Histological Evaluation of Ovine Mammary Tissue Xenografted into Cyclosporine Treated Mice

by

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(ABSTRACT)

Cyclosporine (CsA), a fungal peptide with remarkable anti-lymphocytic properties, has been shown to be an effective immunosuppressive agent. In three separate experiments, mice receiving CsA at various doses (8 mg/kg/day in the first two experiments and 70-100 mg/kg/day in the third), were implanted with ovine mammary tissue expants at various sites (subcutaneous dorsum, ovarian fat-pad, and cleared mammary gland fat-pad). After different periods of time, (day 3, 7, 14, 21, and 35) implants were removed, fixed, stained and embedded. Epithelial morphology of the implants was evaluated via a grading system that classified epithelium from 1 (poor) to 5 (excellent). Leukocyte type and number was determined in subepithelial stroma of implants in the third experiment. In the first two experiments, histological evaluation revealed that the transplanted tissue did not survive, regardless of site of implantation or developmental stage of donor tissue. In experiment three higher, dosages of CsA and different donor tissue were used, this resulted in significantly higher ($P < .05$) mean epithelial scores (indicative of healthier epithelium) for two CsA treated groups vs two control groups (3.75 and 2.33 vs 2.0 and 1.25, respectively). Within each treatment group

(CsA and control), one group of mice received exogenous estrogen and progesterone (E/P) implants. CsA treated mice receiving E/P had significantly higher ($P < .05$) epithelial scores than those not receiving hormones (3.68 vs 2.83). The reverse was true for lymphocyte number (18.9 vs 40.5 cells per 117,600 um^2 of subepithelial stroma) which were significantly less in CsA + E/P treated mice. The overall correlation between lymphocyte number and epithelial score was -.55 ($P < .0001$). These results indicate that ovine mammary tissue xenografted into mice treated with high doses of CsA, are capable of surviving for extended periods of time (at least 35 days).

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Many thanks to my wife Wendy, it is was through her love and patience that I found the strength to complete this manuscript (thanks babe!).

Finally I thank God for giving me the strength and health to complete this manuscript.

I would like to dedicate this manuscript to my father who passed away in June, 1981. I truly regret he was not here to share in this happy event.

Table of Contents

List of Tables

List of Illustrations

INTRODUCTION

Understanding the regulation of mammary gland development is a key in efforts to increase productivity, in both the dairy and beef industries. The dairy industry depends directly on milk synthesis and secretion, and growth of beef calves is directly related to milk production of the dam. Milk production, in turn is limited by the number of functional epithelial cells in the udder. The study of growth and differentiation at the cellular level in bovine mammary gland in vivo is difficult because of the expense of purchasing, housing, feeding and caring for these animals. Use of explant organ culture has been important in the study of mammary secretory cell differentiation, but has met with only limited success when applied to study of mammary parenchymal gland growth and morphogenesis. Recently, the athymic nude mouse has been used as an *ex vivo in vivo* model for the study of mammary gland growth because these animals do not reject foreign tissues. This system, though superior in many aspects to in

vitro procedures, is still limited by the cost, care and inherent susceptibility to disease of these immune deficient mice.

With the recent development of the immunosuppressive drug cyclosporine, the intriguing possibility exists for the development of an alternative model system to that of the athymic nude mouse model in studying growth and development of the mammary gland. If successful, a model of this type, would offer distinct time, cost and logistical advantages over the nude mouse and to strict in vitro methods. Therefore, the objectives of the present study were: (1) to determine whether ovine mammary tissue xenografted into mice treated with cyclosporine would survive, (2) to determine the most appropriate site for xenograft implantation, and (3) to determine whether xenografted tissue would respond to endogenous and exogenous hormonal stimulation.

REVIEW OF LITERATURE

Cyclosporine: History

In 1969/1970 at the Microbiology Department of Sandoz at Basle Switzerland, two new strains of fungi imperfecti were isolated from soils collected in Wisconsin (U.S.A.) and Hardanger Vidda (Norway). One was *Cylindrocarpon lucidum* Booth and the other strain was *Tolypocladium inflatum* Gams. Both of these fungi synthesize cyclosporine A (CsA), a hydrophobic undecapeptide.

Tolyplocladium inflatum Gams is now used for the large scale production of CsA by fermentation. The fungus *Cylindrocarpon lucidum* Booth has been abandoned as a producer of CsA because it grows only in surface culture, whereas *Tolyplocladium inflatum* Gams grows in submerged culture (White, 1982)

In 1972, Borel et al., discovered the marked immunosuppressive effect of crude extracts containing CsA. A modified mouse test model based on inhibition of hemagglutinin formation against sheep erythrocytes and survival time following inoculation with the murine leukemia cells was used to measure immunosuppression. These researchers showed that CsA inhibited hemagglutination, but had no effect on the survival of leukemic mice.

Following development of procedures for purification of CsA from culture homogenates (Ruegger et al., 1976), further production changes improved yields (Dreyfuss et al., 1976) and permitted the start of pharmacological work using pure CsA. By 1975, the structure of CsA was elucidated by both chemical degradation and x-ray analysis (Ruegger et al., 1976; Petcher et al., 1976) and confirming synthesis of CsA was subsequently achieved (White, 1982). The very first experimental animal studies performed with CsA outside Sandoz, involved experiments with heart allografts in the rat (Kostakis et al., 1977). Publication of this work boosted world wide interest in CsA. This interest is reflected by the yearly increase in the number of both experimental and clinical papers, reviews and abstracts published on CsA (i.e. 9 in 1978 and more than a thousand in 1986).

Cyclosporine: Mechanism of Action

Effectiveness of immunosuppression as a clinical or experimental tool can be divided into three developmental phases (White, 1982). The first phase involved use of drugs that exerted indiscriminate cytotoxicity against all dividing cells including immunocompetent cells (i.e. cyclophosphamide, azathioprine, methotrexate, etc.). The next phase was illustrated by the use of drugs that eliminated immunocompetent cells (i.e. lymphocytotoxic drugs; such as antilymphocyte serum, L-asparaginase, and steroids) The last phase is characterized by selective immunoregulation requiring compounds or methods which specifically modulate defined subpopulations (i.e. T-cells) of immunocompetent cells. CsA is the first drug that fulfills this last requirement. Thus, CsA provides the means for regulation of the lymphocytic response to a tissue graft without completely laying waste to the antibacterial defenses of the recipient.

The immune system provides the body with a defense system against invading pathogens. This system is further classified into specific and non-specific resistance. Skin and mucous membranes possess certain mechanical and chemical factors that non-specifically combat the initial attempt of a microbe to cause disease. Mechanical factors include presence of keratin or mucous membranes and production of secretions (i.e. tears, saliva, sebum, perspiration, urine). Chemical factors include variation in composition of secretions and body fluids (i.e. pH of gastric juice, presence of unsaturated fatty acids and lysozyme). Specific resist-

ance includes humoral and cell-mediated immunity. Humoral immunity depends on the production of antibodies by plasma cells, which develop from B-lymphocytes, and subsequently aid in destruction of antigens. Cell-mediated immunity includes that component of the immune system that destroys antigens by sensitized lymphocytes (T-cells). It is cell-mediated immunity that is affected by CsA.

Moreover as related to this project, it is T-cells which are responsible for tissue rejection in unsuccessful transplants. Until recently, immunosuppresive therapy suppressed not only the recipients immune rejection of the donor organ (tissue), but also capacity to respond to nearly all other antigens. This caused recipient's to become very suseptible to infectious disease.

CsA usefulness stems from its modification of T-cell function (Lafferty, 1983). Although it was once thought that presence of antigen alone could cause the activation of

T-cells (Medawar, 1963), it is now clear that two signals are required for T-cell activation (Lafferty et al., 1974; Lafferty and Cunningham, 1975; Cunningham and Lafferty, 1977; Larsson and Coutinho, 1979; Talmage et al., 1977). One signal is provided by antigen binding and the second signal by a molecule that expresses co-stimulator activity. Talmage et al., (1977), used cultured tumor cell lymphocytes to provide evidence for this two-signal model. In tumor cells that have all the antigenic requirements for T-cell activation, presentation of antigen alone failed to induce T-cell response. Similarly, addition of a lymphokine (a

stimulatory molecule released by leucocytes) alone also failed to activate T-cells. Therefore, the process of T-cell activation requires the provision of both signals. Once activated, T-cells secrete a number of different lymphokines (Prystowsky et al., 1982). These include Interleuken 2 (IL-2), Interleuken 3 (IL-3), macrophage-activating factor (MAF), migratory inhibitory factors (MIF), and interferon (IF). Activated T-cells have also been shown (Andrus and Lafferty, 1982; Larsson and Coutinho, 1979) to have receptors for IL-2. This molecule provides the signal required for T-cell proliferation and resultant clonal expansion (Larsson et al., 1980). In contrast to the process of T-cell activation, triggering of activated T-cells requires only one signal; antigen binding to the T-cell receptor (Lafferty et al., 1980). This signal can be provided by specific antigen (the same one used in activating the T-cell initially) or by a mitogen (i.e. IL-2)(Andrus et al., 1980).

The signaling model proposed for CsA's site of action postulates that CsA interferes with the transmission of the antigen-specific signal to the interior of the cell nucleus at some stage following T-cell activation (Andrus et al., 1981). In 1984, Elliot et al., reported that at least part of the immunosuppressive activity of CsA is due to its ability to selectively inhibit accumulation of mRNA coding for IL-2 in human and murine T lymphocytes. The triggering of synthesis of lymphokine encoding mRNA is divided into four steps (Hodgkin et al., 1987): (1) antigen (or mitogen) binds to the surface of the activated T-cell, presumably via its specific receptor; (2) a signal is triggered that is transferred to the cell nucleus; (3) transcription of lymphokine-encoding mRNA occurs; and (4) intact lymphokine is

synthesized and released from the cell. In a 1987 study, Hodgkin and coworkers determined that CsA affects the synthesis of lymphokine encoding RNA by blocking transmission of the antigen signal that activates gene expression in the primed T-cell. Since CsA does not effect the synthesis or export of lymphokine from the activated T-cell (Tutschka et al., 1980; Hess et al., 1982), the drug must interfere with the transission of mitogenic signal to the nucleus of the cell (Hodgkin et al., 1987). However, CsA does not inhibit all the effects of antigen signaling; it appears to be specific for antigen or mitogen-triggered lymphokine production.

One study has suggested a possible intracellular mechanism whereby CsA could be having its affect (Hess and Colombani, 1986). Increased intracellular calcium secondary to mitogen binding and calcium ionophore stimulation is not inhibited by CsA, and this resultant influx of calcium activates calmodulin. However, activated calmodulin avidly binds CsA. Thus, CsA inhibits the ability of calmodulin to activate phosphodiesterase and possibly inhibits the calmodulin-dependent activation of protein kinases and other critical enzymes necessary for T-cell proliferation. Similarily, the presence of calmodulin in the nucleus may account for CsA's ability to inhibit gene activation or suppress activated genes. Therefore, it seems likely that CsA may block calmodulin-dependent inducible mRNA transcription by inhibiting the transduction signal.

To summarize, CsA, by blocking production of IL-2 and preventing clonal expansion of cytotoxic T-cells, inhibits the proliferation of T-cells typically associated with the rejection response.

Cyclosporine: Xenografts

Major types of transplants include isografts, allografts and xenografts. The most successful type of transplant is the isograft. An isograft is a transplant between a genetically identical donor and recipient. They include transplants between identical twins and from one part of the body to another.

An allograft is a transplant between individuals of the same species, but different genetic backgrounds. Examples of allografts include skin transplants and blood transfusions in humans.

A xenograft is a transplant between animals of different species. One must be aware that the further apart the genetic relationship between donor and recipient the less likely it is a graft will survive. With this in mind, Calne (1970) suggested that transplants between widely disparate species should be termed discordant xenografts (i.e. pig-to-dog) and transplants between closely related species should be termed concordant xenografts (i.e. chimpanzee-to-man).

The idea of transplantation across species goes back to pre-historic times, and it can be traced to various literary works in eastern and western mythology. More recently, xenografting in man was carried out in the early 1900's, these cases involved primarily kidney transplants from various donors including pigs, lambs, and monkeys. Needless to say, none of these recipients survived beyond a few days (Council report, 1985). By the 1950's, Medawar and associates (referenced in Council report, 1985) had established the immunologic nature of graft rejection and suggested ways to improve survival through immunosuppression. This led to a resurgence of interest in xenografts in humans. It is apparent that xenografts would provide easy availability of donors, thus alleviating some problems involved in organ procurement. Also, this ready availability of donors would allow the surgical procedures associated with organ transplantation to proceed in a selective, highly organized fashion (Counsil report, 1985). This is in contrast to organ transplantation today that requires almost immediate surgery as donor organs become available.

The use of CsA in heart xenografts has met with limited success. Homan et al., (1981) studied the effect of CsA in a heterotropic cardiac xenograft model in which hamster hearts were implanted into LEW rats. The results indicated that CsA, in doses approaching toxic levels (for rats) of 35 or 50 mg/kg/day for 14 days, prolonged function of the concordant xenografts (median survival time of 11 and 21 days, respectively). This is contrasted with the median survival of 2 days in untreated rats. However, only at the dose of 50 mg/kg/day did CsA significantly reduce histological features of rejection. It was concluded from this

study that the near toxic doses required for CsA to have an effect would make CsA of little value in transplantation of vascularized organ xenografts.

Perhaps the most famous case involving CsA and a xenograft was that of Baby Fae. This case involved transplantation of a baboon cardiac xenograft to a human neonate (Bailey et al., 1985). In this case graft failure appeared to have resulted from a "progressive, potentially avoidable humoral response," and showed only traces of cell-mediated rejection. This seems to indicate that graft failure was not due to ineffectiveness of CsA.

The effect of CsA on rat-heart xenograft to mouse, and mouse-heart to rat was also studied (Sugimoto et al., 1985). Results indicated CsA, at 20 mg/kg/day in the rat-heart to mouse model, significantly increased survival of the heart xenograft (14.4 +/-3.2 days) compared to controls (5 days). A dose of 50 mg/kg/day in the same model resulted in high mortality (3 of 5 mice died in less than 5 days). However one mouse survived the 14 day CsA course, resulting in prolongation of its rat-heart xenograft to day 27. In contrast, results of the mouse-heart xenograft to rat showed that CsA had no effect in increasing its survival compared to controls. These researchers concluded that CsA, though effective in some allograft survival, is only marginally effective in prolonging cardiac xenografts in rodents.

The limited success of these cardiac xenografts CsA has led to studies in which CsA was used in combination with other immunosuppressive regimes

(Reichenspurner et al., 1986; Knechtle et al., 1986a; Sadeghi et al., 1987; Reemtsma and Rose, 1987; Adachi et al., 1987).

Using a monkey donor, and baboon recipients (Sadeghi et al., 1987), CsA-steroid treated baboons showed significant cardiac xenograft survival compared to controls (61 days vs 6 days). However, CsA and steroids given separately had no effect on xenograft survival. CsA used in combination with total-lymphoid irradiation (TLI) was shown to markedly prolong (>100 days) survival of cardiac xenografts in closely related species (Knechtle el al., 1987). These researchers also concluded that choice of species critically affected xenograft survival.

In 1985, Nakajima and co-workers looked at the effect of CsA on hamster pancreatic islet graft survival in rats injected intrahepatically with the islets. Consistent with findings of Homan et al. (1981), results indicated that in xenotransplantation, high doses are required to prevent graft rejection. However, despite continuous CsA treatments, restoration of T-cell responsiveness to IL-2 was found and this recovery probably led to cell mediated islet xenograft rejection. In a similar study Selawry et al. (1986), xenografted hamster islets into CsA treated BB/W rats (spontaneously diabetic strain of rat). However, in contrast with Nakajima et al., islets were placed in the cryptorchid testis as well as in the liver. This result supported an earlier study (Selawry and Whittington, 1985), which showed that organ site used for injection of isolated adult islets affects the outcome of allografts in minimally immunosuppressed rats. Results from this study also indicated that islet xenograft survival can be achieved when

transplants are placed inside the cryptorchid testis of the CsA immunosuppressed BB/W rat, but are consistently rejected when placed in the liver. One result of particular interest from this study that was the apparent differential side effects of CsA depending upon the organ site used for the injection of the islet xenograft. Those animals which received transplants into the liver were especially susceptible to the toxic effect of CsA.

Perhaps the most relevant study to our proposed model, investigated the effect of CsA in human to rat skin xenografts (Biren et al., 1986). Animals were maintained on CsA 25 mg/kg/day for 50 days followed by 12.5 mg/kg 2 times per week. Controls received an equivalent volume of vehicle. All animals receiving split-thickness grafts and treated with CsA, maintained their grafts significantly longer (up to 255 days) than controls (5 to 10 days).

Xenografts: Athymic Nude Mouse

To my knowledge no one has transplanted mammary tissue into mice treated with CsA. However, the athymic nude mouse, a mutant born without a thymus and thus without a completely functional immune system, has been used as a "living" incubator to study the growth of xenotransplanted mammary tissue

(Welsch et al., 1979; Sheffield and Welsch, 1986; McManus and Welsch, 1981; McManus et al., 1978; Outzen and Custer, 1975; Jensen and Walling, 1976; McManus and Welsch, 1980).

This mouse, because of its unique qualities, has offered researchers an opportunity to study the growth and morphogenesis of cancerous human breast tissues. A "living test tube" of this type is needed because experimental investigation of neoplastic progression in humans is limited by ethical and moral restrictions. As mentioned earlier, the study of growth and differentiation at the cellular level in the bovine mammary gland in vivo is difficult and expensive. Thus, the nude mouse provides researchers opportunities not feasible in the past.

Cleared mammary fat pads (CFP) of nude mice, were used to determine if this site was receptive to normal and neoplastic human mammary tissues (Outzen and Custer, 1975). Human breast biopsy specimens, three nonmalignant but abnormally proliferating and one a mammary adenocarcinoma, were transplanted into CFP of nude mice. Results indicated that in each case the mammary explant was not only maintained but proliferated and spread into the host's fat pad stroma.

In another study (Jensen and Wellings, 1976), 217 transplants from 19 cancer associated and 13 non-cancer associated human breasts were transplanted into CFP of athymic nude mice. Of these, 151 survived and either maintained original morphology or showed some degree of differentiation. McManus and co-workers (1978) took biopsy specimens from patients with benign human breast tumors,

and placed them subcutaneously (s.c.) in athymic nude mice. All xenografts were accepted and maintained. In addition, treatment with human placental lactogen was found to directly stimulate DNA synthesis as indicated from tritiated thymidine incorporation into ductal epithelium. Similarly, McManus and Welsch, (1981) showed that xenogafted normal human breast tissue exhibited increased DNA synthesis in response to estrogen (E) and implanted rat pituitary tumor (secretes large amounts of prolactin and growth hormone).

These studies indicate that not only is the athymic nude mouse able to maintain the normal morphology of human mammary xenografts, but transplanted tissue is capable of responding in predictable fashion to hormone stimulation.

Welsch et al., (1979) transplanted mammary tissue slices from a pregnant heifer into female athymic nude mice. Treatment of the host animals with E, progesterone (P), bovine growth hormone (GH), and bovine prolactin (Prl) resulted in a significant increase in bovine mammary epithelial DNA synthesis. Subsequent treatment with hydrocortisone, GH, and Prl induced alpha-lactalbumin synthesis in the grafted tissues. Sheffield and Welsch (1986) extended these observations by transplanting mammary epithelium from five Holstein cows as s.c. slices or as collagenase dissociated epithelial cells into CFP of nude mice. In each experiment mammary tissues maintained normal morphology, while cells injected into CFP formed hollow, multilayered, spherical "organoids". Autoradiography, used to visualize [^{3}H] thymidine labeled cells,

indicated growth of epithelium within slices and organoid areas of hormone treated host animals.

Mammary Isografts in Mice

Hoshino (1962) described the growth potential of normal mammary isografts in virgin mice. Survival of transplants within genetically similar strains of mice was not affected by age or sex of donor or tissue site of transplantation. The extent of growth of transplants was however influenced by 1) amount of adipose tissue at transplantation site, 2) hormonal environment, and 3) period of transplantation.

In 1970, Hoshino reported that mammary tissue, isografted from adult male donor mice into mammary gland free fat pads of adult female hosts regenerate and differentiate in successive stages morphologically and functionally indistinguishable from the intact mammary tissue of the host. Hoshino (1983) subsequently showed that mammary tissue rudiments from 9 day old neonatal mice grew and became morphologically fully differentiated when transplanted into pregnant hosts.

These studies indicate that the isografted mouse mammary tissue is not only capable of surviving, but also can grow, differentiate, and synthesize milk components. Thus, results from these studies bode well with the idea of doing similar studies with xenotransplanted mammary tissue.

Rejection

Disparate skin grafts are typically rejected in mice via the lymphocytic or the vascular pathway. In lymphocytic rejection, host lymphocytes infiltrate the graft and gradualllly destroy its epithelial components. In vascular (hyperacute) rejection, infiltrating lymphocytes are few or absent; however, the graft vessels are engorged with red blood cells, and hemorrhages are widespread. The epithelial elements necrose, as a result of ischemia. Both modes of rejection are considered T-cell mediated (Eichwald et al., 1985).

The term hyperacute rejection has traditionally been applied to organ transplants in which rejection is apparent within 1 hour after connecting a blood supply. Hyperacute rejection is always associated with a presensitized recipient and obstructive vascular damage (Williams et al., 1973). A similar phenomenom is seen with skin allografts in mice. The rejected grafts described as "white grafts" are considered an analogue of hyperacute rejection in organ transplants (Kissmeyer-Nielson et al., 1966). Hyperacutely rejected organs and white grafts

both feature a sensitized host, vascular damage and rapid rejection. However, organs are rejected hyperacutely through a complement mediated mechanism via binding of serum antibody-antigen complex to the endothelium of the transplant, whereas the white graft reaction is cell mediated (Eichwald and Dolberg, 1977).

White grafts are associated with failure to form a blood supply from host to graft because of obliterative changes in graft vessels. This is especially likely if the genetic barrier between the donor and host is large.

Rapid rejection of skin grafts by hosts, accompanied by vascular damage to the graft is seen in other forms of hyperacute rejection of skin grafts. Such grafts are characterized by hemorrhage and necrosis, and because of their gross and microscopic appearance, they are referred to as red grafts (Eichwald et al., 1966). Thus Reisfeld and Kahan (1970) stated that skin graft rejection could be exibited as responses ranging from accelerated lymphocytic infiltration to necrotic red grafts to white grafts.

To better characterize events associated with rejection Eichwald and Dolberg (1977) sensitized mice by injecting spleen cells 5 days prior to allografting skin. After 6 days, grafts were removed and evaluated histologically. Results indicated that red graft vessels became markedly dilated and massive hemorrhage took place within the graft. Microscopically the venular and capillary endothelial cells were destroyed and erythrocytes were in direct contact with the basement membrane. Endothelial cell remnants were shredded and fragments of shed

endothelium appeared to float freely within the vascular lumen. White grafts seemed to be rejected quicker in sensitized mice compared to weaker sensitizaton which seemed to be associated with red graft rejection.

Rejection of cardiac xenografts in rats was determined to be complete when palpation of the thoracic cavity indicated the graft was no longer beating (Homan et al., 1981; Knechtle et al., 1987). Histological examination of the rejected grafts showed interstitial hemorrhage, edema, myocardial necrosis, mucoid degeneration, and neutrophilic and mononuclear cell infiltration.

Rejection of pancreatic islet xenografts in rats was determined when rats became hyperglycemic (Selawry et al., 1986; Nakajima et al., 1985). Histological examination of the rejected grafts indicated cell-mediated rejection response.

In more closely related work, Biren et al. (1986) indicated that CsA treated rats receiving full-thickness human skin xenografts did not maintain their grafts any longer than controls (mean 6.4 days). Controls showed visible signs of immunologic rejection (edema, dusky color) and both CsA and controls showed histological evidence of epidermal death. However, rejection of full thickness grafts in CsA treated mice was thought to be a result of insufficient vascularization and resultant ischemia rather than overt immunological rejection.

In contrast CsA treated rats maintained split-thickness skin grafts for prolonged periods compared with controls. In fact, no grafts were rejected in CsA treated

rats receiving split-thickness grafts (i.e. all rats died naturally 72-255 days post-transplantation with viable grafts intact). Autoradiographic analysis of tritiated thymidine incorporation in incubated biopsy specimens of grafts at several periods indicated labeling rates of 3-4% until the animals died.

MATERIALS AND METHODS

Experiment 1

Objectives:

1. To determine whether ovine mammary tissue zenografted is capable of surviving in CsA mice.

2. To determine the most appropriate site of implantation of these xenografts.

Preparation of Donor Tissue

A female lamb (approximately 4 months of age) was sacrificed. Immediately following sacrifice, the udder was removed and transported to the lab in a insulated container (approximately five minutes). Mammary parenchyma was located in the excised glands, aseptically removed and placed in a container containing sterile medium 199 (Gibco; Grand Island Biological Co., Grand Island, NY) supplemented with penicillin (100 units/ml, streptomycin (100 ug/ml), and fungizone (0.25 ug/ml)(MAB). Under a laminar flow hood, mammary tissue was aseptically cut into approximately 0.2 mm thick slices (approximately 2 x 2 cm) using a Stadie-Riggs hand microtome (Thomas Scientific Co.; Philadelphia, PA). Slices were then cut into pieces approximately 2 x 2 mm and placed in sterile petri dishes with MAB on a 37 C warming plate. Pieces were then aseptically rinsed two times with sterile MAB and maintained in fresh MAB at 37 C until transplanted (generally no longer than 1h).

Preparation of Recipients

Eighteen three week old female mice (IRC albino white mice) were anesthetized with metofane (methoxyflurane; Pitman-Moore, Inc., Washington Crossing, NJ), and the epithelial portion of the fourth inguinal mammary glands were surgically removed as described by De Ome et al. (1959). This left a gland free fat-pad (MGF) with an intact blood supply as a transplantation site. Mice were allowed

to recuperate for at least two weeks. Intact mice of similar age (3 to 4 months) were obtained for use as recipients of transplants to ovarian fat-pads (n = 18) or dorsal subcutaneous region (n = 18).

Surgeries

Concurrent with donor tissue preparation, 54 mice were prepared for surgery. All surgical procedures were aseptically performed under a laminar flow hood. Mice were anesthetized with metofane during surgery.

Mice were chosen at random and 2 mammary xenografts were placed subcutaneously in each of the 18 mice. After cleaning the incision site with 70% ethanol (V/V; in water); a 1 cm incision was made on the midline through the skin on the dorsal body surface, approximately 1 cm posterior to a line between each scapula. Through this incision, two small pockets were made laterally using a blunt probe. Small forceps were then used to insert 1 explant in each pocket. Making sure that the explant was not "squeezed" back up to the incision, the skin was then pulled back together, and the incision closed with a 9 mm stainless steel wound clip.

Incisions were made along the dorsal mid-line of the lumbar region of mice that received xenografts in the ovarian fat-pad. This incision was made through the skin, and internal and external abdominal oblique muscles, to expose the ovarian

fat-pad. A small incision was made in the fat-pad using a micro scapel and the explant was inserted using small forceps. The incision through the two muscle layers was sutured with 4/0 chromic gut, and the skin was closed using a 9 mm stainless steel wound clip.

In the MGF mice, an incision was made to re-expose the cleared mammary fat-pad. The same procedure was used for placing grafts in the MGF as was used in the ovarian fat-pad. Incisions in the skin were closed with 9 mm stainless steel wound clips.

Assignment to Treatments

After surgery mice in each transplant classification were randomly separated into three groups (to be sacrificed on day 7, 21, and 35) of six each. Of these six, four mice were selected for CsA treatment (8 mg/kg/day) and two were selected as controls. Powdered CsA (0.240 g) (OL 27-400, batch # 84097, Sandoz Inc., Pharmaceutical Division; East Hanover, NJ) was dissolved in 10 ml of Tween 80 (Polyoxyethylene Sorbitan Mono-oleate, Sigma Chemical Co.; St. Louis, MO) and 40 ml anhydrous alcohol (final concentration was 4.8 mg/ml). Injections (50 ul) were administered subcutaneously (Black et al., 1985). Controls received a comparable injection of excipient. (See Appendix A)

Xenograft Recovery

Mice were sacrificed via cervical dislocation on 7, 21, and 35 days post-transplantation and xenografts were recovered. Six per classification (i.e. 4 from CsA and 2 from controls) were killed on each of the previously specified days.

After sacrifice, mice were pinned out on a dissection tray, and an incision was made along the previous incision in mice with subcutaneous xenografts. This incision was then extended along the spinal column cranially to the base of the skull and caudally to approximately the first lumbar vertebra. The skin was then reflected to expose the explant. Location of the explant was variable (less than 1 cm from original incision to >3 cm). Explants were removed from the mouse by picking up the connective tissue surrounding the xenograft with mouse tooth forceps and dissecting the explant free using iris scissors.

Mice with xenografts in the ovarian fat-pad were pinned out on a dissecting tray with the ventral body surface exposed. An incision was then made down the mid-line through the skin and under-lying muscle. The ovarian fat-pad was located; using forceps and iris scissors, the ovary and oviduct was dissected from the fat-pad. Gentle palpation of the fat-pad revealed the location of the explant, then most of the surrounding fat was removed leaving the xenograft.

Mice with xenografts in the MGF were also pinned with the ventral body surface exposed. An incision made through the skin re-exposed the fat-pad and gross

examination of the fat-pad revealed the location of the xenograft for subsequent removal. Iris sissors were used to dissect the tissue free from the adipose tissue.

Histological Analysis

Excised xenografts, as well as samples of explants prepared for transplantation, were fixed in modified Karnovsky's fixative (Karnovsky, 1965) for 24 h, then stored in .1 M phosphate buffer, pH 7.3. Tissues were dehydrated through a graded series of ethanol, then embedded in HistoResin (LKB 2218-500 HistoResin) embedding medium (LBK Inc.; Bromma, Sweden). Xenografts were sectioned using a MT 1 ultramicrotome (Sorvall, Newton, CT). Sections were stained with Azure II (Jeon, 1965) for histological observation. Explants not used in transplantation were also fixed, sectioned and stained as described above and served to characterize the morphology of the tissue at the time of implantation.

After slides were coded to prevent observer bias, epithelial cell morphology of histological sections were graded. Grading was based upon a numerical scale that ranged from 1 (poor) to 5 (excellent). Examples corresponding to each grade are illustrated in Figure 1. An overall impression of the epithelium was determined by looking at all areas of epithelium in each piece of tissue in a section (at 100X magnification). This grading system was a modification of the one used in experiment 3 and accounted for morphological differences in 0-time tissue.

Experiment 2

Objectives:

1. To determine whether mammary tissue from a 85d pregnant ewe xenografted into cyclosporine treated mice will respond to exogenous and endogenous hormonal stimulation.

Preparation of Recipients

Mammary gland fat-pads were cleared in forty mice as described in experiment 1.

Donor Tissue

A pregnant (approximately 85 days), non-lactating western cross bred ewe was sacrificed. The udder was removed and explants were prepared as described previously. Previous work (Smith, 1985) has shown that ovine mammary tissue undergoes a period of exponential growth from day 80 to 115 of pregancy.

Therefore, donor tissue taken at day 85 was considered hormonally "primed", and would respond readily to hormonal stimulation.

Surgeries

Each of the 40 mice received two xenografts, one in the MGF and another in the ovarian fat-pad as described in experiment 1. Ten of the 40 mice received a estrogen + progesterone (designed to release 1 ug/day and 1 mg/day of estrogen and progesterone, respectively) and a prolactin implant (designed to release 0.24 mg/day, NIH B6) (Inovative Research, Rockville, MD; Catalog # HH111)) at 30 days post-transplantation. A 1 cm incision was made through the skin on the midline of the ventral body surface at the base of the sternum after the area had been cleansed with 70% ETOH. A blunt probe was inserted into the incision and a small pocket was made anteriorly. Small forceps were used to place the implants in the pocket. A 9 mm stainless steel wound clip was used to close the incision.

Assignment to Treatments

Immediately following surgery, all mice started daily injections of CsA (8 mg/kg/day) as described previously. After 30 days (post-transplantation) 10 mice

were randomly selected to receive hormonal implants as described previously. In addition 10 mice were also selected to be bred on day 30 (daily injections of CsA were continued on all mice until sacrifice). (Appendix B)

Xenograft Recovery

Twenty mice were sacrificed on day 30, 10 mice that received hormonal implants were sacrificed on day 51, the remaining mice were sacrificed within 24h of the time their pups were born (four mice apparently not pregnant were sacrificed on day 51). All xenografts were fixed, embedded, stained and evaluated as described in experiment 1.

Experiment 3

Objectives:

1. To determine whether ovine mammary tissue xenografted into CsA treated mice (high dose) is capable of surviving.

Hormone Pretreatment of Donor

A multiparous, nonpregnant, nonlactating western cross-bred ewe was pretreated with daily injections of estradiol 17-B plus progesterone (0.1 and 0.25 mg/kg/day) for seven days, followed by three days without injection (McFadden, 1985). Estradiol (250 mg) and progesterone (625 mg) (Sigma Chemical Co., St. Louis, MO) were dissolved in 20 ml of benzlbenzoate (Sigma) over low heat and mixed with 80 ml of corn oil to achieve a 20% benzylbenzoate solution (2.5 mg/ml estradiol; 6.25 mg/ml progesterone final solution concentration). The daily dose of steroids consisted of a 3 ml volume (ewe body weight was 75 kg) of this solution given via subcutaneous injection on the ventral body surface, under right and left fore legs, alternately. The ewe was sacrificed on day ten after the initial injection.

Preparation of Donor Tissue

Immediately following sacrifice, the udder was removed and transported to the lab and prepared into explants as described in experiment 1.

Surgeries

Concurrent with donor tissue preparation, forty-two virgin female mice (approximately 6 months of age) were prepared for surgery. Mice were chosen at random and 2 mammary xenografts were placed subcutaneously in each of the 42 mice as described in experiment 1.

Twenty-one of the 42 mice also received estrogen + progesterone implants as described in experiment 2. This same procedure was repeated at 21 days post-transplantation for 3 mice scheduled for sacrifice at +35 days.

Assignment to Treatments

Immediately following surgery 15 of the nonhormone-implanted mice were randomly chosen to receive CsA (OL 27.400 Cyclosprorine, Batch 093L5), and 15 of the hormone-implanted mice were randomly chosen to receive CsA. The remaining six nonhormone-implanted mice and six hormone implanted mice were used as controls. (Appendix C)

Cyclosporine Dose and Administration

CsA, dissolved in olive oil as obtained from Sandoz Pharmaceuticals (100 mg/ml), was administered daily in a 40 ul injection volume. Dose was approximately 100 mg/kg of body weight for the first seven days and was reduced to 70 mg/kg thereafter. Daily injections were administered subcutaneously, taking care to avoid areas of xenografted tissue or hormone implants. CsA doses were increased in an attempt to overide histological features of rejection (Homan et al., 1981).

Xenograft Recovery

Mice were sacrificed via cervical dislocation on 3, 7, 14, 21, and 35 days post-transplantation and xenografts were recovered as described in experiment 1. Three mice per group (i.e. 3 from CsA and 3 from CsA plus hormone implant) were killed on each of the previously specified days. Three control mice from each hormone and nonhormone treated groups were killed on days 7 and 21 post-transplantation.

Histological Analysis

Excised xenografts were fixed, embedded, and stained as described in experiment 1. Sections (about 2 um thick) were cut on a Leitz Rotary microtome (Wetzler, West Germany) modified to accept long edge glass knives, and three to four sections were mounted per slide. Explants not used in transplantation were also fixed, sectioned and stained as described earlier and served to characterize the morphology of the tissue at the time of implantation (0-time).

After slides were coded to prevent observer bias, epithelial cell morphology of histological sections were graded. Grading was done on a numerical scale that ranged from 1 (poor) to 5 (excellent). An overall impression of the epithelium was determined by looking at all areas of epithelium in each piece of tissue in a section (at 100X magnification). Epithelium in apparent ducts was used in determining the numerical grade for the tissue sample. Epithelium in larger ducts and presumptive gland cistern was not utilized in the grading procedure, due to inconsistent morphological appearance of epithelium in large ducts of 0-time tissue.

Examples of each of the five epithelial scores are shown in Figure 1. Xenografts receiving a grade of 5 (Figure 1A) were characterized by ductal epithelium that exhibited normal morphology. Epithelial cells were normal in shape and organization, they showed no indication of vacuolization and no apparent cell lysis. This epithelium was indistinguishable from epithelium seen in 0-time tissue.

Figure 1. Photomicrographs of tissue that is representative of the five epithelial scores used in evaluating xenograft epithelium (600x).

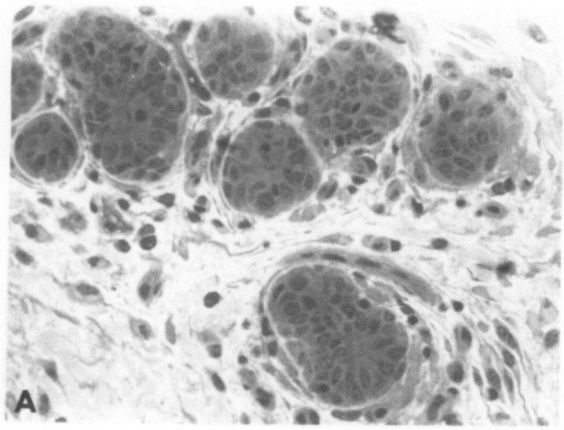
Figure 1A. Representation of epithelium that typically received a score of 5, characterized by normal, non-secretory, ductular epithelium. The epithelial cells are typically compact with irregular shaped nuclei, a small nuclear to cytoplasmic ratio and relative absence of cellular vacuoles. (This tissue also represents the appearance of tissue at the time of transplantation in experiment 3)

Figure 1B. Representation of epithelium that typically received a score of 4, characterized by normal, non-secretory, ductular epithelium and a small amount of vacuolization at the basal end of the epithelial cells.

Figure 1C. Representation of epithelium that typically received a score of 3, characterized by epithelial cells with a considerably greater amount of cellular vacuolization.

Figure 1D. Representation of epithelium that typically received a score of 2, and was characterized by some loss of ductular organization, occasional cell degeneration, cell lysis and presence of scattered pycnotic nuclei within the epithelial lining.

Figure 1E. Representation of epithelium that typically received a score of 1, characterized by epithelial cells that have degenerated, presence of lysed cells, cellular debris and general loss of ductular organization.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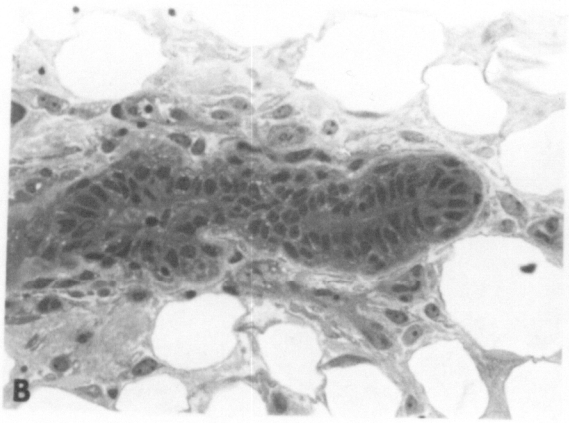
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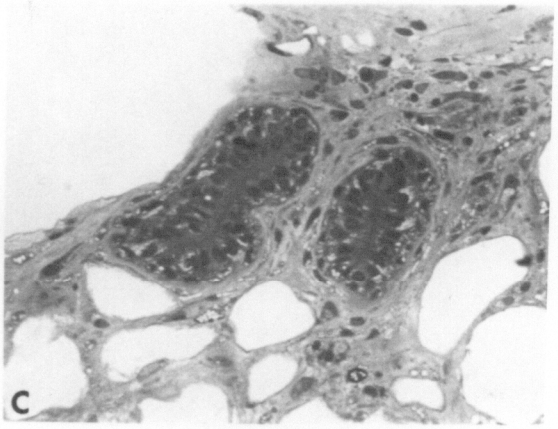
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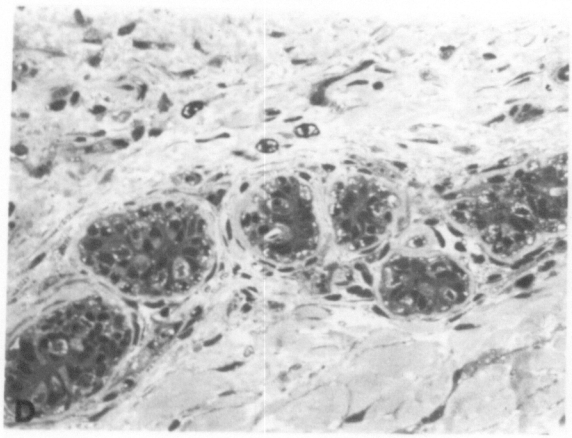
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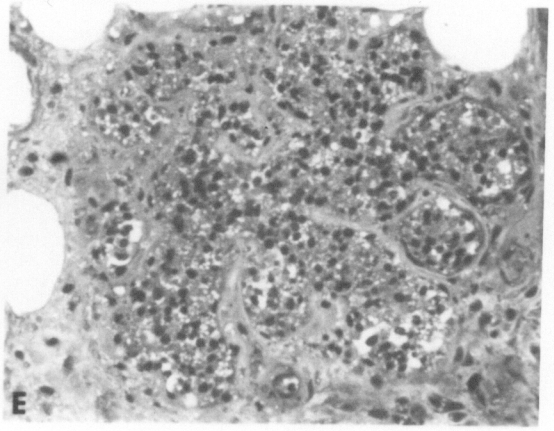
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Epithelium receiving a grade of 4 (Figure 1B) was similar to that receiving a grade of 5; however, there was a small amount of vacuolization seen in the basal end of the cell, and nuclei appeared normal and ductular organization was intact. In grade 3 (Figure 1C) epithelial cells exhibited considerably greater occurence of vacuolization, giving the cells a lacy appearance adjacent to the basement membrane. A loss of some ductular organization, with apparent areas of epithelial cell lysis, presence of pycnotic nuclei, and areas of basement membrane denuded of epithelial cells characterized epithelium receiving a grade of 2 (Figure 1D). Individual epithelial cells were difficult to recognize, pycnotic nuclei were common and ductular organization was disrupted in xenografts graded 1 (Figure 1E).

Leucocyte infiltration was also determined in the subepithelial stroma of histological sections. A 10 x 10 um grid placed in the eye piece of an Olympus BH-2 microsope (Olympus Corporation, Precision Instruments Division; Lake Success, NY) was used to determine leukocyte numbers in a defined area of stroma (examined at 400x).

Cell counts were determined by placing the edge of the grid on the outer edge (adjacent to the basal lamina) of epithelium, making sure that at least half of the grid was transposed over stromal tissue directly adjacent to the epithelium. Leucocytes quantified in the transcribed area included lymphocytes, polymorphonuclear leucocytes (PMN), mast cells, plasma cells, and macrophages.

In each coded slide, three randomly chosen areas of the subepithelial stroma were evaluated. Total tissue area quantified was 117,600 um^2 per sample.

LONG-TERM CULTURE

Objectives:

1. To compare the morphology of ovine mammary epithelium from long-term culture to that recovered from cyclosporine treated mice at similar times (day 3, 7, 14, 21, and 35).

Hormone Pretreatment of Donor

Donor tissue was the same used in experiment 3.

Preparation of Donor Tissue

Preparation of donor tissue was the same as experiment 3, except Stadie-Riggs slices were cut into strips approximately 3 x 10 mm.

Culture Medium

The medium utilized in explant culture was medium 199 containing Earle's salts, sodium acetate (10 mM) sodium bicarbonate (26.2 mM), L-glutamine, and 25 mM Hepes buffer (Gibco). This medium was supplemented with Penicillin G (100 Units/ml of medium), Sodium Streptomycin Sulfate (100 ug/ml of medium), and Fungizone (Amphotericin B, 2.5 ug/ml of medium). In addition, 5% bovine serum (Hyclone Laboratories; Lot # 1111540, Logan, UT) was added (50 ul/ml of medium). Final culture media was prepared by adding bovine insulin (0.5 ug/ml; Eli Lilly and Co., Indianapolis, IN), L-triiodothyronine (T3) at 1 ng/ml of medium, estradiol 17-B at 1 ug/ml, and progesterone at 0.1 ug/ml of medium (McFadden, 1985). Unless indicated otherwise, reagents were obtained from Sigma Chemical Company (St. Louis, MO).

Culture Incubation Protocol

Fifteen tissue strips were placed inside sterile stainless steel grids previously molded into envelopes (1 strip per envelope). The tissue filled envelopes were then inserted inside sterile plastic 15 ml culture tubes containing 8 ml of sterile medium as described. The envelope was placed mid-way in the tube and remained there during incubation. An atmosphere of 57% N_2, 5% CO_2, and 38% O_2 was placed over the media and the tubes were capped. Tubes were then

placed on a Thermolyne Speci-Mix tube rocker (Thermolyne Corporation, Dubuque, Iowa) in a 37 C incubator.

Tubes were removed from the incubator every 7 days and media changed. Under a laminar flow hood, old media was aspirated with sterile Pasteur pipets and replaced with fresh, sterile media. The atmosphere was restored and the tubes were returned to the incubator. Tissues and media were handled aseptically. Procedures were essentially as described by Harbell et al. (1977).

Histological Evaluation of Cultured Tissue

Tissue was harvested on 3, 7, 14, 21, and 28 days after start the start of incubation. Strips were fixed in modified Karnovsky's fixative for 24 h, then embedded, sectioned, processed and evaluated as described earlier.

Statistical Methods

Because of clear negative results obtained in experiments 1 and 2, statistical analysis was not done. In contrast, data from experiment 3 was statistically evaluated as described below.

Dependent variables were analyzed using the general linear models (GLM) procedure of SAS (SAS, 1982). Two (day 7 and 21) of the five time periods which

included mice receiving all four treatments (CsA + E/P, CsA, control + E/P, and control), these were analyzed separately and orthogonal contrasts were used as a mean separation procedure (McGilliard, 1987). Contrast 1 compared the mean of CsA + E/P and CsA to the mean of control + E/P and control for epithelial score, PMN, lymphocyte, plasma cell, mast cell, and macrophage number. Contrast 2 compared the mean of CsA + E/P and the mean of CsA for all measurements. Contrast 3 compared the mean of control + E/P and mean of control for all measurements. A second analysis was done to allow comparison of the two treatments involving CsA that included all sample times.

Correlations between lymphocyte number and epithelial score, and PMN number and epithelial score for both treatments containing CsA over all times were also calculated using SAS (Pearson correlation coefficients). Pearson correlation coefficients were calculated for lymphocyte number and epithelial score, and PMN number and epithelial score for all treatments irrespective of sample time.

Because the major interest of this study was to determine whether xenografted tissue could survive in CsA treated mice, epithelial scores for both CsA treatments were compared to 0-time tissue using Dunnett's test (Gill, 1978) for comparisons at each of the five time periods.

RESULTS

Experiment 1 and Experiment 2

Mean epithelial score for all sample treatments at all times (regardless of transplantation site) was 1, with the exception being CsA treated mice at day 7 post xenografting in experiment 1. In this treatment group, five subcuataneous xenografts were recovered from 3 mice, of these five, four had epithelial scores of 1, and one, a score of 2.

Examples of 0-time tissue, and tissue taken at 7, 21, and 35 days following subcutaneous transplantation for experiment 1 are shown in figure 2. Tissue representing 0-time (Figure 2A) was typically comprised of ductular epithelium distributed sparingly throughout a network of stromal and adipose tissue. In xenografts recovered on days 7, 21, and 35 (Figure 2B, 2C, and 2D), epithelium typically showed a progressive loss of cellular organization, cell lysis with presence

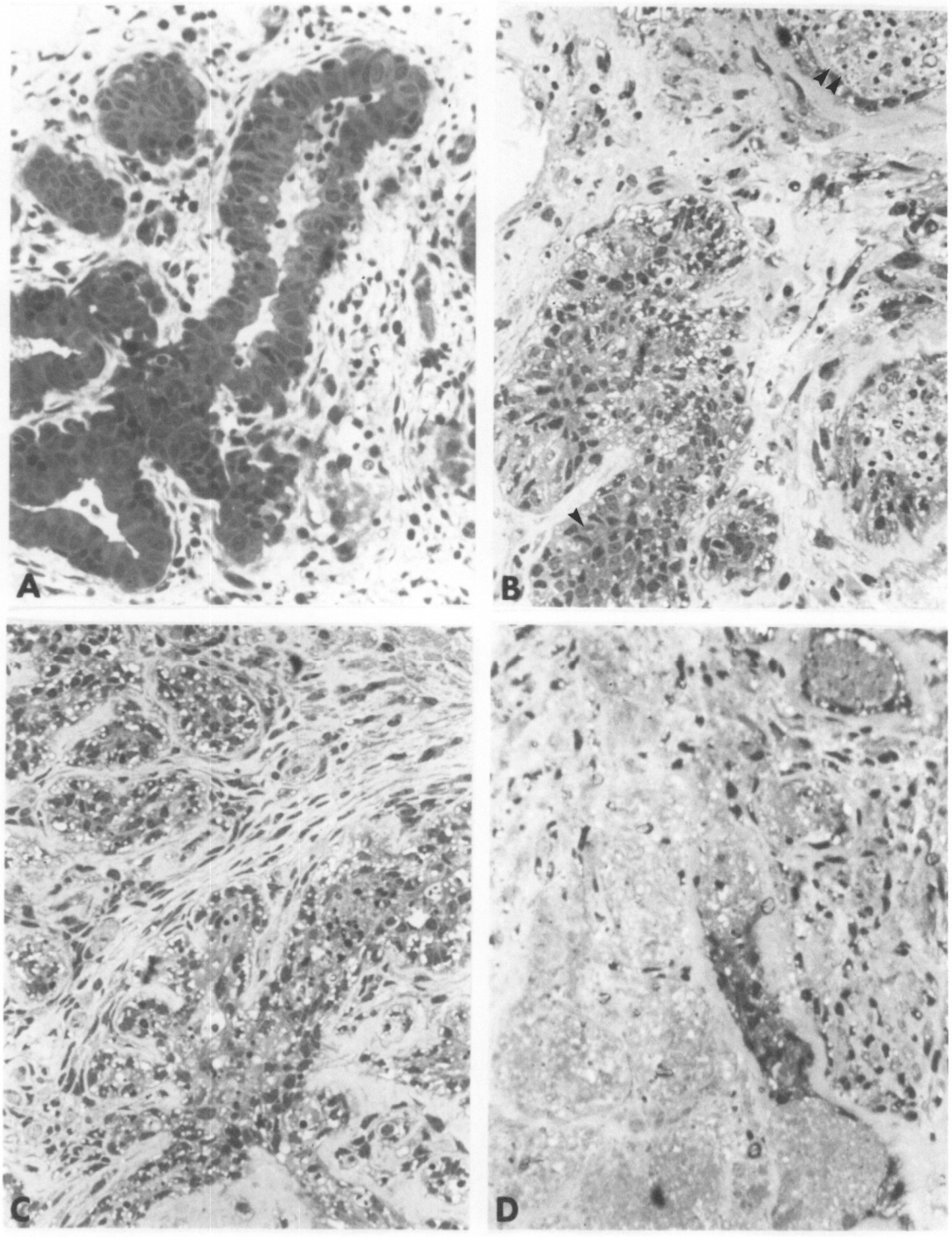
Figure 2. Photomicrographs representing 0-time tissue, and xenografted tissue taken at day 7, 21, and 35 in experiment 1. (600x)

Figure 2A. Tissue prepared at 0-time, characterized by non-secretory ductular epithelium.

Figure 2B. Ductular epithelium xenografted subcutaneously in a cyclosporine treated mouse for 7 days, characterized by presence of areas with heterogenous epithelial cell morphology. Some areas exhibited intact epithelial cells with limited vacuolization (single arrow); while adjacent ductular remnants contained disorganized epithelial cells with pycnotic nuclei and luminal spaces filled with cellular debris (double arrows).

Figure 2C. Ductular epithelium xenografted subcutaneously in a cyclosporine treated mouse for 21 days. Morphological characteristics are essentially equivalent to those observed in xenografts recovered on day 7 post-transplantation.

Figure 2D. Ductular epithelium xenografted subcutaneously in a cyclosporine treated mouse for 35 days. Tissue is characterized by complete loss of cellular organization, presence of pycnotic nuclei, degeneration of epithelial components and almost unrecognizable ductular structure.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of abundant cellular debris, pycnotic nuclei, and a general degeneration compared with 0-time tissue. The degree of severity of these degenerative changes appeared to increase over time.

Figure 3 represents 0-time tissue and subcutaneous xenografts recovered on day 30 and 51 (mice received hormonal implants on day 30 or were bred) in experiment 2. The 0-time tissue taken from a ewe pregnant 85 days, was characterized by pronounced lobular-alveolar development. The individual epithelial cells were obviously secretory, with proteinous secretions in the lumen and lipid droplets in the cytoplasm. Xenografts, recovered on day 30 and 51, showed a complete loss of cellular structure and disappearance of alveolar or ductular organization regardless or treatment of site of transplantation.

Experiment 3

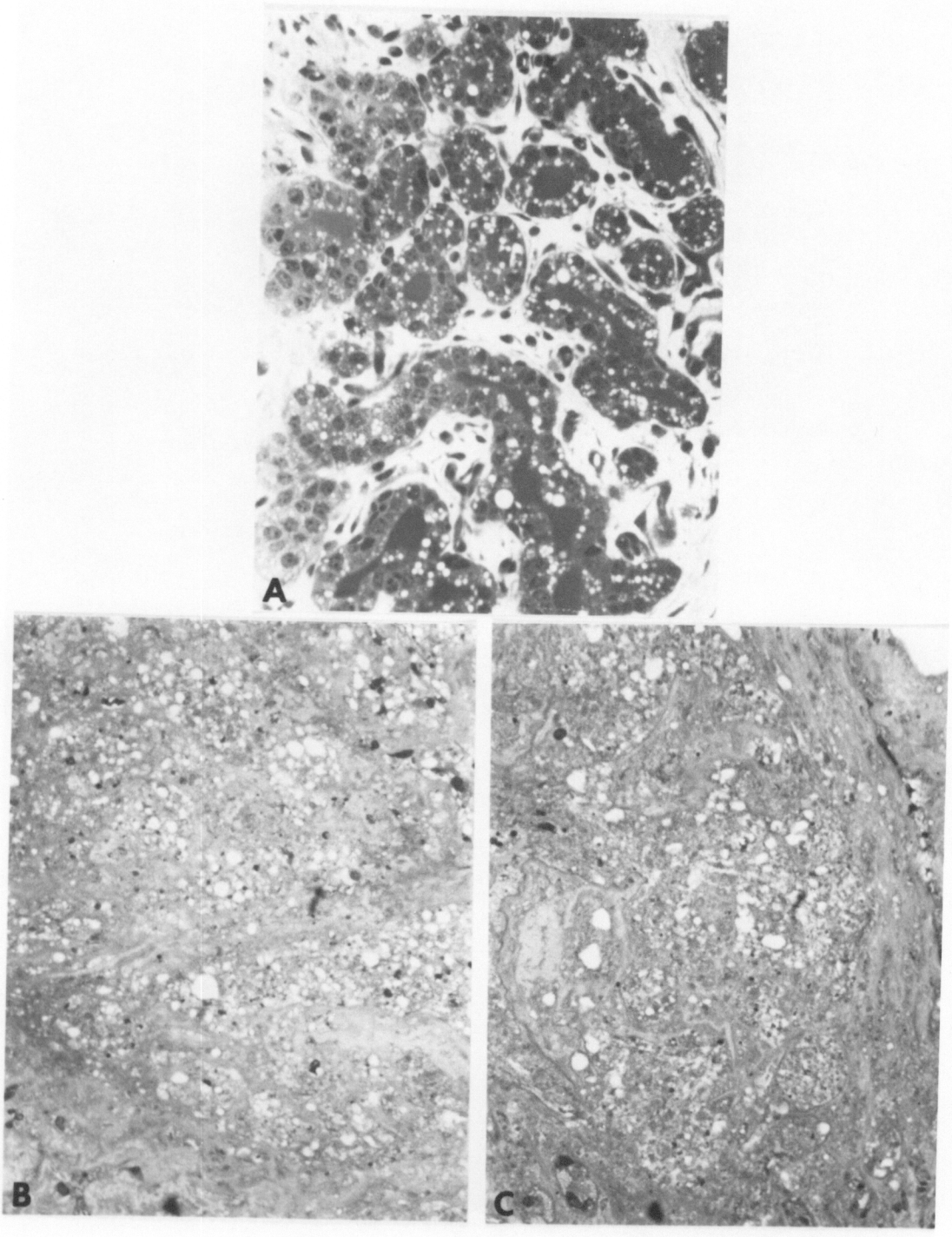
Forty of 42 mice that received xenografts survived and were sacrificed as scheduled. One mouse from the control group receiving E/P was found dead on day 18 (no xenografts were recovered), and a CsA + E/P treated mouse also died on day 25, again no xenografts were recovered (cause of death in both cases was not apparent). In 13 of the 40 surviving mice, only 1 of 2 pieces of xenografted tissue was recovered.

Figure 3. Photomicrographs of 0-time tissue and xenografts recovered on day 30 and 51 in experiment 2. (600x)

Figure 3A. 0-time tissue taken from a ewe pregnant 85 days, characterized by pronounced lobular-alveolar development. Epithelial cells frequently contain small lipid droplets and lumenal areas are densly stained. In comparison with the epithelial cells typical of tissue transplanted in experiment 1, the cells have rounded, basally displaced nuclei and a greater cytoplasm to nuclear ratio.

Figure 3B. Alveolar epithelium xenografted for 30 days in a cyclosporine treated mouse, characterized by complete degeneration of epithelial components and loss of cellular organization.

Figure 3C. Alveolar epithelium xenografted for 51 days in cyclosporine treated mouse that received a estrogen/progesterone and prolactin implant on day 30. The tissue is indistinguishable from that observed in CsA treated mice 30 days post-transplantation.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One CsA + E/P treated mouse, sacrificed at day 14, had a "yellowish" tint to the subcutaneous tissues and muscle tissue. All mice injected with CsA showed obvious signs of discomfort immediately following injection, these included increased respiratory rate and twisting of the body toward the injection site. Reactions subsided approximately 45 minutes post-injection. All CsA treated mice at the later time periods (14, 21 and 35 days) seemed to have experienced some weight loss, although no weights were recorded at the time of sacrifice.

Least squares means for epithelial score, and subepithelial totals for PMN, lymphocyte, plasma cell, mast cell and macrophage numbers for each treatment (+7 and +21 days post-xenografting) are shown in Table 1. Mean epithelial score for xenografts from CsA + E/P and CsA treatments was significantly greater ($P<.05$) than mean of control + E/P and control. Comparison of mean xenograft epithelial score for CsA + E/P and CsA indicated a significant increase ($P<.01$) due to the addition of E/P. Mean lymphocyte number for CsA + E/P and CsA was lower ($P<.07$) than in controls. Similarly, mean plasma cell number for CsA + E/P and CsA was also significantly lower ($P<.05$) than in controls. However, mean plasma cell number in CsA + E/P was greater than in CsA treated mice. Mice differed ($P<.01$) in mean epithelial score, mean lymphocyte ($P<.001$) and plasma cell number ($P<.05$) within each time and treatment combination indicating variations in capacity of individual recipients to "support" transplanted tissue regardless of exogenous treatments.

Table 1. Comparison of mean treatment effects[1]

	Treatment				Contrast[2]		
Measurement	CsA+E/P[3]	CsA	Con+E/P[4]	Con	1	2	3
Escore[5]	3.75	2.33	2.00	1.25	A	B	-
SE[6]	0.15	0.14	0.19	0.13			
PMNs[7]	23.33	36.25	39.17	41.08	-	-	-
SE	18.01	16.98	22.45	15.88			
Lymphocytes	13.08	34.75	44.08	87.92	C	-	-
SE	8.10	7.64	10.10	7.14			
Plasma cells	2.75	1.50	4.00	9.42	B	-	C
SE	0.66	0.63	0.83	0.59			
Mast cells	3.92	5.08	4.25	4.42	-	-	-
SE	2.19	2.01	2.73	1.93			
Macrophages	1.75	1.00	4.00	2.75	-	-	-
SE	0.62	0.58	0.77	0.55			

[1] Least squares mean of treatments.
[2] Contrast 1: mean of CsA+E/P and CsA vs mean of Con+E/P and Con.
2: CsA+E/P vs CsA.
3: Con+E/P vs Con.
[3] Cyclosporine + estrogen/progesterone.
[4] Control + estrogen/progesterone.
[5] Epithelial score.
[6] Standard error.
[7] Polymorphonuclear neutrophil leukocyte.

A,B,C - Significance of contrasts: A=P<.05, B=P<.01, C=P<.07.

Leucocytes were quantified in each of three randomly selected areas of subepithelial stroma in histological sections of each recovered implant. Data reported as number of cells per 117,600 um^2 of subepithelial stroma.

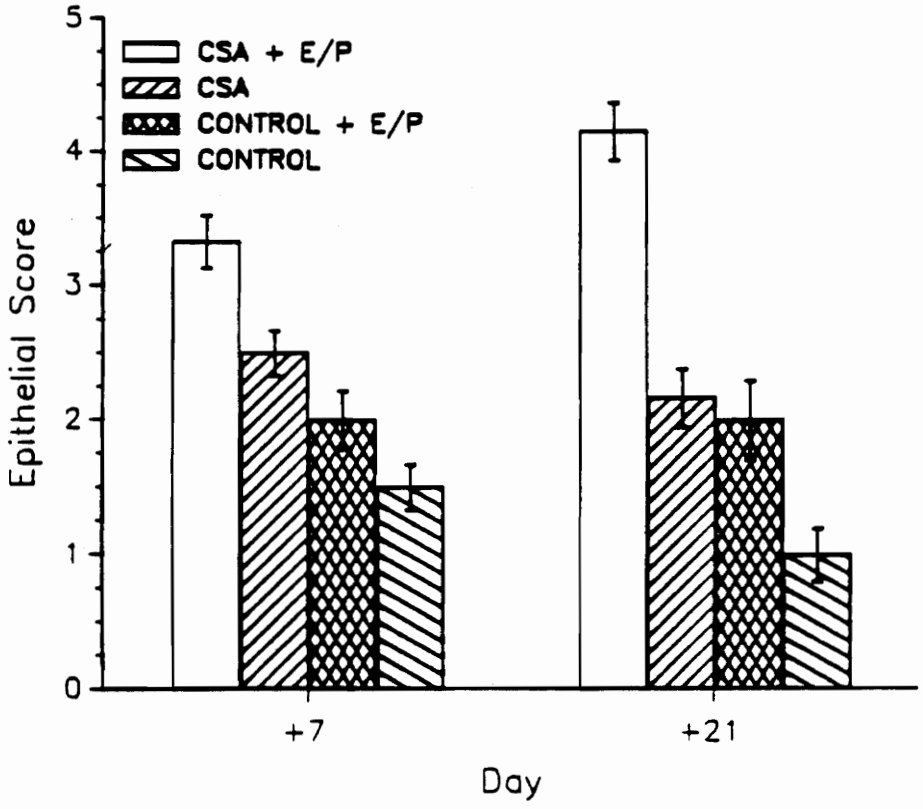

Figure 4. Mean epithelial scores of xenografts from cyclosporine + estrogen/progesterone (CsA + E/P), cyclosporine (CsA), control + estrogen/progesterone and control treated mice on day 7 and 21.

Xenografts recovered from mice treated with CsA + E/P had higher mean epithelial scores at day 7 and 21 than those from mice treated with CsA, control + E/P and control (Figure 4). Also, mean epithelial scores of xenografts at 7 and 21 days in CsA treated mice were higher than both control treatments. Statistical analysis comparing all 4 treatments were made at day 7 and 21 because there were no control treatments at the other time periods.

Morphological comparisons of xenografts recovered from CsA + E/P, CsA, control + E/P, and control treated mice at day 7 and 21 are shown in Figures 5 and 6, respectively. On day 7, ductular epithelium recovered from mice treated with CsA + E/P showed normal cellular organization (generally equivalent to 0-time tissue) and few if any signs of degeneration. CsA treated mice, although not as consistent as the CsA + E/P treated mice, also showed few signs of degeneration and little loss of cellular organization. This is in contrast to the control treatments, which showed frequent loss in cellular organization and varying degrees of epithelial degeneration. Again, as seen in the two CsA treated groups, xenografts from mice that received the hormonal implants tended to have better preserved epithelium. This pattern was also evident in comparison of xenografts recovered on day 21. That is, the mice receiving the hormonal implants had better preserved epithelium. Again, in the CsA + E/P treated mice, the epithelium was essentially indistinguishable from 0-time and frequently appeared to be better maintained than in xenografts recovered on day 7 and 14 in the same treatment group (see Figures 5 and 8). This was not the case for xenografts taken from CsA treated mice on day 21, their epithelial components, though not com-

Figure 5. Comparison of xenografts recovered from cyclosporine + estrogen/progesterone, cyclosporine, control + estrogen/progesterone and control treated mice at day 7 post-transplantationin experiment 3. (600x)

Figure 5A. Xenographs recovered from a cyclosporine + estrogen/progesterone treated mouse, characterized by healthy, normal ductular epithelium, and a small amount of vacuolization at the basal end of the epithelial cell.

Figure 5B. Xenographs recovered from a cyclosporine treated mouse, characterized by healthy epithelium however, there is some vacuolization present around the basement membrane. The tissue closely resembles that observed in Figure 5A.

Figure 5C. Xenographs recovered from a control + estrogen/progesterone treated mouse. Epithelium has undergone some degeneration with some cell lysis.

Figure 5D. Xenographs recovered from a control mouse, characterized by occasional degeneration of epithelial components, cell lysis, and presence of scattered pycnotic nuclei.

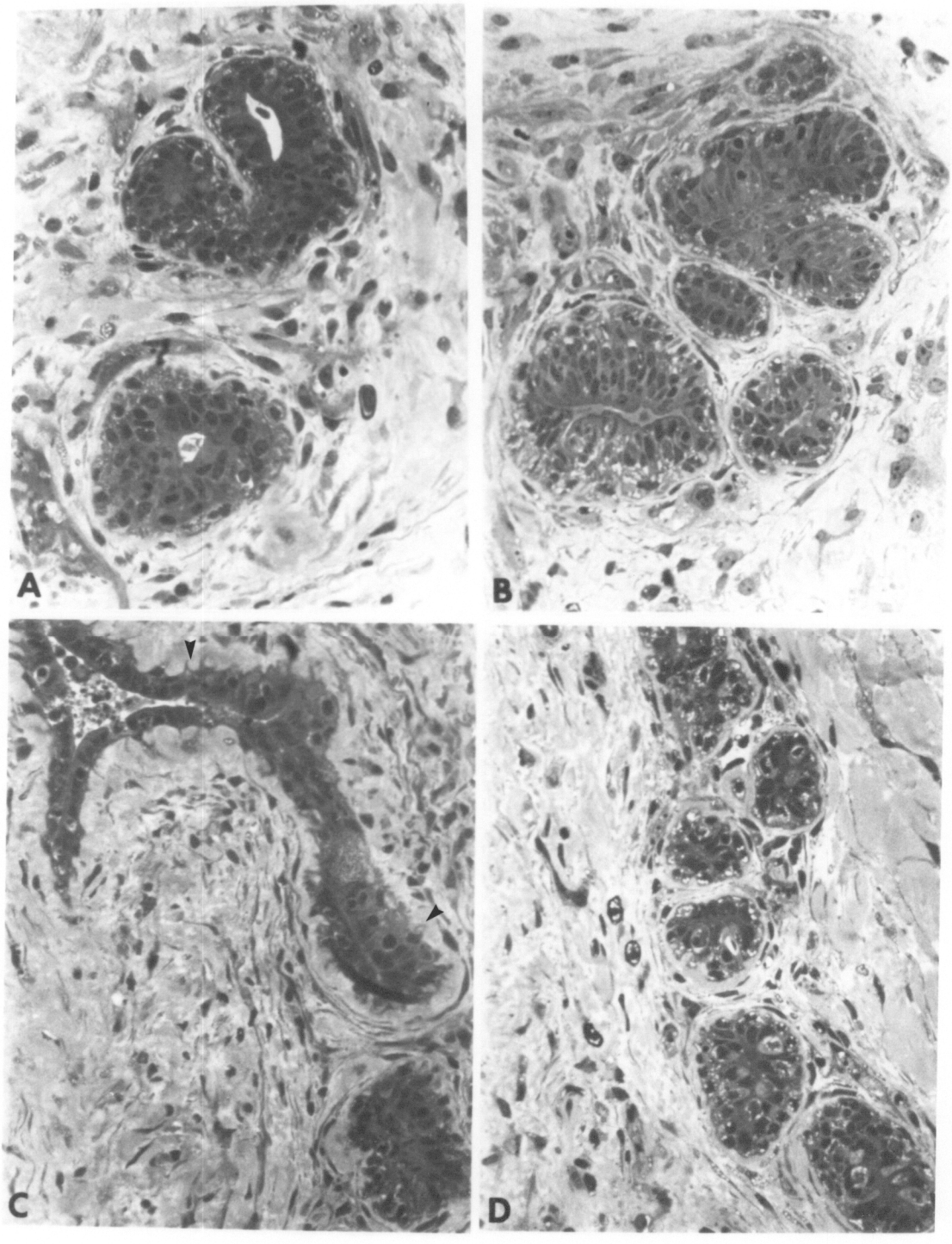
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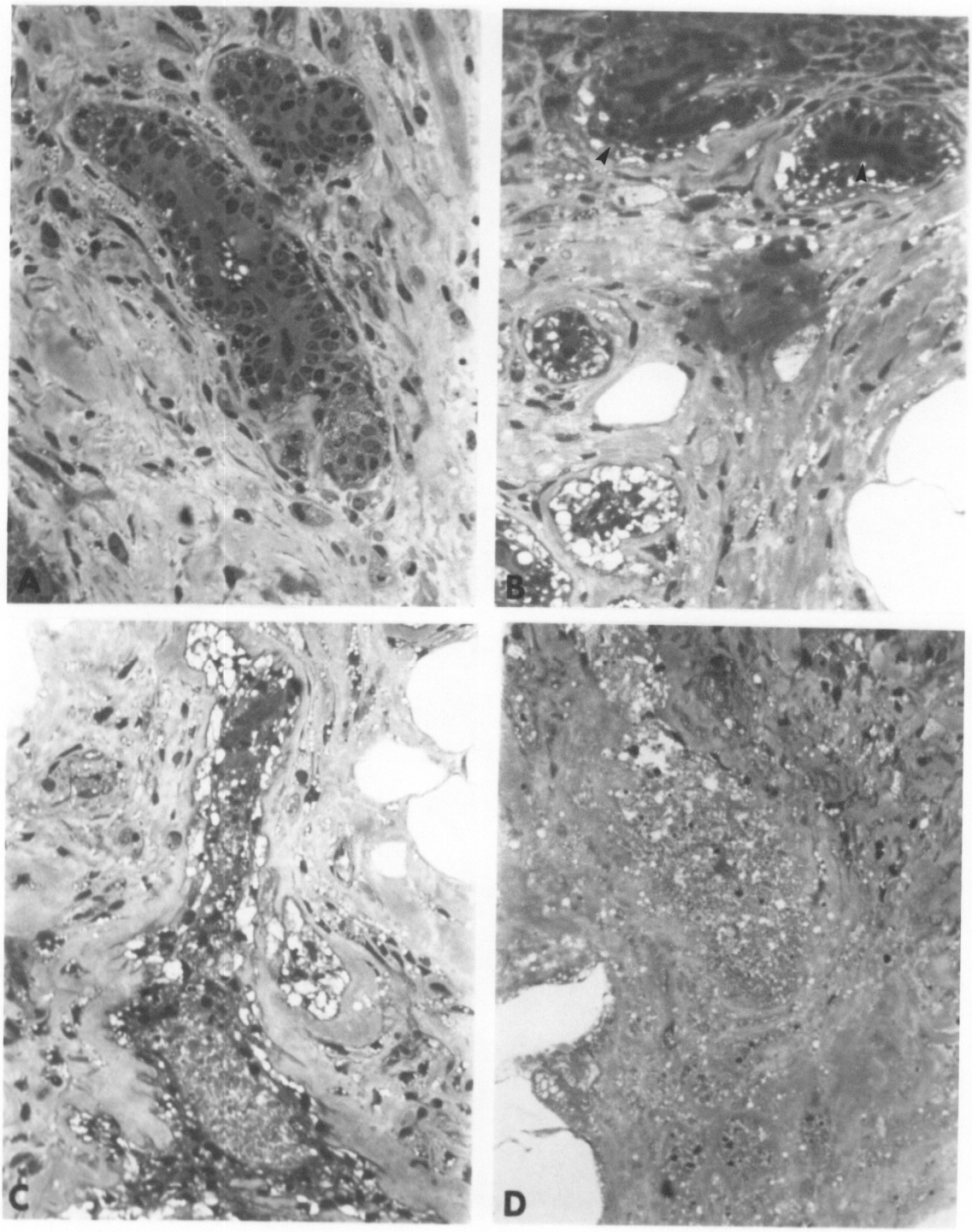
Figure 6. Comparison of xenografts recovered from cyclosporine + estrogen/progesterone, cyclosporine, control + estrogen/progesterone and control treated mice at day 21 post-transplantation. (600x)

Figure 6A. Xenograft recovered from a cyclosporine + estrogen/progesterone treated mouse, characterized by healthy epithelium that shows few signs of degeneration or loss of cellular organization. A small amount of vacuolization can be seen at the basal end of the cell.

Figure 6B. Xenograft recovered from a cyclosporine treated mouse, characterized by some areas with relatively healthy appearing epithelium (arrow) and some areas with apparent cell lysis, loss of cellular organization and epithelial degeneration, and large vacuoles located around the basement membrane.

Figure 6C. Xenograft recovered from a control + estrogen/progesterone treated mouse, epithelium is characterized by complete degeneration of epithelial components, cell lysis, and loss of cellular organization. However, remnants of ductular structures are evident.

Figure 6D. Xenograft recovered from a control mouse, characterized by complete degeneration of epithelial components, cell lysis, loss of cellular organization, and ductal remnants are barely recognizable.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pletely degenerated like the controls at this time period, did show some signs of cell lysis and loss of organization.

Least squares means comparing epithelial score, PMN, lymphocyte, plasma cell, mast cell and macrophage numbers for xenografts taken from CsA + E/P and CsA treated mice at all sample times are shown in Table 2. Mean epithelial score and lymphocyte number were significantly different ($P<.05$). Also, mean PMN number for tissues from CsA + E/P treated mice (15.0) was less than for CsA treated mice (34.8). Comparison of mean xenograft epithelial score in CsA + E/P and CsA treatments was also made to mean epithelial score of 0-time tissue (Figure 7). Mean xenograft epithelial scores for CsA + E/P treated mice were not different from 0-time for any of the 5 time periods. However, at 7, 21, and 35 days, for CsA treated mice mean epithelial score was significantly lower ($P<.05$) than for 0-time tissue.

In Figures 8 and 9, the relationship between the different recovery periods and lymphocytes and PMNs in the subepithelial stroma of xenografts are shown for each of the four treatments (mean 0-time lymphocyte and PMN number were 13.6 and 2.5, respectively). Comparing lymphocyte number at each of the different times for the two CsA treatments, both were low and equal on day 3. In the CsA + E/P treatment group, lymphocyte numbers remained consistently low through day 21, then increased by day 35. In contrast, in the CsA treated group, lymphocyte number progressively increased. Control treatments showed a parallel increase in lymphocyte numbers from day 7 to day 21. However, like the

Table 2. Comparison of CsA + E/P[1] and CsA treatment means[2].

	Treatment			
Measurement	CsA+E/P	SE	CsA	SE
Escore	3.68	0.15	2.83[a]	0.14
PMNs[3]	15.02	9.90	34.80	9.06
Lymphocytes	18.90	4.50	40.53[a]	4.12
Plasma cells	2.5	0.63	2.2	0.58
Mast cells	4.82	1.20	5.1	1.10
Macrophages	1.63	0.43	1.4	0.39

[1]Cyclosporine + estrogen/progesterone.
[2]Least squares mean of treatments.
[3]Polymorphonuclear neutrophil leukocyte.

[a]Means bearing different superscripts within a row differ($P<.05$).

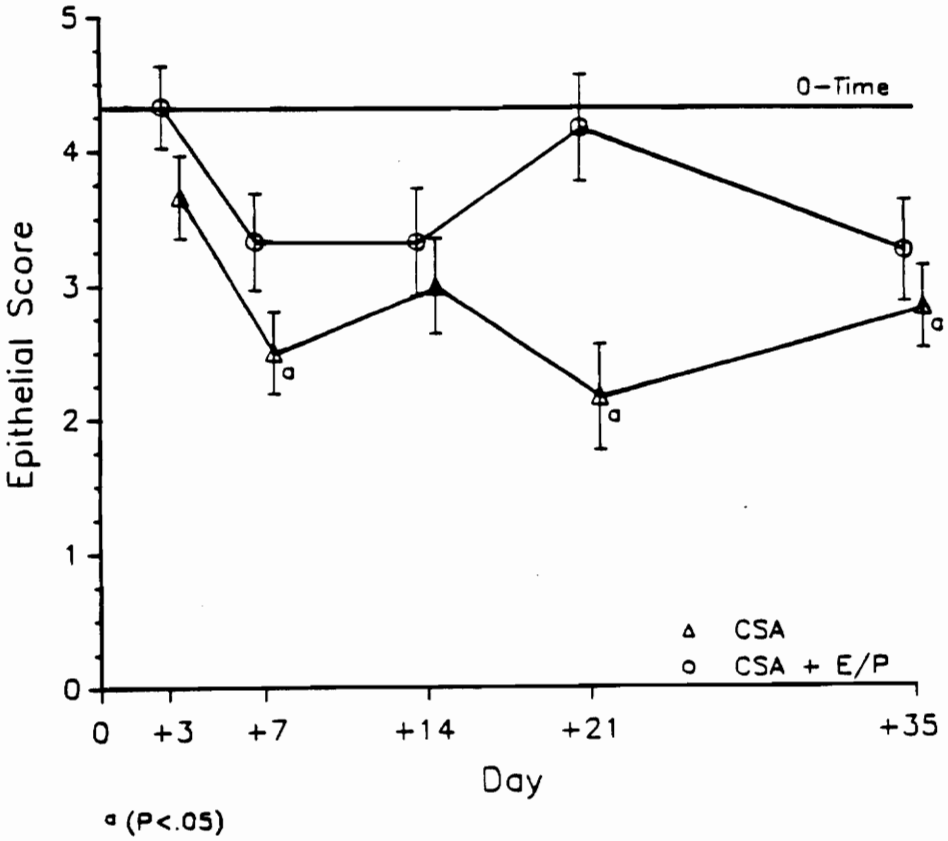

Figure 7. Comparison of mean epithelial scores for all times in cyclosporine + estrogen/progesterone (CsA + E/P), and cyclosporine (CsA) treated mice vs mean epithelial score of 0-time tissue (top line).

[a] ($P < .05$) Probability that mean treatment epithelial scores are different from mean 0-time epithelial scores (Dunnett's test; Gill, 1978).

0-time Standard Error of the mean = +/- 0.21

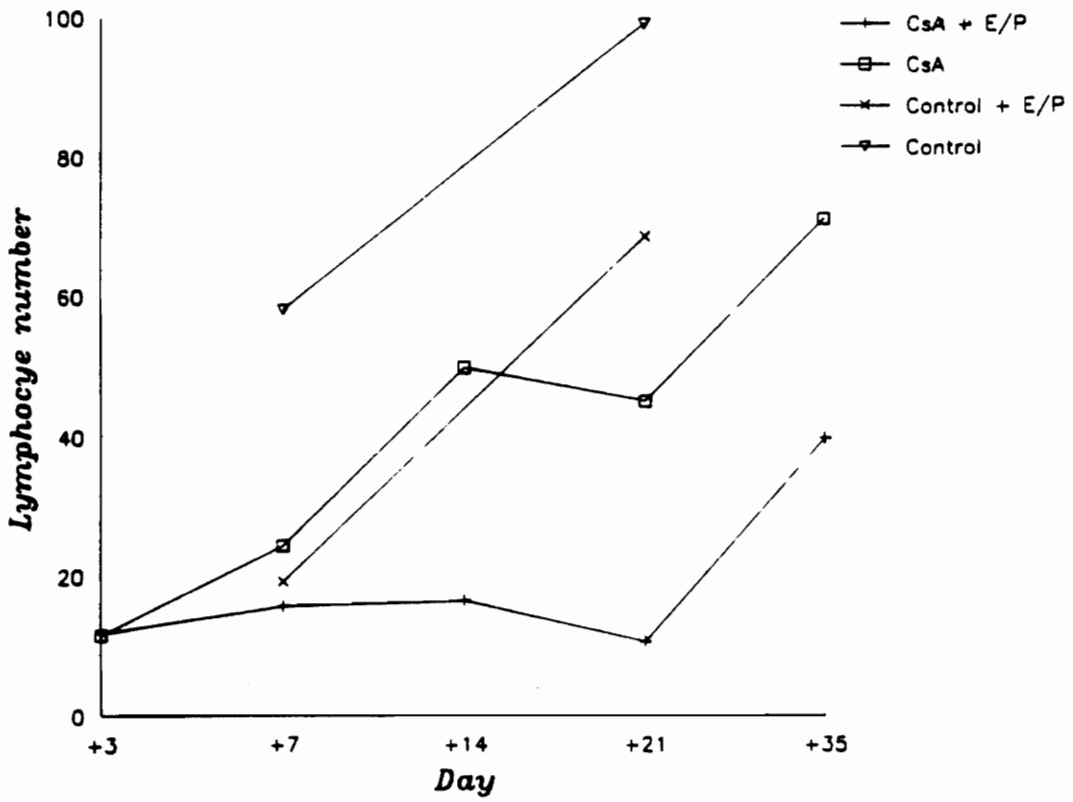

Figure 8. Relationship between time and number of lymphocytes found in subepithelial stroma of xenografts recovered from mice treated with cyclosorine + E/P (CsA + E/P), cyclosporine (CsA), control + E/P, and control (Data reported as number of cells per 117,600 um^2 of subepithelial stroma)

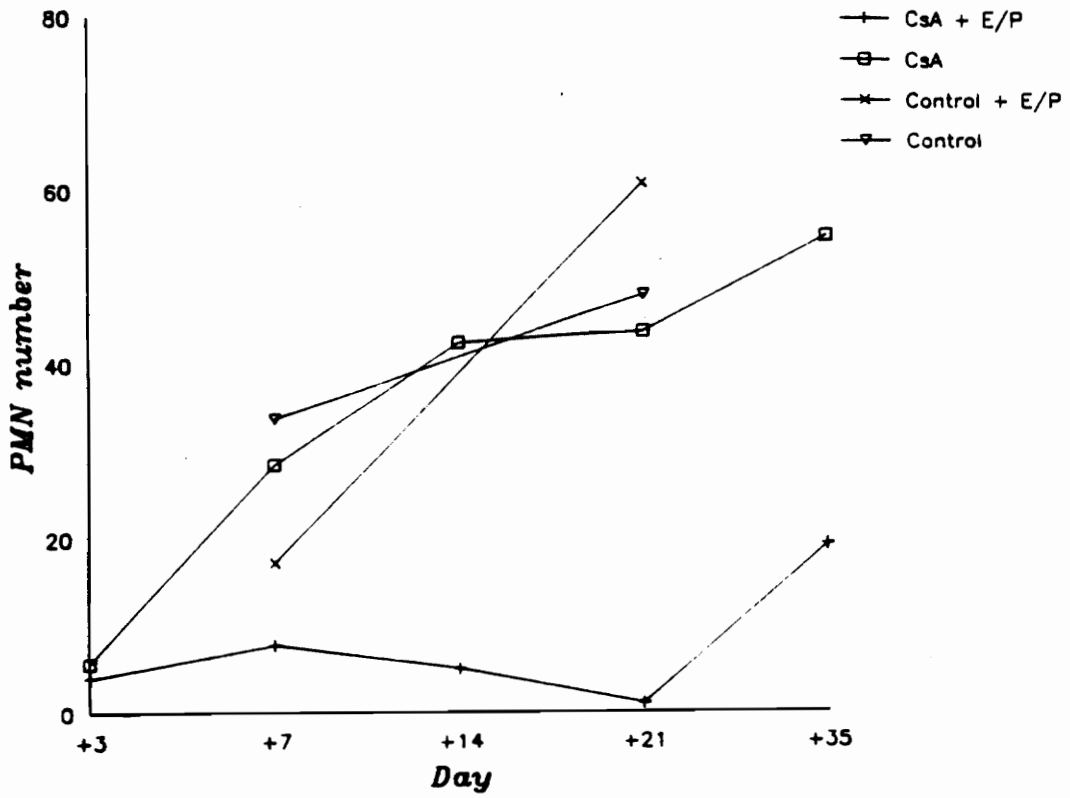

Figure 9. Relationship between time and number of polymorphonuclear neutrophil leukocytes (PMN) found in subepithelial stroma of xenografts recovered from mice treated with cyclosorine + E/P (CsA + E/P), cyclosporine (CsA), control + E/P, and control. (Data reported as number of cells per 117,600 um^2 of subepithelial stroma)

hormone treated mice, among those given CsA the control + E/P treated group had lower lymphocyte numbers at both time periods.

General trends in PMN number were approximately the same as seen with subepithelial lymphocyte numbers. However, there was a greater disparity in PMN numbers in the CsA treated groups than that seen in the comparison of lymphocyte numbers.

In Figures 5 and 6, examples of epithelium in xenografts recovered at day 7 and 21 for all four treatments can be seen. Examples of epithelium recovered from CsA + E/P and CsA treated mice at the other time periods (day 3, 14, and 21) can be seen in Figure 10. Epithelium seen in recovered xenografts from CsA + E/P and CsA treated mice maintained near normal morphology when compared to 0-time, with the exception of the CsA treated group at day 14. In this group, the epithelium appeared to have normal cellular organization, but there was evident vacuolization of individual eithelial cells around the basement membrane. It is especially interesting to note the apparent maintenance of the epithelium in the zenografts recovered at day 35. Moreover, in contrast with earlier periods, there was no apparent greater preservation of the epithelium in mice treated with E/P in addition to CsA.

Correlation coefficients for epithelial score and lymphocyte number, and epithelial score and PMN number for each of the five time periods are shown in Table 3. Correlations comparing epithelial score and lymphocyte number were

Figure 10. Photomicrographs of epithelial tissue recovered from mice treated with cyclosporine + estrogen/progesterone and cyclosporine at days 3, 14, and 35 post-transplantation in experiment 3. (600x)

Figure 10A. Ductal epithelium of xenografts recovered from a mouse treated with cyclosporine + estrogen/progesterone at day 3. The epithelial cells are typically compact with irregular shaped nuclei, a small nuclear to cytoplasmic ratio and relative absence of cellular vacuoles.

Figure 10B. Ductal epithelium of xenografts recovered from a mouse treated with cyclosporine at day 3. The ductular epithelium closely resembles that seen in Figure 10A, with the exception of having slightly more vacuolization.

Figure 10C. Ductal epithelium of xenografts recovered from a mouse treated with cyclosporine + estrogen/progesterone at day 14. The ductular epithelium closely resembles that seen in Figure 10B, however vacuoles located at the basal end of the cell appear larger.

Figure 10D. Ductal epithelium of xenografts recovered from a mouse treated with cyclosporine at day 14. The epithelium appears to have degenerated slightly, but epithelial cell organization is intact and no cell lysis or pycnotic nuclei are evident.

Figure 10E. Ductal epithelium of xenografts recovered from a mouse treated with cyclosporine + estrogen/progesterone at day 35. The ductular epithelium closely resembles that recovered at day 3 (Figure 10A and 10B), but there is apparent leukocyte infiltration in the subepithelial stroma (arrow).

Figure 10F. Ductal epithelium of xenografts recovered from a mouse treated with cyclosporine at day 35. The ductular epithelium closely resembles that recovered at day 3 (Figure 10A and 10B), but there is apparent leukocyte infiltration in the subepithelial stroma (arrow).

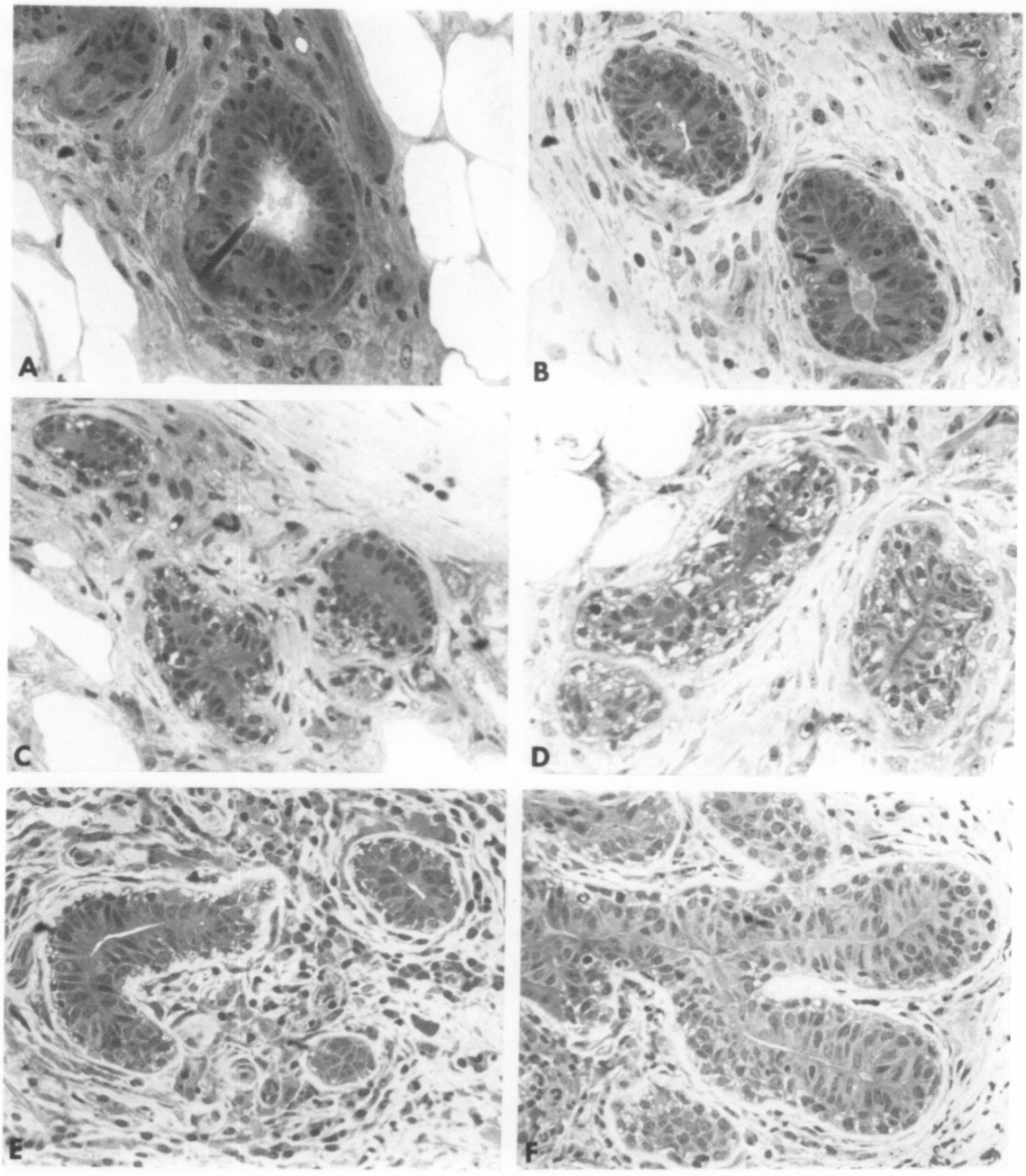
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Table 3. Pearson correlation coefficients comparing epithelial score (Escore) and lymphocyte number (Lym), and epithelial score and polymorphonuclear neutrophil leukocyte (PMN) number.

		Day				
		+3	+7	+14	+21	+35
[1]Escore-Lym	r =	-.39	-.60[b]	-.65[c]	-.53	-.35
[2]Escore-Lym	r =	-.39	-.51[b]	-.65[c]	-.66[a]	-.35
[1]Escore-PMN	r =	.03	-.50	-.78[b]	-.53	.23
[2]Escore-PMN	r =	.03	-.34	-.78[b]	-.59[b]	.23

[1]Comparison without control treatments.
[2]Comparison includes control treatments.

[a](P<.01) Probability that correlation does not = 0.
[b](P<.05) Probability that correlation does not = 0.
[c](P<.10) Probability that correlation does not = 0.

negative for each time period regardless of whether the comparison included control treatments. In both cases there was a significant negative correlation between epithelial score and lymphocyte number at day 7 ($P<.05$) and day 14 ($P<.1$). However, the negative correlation at day 21 was significant ($P<.01$) only when the calculation included the control treatments.

Correlations comparing epithelial score and PMN number were negative for all times except for the first and last time periods (day 3 and 35) regardless of inclusion of control treatments. In both cases, at day 14 the correlation was strongly negative ($r = -.78$: $P<.05$). As was the case with epithelial score and lymphocyte number, the correlation at day 21 was negative when comparison did not include control treatments, but was significant ($r = -.59$; $P<.05$) only when the correlation included the control treatments.

Overall correlations between epithelial score and lymphocyte number, and epithelial score and PMN number across all time periods and treatment combinations are shown in Figure 11 and Figure 12. Correlations between epithelial score and lymphocyte number, and epithelial score and PMN number were negative ($r = -.55$; $P<.0001$ and $r = -.42$; $P<.001$, respectively) in both cases.

Culture

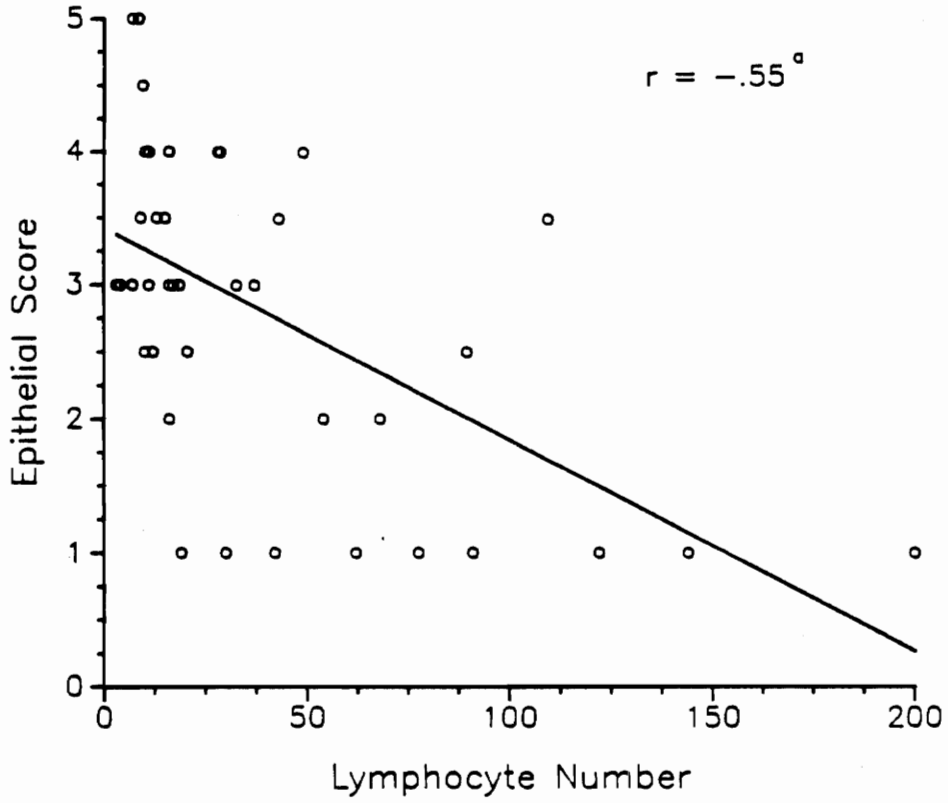


Figure 11. Correlation between lymphocyte number and epithelial score for all times and treatments.

[a] ($P < .0001$) Probability that correlation does not = 0.

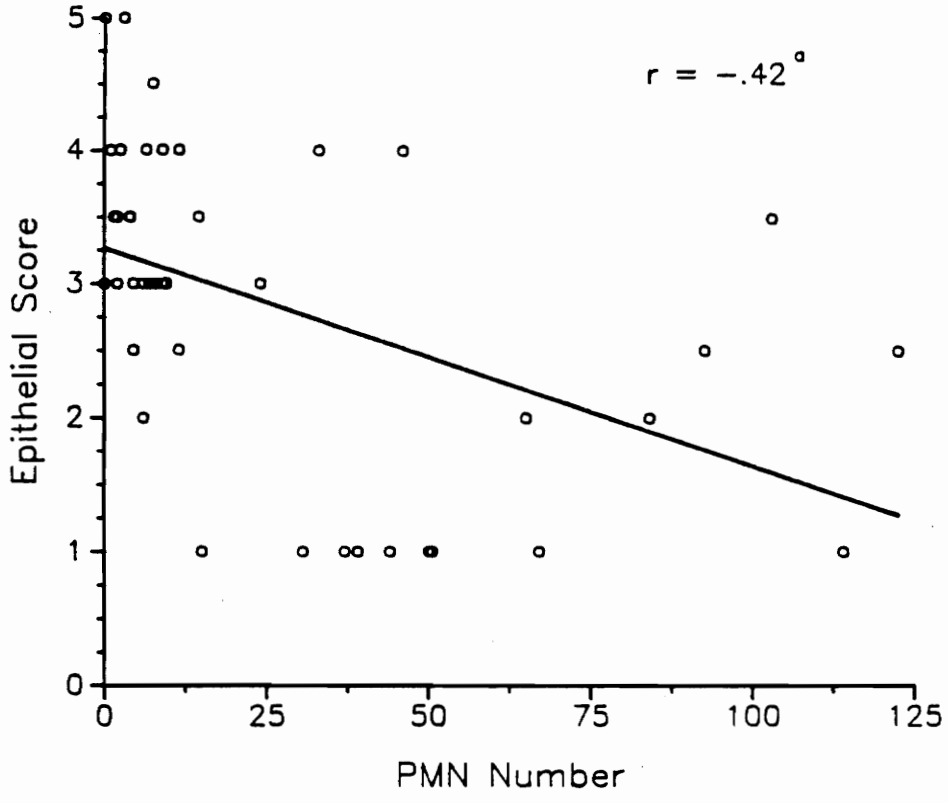


Figure 12. Correlation between polymorphonuclear neutrophil leukocyte (PMN) number and epithelial score for all times and treatments.

[a] ($P < .001$) Probability that correlation does not = 0.

Table 4. Summary of epithelial scores from culture.

Day	Tube Number 1	2	3	Mean
3	4	5	4	4.3
7	4	3	1	3.5**
14	1	1	1	1.0
21	1	1	1*	1.0
28[1]	1*	1*	1*	1.0

[1]Tissue was harvested one week early due to contamination of media.

*Culture tube contaminated.
**Mean does not include Escore of contaminated tube.

Epithelial scores for cultured ovine mammary explant strips are presented in Table 4. Culture strips recovered at day 3 and 7 had mean epithelial scores of 4.3 and 3.5, respectively. Mean epithelial scores at all later times was 1.

A representation of cultured ovine mammary gland strips recovered at days 3, 7, 14, 21, and 28 are shown in Figure 13. Cultured strips harvested on day 3 showed a great degree of variation in epithelial morphology. Epithelium that was located toward the outside of the strip (that in close proximity to media), showed normal morphology generally comparable to that of 0-time. However, the epithelium located toward the inside of the strip was always completely degenerated. This was often seen within the same duct as it traversed to the interior of the strip. This same pattern was also seen in strips recovered on day 7, though the epithelium that was located toward the outside was also beginning to show signs of degeneration. Epithelium in strips recovered on day 14, 21, and 28 was completely degenerated regardless of location within the tissue piece. It should be mentioned that all 3 tubes of the later time period were contaminated, though it is unlikely that this would have made a difference in the appearance of the epithelium, considering the two previous time periods contained no viable tissue. When scoring cultured strips of tissue only the epithelium in the outer-most, region was evaluated.

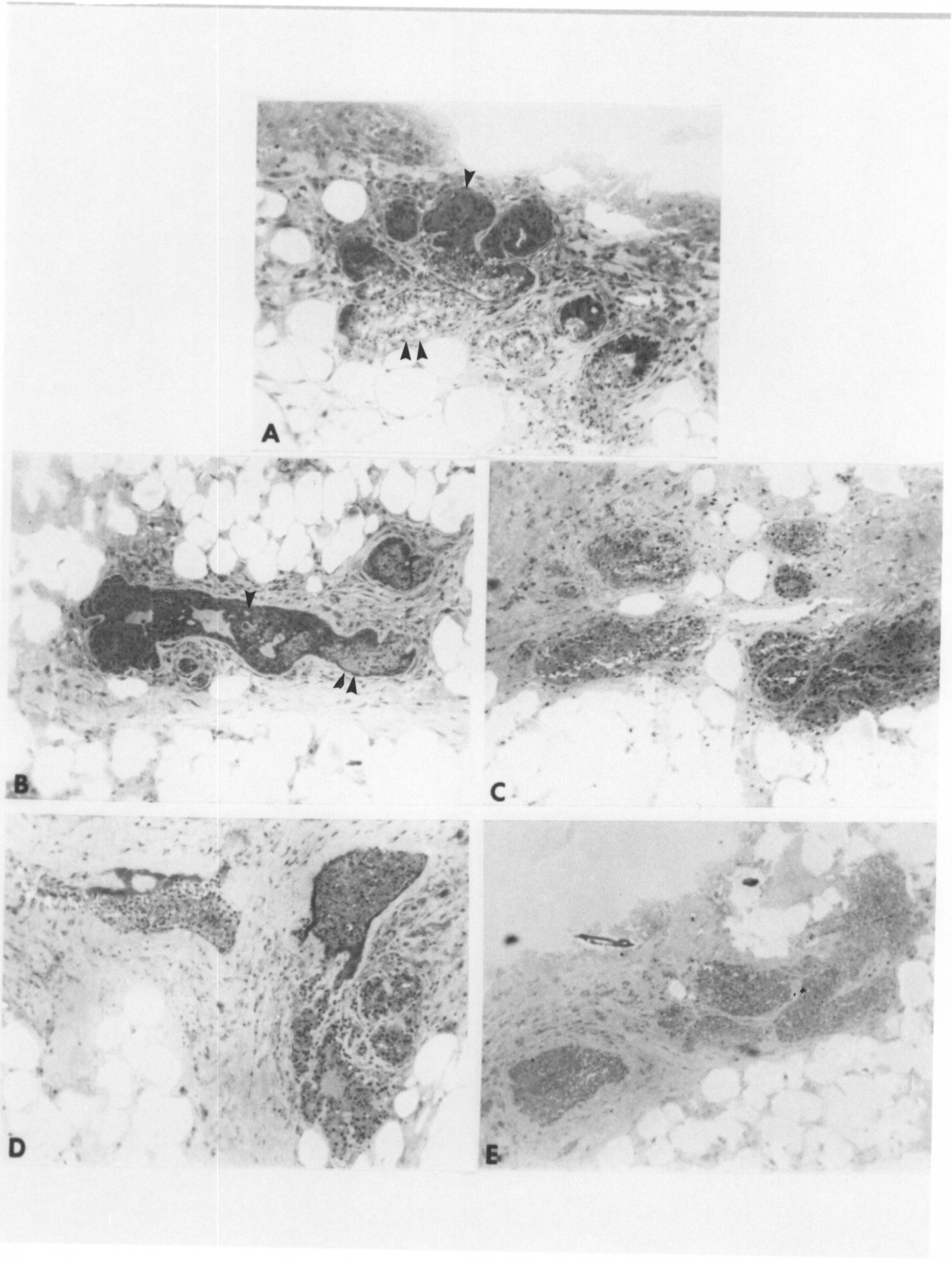
Contamination was observed in 1 of 3 culture tubes at day 7 and 21. Explant strips in the last 3 culture tubes were harvested at day 28 (1 week prior to scheduled harvest) due to contamination.

Figure 13. Representative photomicrographs of in vitro cultured ovine mammary gland strips recovered at days 3, 7, 14, 21, and 28. (250x)

Figure 13A. Culture strip harvested after 3 days in culture, epithelium is characterized by normal healthy tissue located on the outside of the strip (arrow), and degenerted epithelium located towards the interior of the strip (double arrow).

Figure 13B. Culture strip harvested after 7 days in culture, characterized by epithelium that shows signs of degeneration on the outer edge of the strip (arrow), while that on the interior has completely degenerated (double arrows).

Figures 13C, 13D, and 13E. Culture strips harvested after 14, 21, and 28 days in culture, respectively. Epithelium is characterized by complete degeneration of epithelial components, cell lysis, pycnotic nuclei, and complete loss of cellular organization.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DISCUSSION

The success of recent studies using athymic nude mice as a model for studying mammary gland growth in humans (Outzen and Custer, 1975; Jensen and Wellings, 1976; McManus et al., 1978; McManus and Welsch, 1981) and the bovine (Welsch et al., 1979; Sheffield and Welsch, 1986) has created much interest. The possible uses of this model in cancer research and ruminant mammary growth and development are great. Results from recent studies of xenograft transplantation have also stimulated renewed interest because of use of CsA. Although discordant organ xenografts typically have poor success rates; the success of pancreatic islet (Nakajima et al., 1985; Selawry et al., 1986) and discordant skin xenografts (Biren et al., 1986) has shown promise in the use of CsA for at least some applications. Thus, these data gave rise to the idea of using CsA treated mice as recipients of ruminant mammary tissue in hopes of mimick-

ing the results others (Welsch et al., 1979) have seen following transplantation of mammary tissue into athymic nude mice.

There were two primary objectives of experiment 1. First, we intended to demonstrate that ovine mammary tissue could be xenografted into CsA treated mice for an extended period of time (up to 35 days). Secondly, three different sites in these mice were selected to receive xenografts (ovarian fat-pad, MGF, and subcutaneously) to determine the most appropriate site for transplanting. Results from this experiment indicated failure of xenografted mammary tissue to survive (Figure 2). Also, due to a lack of survival or morphological differences in any of the three transplantation sites selected, no determination was possible as to which would be most appropriate. Thus, we failed to meet our objectives.

In the second experiment, ovine mammary tissue was again transplanted into mice. However, in this experiment donor tissue was more developed (i.e. tissue from lamb vs pregnant ewe) compared to that used in experiment 1 (Figure 2A and 3A). Objectives of this study were to determine whether "pregnancy primed" mammary tissue could survive for an extended period of time. Also, if the tissue did survive would it respond to either exogenous or endogenous hormonal stimulation. Also, to determine if morphological differences could be seen in epithelium at the two transplantation sites within the same mouse (ovarian fat-pad and MGF) Again, as in experiment 1, the failure of all xenografts to survive made determination of these objectives impossible (Figure 3).

The negative results from the first two experiments led to a re-evaluation of the study. It was obvious that changes were needed in the protocol if the proposed model was to succeed. At this point it was decided that we would again utilize tissue less developed than in the mid-pregnant ewe and increase the dose of CsA. Although dose rates for CsA (8 mg/kg/day) had been adapted from reports concerned with limb transplantation allografts between strains of rats (Black et al., 1985); we reasoned that the discordant xenografts we were attempting might require much larger doses of CsA for survival of the implant. Our conclusions were supported by reports which appeared as our work was in progress. Specifically, Sugimoto et al. (1985) reported use of very high doses of CsA (50 mg/kg/d) even in concordant xenografts.

The objective of experiment 3 was to determine whether ovine mammary tissue could be successfully xenografted subcutaneously into mice receiving high doses of CsA. The subcutaneous transplantation site was chosen because, although there was no difference in morphology of the epithelium recovered from the different sites in experiment 1, successful recovery of xenografts was more frequent in those that were subcutaneously transplanted.

Mean epithelial scores for CsA + E/P and CsA were significantly greater than the mean of control + E/P and control (Table 1). This indicated that xenografted epithelium recovered from mice treated with CsA maintains its morphology better than that recovered from mice that did not receive CsA.

It is clear that in tissue harvested at day 7 and 21 (Figure 5 and 6) that CsA treated mice had epithelium of normal morphology, but that degenerative alterations were typical of the epithelium of control treatments. This was especially evident in epithelial score at day 21 in those mice receiving CsA + E/P treatments (Figure 4).

Lower lymphocyte numbers in the subepithelial stroma of CsA treated mice vs controls (Table 1) indicate that CsA did prevent the increased numbers of lymphocytes typically associated with cell-mediated graft rejection or response to degeneration. However, within each treatment (CsA and control) the trend was for the group receiving hormones to have lower numbers of lymphocytes and higher epithelial scores. This is likely a reflection of better maintenance of the epithelial cells in steroid treated mice. Since transplanted epithelium in mice that did not receive exogenous ovarian steriods degenerated, the chemotaxic effects of debris from cell lysis would likely have attracted more lymphocytes into the area, and thus set up a positive cycle of damage, more leukocyte migration and damage by the leukocytes. Conversely, in those receiving hormones the epithelial components were better maintained and thus lacked the added chemotaxic effects attributed to cellular debris.

Similar patterns can be seen in plasma cell number as was seen in epithelial score and lymphocyte number, but reasons for these similarities may not be the same. Significant differences in plasma cell numbers for the two time periods could be explained by increased stress on CsA treated mice due to near toxic doses of CsA.

That is, stress increased the release of corticosteroids from the adrenal cortex which in turn could have caused suppression of the immune system and more specifically decreased numbers of plasma cells. Similar effects could also have been responsible for reduction in lymphocyte numbers in these treatment groups. Alternatively, reductions in lymphocytes and plasma cells may reflect direct effects of CsA in prevention of proliferation of lymphocytes and differentiation of plasma cells from B-lymphocytes.

Comparisons of epithelial score and lymphocyte numbers for CsA + E/P and CsA treated mice for all times (Table 2) show a similar relationship to that seen when just two times (+7 and +21 days) were used in the analysis. These data indicate a consistent positive effect of the inclusion of estrogen and progesterone with CsA treatment. Similar trends are also seen when mean epithelial scores for each of these two treatments are compared to 0-time mean epithelial score (Figure 7). Only CsA treated mice had epithelial scores that were statistically different from mean 0-time epithelial score. That is not to say that epithelium seen in CsA + E/P treated mice appeared exactly as 0-time, but it does indicate that it more consistently resembled it.

To further support the positive effect CsA + E/P had on limiting lymphocyte and PMN number, data were plotted to illustrate time trends in both of these parameters (Figure 8 and 9). At 3 days post-transplantation, both CsA treatments showed nearly identical numbers of both lymphocytes and PMNs. As time progressed to 14d post-transplantation CsA treated mice exhibited a generally

steady increase in leukocyte number followed by a leveling off through day 35. In CsA + E/P treatments leukocyte number was minimal until day 35. This response may indicate a loss in effectiveness of CsA at these later time periods. This supports findings of other researchers (Nakajima et al., 1985; Homan et al., 1981) in which use of high doses required to prevent graft rejection progressively led to a restoration of T-cell responsiveness to IL-2 and eventually to rejection of the xenografts.

While CsA treatments showed a consistent degree of disparity in lymphocyte and PMN number over time, it is hard to make inferences about the two control treatments, since there were only two times periods. Nonetheless, there were evident increases in numbers of both lymphocytes and PMNs by day 21. Furthermore, correlations between epithelial score and lymphocyte number and epithelial score and PMN number became significant and more negative at day 21 when control treatments were included in the analysis. This was also true for the correlation between epithelial score and lymphocyte number at day 7, but that was not the case with epithelial score and PMN number. This indicates that there exists a strong negative relationship between epithelial score and lymphocyte number, and epithelial score and PMN number (Figure 11 and 12).

Photomicrographs of epithelium taken from each of the five recovery periods for each of the CsA treatments (Figure 5, 6,and 10) indicated somewhat surprising trends. It appeared that the epithelial components of these transplants gradually degenerated between day 14 and 21. But in almost all cases epithelium appeared

healthier (higher epithelial score; similar to 0-time) at day 21 than at day 14 and astoundingly epithelium harvested at day 35 was superior to samples obtained on the two prior recovery periods. It may be that early degeneration of epithelium is due to a lack of nutrients to the explant, resultant from a lack of a formal blood supply. As the xenograft becomes established, possibly between day 14 and 21, channels for nutrient sources are established (i.e. vascularization), the weakened epithelium regenerates and improves its morphological appearance.

In the long-term cultrue experiment that was conducted along with experiment 3, results from the first two time periods were encouraging. Mean epithelial scores (Table 4) were not different from 0-time for tissue harvested at day 3. However, the epithelium quantified was located in the outer portion of the culture strip. This was due to the total degeneration of interior epithelial components (Figure 13A and 13B). In the first two recovery periods (day 3 and 7), ducts located to the outside showed near normal morphology. However, within the same duct epithelium would quickly degenerate as the duct headed toward the interior of the strip. This seems to indicate that epithelium that was not in close proximity to the nutrient media quickly degenerated (less than 3 days). All epithelium completely degenerated by day 14, regardless of its location within the strip.

The success of experiment 3 compared to the two previous experiments can be explained in two ways. The first is that differences in the developmental stage of the donor tissue is responsible. However, this seems unlikely because when the morphology of the 0-time tissue in experiments 1 and 3 are compared (Figures

1A and 2A) they are very similar. In experiment 1 donor tissue was taken from a four month-old ewe lamb and in experiment 3 donor tissue was taken from a estrogen primed, non-lactating ewe. However, both showed primarily ductular epithelium with large amounts of subepithelial stroma and adipose tissue. It is likely however, that results from experiment 3 would not have been as positive had more mature donor tissue been used. This assumption is based on a comparison of the epithelium recovered from CsA treated mice after 35 days in experiment 1, and that recovered from CsA treated mice after 30 days in experiment 2 (Figure 2D and Figure 3B). Xenografts recovered in experiment 1, though degenerated, did show traces of old ducts, whereas those recovered in experiment 2 showed almost no alveolar remnants. This conjecture is even further supported by the fact that xenografts recovered in experiment 1 had been in the mice for five more days than those in experiment 2. It also seems logical that more developed, single-layered, secretory alveolar epithelium that has had its nutrient source abruptly severed would be less tolerant of its harsh new environment than bilayered, undifferentiated ductular epithelium.

A more likely explanation for the success of experiment 3 is due to the increased dose of CsA. The large dose that the mice received in the later experiment was likely sufficient to overide the strong cell-mediated rejection response that would normally accompany a discordant xenograft. This is supported by results from a study conducted by Homan et al. (1981) where only at a dose of 50 mg/kg/day (near toxic) did CsA significantly reduce histological features of rejection of concordant cardiac xenografts in rats.

It is important to note that few studies have been conducted in which near toxic doses of CsA have been administered for long periods of time in an attempt to prevent xenograft rejection. This is because the primary objective of human transplantation is the long-term survival of the recipient. Thus, near toxic doses of CsA, and its potential lethal side effects have made a treatment regime of this nature obsolete.

In summary, data from these experiments, and especially those from experiment 3, indicate the mice treated with near toxic doses of CsA have the potential to be used as an ex vivo in vivo model for studying mammary growth and development. Still, further studies need to be conducted that will show that this xenografted tissue will grow, differentiate, and lactate (produce milk constituents) in response to hormonal stimulation.

SUMMARY

Although experiments 1 and 2 were relatively unsuccessful, there were some conclusions that could be made from these two studies that can be of use in future studies. Mice given low doses of CsA are not capable of maintaining an environment suitable for the survival of discordant mammary xenografts. This is true regardless of transplantation site. However, if percent recovery of implants is important, the subcutaneous site is the best alternative. Immature donor mammary tissue is the most appropriate choice when deciding what type of tissue to xenograft, if long term survival is the goal of the experiment.

Results from experiment 3 indicated that a high dose of CsA was effective in increasing the survival of ovine mammary xenografts. Higher mean epithelial scores and lower mean lymphocyte numbers of CsA treated mice vs controls, provide quanitative evidence that support histological observation of CsA's effectiveness. The most unexpected result of this study was the positive effect estrogen/progesterone had on the survival of xenografted mammary epithelium.

Further studies need to be conducted involving the use of other hormones in combination with CsA, with the goal of finding a combination that will maximize survival. Also, our model could possibly be further improved by the use of other immunosuppressive therapies in conjunction with CsA (i.e. total lymphoid irradiation or corticosteroids).

Although the objectives of the first two experiments were not met, results from the last study provide incentive for further studies to be conducted to acheive these goals. That is, what is the most appropriate site of implantation, will xenografted tissue respond to exogenous and endogenous hormonal stimulation? If the tissue does respond will it grow into a host fat-pad? These and many more questions need to be answered in future studies. However, with the success of experiment 3, I feel that "the door has been opened" for the further development of this model for use in studying growth and development of mammary tissue.

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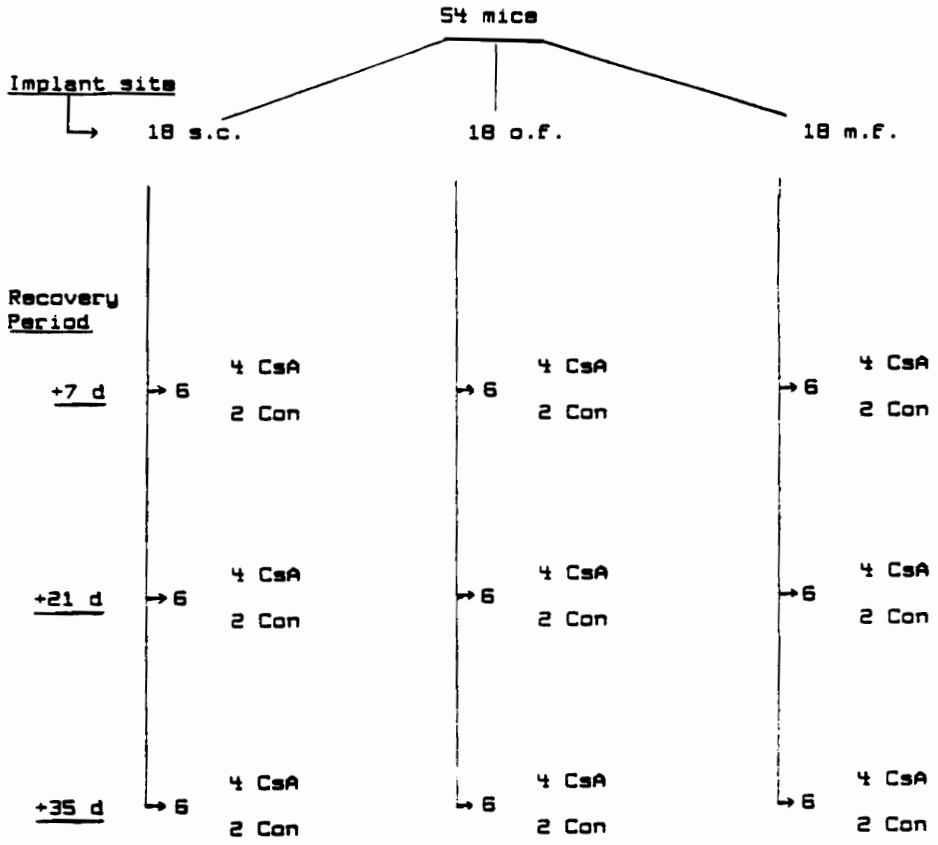
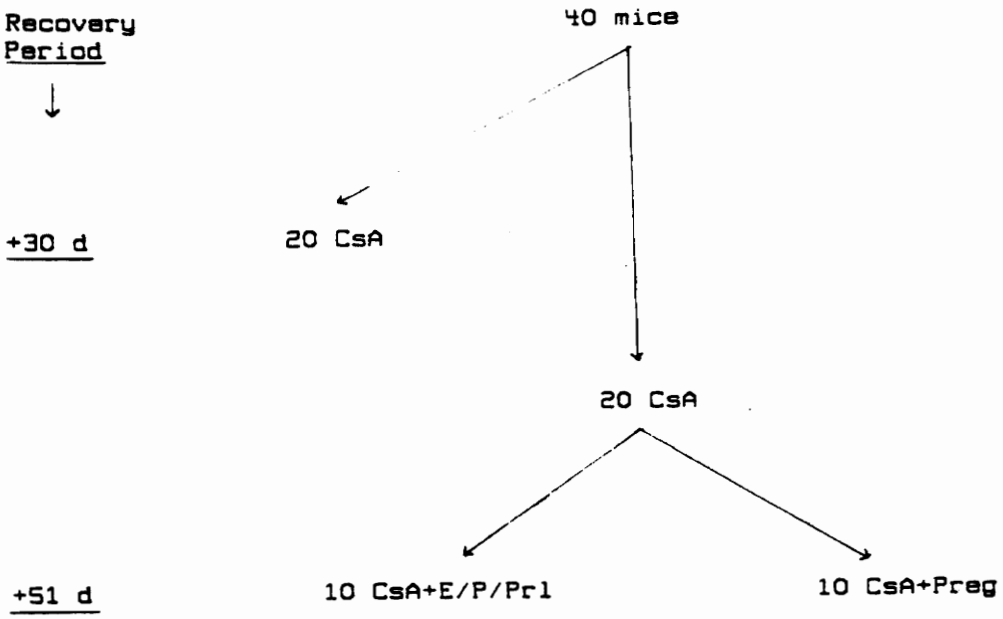
Appendix A. Experiment I



s.c. - subcutaneous
o.f. - ovarian fat-pad
m.f. - mammary gland fat-pad
CsA - cyclosporine
Con - control

Appendix B. Experiment II



CsA = Cyclosporine
E = Estrogen
P = Progesterone
Prl = Prolactin

Appendix C. Experiment III

# of mice & Treatment	Recovery Period (day)				
	+3	+7	+14	+21	+35
15 CsA + E/P	3	3	3	3	3*
15 CsA	3	3	3	3	3
6 Con + E/P		3		3	
6 Con		3		3	

CsA + E/P = Cyclosporine + Estrogen/Progesterone
CsA = Cyclosporine
Con + E/P = Control + Estrogen/Progesterone
Con = Control

* Received another Estrogen/Progesterone implant at day 21

Vita

Thomas Earl Daniel Jr. was born in Danville, Virginia on February 2, 1959. After graduating from Halifax County High School, South Boston, Virginia in June, 1977, he attended Ferrum College until the spring of 1979. He later attended Virginia Polytechnic Institute and State University and received the Bachelor of Science degree in Dairy Science in March, 1986. The author began his graduate study toward the Master of Science in Dairy Science in April, 1986 at Virginia Polytechnic Institute and State University, with expected completion in February, 1988. The author is a member of Gama Sigma Delta and Phi Kappa Phi Honor Societies. Honors received include award of a Graduate Assistantship.

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