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Our central hypothesis is that breast ductule epithelial cells possess excretory transporters which could transport hydrophobic carcinogenic molecules to the lumen of the ductules. These deposited carcinogens could diffuse back through the apical membrane and attack DNA of the lining cells. So far, all of our data support the conclusion that human breast ductules possess one of these excretory transporters, Mrp2, which transports anionic, hydrophobic, xenobiotic molecules. First, immunocytochemical studies show that the apical membrane of ductules epithelial cells possesses Mrp2. Second, our transport studies show that freshly isolated ductules transport sulforhodamine 101, a substrate for the Mrp2 transporter, into their lumens and that this transport is stimulated by estrogen. This transport process is tremendously enhanced by exposure of ductules to estrogen. Since lifetime exposure to estrogen is considered the major risk factor in breast cancer, this estrogen regulation of the transport phenomenon may be especially significant. During this grant we have established the above points, and developed the model systems and methods to study this transport phenomenon with fresh ductules. Our tissue culture methods should now allow us to study ductule epithelial cells as flat sheets in Ussing chambers to further understand the regulation of Mrp2 transport in breast tissue.

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Karl Karnaky, Jr. January 15, 1999

PI - Signature Date

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(5) INTRODUCTION

BACKGROUND

Overview

We proposed to examine the possibility that a well-described membrane transporter, Pgp, could continually or episodically (under the influence of various steroid hormones or environmental factors) transport toxic molecules into the lumen of breast epithelia over long periods of time (years). This process could subject breast epithelia to extremely high concentrations of toxic molecules, much higher than predicted from blood concentrations.

Transporters of endogenous and xenobiotic, hydrophobic molecules

One strategy organisms use to limit the toxic effects of xenobiotic is excretory transport. Energy-dependent transport of xenobiotics can be seen at multiple levels. At the cellular level, plasma membrane transporters use the energy released from ATP splitting to drive potentially toxic chemicals out of the cell. These xenobiotic transporting ATPases are members of the ATP-binding cassette family of membrane proteins, which includes the multidrug resistance (MDR; Ford and Hait, 1990; Germann et al., 1993) transporters, also called P-glycoproteins, and the MDR-associated proteins (mrp; Cole, et al., 1994). It was subsequently shown that exposure to a surprisingly wide range of chemotherapeutic agents results in overexpression of one or both transporters, and the resulting increase in xenobiotic export capacity is one mechanism underlying the phenomenon of multidrug resistance seen in certain tumors (Bellamy, 1996). Such transporters are expressed in normal tissues. For P-glycoprotein, the highest levels of expression are found in transporting epithelia, e.g., liver, intestine, and renal proximal tubule (Fojo et al., 1987; Thiebaut et al., 1987). For mrp, liver and kidney express an epithelia-specific isoform (canalicular multispecific organic anioin transporter, cMOAT, or Mrp2; Buchler et al., 1996; Schramm et al., 1995). In epithelia, P-glycoprotein and Mrp2 are inserted in the plasma membrane in a polar manner, being localized to the pole of the cell facing the compartment into which xenobiotics are being excreted, i.e., canalicular and luminal membranes. This puts these transporters in the proper location to mediate the pumping of xenobiotics into urine and bile. As with tumor cells in culture, P-glycoprotein levels in epithelial have been shown to increase in animals chronically dosed with substrate [cyclosporin A (CSA)], suggesting that rates of xenobiotic excretion would be similarly increased (Jette et al., 1996). Thus, at the cell, tissue, and organism levels, ATP-driven xenobiotic transporters can both protect against toxic chemicals and limit the usefulness of

therapeutic drugs.

Because there is immunocytochemical evidence for Pgp in breast epithelium with four different Pgp antibodies (Pavelic et al., 1993), we first pursued the data for the presence and function of this transporter in breast tissue.

The Permeability glycoprotein, or Pgp

Pgp is a well-studied membrane protein which can transport certain hydrophobic, slightly cationic molecules such as anthracyclines (e.g., daunomycin), etoposides, and Vinca alkaloids such as vinblastine. As we stated above, the concept of the Pgp started with the phenomenon of multidrug resistance, and was first described in mammalian cancer cells. The list of transported substrates is long and includes colchicine, daunomycin, vincristine, vinblastine, mithramycin C, ethidium bromide, and gramicidin D (reviewed in Fojo, 1991). Recent additions to this list are benzo-a-pyrene (Yeh et al., 1992), and the neurotoxic insecticide, ivermectin (Schinkel et al.,1994). A large number of structurally diverse pesticides inhibit Pgp transport (Bain and LeBlanc, 1996). Pgp is induced in several tissues of the rat by simple gavage of the pesticide, chlorpyrifos (Bain and LeBlanc, 1996).

The Pgp is located in the apical membrane of proximal tubule cells, and the apical membrane of enterocytes of the small and large intestine, the bile canaliculus of the liver, the gallbladder, the luminal membrane of brain and testicular capillaries, and in the adrenals (where it may transport steroid hormones)[reviewed in Fojo, 1991].

Presence of transporters in breast epithelium

Some studies report significant levels of Pgp expression (as detected by immunocytochemistry) may be present in breast cancer **before** exposure to drugs associated with multidrug resistance (Wishart et al., 1990). In that study, heterogeneous expression in epithelial cells was detected with both C219 (26 of 29 patients) and MRK16 (12 of 29 patients). In another study of breast cancer patients, no or minimal reactivity was found in specimens coming from the untreated patients (12 cases) or from patients treated with substances not involved in the multidrug resistance phenomenon (4 cases) [Schneider et al., 1989]. In contrast, three out of seven tumors from patients treated with multidrug resistance related substances showed clear reactivity (positive staining in more than 20% of the tumor cells). It is generally assumed that increased expression of Pgp after the start of chemotherapy occurs because at least some cells present in the epithelium expressed Pgp (Gottesman and Pastan, 1993). These were then selected for when chemotherapy began. Other mechanisms are possible, however, such as a direct effect of chemotherapy or a natural process which is part of the progression of the cancer. As stated above, because there is immunocytochemical

evidence for Pgp in breast epithelium with four different Pgp antibodies (Pavelic et al., 1993), we first pursued data for the presence and function of this transporter in breast tissue.

The nonlactating breast secretes fluid and molecules

The breast contains a most unusual exocrine gland: one that might never or might only rarely drain its products. Keratotic material normally plugs the lactiferous sinuses in nonlactating women, so nipple secretions are not clinically appreciated. However, the keratotic plug can be removed and a simple nipple aspirator device can be used to obtain fluid in a large proportion of women. In a study of 606 normal nonlactating women, 70% of the Caucasian women and 48% of women of all races gave nipple aspirate fluid (Petrakis et al., 1987a). In this same study, another group of women (numbering 103) with suspicious breast lesions had an even greater percent producing nipple aspirate fluid (88%). 21% of the women in this group who were later found to have carcinoma, and 72% of those had originally produced nipple aspirate fluid.

These nipple aspirate fluids represent secretions of both endogenous and exogenous substances (King and Goodson, 1991). The secretion also contains exfoliated epithelial cells and nonepithelial cells of blood or immune system origin. Endogenous substances include lactose, alpha-lactalbumin, immunoglobulin, cholesterol, fatty acids, and a number of steroids (Petrakis et al., 1987a). Exogenous substances include technetium, barbiturate, fatty acid, caffeine, pesticides, and nicotine and cotinine related to cigarette smoking (Petrakis et al., 1987a). Mutagenic activity with the Ames salmonella test was exhibited by 10% of the nipple aspirate fluid samples. Petrakis et al. (1987b) studied estrogen (estrone and estradiol) levels in serum and nipple aspirates of breast fluid in relation to reproductive and menopausal characteristics in 104 normal women. In general, breast fluid estrogen levels were 5 to 45 times higher than serum levels. Breast fluid estrogen mean levels were lower in premenopausal parous women than in nulligravidous or nulliparous women whereas serum levels did not differ in these 3 groups. Breast fluid estrogen levels were positively correlated with months since last birth or since last breast-feeding. Estrogen levels were low in nipple aspirates of breast milk but gradually increased in breast fluid of non-lactating women over a period of several years after cessation of lactation. On the other hand, serum estrogen levels did not increase with months since last breast-feeding. Petrakis et al. (1987b) suggested that the prolonged low levels of breast fluid estrogens following full-term birth and lactation may, in part, provide a mechanism by which parity reduces breast cancer risk.

Cholesterol and its oxidation products, 5,6 alpha and beta epoxides and their common hydrolysis product cholesterol triol, were measured in nipple aspirate fluid by Gruenke and associates (Gruenke et al., 1987). Both these products have been

implicated in oncogenic behavior in a number of different studies demonstrating induction of sarcomas, in vitro transformation of embryo hamster cell lines, chromosome damage and inhibition of DNA repair, and mutagenic activity.

That breast epithelia secretes fluid and many types of molecules into the lumen is well established. It is also clear that very high levels of estrogen are present in these secretions. It has been shown that the estrogen metabolite, 17 beta-estradiol glucuronide, is a substrate for Pgp (Gosland et al., 1993), prompting us to ask the specific question as to whether estrogen is transported by breast ductule epithelium. Most of the research on breast secretions was done before it was known that Pgp is present in breast epithelium.

Effect of lactation on risk of breast cancer

Numerous studies have reported a beneficial effect of lactation on the risk of breast cancer in premenopausal women in Western societies (Byers et al., 1985; McTiernan and Thomas, 1986; Kelsey and Berkowitz, 1988). Indeed, a study of risk factors for breast cancer in Chinese women in Shanghai showed a high degree of reproducibility of most established menstrual and reproductive breast cancer risk factors of Western populations in this low risk developing country (Yuan, et al., 1988).

Relationship of transporter expression and function to levels of estrogen and certain pesticides

Because estrogen can reach such high levels in breast epithelial fluid (Petrakis et al. 1987b) and progesterone has been found to be an inhibitor of Pgp transport (Ichikawa-Haraguchi et al., 1993; Van Kalken et al., 1993; Ueda et al., 1992) and a substrate of Pgp (Qian and Beck, 1990) it is reasonable to question the role of these two hormones in Pgp function in breast epithelium. Interestingly, the estrogenic, organochlorine pesticides, DDT and DDE, in nM concentrations, led to increased Pgp expression, multidrug resistance, and drug efflux in MCF-7 breast cancer cells (Fine et al., 1997). Lanning et al. (1996) showed that the organophosphorus insecticide, chloroprifos oxon, introduced by gavage into Fisher 344 rats, increased Pgp expression after 72 hours, in the kidney, adrenal, liver, jejunum, and stomach compared to control tissues.

The Mrp gene has an estrogen responsive element (Zhu and Center, 1994). To my knowledge, the only report of Mrp2 regulation by hormones is our study showing that this transporter is downregulated by endothelin-1 in the shark rectal gland. (Karnaky et al., 1999).

IT IS IMPORTANT TO NOTE THAT WE WERE NOT ABLE TO

FIND ANY EVIDENCE FOR THE Pgp TRANSPORTER IN HUMAN BREAST TISSUE. Neither transport studies, in which freshly isolated breast ductules were incubated in daunomycin, nor immunocytochemcial studies demonstrated the presence of Pgp presence or any evidence for its function. **HOWEVER, WE DID DISCOVER THE PRESENCE AND FUNCTION OF A SECOND TRANSPORTER, mrp. THIS WORK ON HUMAN BREAST TISSUE WAS BEGUN BECAUSE OF A SECOND, INDEPENDENT, RESEARCH PROJECT ON THE RECTAL GLAND OF THE DOGFISH SHARK. THIS SHARK RESEARCH WAS CONDUCTED AT THE MT. DES. ISL. BIOL. LAB. AND WAS SUPPORTED BY A SENIOR FELLOWSHIP FROM THE SALSBURY COVE RESEARCH FUND OF THAT LABORATORY. HERE, WE RELATE THE STORY OF THAT DISCOVERY.**

Discovery of Mrp2 in human breast ductules

Recent evidence indicates that aquatic organisms possess one or more MDR-like transport mechanisms that allow them to restrict xenobiotic uptake and increase chances of survival in polluted environments (reviewed in Kurelec, 1997). For example, Western blotting shows the presence of proteins immunologically related to P-glycoprotein in tissues from marine and freshwater invertebrates and vertebrates. Binding assays with membrane fractions show xenobiotic binding with the broad specificity characteristics of P-glycoprotein. Also, the ability of sponges, marine mussels, freshwater clams, and marine worms to limit accumulation of xenobiotics is reduced after exposure to P-glycoprotein modifiers, e.g., verapamil. Finally, several studies show induction of the multixenobiotic resistance phenotype in organisms exposed to polluted water (Kurelec, 1997). Together, these findings make a case for the presence of one or more xenobiotic excretion systems in these organisms. Such transport systems are present at some basal level in animals maintained in unpolluted environments and they appear to be upregulated on exposure to pollutants.

In contrast to mammals, where high levels of P-glycoprotein and Mrp2 are found in the luminal membranes of the epithelial cells of the renal proximal tubule, intestines, and liver (Buchler et al., 1996; Schaub,, et al., 1997; and Thiebaut et al., 1987), we know little about the tissue distribution of xenobiotic transporters in aquatic animals. In the liver this transporter appears to be responsible for the excretion of bile acid sulfates and glucuronides (Suchy et al., 1997). In killifish kidney proximal tubules, confocal microscopy demonstrated secretion of fluorescent methotrexate into the lumen; this

transport was inhibited by leukotriene C_4 (Masereeuw et al., 1996). Limited data from teleost fish do indicate that P-glycoprotein and Mrp2 are present in renal and hepatic tissue, where they could mediate the excretory transport of xenobiotics (Hemmer eet al., 1995; Masereeuw et al., 1996; Schramm et al., 1995). However, aquatic organisms possess other excretory organs. One such organ is the rectal gland of marine elasmobranchs. This gland is a specialized, salt excretory organ composed of numerous blind-ended, branched tubules that empty into a central duct (Forrest, 1996; Silva et al., 1996). Rectal gland tubules contain only a single cell type, a columnar epithelial cell. The NaCl concentration of the fluid secreted by the shark rectal gland is higher than plasma, thus contributing to the ability of these hyperosmoregulators to volume and ion regulate. To date, NaCl and fluid excretion have been the only described function of the elasmobranch rectal gland. In our publication (Miller et al., 1998) we used isolated rectal gland tubules and confocal microscopy and described a second function of the shark rectal gland, xenobiotic excretion. We discovered the presence and function of a Mrp2-like transport activity.

Shark rectal gland: Xenobiotic transport in isolated rectal gland secretory tubules

In initial experiments, tubular fragments were exposed to a variety of fluorescent xenobiotics and cellular and luminal fluorescence visualized using confocal microscopy. Previous studies with teleost renal proximal tubules had shown that the substrates chosen were transported by the organic anion system (fluorescein and sulforhodamine 101), the organic cation system (daunomycin and rhodamine 123), Pgp (NBDL-CSA, rhodamine 123 and daunomycin) and Mrp2 (sulforhodamine 101 and fluorescein-methotrexate) [reviewed in Masereeuw et al., 1996]. Three patterns of xenobiotic accumulation were found in rectal gland tubular fragments: 1) fluorescein did not accumulate within the cellular or luminal compartments; 2) NBDL-CSA, rhodamine 123 and daunomycin accumulated within the cells, but luminal fluorescence was always lower than cellular fluorescence; 3) sulforhodamine 101 and fluorescein-methotrexate did not accumulate to high levels within rectal gland cells, but luminal fluorescence was substantially higher than cellular or medium fluorescence. Thus, rectal gland tubule fragments were able to selectively concentrate fluorescent xenobiotics in the luminal space. Of the five fluorescent substrates for renal xenobiotic transport systems, only those handled by Mrp2 showed concentrative secretion into the lumens of rectal gland tubules.

We used confocal microscopy and digital image analysis to characterize the mechanism driving sulforhodamine 101 transport from medium to tubular lumen. This substrate was chosen rather than fluorescent methotrexate because of its

resistance to photobleaching and reduced dependence of fluorescence to changes in medium pH. Cellular fluorescence intensity rose rapidly with time and reached a steady state value that was about two thirds of the level in the medium. Luminal fluorescence also rose rapidly, but, at steady state, mean luminal fluorescence intensity was about 8 times higher than cellular fluorescence and 5 times higher than in the medium. Addition of 0.1 KCN had little effect on cellular fluorescence.

Next we examined the effects of changing medium sulforhodamine 101 concentration on steady state medium, cellular and luminal fluorescence. As one would expect, medium fluorescence increased linearly with sulforhodamine 101 concentration. Cellular fluorescence was also a linear function of medium sulforhodamine 101 concentration, but, at each concentration, cellular fluorescence was significantly lower than medium fluorescence. Luminal fluorescence exceeded medium and cellular fluorescence. Luminal fluorescence increased with medium sulforhodamine 101 concentration but, unlike medium and cellular fluorescence, those increases were less than proportional. That is, the data for 5μM and 10μM sulforhodamine 101 fell below the extrapolated line drawn through the origin and the 1μM sulforhodamine 101 point.

Having demonstrated that sulforhodamine 101 transport into rectal gland tubular lumens was concentrative, energy dependent and saturable, we used chemicals known to be handled by specific transport systems as tools to characterize specificity limits of the mechanism involved. PAH and TEA, model substrates for the classical renal organic ánion and organic cation transport systems, respectively, did not affect sulforhodamine 101 transport. Neither did verapamil, a substrate for both the renal organic cation transport system and Pgp.

In liver, secretion of large amphiphilic organic anions from hepatocyte to bile canaliculus is mediated by one or more multispecific organic anion transporting ATPases (Mrp2 or cMOAT). A similar transporter has been proposed for luminal membrane of renal proximal tubule (Masereeuw et al., 1996). It is important to note that these anion-transporting ATPases are distinct from the Pgp that is also present at high levels in the canalicular membrane of hepatocytes and in the luminal membrane of renal proximal tubule cells (Hemmer et al., 1995). The organic anion transporting ATPases are sensitive to inhibition by a variety of anionic compounds. Of these, cysteinyl leukotrienes (LTC) have a particularly high affinity for the transporter, with LTC_4 exhibiting a K_m of 250 nM (Masereeuw et al., 1996). Transport by these ATPases is also inhibited by cyclosporine A (CSA, K_i 3μM) and by certain GSH conjugates. To determine whether a transporter with similar specificity might be operating in rectal gland tubules, we measured the effects of LTC_4, CSA, and chlorodinitrobenzene (CDNB) on sulforhodamine 101 transport. Both CSA and LTC_4 significantly reduced luminal fluorescence, but had no significant effects on cellular fluorescence. Luminal fluorescence was also reduced

in a concentration-dependent manner when tubules were incubated in medium containing CDNB. This molecule enters the cell by simple diffusion, and is conjugated to GSH. The resulting conjugated compound is a high affinity competitor for multispecific organic anion transporting ATPases such as Mrp2.

Shark rectal gland: Sulforhodamine 101 secretion by perfused rectal gland

To determine whether sulforhodamine 101 was secreted into luminal fluid in the intact rectal gland, glands were perfused with medium containing 1μM sulforhodamine 101 and secreted (duct) fluid analyzed for sulforhodamine 101 (fluorometer) and chloride (chloridometer). After an initial 30 min period, duct fluid volume and chloride excretion stabilized. From 30 min on, duct fluid chloride concentration averaged about 350μM, which was significantly higher than the chloride concentration of perfusate or venous effluent. Chloride secretion in these unstimulated glands averaged 1μEq/min/g, in agreement with previously published values (see Valentich et al., 1995).

When 1μM sulforhodamine 101 was added to the gland perfusate, dye could be detected in duct fluid. After a short delay, the concentration of sulforhodamine 101 in duct fluid rose and eventually reached a plateau. At steady state, the sulforhodamine 101 excretion rate averaged 4 nmol/min/g and the sulforhodamine 101 concentration in the duct fluid averaged about 1 μM. When 25 μM CDNB was added to the perfusate along with sulforhodamine 101, steady state dye excretion was reduced by over 75%. CNDB has at most a small effect on chloride secretion rate. Thus, the intact glands exhibited the same selective and CDNB-sensitive excretory transport of sulforhodamine 101 as isolated tubule fragments.

Shark rectal gland: Immunolocalization of Mrp2

Frozen sections of rectal gland were exposed to a polyclonal antibody to Mrp2 and a fluorescent secondary antibody. Epifluorescence micrographs of this tissue showed strong staining at the luminal plasma membrane and an absence of staining at the basolateral membrane or within the cells.

(6) BODY

This proposal has brought together several powerful techniques to elucidate potentially harmful transport processes in breast epithelium

In our view it is imperative that to understand normal breast epithelial function we will need to study ductules freshly dissected from the animal or in primary cultures,

rather than cancer cell lines. To achieve this important goal, our research team includes Dr. Debra Hazen-Martin, who has vast experience in primary tissue culture of both normal and cancerous tissues. We have studied breast epithelia in two different tissue preparations.

Technical Objectives

Task 1: Do glandular epithelia of freshly isolated breast tissue transport Pgp substrates? (functional expression)

Ductule Isolation Method: Breast tissue is received from reduction surgery and placed into M199 (Gibco) with 5% FBS (Sigma) and Penicillin-Streptomycin [100 I.U./ml and 100 μg/ml, respectively]. Tissue may be stored overnight at 4 °C and remain viable. Dense stromal tissue is cut into approximately 5 mm X 5 mm pieces and placed into M199 with 5% FBS and 2000 U/ml Type I collagenase (Sigma). Vials of mixture are placed into 95% O_2 and 5% CO_2 gassed bubbles and rocked on high speed for 3 hours, and regassed every three hours. When stromal areas have dissociated, the mixture is centrifuged at 1000 r.p.m. for 1 min. The fat layer is removed and the remaining mixture is poured into a 60 mm culture dish. Fresh media is added and ductules are viewed by dissecting scope. Ductules are pipeted into fresh media to be used for transport studies. These ductules are incubated overnight in normal M199 or with M199 containing 10^{-8} M β-estradiol and used for fluorescent and inhibition studies by confocal microscopy.

Results: we could find no evidence that freshly isolated tubules transport Pgp substrates. Even after repeated attempts, the fluorescent Pgp substrate, daunomycin, never appeared in the lumen.

However, following the discovery of a second type of transporter, the Mrp2 transporter, in the dogfish shark rectal gland secretory tubules using Texas Red (see above), we immediately tried this substrate on human breast ductules. As can be seen by **Figure 1 (below in APPENDICES)**, we observed Texas Red in the lumen of ductules if, and only if, we pretreated the ductules with estrogen or progesterone. These data strongly suggest that Mrp2 transport in the breast ductules is under hormonal control. We also asked the crucial question as to whether immunocytochemical methods would demonstrate Mrp2 in fresh isolated breast ductules which had not been subjected to collagense digestion and to hormone treatment. As can be seen by **Figure 2 (below in APPENDICES)**, Mrp2 is present in the apical membrane region of ductual epithelial cells from freshly isolated

tissue which has not been subjected to the collagenase treatment.

Experimental problems: 1) low number of surgical tissue available to study; 2) some samples of breast tissue are simply too fibrous to study. My technician discovered several of these and found that they were just too tough and did not digest at all with our collagenase procedure. We learned to detect these with a simple dissection procedure so that we did not need to waste expensive collagenase on these rare fibrous samples. The major reason for slow progress on this grant is low number of surgical tissue available to study from our hospital.

Task 2: Do primary cultures of glandular epithelia transport Pgp substrates? (functional expression)

We have devoted considerable time to this very important technique of tissue culture of breast epithelial cells.

Cell culture methods: Breast tissue form reduction surgery is placed into holding medium consisting of Dulbecco's modified Eagle's medium (DME) supplemented with 15% fetal bovine serum, 0.5 μg/ml Fungizone, and Penicillin-Streptomycin [100 I.U./ml and 100 μg/ml, respectively]. All above reagents from Gibco, N. Y. Under a biohazard hood, stromal tissue is minced and placed into a dissociation mixture of DME with 5% FBS supplemented with selenium (ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), collagenase type I (200 U/ml), hyaluronidase (100 U/ml), fungizone, and Pen-Strep. [Enzymes from Sigma and ITS from Collaborative Research]. Tissue is placed in a 37 °C bath on a magnetic stir plate on low speed, overnight. Under sterile conditions, dissociated mixture is poured into 50 ml tubes and centrifuged for 10 min at 1000 r.p.m. Fluid is drawn off and the pellet is resuspended with fresh growth medium and plated into collagen coated flasks (Corning T-25 flasks coated with Vitrogen 100 collagen from Collagen Biomaterials). Growth medium consists of 1:1 DME and Ham's F-12 growth medium (Gibco) supplemented with previously stated concentrations of insulin, transferrin, selenium, Pen-Strep, fungizone, hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), epidermal growth factor (10 ng/ml), and sometimes bovine pituitary extract (30 μg/ml). All growth supplements were obtained from Collaborative Research, unless otherwise stated. Cultures were fed 3 times a week and may become confluent within 2 weeks. **Figure 2c (below in APPENDICES)** shows cells grown on plastic tissue culture dish.

Results: Cultures grown on Transwells inserts:

The next step in the process to study transport by breast ductule epithelial cells is

to make monolayer cultures suitable for study in the Ussing chamber. These cultures must be grown on some sort of permeable substrate. We have been successful in making cultures with high electrical resistance. Cells are harvested from the plastic flask and plated on commercially available Transwell insert supports and allowed to form confluent cultures. Several of these cultures exhibited resistances of approximately 1000 $\Omega.cm^2$ when this electrical property was measured with an EVOM electrical device. This is a special device commonly used to measure electrical properties of cultured cell in this type of insert. 1000 $\Omega.cm^2$ is an excellent resistance value. We then mounted two of these high resistance epithelial in our special teflon Ussing chambers. For this procedure we needed to use a scalpel blade to cut the epithelial culture from the Transwell. When we mounted these cultures in Ussing chambers they had lost about 80-90% of their resistance. Although not ideal, these cultures should be suitable for studying Mrp2-mediated transport under very carefully controlled in vitro conditions. These cultures respond to the addition of forskolin, which stimulates the short-circuit current. This finding signifies that the epithelial cells are polarized, with NaCl transport proteins in the correct location on cell membranes. Likewise, the Na pump inhibitor, ouabain, decreased the short-circuit current to almost zero. This latter observation also means that the Na pump is on the basolateral membrane, its usual location. Although we have not had time to examine the transport of Mrp2 substrates as yet, these preparations appear to be suitable for these studies, which we will do in the current grant.

One of my colleagues, Dr. Larry Renfro, has revealed to our group that in his experience, Pgp function does not appear in tissue cultured winter flounder proximal tubule epithelial cells **UNLESS** the cultured cells are grown on a contracting collagen gel, which gives the cells a more columnar shape (Sussman-Turner and Renfro, 1995).

Contracting gel method:

Rat tail collagen is made and its weight is determined. Depending on this amount, the collagen is added to 0.1% acetic acid, NaOH/Hepes buffer, and 10X M199. Gels are quickly poured in tissue culture dishes or plates and allowed to polymerize. Growth media is added and rinsed 3 times for twenty min. each. Cells are typically triple plated to assure confluent attachment. After a few days of attachment, the gel can be released by rimming the edge with a sterile Pasteur pipet. Contraction occurs according to the concentration of collagen. Theoretically, epithelial cells grown on these gels can be used in Ussing chambers for experimental evaluation of Mrp2 substrate.

Experimental problems: These cultures grown on contracting gels did not form monolayers of tighly joined (confluent) cells. We do not know why this happened, and are continuing to work with this method.

Task 3: Do breast epithelial cell lines transport Pgp substrates?

The work described above has taken the full two years of the grant, and we have not been able to get to this task. We are working on it in the current Army Breast Cancer Grant.

Task 4: In any of the cultured cells above, is the transport rate for Pgp substrates influenced by estrogen and progesterones at concentrations normal for blood during a monthly menstrual cycle?

As shown above (Figure 1), estrogen and progesterone DO influence the transport of Mrp2 substrates to the lumen of breast ductule cells. Since we have not been able to study transport of Mrp2 substrates across cultured breast epithelial cells, we have not been able to complete this task. However, as stated above, at the end of the grant period we have had several primary breast epithelial cell cultures which gave adequate resistance in Ussing chambers. We will attempt this in the current Army Breast Cancer grant.

Task 5: What is the location of the Pgp in the tissues used for transport studies above?

We have localized Mrp2 to the apical membranes in our cultures using antibodies to an epitope of the human form of this transporter kindly gifted by Dr. Dietrich Keppler. This apical localization is exactly where we predicted the transporter would be located. In this location Mrp2 could transport carcinogens to the lumen of breast ductules. **Figure 3 (below in APPENDICES)** demonstrates this apical localization. As a check on our techniques (a positive control), we have also used this same antibody to conduct immunocytochemistry on an intestinal cell line, CaCo2, which possesses Mrp2. As shown in **Figure 4 (below in APPENDICES),** this cell also exhibits apical membrane localization.

Task 6: What is the expression of Ppg in breast tissue used for transport studies?

At the end of this grant period, we have finally obtained our first Western blot of breast epithelial cell cultures from 5 patients and compared them to the staining pattern of the intestinal cell line, CaCo2, which is known to possess Mrp2. Both the CaCo2 and all five specimens of breast cultured epithelial cells showed the same staining

pattern (a band at approximately 190 kDa, using Keppler's antibodies to human Mrp2).

Task 7: Can we detect the mRNA for Pgp in freshly isolated tissues, primary cultures, and breast epithelial cell lines?

We have been unable to get to this phase of the experiments but will be doing it soon during the current Army Breast Cancer grant.

Recommendations in relation to the Statement of Work

As we stated in the original grant proposal, "....it is extremely difficult to give a precise timetable for this research, because part of it depends on the availability of surgical tissue from breast reduction or similar types of procedures...." The frequency of surgical procedures did in fact turn out to be a rate-limiting step in the research progress. Even though we were told that there were several operations per week, it was rarely that, and sometimes the rate was only two operations in one month. This problem could only be overcome by seeking additional sources of tissue at other area hospitals. However, the only tissue which will give meaningful data to test our hypothesis is FRESH tissue. Transformed cell lines will not give us information about processes occurring over decades in the life of healthy breast tissue. In summary we have been able to make progress in all of our Tasks with the exception of the measurement of mRNA in breast epithelial cells and the study of commercially available cultures of breast epithelial cells. We still believe that the best possible tissue for these studies is the freshest tissue we can obtain, directly from surgeries here in Charleston.

(7) CONCLUSIONS

Our central hypothesis is that breast ductules possess excretory transporters which could transport hydrophobic carcinogenic molecules to the lumen of these ductules. The consequence of this could be very harmful, since these deposited carcinogens could diffuse back through the apical membrane and attack DNA of the lining cells. This transport process would be especially harmful if it were enhanced by the presence of estrogen. So far, all of our data support the conclusion that human breast ductules possess one of these excretory transporters, Mrp2. First, immunocytochemical studies show that the apical membrane of ductules epithelial cells possesses Mrp2. Second, our transport studies show that freshly isolated ductules transport sulforhodamine 101, a substrate for the Mrp2 transporter, into their lumens. This transport process is tremendously enhanced by exposure of ductules to estrogen.

Since lifetime exposure to estrogen is considered the major risk factor in breast cancer, this estrogen regulation of the transport phenomenon may be especially significant. This apparent regulation b estrogen may be significant in terms of dietary intake of xenoestrogens and with regard to medications such as tamoxifen. Third, our preliminary Western blot data show that our breast epithelial cells cultured in flasks possess Mrp2. During this grant we have established the above points, and developed the model systems and methods to study this transport phenomenon with fresh ductules. However, it is critical that we develop even more refined methods, namely tissue culture methods so we can study ductule epithelial cells as flat sheets in Ussing chambers. We have not fully completed this methodology to the point of demonstrating transport of Mrp2 substrates and its regulation by estrogen. However, we have now achieved cultures which have a high resistance, and are ready for those transport studies. We have overcome a number of technical challenges during these two years and are ready to extend our findings.

We should close by emphasizing that it was work on the shark rectal gland by a colleague, Dr. John Valentich, which first stimulated my interest in the xenobiotic exporters. As detailed above, the discovery of sulforhodamine 101 transport in the shark rectal gland model prompted our attempt to look for Mrp2-mediated transport in human breast tissue. Just last summer, working the Mt. Des. Isl. Biol. Lab., we discovered that endothelin-1 downregulates Mrp2 and that $ZnCl_2$ and $CdCl_2$ upregulate this transporter in shark rectal gland tubules (Karnaky et al., 1999). We will continue to study Mrp2 transport processes in the shark rectal gland to aid in our understanding of the potential role in carcinogenesis of similar transport processes in human breast ductules. I have funding for that study through a Senior Fellowship (New Investigator Award) from the Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine.

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APPENDICES

FIGURE 1

Two different ductules (one in the top panel and one in the bottom panel) isolated from human breast tissue. Both of these ductules were exposed to estrogen (10^{-8} M for 24 hr), and then the ductule in the bottom panel was exposed to 2.5μM Texas Red for 90 min. Finally the ductules were photographed with a CONFOCAL FLUORESCENCE MICROSCOPE. The ductule in the top panel shows that there is NO autofluorescence in this tissue (it would require a phase contrast microscope to actually see the tissue). The branched tubule in the bottom panel has a bright intensity in the ductule lumens caused by Texas Red. Interestingly, there was NO transport in tissue not exposed to hormones, and there was NO autofluorescence in tissue NOT exposed to hormones or fluorescent substrates. These observations are strong evidence that the secretion of the Mrp2 substrate, Texas Red, is under hormonal regulation.

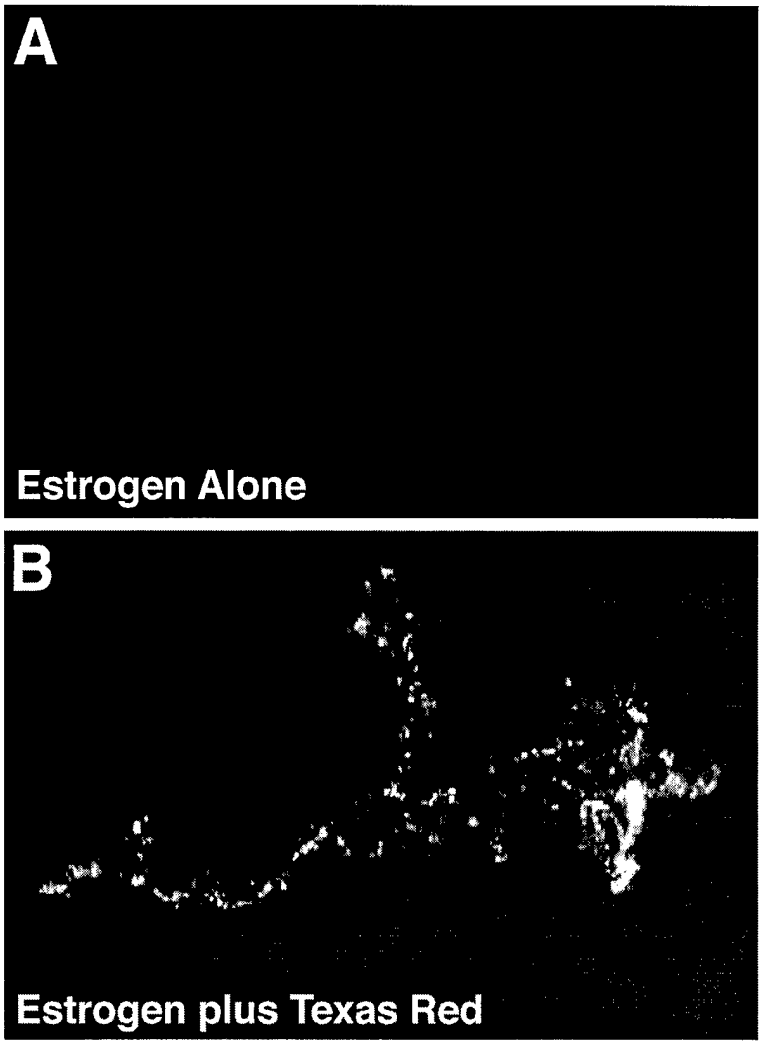

Figure 1

FIGURE 2

Breast tissue frozen immediately after receipt from the operating room. The tissue was frozen in OCT compound and frozen sections cut with in a cryostat. These serial sections were lightly fixed with formaldehyde, and processed for immunocytochemistry with an immunoperoxidase method. In Panel A (control), the primary antibody to the human form of Mrp2 (kind gift of Dr. Dieterich. Keppler, Heidelberg, Germany), was left out of the reaction mixtures. In Panel B, the Mrp2 antibody was present. As can be seen in Panel B, a strong reaction is present in the apical membrane of this breast ductule. The control tissue lacks this apical localization.

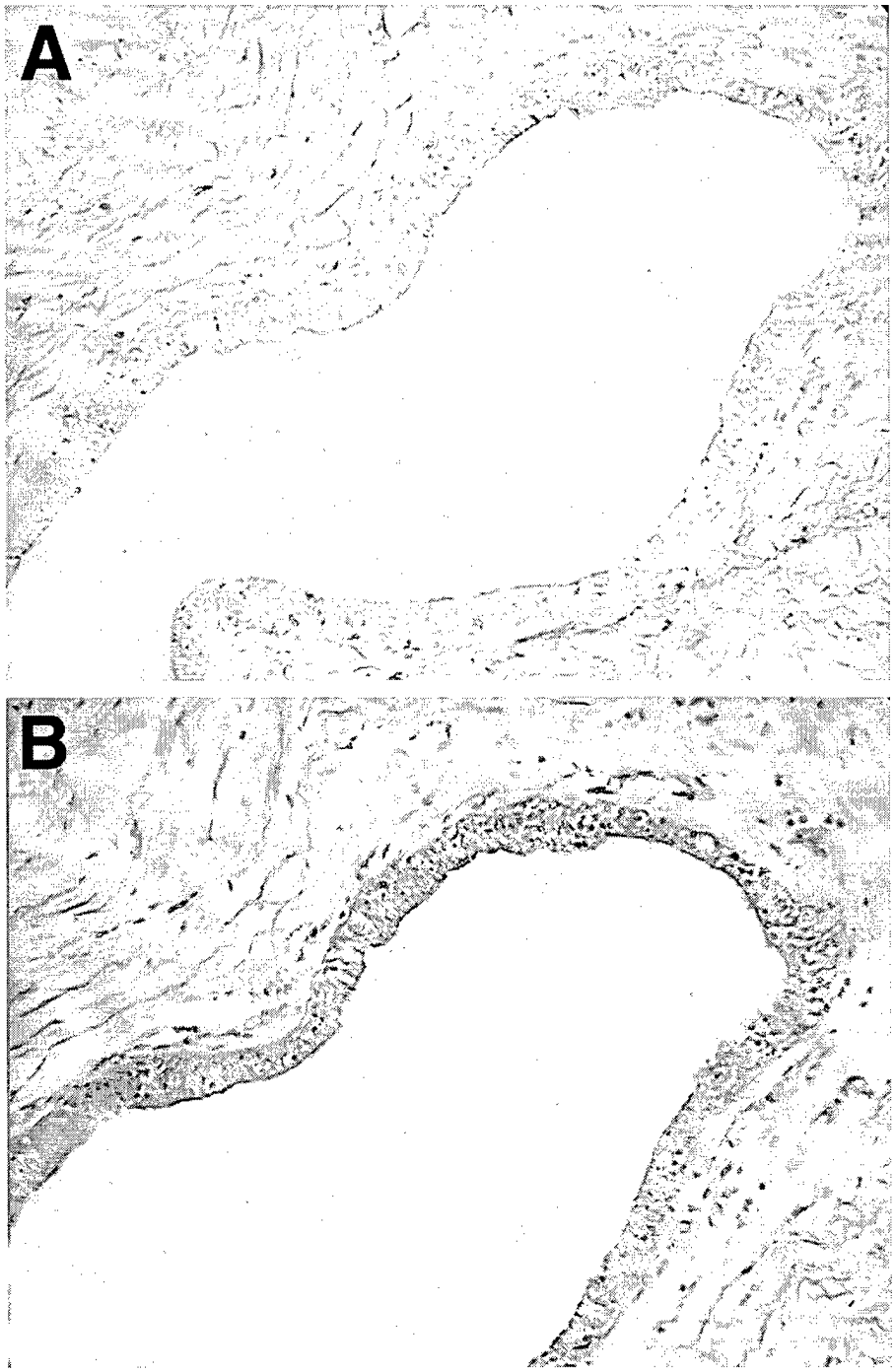

Figure 2.

FIGURE 2c

This figure shows a confluent layer of breast epithelial cells after twelve days of growth on a plastic tissue culture dish. When we detach these cells from this plastic dish with trypsin, they are reseeded on Transwells, where they become confluent again.

Figure 2c

This micrograph shows confluent breast ductule epithelial cells after twelve days of growth on a plastic dish. The dish was originally seeded with freshly isolated organoids from collagenase-digested breast tissue. Next these cells will be detached from this surface and seeded on collagen coated substrates such as Transwell, where they become confluent again, and establish an electrical resistance of several hundred ohm-cm2.

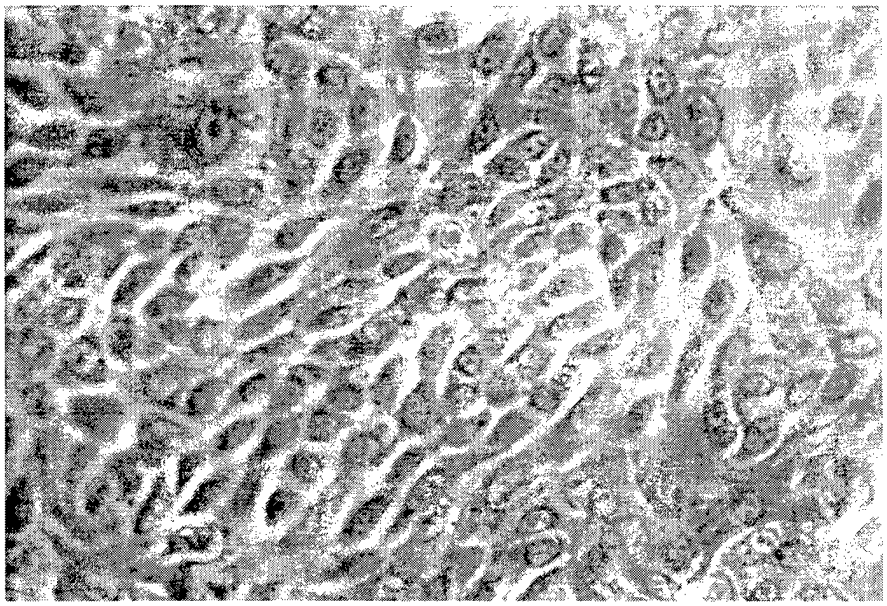

FIGURE 3

A culture of breast epithelial cells grown in on the substrate of a Transwell. These cells were fixed with paraformaldehyde and processed for immunocytochemistry with an antibody to human Mrp2. These cultures were examined en face with the CONFOCAL FLUORESCENCE MICROSCOPE. Panel A is the control, and shows only the smallest amount of background autofluorescence. Panels B and C are two different views of these cultures and shows a large amount of immunoreactivity, apparent only at the apical surface. The cells are very thin, giving the impression that cytoplasm is also stained.

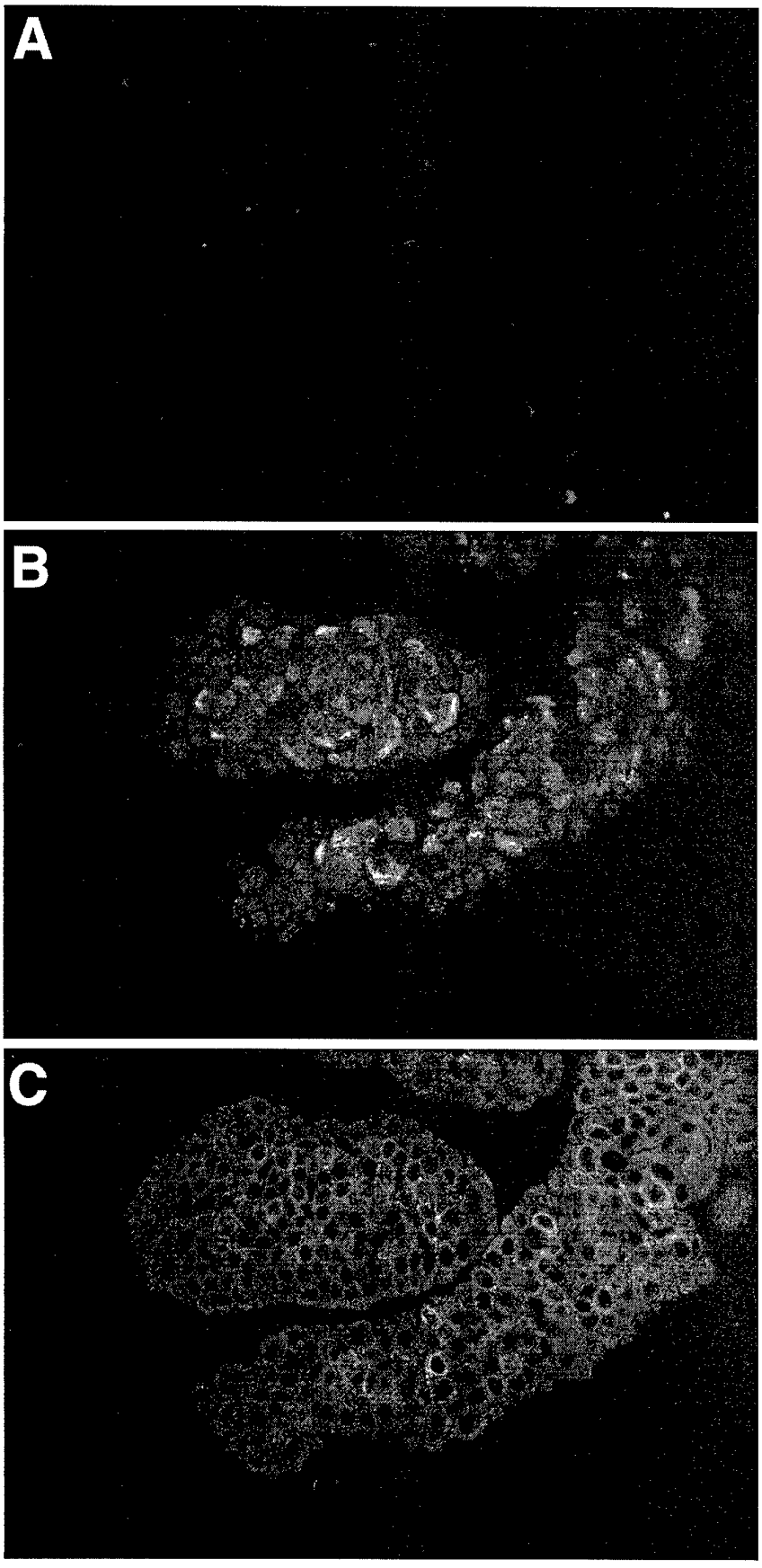

Figure 3

FIGURE 4

As a positive control, we used the same immunocytochemical method on cultures of CaCo2 cells, an adenocarcinoma cell line derived from the small intestine, grown on Transwells. Panel A is the control, and Panels B and C are two different views of one of these cultures. Labelling is most intense in the apical membrane region of the cells.

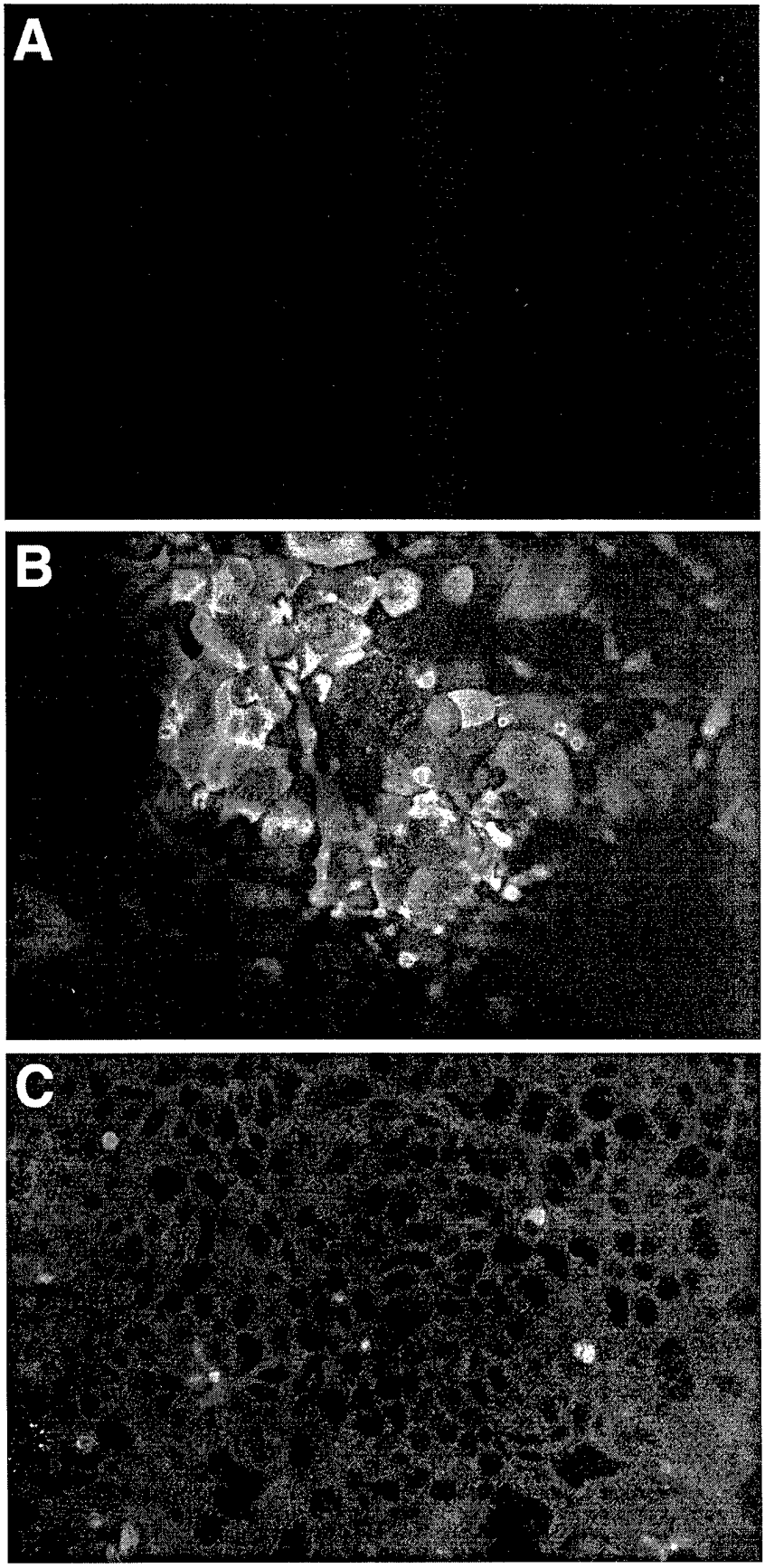

Figure 4

(11) BIBLIOGRAPHY: NO PUBLICATIONS OR ABSTRACTS AS OF THE TIME OF THIS REPORT

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