted to a pH of 10.5 with 2 M Tris solution to enhance its stability. PGE₁ was reconstituted in a stock solution of 1 mg in 100 μ L of ethanol. This solution was then diluted to the appropriate concentration in 0.9% sodium chloride for infusion.

All prostaglandins, freshly reconstituted for each experiment, were administered by constant infusion through the catheters placed into the aorta, and the dosage was titrated according to blood pressure tolerance. Control animals for the prostaglandin experiments received infusions of the particular prostaglandin vehicles.

PAF Antagonist: SRI 63-441 was provided by the Sandoz Research Institute, Sandoz, Inc. (East Hanover, NJ). For all studies reported here SRI 63-441 was reconstituted as a 10 mg/mL solution in 0.68% sodium acetate and 0.9% sodium chloride (pH 5.3). Before its administration, this solution was placed in a warm water bath (24-25 C) for 5 minutes and then administered as a bolus intravenous injection. Control dogs received the sodium acetate buffer intravenously.

Experimental Design

Four experimental groups are reported: Group I: the administration of SRI 63-441 alone was compared with controls; Group II: the combination of SRI 63-441 and PGI₂ was compared with controls; Group III: the combination of SRI 63-441 and PGE₁ was compared with controls; Group IV: the combination of SRI 63-441 and PGE₁ was compared with PGE₁ alone.

Both prostaglandins (PGI₂, PGE₁) were administered at a dose of 0.5-1.0 ug/kg/min (depending on systemic blood pressure) by continuous intra-arterial infusion commencing at 30 minutes before revascularization. SRI 63-441 was administered at 5

mg/kg/dose by intravenous bolus beginning at 5 minutes before and then 5 minutes after, and then every 15 minutes after revascularization. For each study, experiments were performed on control and test animals simultaneously.

Kidney survival was assessed for each experiment as described above. Urine was collected for the entire transplant period, and the total volume was used as an indicator of renal function. For six experiments in Group II and for three experiments in Group IV, platelet counts in the renal artery, renal vein, and peripherally were determined serially (every 10 minutes for the first hour and then every 30 minutes for up to 4 hours in the treated groups). Serial kidney biopsies (every 15 minutes) were performed in an extra group of animals that are not included in survival statistics. Biopsies were also done at the end point of the experiment, (using the criteria outlined above) in all groups studied.

C. RESULTS

Renal Xenograft Survival

Figure 4 depicts the survival for transplanted porcine kidneys in the four groups studied. The control dogs in all experiments consistently demonstrated violent rejection of the pig kidney within a mean time of approximately 30 minutes. The administration of SRI 63-441 as a single agent at the dosage and schedule described here did not result in a significant increase in graft survival. The addition of either prostanoid (PGI₂ or

} PGE1) to SRI 63-441 treatment resulted in a marked synergism and a significant increase in kidney survival. SRI 63-441 and PGI2 resulted in a greater than six fold (217.1 ± 39.4 versus 37.4 ± 11.1 minutes, $p < 0.001$) and SRI 63-441 and PGE1 in a greater than seven fold (268.6 ± 27.7 versus 37.2 ± 5.6 minutes, $p < 0.001$) increase in renal xenograft survival when compared with their respective controls. PGI2 or PGE1 infused as single agents had no significant effect on kidney survival. In fact, in Group IV, the combination of SRI 63-441 and PGE1 resulted in a greater than nine fold increase in kidney survival when compared with PGE1 infusion alone (280.4 ± 31.8 versus 31.0 ± 11.1 minutes, $p < 0.001$). PGE1 in this experiment behaved no differently than the control vehicles in the other experiments.

Renal Xenograft Function

Renal function as determined by total urine output for each transplant is depicted in Figure 5. The results parallel those observed for kidney survival already presented above. SRI 63-441 alone did not significantly improve renal function. The combination of SRI 63-441 and PGI2 resulted in a greater than threefold (99.5 ± 30.4 versus 29.5 ± 13.7 mL, $p < 0.05$) and of SRI 63-441 and PGE1 in a greater than 20-fold (436.2 ± 134.8 versus 21.2 ± 7.3 mL, $p < 0.02$) increase in total urine output. Neither prostanoid alone resulted in a significant increase in urine output. The combination of SRI 63-441 and PGE1

resulted in a 12-fold increase in total urine output (370.9 ± 78.9 versus 32.7 ± 6.4 mL, $p < 0.003$) when compared with PGE1 alone, which did not differ significantly from vehicle controls.

Platelet Studies

The serial platelet counts for six experiments in Group II (SRI 63-441 + PGI2 versus controls) and three experiments in Group IV (SRI 63-441 + PGE1 versus PGE1) are depicted in Figures 6 and 7, respectively. There was a significant difference between the renal artery and renal vein platelet counts in Group II for both control and treated animals, with a more pronounced gradient evident in the control group. No significant gradient in platelet count between renal artery and vein was observed in Group IV dogs.

Histological Evaluation

Microscopically, in the serial experiment, baseline biopsies from both control and treated animals contained normal glomeruli, with normal cellularity, and patent, empty capillary loops. After reperfusion, both kidneys exhibited progressive obliteration of capillary loops by eosinophilic granular material mixed with red blood cells. Similar thrombi were found in arteries and arterioles as early as 15 minutes. Tubules remained well preserved until 1 hour. There was progressive edema, then congestion, then hemorrhage in the interstitium. At the

} endstage, when renal function ceased, all specimens showed diffuse thrombosis of glomeruli, arteries, and vasa rectae. There were interstitial hemorrhages and early changes of ATN.

Sequential Biopsies: Biopsy specimens were taken at 5, 45, and 90 minutes. Qualitatively, the glomerular changes were similar in the control and treated groups. However, the development of glomerular thrombosis proceeded at a slower rate in the treated kidneys. Table IV shows the results from one experiment. The control animal reached end-stage with thrombosis in 94% of the glomeruli at 45 minutes, whereas the process reached completion in the treated kidney at 90 minutes. At endstage, red blood cell thrombi were more prominent in the treated kidney.

Necrosis of tubular epithelial cells was noted in the control kidney with blood in the lumens at 60 minutes. These changes persisted until the end of the experiment. It is important to note that significant necrosis was not noted in the treated kidney.

There was a difference between the two kidneys in the amount and onset of interstitial hemorrhage. Focal hemorrhages were noted in the control kidney at 15 minutes; this progressed to diffuse hemorrhage by 45 minutes and persisted. In contrast, the treated kidney showed only focal hemorrhages at 30 minutes, and again at 90 minutes. There were no significant differences between the control and treated kidneys with regard to the presence of thrombi in arteries and arterioles.

Endstage Biopsies: The endstage biopsies obtained after implantation represented a later stage than the sequential biopsies. In these sections, RBCs were a prominent component of the glomerular thrombi in the treated kidneys, whereas in the control groups the thrombi were composed of mostly granular eosinophilic material consistent with platelet-fibrin thrombi, similar to the changes noted in the sequential biopsies. There was more interstitial hemorrhage in the treated dogs than in the controls in five cases, and less hemorrhage in four cases. Three of the treated kidneys and none of the control kidneys exhibited up to 20% glomeruli free of thrombi.

VI. THE EFFECT OF SRI 63-441 ON CAT-TO-RABBIT RENAL
XENOTRANSPLANTATION

A. MATERIALS AND METHODS

Animals

Female, purpose bred for research cats that weighed 4.0-5.0 kg were purchased from Liberty Labs, Liberty Corner, NJ, and served as donors. Female Checkered Flemish giant rabbits weighing 4.0 to 6.2 kg were purchased from Zivic Miller Labs, Allison Park, PA, and served as recipients. All animals were housed in the central animal facility and were allowed to acclimatize for at least one week prior to experimentation. They received standard animal chow and water ad libitum and were fasted for 24 hours before surgery.

SURGICAL PROCEDURES

Orthotopic Renal Transplantation

Donor bilateral nephrectomy was performed en bloc taking the aorta, vena cava, renal vessels and ureters. The kidneys were flushed in situ, through an infrarenal aortic cannula, with 400-500 cc of cold (4°C) Ringers Lactate. Each donor provided 2 kidneys for transplantation: Control versus SRI 63-441 treated.

In the recipient, the left lumbar fossa was prepared for anastomosis. Orthotopic kidney transplantation was then performed by anastomosing the renal veins using a microvascular cuff technique, and the renal arteries by an end-to-side aorta-

The ureters were cannulated with a 20-22 gauge catheter and approximated to the skin for urine collection.

PHARMACOLOGIC ADMINISTRATION

SRI 63-441 was prepared in an identical manner as described in the previous section and administered as a single intravenous bolus beginning 5 minutes before revascularization and at a dosage of 4 mg/kg. Controls received the drug buffer intravenously.

Experimental Design

A total of 15 control procedures were performed. SRI 63-441 was administered to an additional 6 rabbits. Each animal treated with SRI 63-441 had a paired control procedure performed at exactly the same time.

B. RESULTS

Renal xenograft survival as determined by kidney color, consistency and cessation of urine formation; and function as measured by urine output are given in Table V, for both groups studied. The urine output of treated animals at one and two hours after revascularization was significantly better than in untreated controls, in fact, controls in this model consistently produce very little, if any urine. Although the biological

importance of the almost two-fold increase in kidney survival observed in treated animals is not completely apparent, the results were statistically significant.

Histological Evaluation

Representative histologic sections from these experiments are presented in Figure 8 (Control), and Figure 9 (SRI 63-441 treated). In the control animals, all except one kidney showed normal glomeruli and varying degrees of tubular vacuolization, or ATN prior to 40 minutes.

In the one animal 10-14% of the glomeruli in each of the kidneys in different recipients contained thrombi by 30 minutes. At 40 minutes glomerular thrombosis ranged from 2 to 8%. In end-stage specimens taken at 1 hour or later, the percentage glomerular thrombosis ranged from 2 to 14%, so it appeared that the end-stage was reached between 40 and 60 minutes. Eosinophils were frequently noted in all specimens. Polys and necrosis were seen in only one specimen.

Kidneys from animals receiving SRI 63-441 showed only mild tubular vacuolization at one hour, compared to overt ATN in the control kidneys. At 1 1/2 hours both kidneys were the same, with at most 3% glomerular thrombosis and ATN.

VII. SUMMARY

At the single dosage and schedule of SRI 63-441 evaluated in these studies, inhibition of PAF alone was not sufficient to improve renal xenograft survival or function in the pig-to-dog model, although a trend did exist, but did significantly improve these parameters in the cat-to-rabbit model. These findings may have some implication into a better understanding the process and biology of HAR in different species combinations. From studies in rats and other recent studies in dogs, we have determined that the critical consideration for the efficacy of SRI 63-441 is to administer it at a much higher dose and before revascularization. However, the addition of either one of two prostanoids, PGI₂ or PGE₁, resulted in a synergistic response and a manyfold increase in renal xenograft survival and function in the pig-to-dog model. Although a higher dosage and more appropriate scheduling of SRI 63-441 may improve the effectiveness of single drug therapy, the need for multiple drug treatment eluded to above may be necessary to adequately modulate this violent inflammatory process.

There are many reasons for choosing PGI₂ and PGE₁ as complimentary agents to SRI 63-441. They both inhibit platelet function by stimulation of receptor-coupled adenylate cyclase and increase intracellular cAMP, a receptor mechanism different from that required for SRI 63-441 inhibition (47). To varying degrees, they both exhibit other properties that establish them as excellent therapeutic candidates for such inflammatory

processes. These biological activities include vasodilatation, fibrinolytic action, inhibition of the generation of certain deleterious metabolites of the arachidonic acid cascade, inhibition of oxygen-free radical generation, and the property known as cytoprotection (47). Moreover, both these prostaglandins have been evaluated in other models of hyperacute rejection. Treatment with PGE1 prolonged hamster-to-rat cardiac xenograft survival (48). PGI2 administration significantly prolonged kidney survival in both a model of hyperacute renal allograft rejection in presensitized dogs and in cat-to-dog xenografts (30,48) but had no effect on guinea pig-to-rat cardiac xenograft survival (48). Neither PGI2 nor PGE1, when administered alone as a constant infusion without combination SRI 63-441 treatment, had a significant effect on pig-to-dog renal xenograft survival or function as described in our studies. Furthermore, the therapeutic effectiveness of these prostaglandins in combination with SRI 63-441 was evident only when they were infused intra-arterially; peripheral administration of prostaglandins did not exhibit synergism with SRI 63-441 (unpublished results). This is most likely due to inactivation of prostaglandins as they pass through the lungs (47).

The pig-to-dog renal xenograft model evaluated in these studies probably represents the most severe animal model of hyperacute rejection known. Rapid and violent kidney rejection is the rule and there have been only a few reports of successful

abrogation of this process. Shons and Najarian reported a significant increase in pig renal xenograft survival from a mean of 5 minutes in controls up to a mean of 45 minutes in dogs treated with a variety of antiplatelet agents (29). Merkel et al. reported renal xenograft survival times of 120, 90, and 180 minutes in three dogs that had preoperative plasmapheresis (49). Kux et al. reported a mean rejection time of 85 minutes in 15 kidneys treated with intra-arterial citrate infusion; these experiments were limited by significant toxicity (50). The control xenografts for the latter two sets of experiments rejected in less than 10 minutes (20,49).

Consistent and reproducible results were achieved in the same model in the current studies by combining SRI 63-441 with either PGI₂ or PGE₂. Pig kidneys surviving for 3-4 hours were consistently achieved with maintenance of good urine output in all combination treatment groups. There were three kidneys in this series of experiments that functioned satisfactorily for 6-9 hours. The results in the cat-to-rabbit model, although less striking, were significant and further emphasize that this phenomenon is not species specific.

CONCLUSIONS

These studies further confirm the central role of plate-activating factor in the inflammatory cascade that destroys hearts and kidneys undergoing hyperacute rejection. Furthermore,

} these studies introduce a novel, reproducible, and potentially effective therapeutic approach to this difficult problem, which relies on a better understanding of the basic pathophysiologic process of inflammation and which utilizes as its basis, a synthetic receptor antagonist that specifically inhibits the potent and unusually diverse spectrum of biologic activities of one of the key mediators of classical inflammation.

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TABLE I

THE EFFECT OF SRI 63-441 ON CARDIAC ALLOGRAFT SURVIVAL IN PRESENSITIZED RECIPIENTS

Group	Treatment	N	Graft Survival Time (minutes)	Mean +S.E.
1	Control-no treatment	74	2,3,3,4,4,5,5,6,7,8,8,10,15,15,16,17,17,17,20,21,28,30,30,30,31,32,34,35,37,38,40,45,45,49,50,50,58,59,62,64,65,66,74,78,87,90,90,92,100,113,120,140,180,184,211,340,349,375,383,395,420,450,480,600,600,600,607,870,960,1200,1266,1440,1440	216+40.0
2	SRI-63-441 5mg/kg	4	17,37,70,600	181+140 ^a
3	SRI 63-441 10mg/kg	10	5,28,61,70,86,205,1628,1709,3150,5760	1270+602 ^a
4	SRI 63-441 15mg/kg	20	39,108,129,180,184,220 ⁺ ,240,455,600,720,1680,2880,3900,4320,4320,5040,5760,5760,5760,7200	2475+560 ^b
5	SRI 63-441 20mg/kg	8	40,59*,60 ⁺ ,120 ⁺ ,360 ⁺ ,745,2880,6480	1343+808 ^a

* died postoperatively

+ died with pulsating grafts

^a p value N.S.

^b p value < 0.001 (t test)

TABLE II

EFFECT OF CYCLOSPORINE ALONE OR IN COMBINATION WITH SRI 63-441 ON
CARDIAC ALLOGRAFT SURVIVAL IN PRESENSITIZED RATS

Group	Treatment	N	Graft Survival Time (minutes)	Mean \pm S.E.
1	Control-no treatment	74	2,3,3,4,4,5,5,6,7,8,8, 10,15,15,16,17,17,17,20, 21,28,30,30,30,31,32,34, 35,37,38,40,45,45,49,50, 50,58,59,62,64,65,66,74, 78,87,90,90,92,100,113, 120,140,180,184,211,340, 349,375,383,395,420,450, 480,600,600,600,607,870, 960,1200,1266,1440,1440	216 \pm 40.0
11	CSA single administration- 6 hours	10	226,235,270,330,360,615, 956,3600,4320,4320	1523 \pm 566 ^a
12	CSA multiple administration	9	5,15,244,250,570,629, 2880,5760,15840	2190 \pm 1736 ^b
13	CSA multiple+ SRI 63-441 10mg/kg	10	61,76,80,105,125,140, 304,517,7200,7200	1581 \pm 938 ^b
14	CSA multiple+ SRI 63-441 15mg/kg	10	15,40,52,101,115,120, 630,1140,7200,8640	1805 \pm 1031 ^b

^a p value < 0.05 (t test)

^b p value N.S.

TABLE III

THE EFFECT OF PROSTAGLANDIN (PGE₂) ALONE OR IN COMBINATION WITH SRI 63-441 ON CARDIAC ALLOGRAFT SURVIVAL IN PRESENSITIZED RATS

Group	Treatment	N	Graft Survival Time (minutes)	Mean ±S.E.
1	Control-no treatment	74	2,3,3,4,4,5,5,6,7,8,8,10,15,15,16,17,17,17,20,21,28,30,30,30,31,32,34,35,37,38,40,45,45,49,50,50,58,59,62,64,65,66,74,78,87,90,90,92,100,113,120,140,180,184,211,340,349,375,383,395,420,450,480,600,600,600,607,870,960,1200,1266,1440,1440	216±40.0
6	PGE ₂ alone	10	55,64,75,83,150,200,227,240 ⁺ , 7200,10080	1837±1154 ^a
7	PGE ₂ +SRI 63-441 15mg/kg	10	3,12,13,22,46,95,110 ⁺ , 120 ⁺ , 600 ⁺ , 7200	822±711 ^a

⁺ died with pulsating grafts

^a p value N.S. (t test)

TABLE IV
PROGRESSION OF HISTOLOGIC FINDINGS IN SEQUENTIAL
BIOPSIES OF CONTROL AND TREATED KIDNEYS

Time (min)	Controls			Treated		
	Glomerular Thrombosis	ATN	Interstitial Hemorrhage	Glomerular Thrombosis	ATN	Interstitial Hemorrhage
5	26	0	0	13	0	0
15	69	0	focal	46	0	0
30	83	0	focal	60	0	focal
45	94	0	severe	73	0	0
60	91	focal	severe	80	0	0
75	88	focal	moderate	77	0	0
90	78	focal	severe	97	focal	moderate

TABLE V
EFFECT OF SRI 63-441 ON CAT-TO-RABBIT
KIDNEY XENOTRANSPLANTATION

Group	N	Xenograft Survival (minutes)		Urine Output (cc/hour)	
		Individual	Median \pm S.E.	1 hour	2 hours
Control	15	45, 60, 70, 75, 80, 85, 85, 85, 90, 95, 105, 110, 120	84.3 \pm 5.26	0.22 \pm 0.17	0.05 \pm 0.06
SRI 63-441 (4 mg/kg)	6	100, 125, 135, 135, 145, 160	133.3 \pm 8.23*	2.33 \pm 1.10	0.96 \pm 0.35**

* p < 0.002

** p < 0.001

FIGURE LEGENDS

1. Photomicrograph of the myocardium from a heterotopic allograft (ACI --> Lewis) previously sensitized by four successive donor skin grafts (control). Note acute vasculitis and thrombosis. Slight acute inflammation noted in myocardial interstitium. (H+E 550x)
2. Photomicrograph of the myocardium from a heterotopic allograft (ACI --> Lewis) previously sensitized by four successive donor skin grafts and treated with a single bolus of 15 mg/kg SRI 63-441 (IV). Note the lack of vasculitis and thrombosis. There is evidence of repair indicating some damage to myocardium prior to harvest. The extent of repair ranged from mild to moderate in degree. (H+E 550x)

3. Photomicrograph of the myocardium from a heterotopic allograft (ACI --> Lewis) previously sensitized by four successive donor skin grafts. This animal was treated with multiple administrations of 1.28 mg/kg/day FK 506 (IM) and a single bolus of 1.5 mg/kg SRI 63-441 (IV) at four minutes prior to organ revascularization. Note the absence of vasculitis and thrombosis. Endothelial swelling is prominent, however, as is the mild acute cellular rejection evident in the remaining myocardium.

- 4.* Survival of renal xenograft after pig-to-dog kidney transplantation assessing the effect of single and combination treatment with a PAF receptor antagonist (SRI 63-441) and two prostanoids (PGI₂ and PGE₁). Results are expressed as total survival time (mean minutes \pm SE).

- 5.* Function of renal xenograft after pig-to-dog kidney transplantation from experiments described in Figure 1. Results are expressed as total urine output for the entire transplant period (mean milliliters \pm SE).

- 6.* Platelet counts (mean \pm SE) determined for the renal artery (RA), renal vein (RV), and peripherally (P) for six sets of experiments in Group II (SRI 63-441 + PGI₂ versus control).

- 7.* Platelet counts (mean \pm SE) determined for the renal artery (RA), renal vein (RV), and peripherally (P) for three sets of experiments in Group IV (SRI 63-441 + PGE1 versus PGE1).

8. Control renal xenograft (cat-to-rabbit): Microscopic section of glomerulus at 45 minutes post revascularization. Note thrombi in capillary loops. (H+E 480x)

9. SRI 63-441 (4 mg/kg bolus) renal xenograft (cat-to-rabbit): Microscopic section of glomerulus at 45 minutes post revascularization. Note thrombi in capillary lumens particularly over right half. (H+E 480x).

* Reprinted with permission from Makowka et al. *Annals of Surgery* 206(4):482-495, 1987.

FIGURE 1

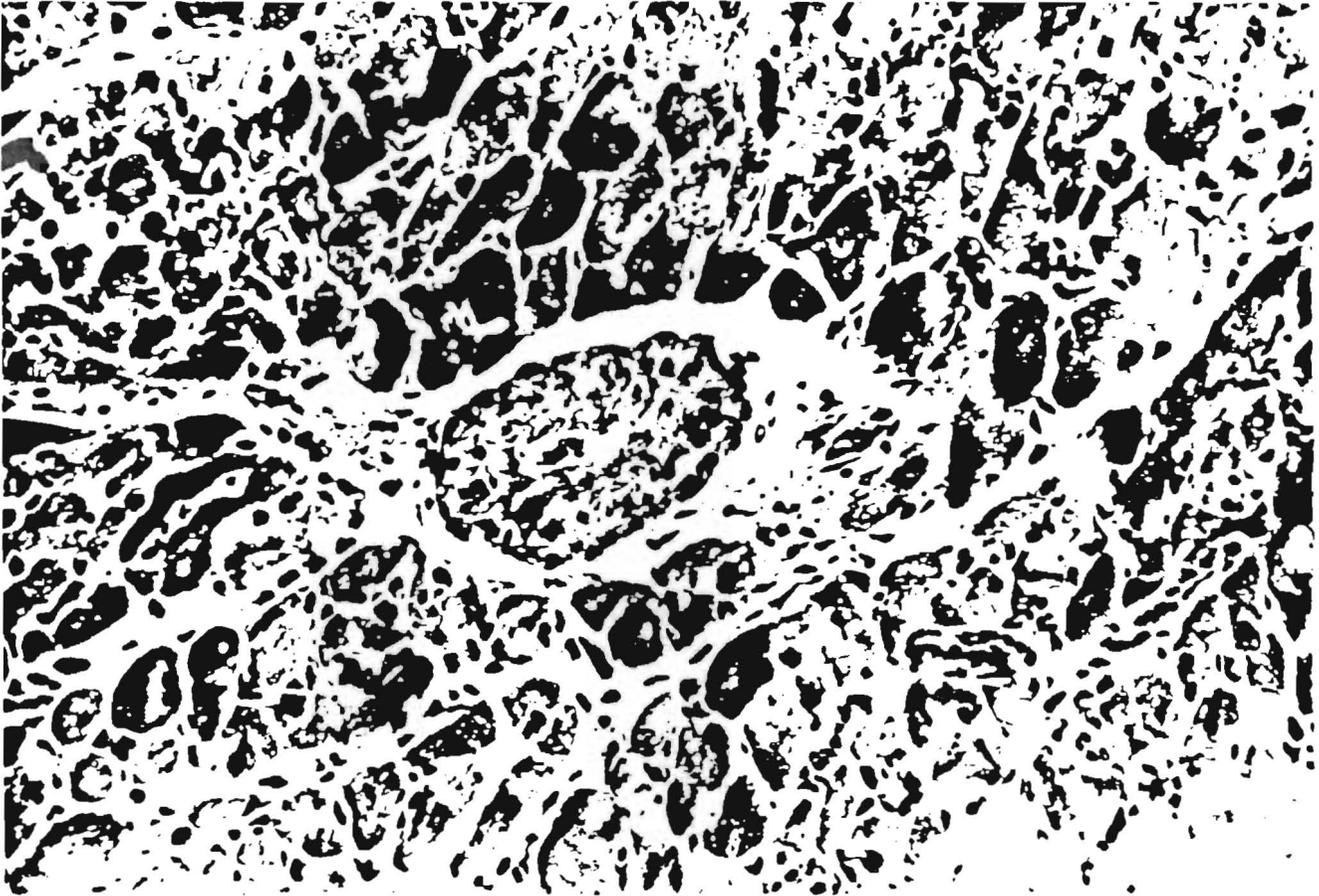


FIGURE 2

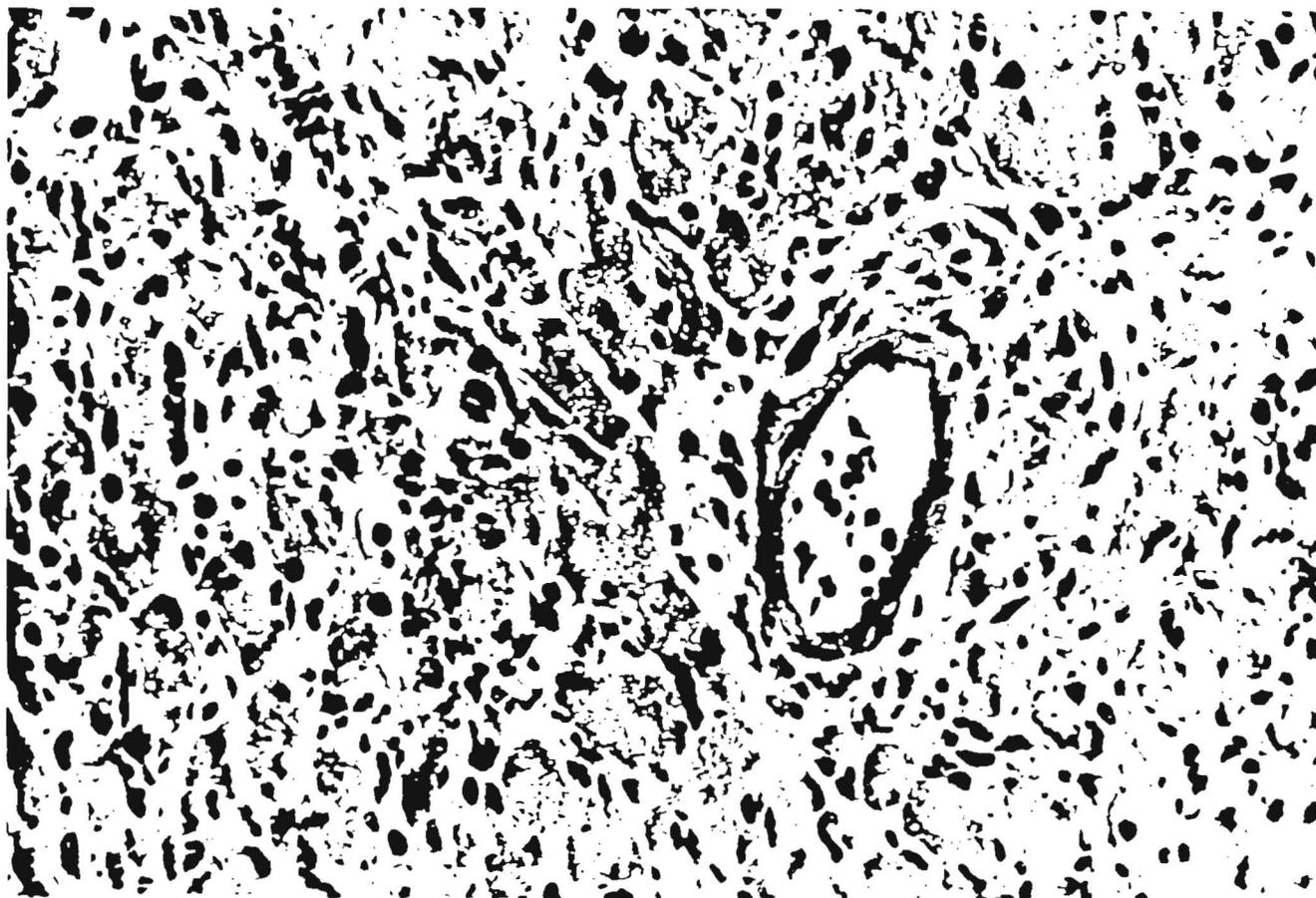


FIGURE 3

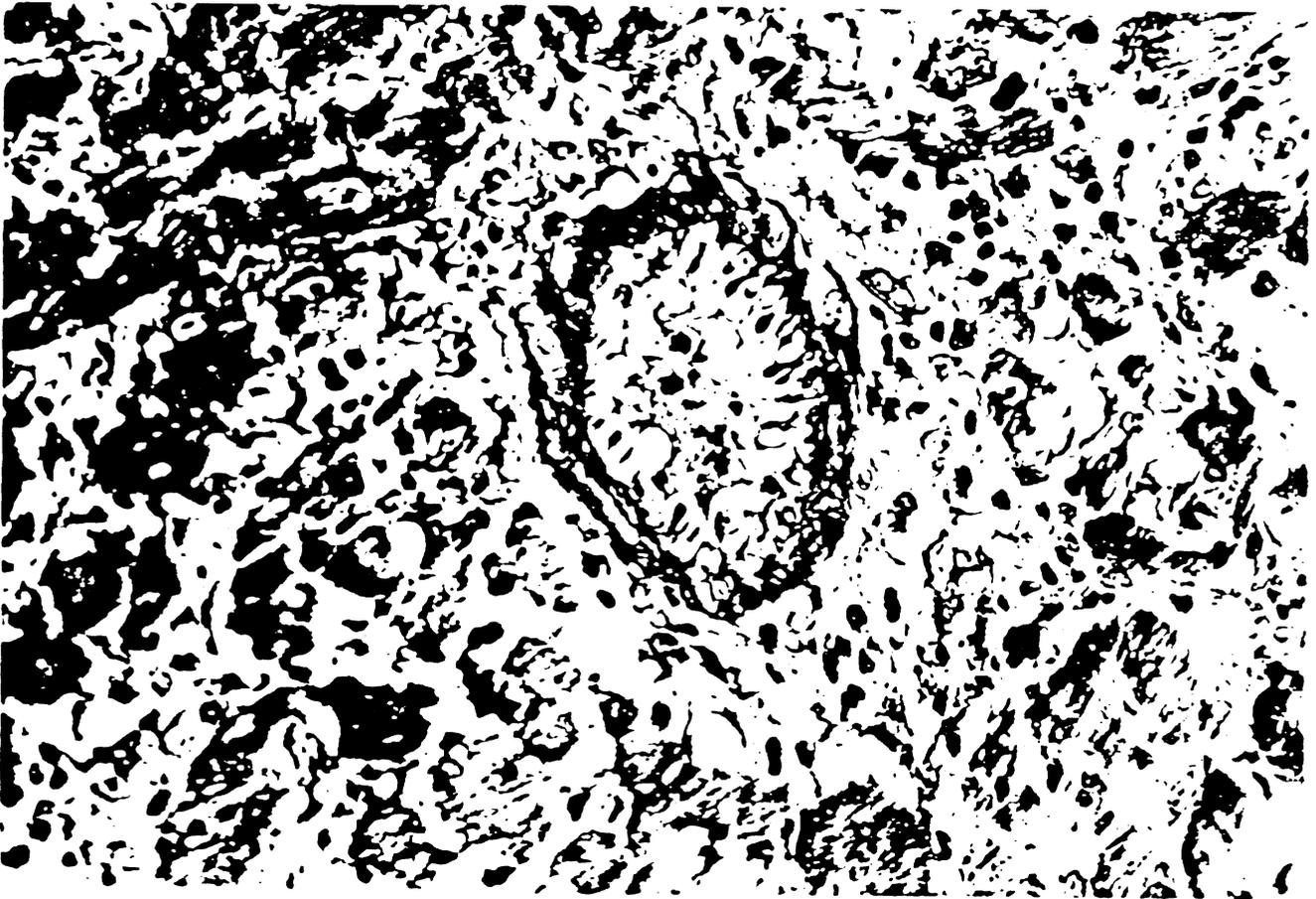


FIGURE 4

RENAL XENOGRAFT SURVIVAL

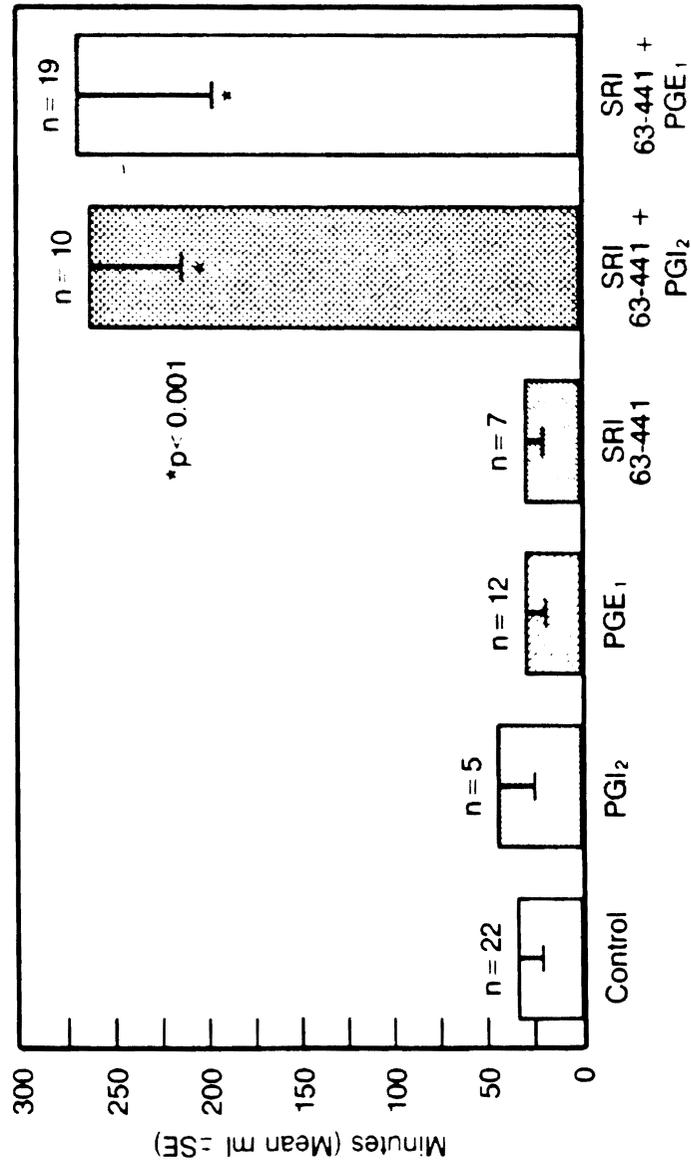


FIGURE 5

RENAL XENOGRAFT URINE OUTPUT

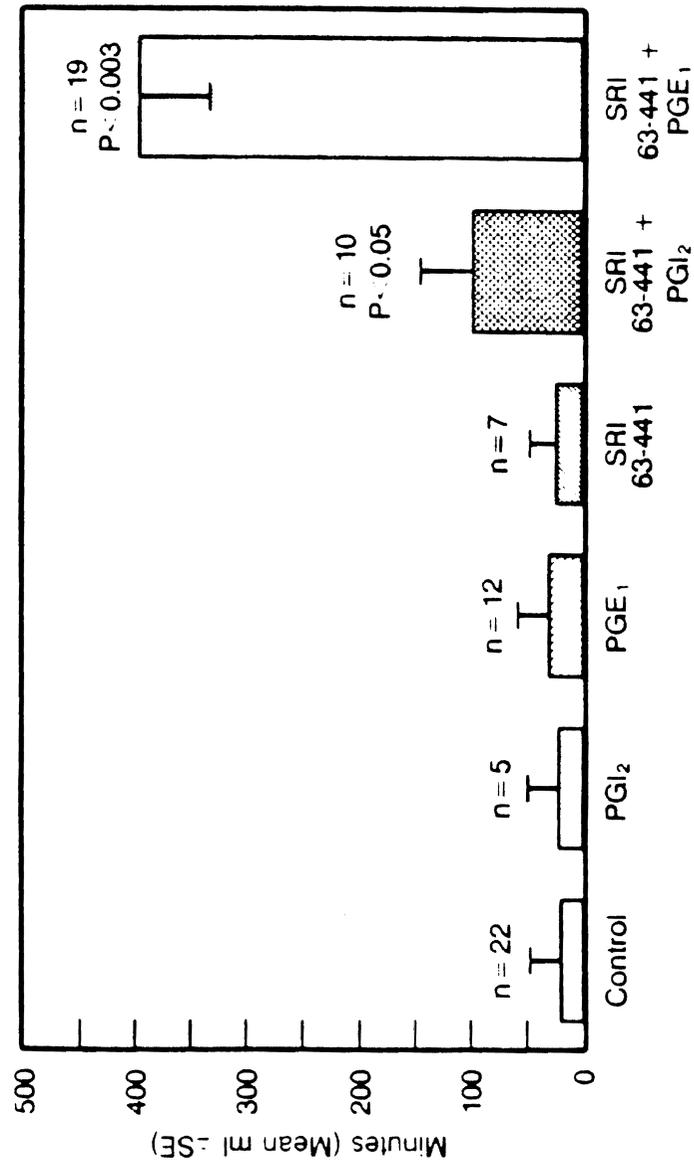


FIGURE 6

PLATELET RESPONSE

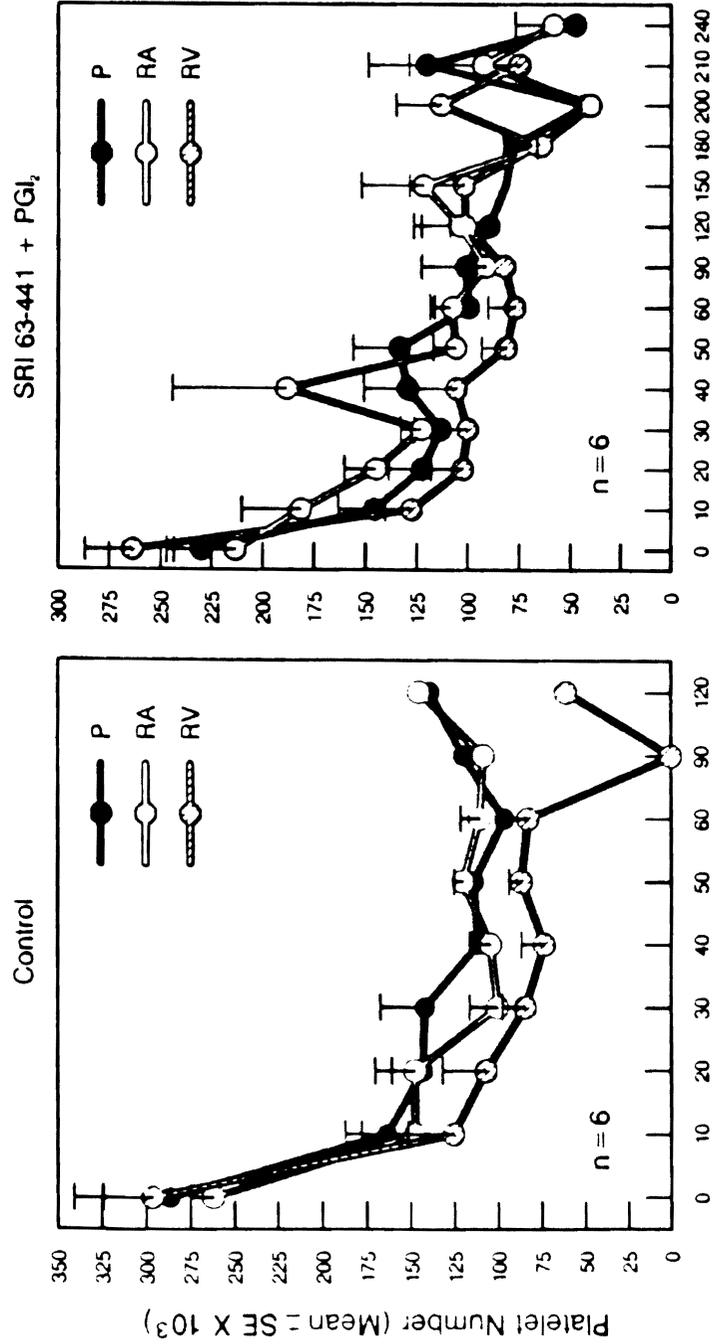


FIGURE 7

PLATELET RESPONSE

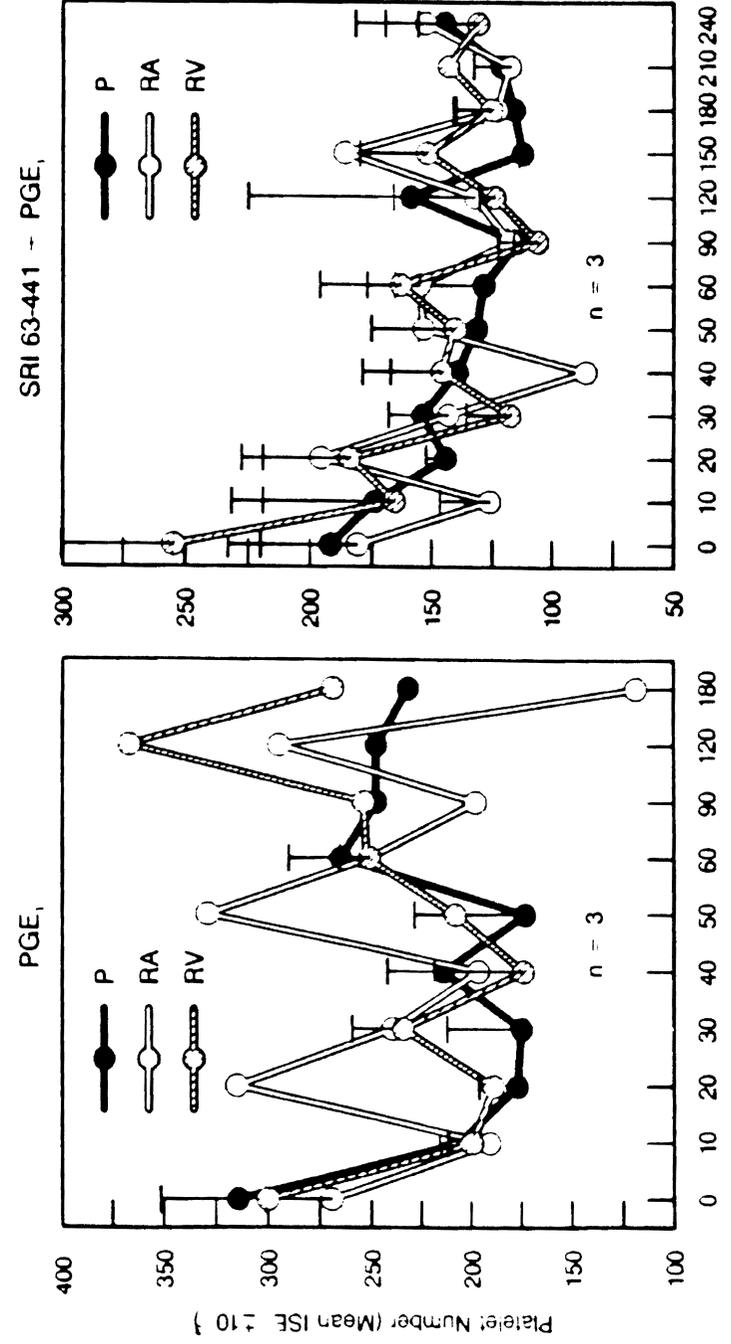


FIGURE 8

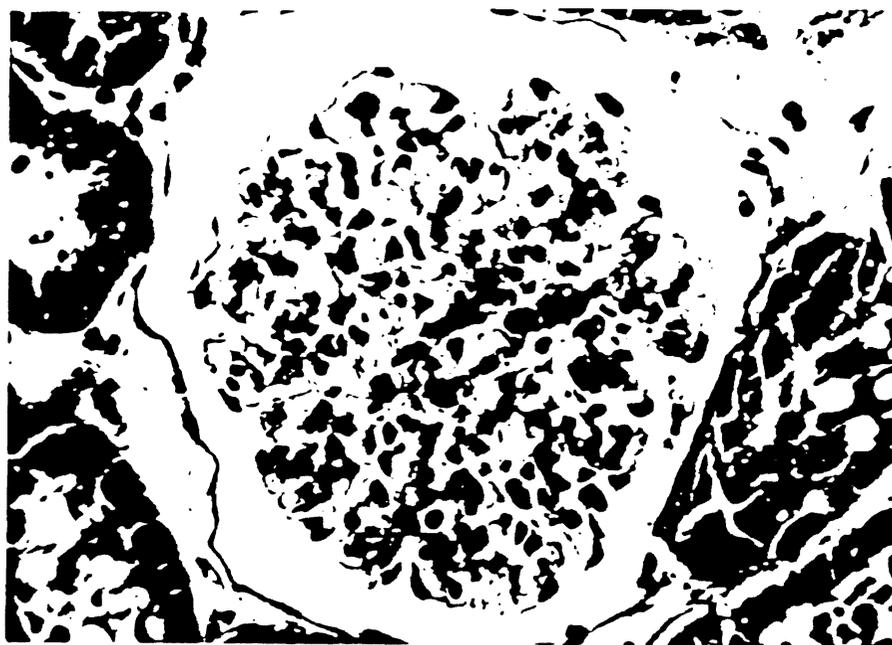


FIGURE 9

