PREECLAMPSIA AND THE ROLE OF UTERINE NATURAL KILLER CELLS

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Preeclampsia and the role of uterine Natural Killer (uNK) cells

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ABSTRACT

The investigation of preeclampsia has been greatly facilitated by the use of pregnant mouse models. In this study, immunodeficient mice (RAG2$^-$/γc$^-$) were used to examine the migration of white blood cells (WBC) and uterine Natural Killer cells to the uterus and spleens of bone marrow engrafted mice. Three weeks after the mice were injected with C57Bl6 or Balbc bone marrow cells, they were mated. Ten days after the appearance of a copulation plug, organs were collected, paraffin embedded and sectioned. Hematoxylin and Eosin (H&E) staining was done to determine basic morphology and confirm successful sectioning technique. Biotinylated anti-CD45 antibody was used to determine the presence of WBC. Dolichos Biflorus Agglutinin (DBA) lectin was used to determine the presence of uterine Natural Killer (uNK) cells. Neither anti-CD45 nor DBA stain yielded significant positive results for the test mice. Currently, there is no other leukocyte-specific antibody available to stain WBC paraffin-embedded sections. Periodic Acid Schiff (PAS) staining for Natural Killer cells showed 2-4/100x field of view in control mice, and 7 of 12 of the spleens from test mice had at least one positive cell, while 4 had levels similar to controls. Follicles were present in most of the positive control mouse spleen sections but not in the test mice. While serum antibody was previously demonstrated in these mice, we found only low numbers of transplanted bone marrow cells in the spleen or uterus of these mice. It appears that engraftment will not be successful in RAG2$^-$/γc$^-$ mice in the absence of further immunosuppressive treatment.

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List of Abbreviations

BM: Bone Marrow

DBA: Dolichous Biflorus Agglutinin

ELISA: Enzyme Linked Immunosorbent Assay

FM: Female

H&E: Hematoxylin and Eosin

HLA: Human Leukocyte Antigen

Ig: Immunoglobulin

IL: Interleukin

KIR: Killer cell Immunoglobulin-like Receptor

MHC: Major Histocompatibility Complex

ML: Male

NADG: N-acetyl-D-Galactosamine

NOD: Non obese diabetes

PAS: Periodic Acid Schiff

PBMC: Peripheral Blood Mononuclear Cells

SCID: Severe Combined Immunodeficiency

TCR: T cell receptor

uNK: uterine Natural Killer

WBC: White Blood Cell

Introduction

In an attempt to understand the role of the immune response during pregnancy, this study investigated reconstitution of genetically immunodeficient mice with normal bone marrow cells and migration of uterine natural killer (uNK) cells into various organs. These cells are believed to be crucial in normal pregnancy development in mice, as it has been noted that mouse models lacking these cells develop characteristics that may mimic symptoms in humans having a condition known as preeclampsia.

Pregnancy

During normal fetal development, cleavage of the fertilized egg begins a few hours after fertilization, during which time the fertilized egg enters a cycle of synchronous cell divisions. These initial divisions form a 64-celled ball of unspecialized cells called a morula. As cleavage continues, the divisions lose their synchronous character, and the cells are now referred to as a blastula/blastocyst when they divide to produce 128 cells. A blastocoele, which is a fluid-filled cavity in the middle of the blastula, forms. The outer layer of cells around the blastocoele of the mammalian blastocyst flattens out to form a trophoblast layer, which has the property of epithelial cells. The inner surface of the trophoblast layer also has an inner mass of cells which later on become a part of the embryo. This trophoblast layer,

however, becomes a part of the placenta after implantation and gives rise to the chorion, which is one of the four extra-embryonic membranes formed during fetal development. Two weeks after fertilization the chorion extends villi (finger like projections) into the uterine wall. These chorionic villi closely associate with endometrial cells, thus initiating placenta formation (Carlson, 1981).

Cleavage continues in this blastula stage, during which cell numbers increase to between 10,000 and 15,000. The blastula then enters gastrulation, which is characterized by formation of three defined germ layers; the ectoderm, endoderm and mesoderm. At about the 6^{th} or 7^{th} day after human fertilization, the blastocyst implants itself into the upper part of the uterine lining. When cells of the trophoblast come into contact with maternal tissue, they begin to rapidly proliferate and form two distinct layers. The inner layer retains a distinct cellular nature and is referred to as the cytotrophoblast, whereas the other layer forms an irregular syncytium (mass of cytoplasm with several nuclei but no distinct cell boundaries) and is called the syncytiotrophoblast. After eleven to twelve days of human pregnancy, the blastocyst has almost completely embedded within the endometrium, the mucous membrane that lines the uterus. The uterine epithelium grows back on the embryo. During the initial implantation stages, the embryo is

nourished by maternal fluids and debris resulting from the destruction of endometrial cells. However, irregular lacunae (small empty cavities) form within the syncytiotrophoblast which become filled with maternal blood emanating from eroded uterine blood vessels. (Carlson, 1981)

The decidua is the epithelium tissue of the endometrium formed when the blastocyst is implanted, and this region is where the maternal arteries that supply blood between the mother and fetus are found. Below the decidua is the metrial gland, which is now known to contain numerous uterine natural killer (uNK) cells during pregnancy. Placental formation is initiated by the decidual reaction. The decidual reaction is when uterine stromal cells around the blastocyst undergo transformation in which they enlarge and become filled with glycogen and lipid droplets. The placenta layer forms above the decidua, and acts as a bed that supplies nutrients and allows for exchange of gases between the mother and the fetus. (Carlson, 1981). To facilitate proper gaseous and nutrient exchange between the fetus and the mother, trophoblast cells from the fetus either form a multinucleated syncytial layer which lines the intervillous blood space, or they form individual extensions called villi. Floating villi are responsible for gaseous, nutrient and waste product exchange, whereas anchoring villi contain trophoblast cells that invade into maternal spiral arteries (one of the corkscrew-like arteries in the

endometrium) to initiate normal blood flow (Aplin et al., 2000). Once these villi attach to the spiral arteries, they replace vascular endothelial cells and transform the smooth muscle cells around the arteries so that they become unresponsive to vasoconstrictive agents (Reviewed in: Kam, et al., 1999). This invasion of the spiral arteries is of great importance as it consequently results in wider aterial diameter and increased blood flow between the fetus and mother. However, in preeclampsia, the anchoring villi fail to invade these maternal arteries to a sufficient depth, thus resulting in poor remodeling of the arteries. The arteries remain significantly constricted and this leads to serious complications during pregnancy (Waite et al., 2002).

Preeclampsia

Preeclampsia is a pregnancy condition seen during the second and third trimester in some women. It is characterized by a variety of symptoms that include hypertension, proteinuria, hyperuremia, general edema in the woman, and decreased perfusion of the choriodecidual space (space between the maternal tissue and the fetal membrane (Perloff, 1998). The latter leads to increased fetal mortality, as well as severe growth retardation in fetuses that survive. Preeclampsia is a common cause of maternal mortality

worldwide, accounting for 20% of pregnancy-related deaths in the United States (Waite et al., 2002).

The cause of preeclampsia is unknown. Genetic linkage to the disease has been demonstrated. Research shows that daughters of women who experience preeclampsia are more likely to develop the disease (Waite et al., 2002). Sons of preeclamptic women have also been known to father children of preeclamptic pregnancies. Ongoing research is aimed at testing to see if a particular gene on human chromosomes affects womens' disposition toward developing preeclampsia.

It has also been noted that an increase in placenta mass usually increases preeclampsia risks, as seen with multiple gestations. Microvascular diseases such as systemic lupus erythromatosus, hypertension and diabetes also lead to a higher incidence of preeclampsia in the affected women, as well as high body mass index and the presence of a urinary tract infection. Preeclampsia is more prevalent in the Black race, but this finding is suspected to be contributed to by some of the above mentioned factors, such as their higher risk for hypertension and high body mass index. Environmental factors have also been shown to contribute somewhat to preeclampsia, with women living at high altitude being at an increased risk (Waite, et al, 2002).

Preeclampsia is noted as a two stage disease, the first being an initial defect in placental development, followed by a second stage characterized by a maternal response to the abnormality. The initial placental defect results from the shallow trophoblast invasion into the maternal arteries. As reviewed by Robilliard, studies done by Zhou et al showed failure of extravillous cytotrophoblasts to invade the maternal uterine spiral arteries to a sufficient depth at the second physiological invasion around the 14-16th week of gestation in humans (Reviewed in: Robillard, 2002). This has been shown to result in smaller uterine arteries in the affected women, resulting in reduced blood between the mother and the fetus. In an attempt to compensate for the decreased blood flow, Robilliard speculates that blood is pumped at a higher rate, resulting in increased blood pressure so that the fetus is constantly supplied with the necessary nutrients for successful development (Robilliard, 2002).

Various studies have been carried out to in an attempt to understand the mechanism of preeclampsia. It has been discovered that there is a lack of tolerance to the fetus, as evidenced by histological changes in the placental beds of preeclamptic women that resemble histologic changes seen in allograft rejection (Reviewed in Waite et al., 2002). However, the mechanisms for allograft rejection and rejection of the developing fetus

differ. Allograft rejection occurs when there is a transplant between two genetically different people. The recipient's HLA (human leukocyte antigen) antigens differ from the donor's, such that the recipient's T cells attack the donor's organ and rejects it (Parham, 2000). In the proposed mechanism for fetal rejection by the maternal immune system, there are few B and T cells present at the maternal fetal interface, therefore these cells do not act to attack the foreign fetal cell (Reviewed in: Waite et al., 2002).

In the decidua, uterine Natural Killer (uNK) cells have a reduced ability to lyse trophoblast and other normal NK target cells (Sladek et al., 1998). A few findings have led to the explanation of why these trophoblast cells (foreign to the host) have not been killed by uNK cells, that carry the same killer and killer cell immunoglobulin-like receptor (KIR) ligands as peripheral (blood and spleen) NK cells. KIR ligands are receptors on NK cells that bind to self MHC I on target cells so that the cell is protected from being killed by the NK cell (Parham, 2000). One study established that these uNK cells are close to, but do not come into direct contact with the trophoblasts, or if they do get into contact, the KIR binds to major histocompatibility complex (MHC I) HLA-A on these cells using the same mechanism used by peripheral NK cells to prevent destruction of the target cell (Reviewed in: Waite et al., 2002). However, another review by Vitale

discusses how MHC I HLA-A molecules have not been successfully detected on trophoblasts, but that HLA-G (non-classical MHC I molecule) and HLA-C have been detected in higher numbers. These are believed to be bound by receptors on the uNK cells (p58 and p70), or in part by the CD94/NKG2 receptor, thus having the same effect. p58 and p70 are receptors found on NK cells that bind to MHC class I molecules, with p70 having a higher affinity for HLA-B (Vitale et al., 1995). CD94/NKG2 is an inhibitory receptor found on virtually all decidual NK cells, but on few peripheral NK cells (Waite et al., 2002). These receptors, after binding to the HLA molecules, inhibit target cell destruction by the NK cell. The appearance of the uNK cells during pregnancy, and the lack of these cells in preeclamptic women have led researchers to investigate their origin and role in the disease process.

Natural Killer Cells

It has been shown that the most abundant immune cells present in the uterus during pregnancy are natural killer (NK) cells (Waite et al., 2002). NK cells are a part of the innate immune response, and are referred to as large granular lymphocytes. They target virus-infected and cancer cells that do not express self MHC molecules (MHC I) on their cell surface (Raulet,

1999). NK cells attach to the target cell through their killer ligand, and cause lysis of the virus infected or cancer cells. Normal cells express MHC I, and therefore the NK cell killer inhibitory receptor (KIR) attaches to MHC I on the target cell, protecting it from lysis (Parham, 2000).

Uterine natural killer (uNK) cells are believed to be different from the normal NK cells found in the periphery (Croy et al., 2000). CD56 and CD16 are cell surface ligands found on most granulocytes and NK cells. When stained with fluorescent molecules, uNK cells show different levels of expression of these ligands. uNK cells in the decidua are marked by the phenotype ($CD56^{bright}$ $CD16^{-}$) compared to the peripheral NK ($CD56^{dim}$ $CD16^{+}$) phenotype. During the early months of pregnancy, these uNK cells are found to be associated with trophoblast cells that later on associate with the maternal spiral arteries (King, 1998).

Role of NK cells in pregnancy

The presence of a high number of uNK cells in the decidua suggests their possible necessary role in implantation (Reviewed in: Waite et al., 2002). Studies in humans done by Burrows *et al.* showed that uNK make up about 30-40% of the total leukocytes in the first trimester, and then these cells decrease during the second trimester. However, rodents have been

found to consistently express these uNK cells for an extended period during gestation (Reviewed in: Hunt et al., 2000).

Three mouse models have been successfully used to investigate the role of uNK cells in preeclampsia. These models all have mutations in various proteins/genes that make them uNK cell deficient. The first, tgE26 mice, have an insertional mutation causing the synthesis of multiple copies of the human CD3E gene (Croy et al., 2000). CD3E is a subunit of the CD3 complex found on thymocytes, T cells and NK1-T cells (a specialized population of alpha/beta T cells that coexpress receptors of the NK lineage) (Bendelac et al., 1997). CD3E is used for signal transduction in T cells to stimulate activation after antigen binding. T cells and NK cells are missing in the mouse, possibly due to the development of anergy (lack of response) as a result of inappropriate signaling. In these mice, the lack of uNK cells does not result in fetal loss. However, absence of uNK cells in these mice has been associated with the development of preeclamptic symptoms. A histochemical study showed that after 7-8 days of gestation, there were detectable abnormalities in the arteries and decidua of these mice. At day 6 in the gestation period, the endothelial cells of the dedicual arteries appeared as normal cuboidal cells, but on the 7^{th} day, they lost this normal histology seen during pregnancy and became tall and columnar. This, therefore,

established a link between uNK cells and their role in maintaining arterial systems during pregnancy. To confirm this, studies by Guimond and coworkers showed that transplantation/engraftment of bone marrow from B and T cell deficient scid/scid mice donors into pregnant tgE26 mice restored the uNK cells as seen through specific staining procedures. This restoration of uNK was linked to a reversal of preeclamptic symptoms in these mice (Reviewed in: Croy et al., 2000).

In the second mouse model, the mice are doubly mutant for p56 lck and IL-2Rβ. p56 is a member of protein tryrosine kinases that can associate with the IL-2Rβ chain and activate signal transduction. IL-2Rβ is a subunit of the IL-2 receptor, which is important in the signal transduction of the IL-2 pathway in T cells and NK cells. A defect in this receptor molecule results in no growth or stimulation of the two cell types. These mice, therefore, lack NK and T cells. Thus, no uNK are detected during pregnancy in these mice, since there are no precursor NK cells. As expected, $p56^{-}$/IL-$2R\beta^{-}$ mice show preeclamptic symptoms which can be reversed by an engraftment of normal bone marrow cells (Croy et al., 2000).

In the last model, mice are doubly mutant for RAG-2 and the common cytokine γ (γc^{-}) receptor subunit. This model, a hybrid between the γc null and RAG-2 null strain, will be used in our studies. The mice have a

defective receptor chain (γc) for IL-2, IL-4, IL-7, IL-9 and IL-15. A defect in the IL-2 and IL-15 receptors results in the lack of NK cells. The γc defect also results in ineffective B and T cell responses. In addition, the mice are mutant for the RAG-2 gene, which is required for the rearrangement of the VDJ regions of the antigen receptors (Ig and TCR) of B and T cells. This strain, therefore, lacks B cells, T cells and NK cells and would be expected to show abnormalities during pregnancy due to the absence of uNK cells (Croy et al., 2000). The advantage of using this model is that it also lacks B cells, unlike the other two models that only lack T cells and NK cells. In addition, the other models have residual low levels of NK cells, while none are seen in RAG2$^-$/γc$^-$ mice. Therefore, there is no T cell, B cell or NK cell immune response that may decrease the chances of a successful bone marrow cell reconstitution.

Using the above mentioned models, the actual role of uterine leukocytes during pregnancy has been studied and has been found to include facilitation of implantation and modulation of arteries in the uterus. In addition to uNK cells, leukocytes found at the decidua in mice, rats and humans include macrophages and eosinophils. The cell numbers are believed to be enhanced by estrogen. The different uterine leukocytes produce different cytokines and regulatory molecules. uNK cells, together

with macrophages, produce tumor necrosis factor alpha (TNFα), nitrogen oxide (NO, facilitated by nitric oxide synthase), and perforin. Most of these cytokines or molecules are used to attack any foreign cells that may prove detrimental in pregnancy development (Hunt et al., 2000). uNK cells are also believed to secrete IFNγ which, in combination with endogenous IFNγ (secreted by T cells and peripheral NK cells), has been noted to improve vascular remodeling in which the lumen to wall ratio increases (Redline, 2000). It is speculated that this cytokine is synthesized at the highest levels at midgestation, after which the uNK begin declining. To investigate the role of this cytokine, studies by Ashkar et al. have been conducted in which $RAG2^{-}/\gamma c^{-}$ mice were either engrafted with bone marrow cells from donors or injected with the IFNγ itself. Bone marrow cells would provide endogenous (uNK-derived) IFNγ, whereas the cytokine injections directly increase IFNγ activity in the mice. Their results showed that decidual integrity during the first days of pregnancy (up to day 14) is regulated by peripheral IFNγ, and that higher levels of the cytokine are required between days 10-12 for vascular remodeling. The highest levels of IFNγ needed at days 10-12 must come from the uNK cells, since a lack of these cells in the $RAG2^{-}/\gamma c^{-}$ mice that only received cytokine injections has been established to cause poor vascular remodeling. From this study, it has therefore been

proven that successful engraftment of bone marrow cells can result in reversal of preeclamptic symptoms, largely due to the IFNγ produced by the uNK cells (Reviewed in: Redline, 2000).

Nitric oxide has also been shown to play a role in the development of maternal uterine vasculature during pregnancy. It is believed to reverse failure of arterial dilation, which is one of the main symptoms in preeclamptic women. In a study conducted by Burnett *et al.*, the tgE26 mouse strain showed thickened and constricted arterial vessels at mid-gestation. They proved that uNK cells are major producers of nitric oxide synthase. Lack of the uNK cells resulted in no nitric oxide production, and consequently abnormal arterial vessel constriction. Production of nitric oxide is important since it stimulates relaxation in uterine arteries (Reviewed in: Hunt et al., 2000). uNK cells have thus been shown to directly, or indirectly stimulate cytokines or enzymes necessary in normal arterial development during pregnancy.

To further understand the role of uNK cells during pregnancy, Guimond and coworkers used TgE26 and Tg2978 (H-2 k defined and H-2 k undefined) for histology studies. The models also differed in their expression of the CD3 gene. The mice were mated, and uteri were harvested at day 14 for staining. Compared to controls used in the study that showed normal

metrial gland development, tgE26 mice proved that a decrease in the number of uNK cells results in no formation of the metrial gland. The fetuses of the TgE26 mice weighed less than those from Tg2978 mice that did not have severe depletion of uNK cells at 3 weeks and 7 weeks of age. In addition, viability of the fetuses was measured at different days of gestation during pregnancy, starting at days 6-8, and continuing up to days 15-17. There was a significant decrease in viability of the TgE26 mice by day 17 when compared to control mice. Viability was determined by looking at the implant size and color (Guimond et al., 1997). This, therefore, helps to confirm the role of uNK cells in normal fetal development.

In another study investigating engraftment of bone marrow from SCID into tgE26 mice, Guimond and coworkers looked at common pregnancy developmental processes like vessel remodeling by determining their wall to lumen ratio, as well as placental sizes in the tgE26 mice. The tgE26 mice were pretreated with 5Fu before they were engrafted with bone marrow cells from SCID mice, and 3 weeks after engrafted they were mated. Uteri were collected at days 10, 12 and 14 days of pregnancy, processed, embedded and stained with PAS. Results obtained from implant sections stained with hematoxylin and eosin show that placenta sizes were larger in tgE26 mice reconstituted with the bone marrow cells compared to tgE26

mice that were not reconstituted. This was observed at different days during pregnancy (days 10-14) and the sizes were also compared to the control mice. The successfully engrafted tgE26 mice also showed higher levels of uNK cells in the metrial gland (40-50% more cells) compared to the unreconsituted tgE26 mice (normally having 1-3.1% uNK cells) that showed no metrial gland development. Finally, the reconstituted tgE26 mice showed a higher lumen-to-vessel wall ratio compared to tgE26 mice that were not reconstituted with bone marrow cells at all. These experiments further prove the importance of uNK cells in normal fetal development (Guimond, 1999).

Origin of uterine Natural Killer Cells

To determine the origin of uNK cells, several studies have been carried out. Some investigators believe that they come from bone marrow cell precursors, while others through their studies show that they proliferate within the decidua. uNK cells have been found to be highly positive for L-selectin, a molecule used by lymphocytes to move from blood into tissue. This would allow the uNK cells to migrate to the uterus, thus further suggesting that they originate from bone marrow precursors. Recent work by Drake *et al.* suggests that the trophoblasts secrete a chemokine MIP-1α that preferentially attracts the uNK cells to the region of trophoblast invasion

(Reviewed in: Waite et al., 2002). Studies by Peel and coworkers show that some uNK cells originate from bone marrow precursors. In their study, they used lethally irradiated mice and rats and reconstituted them with either mouse or rat bone marrow cells respectively. These mice were then given hormonal therapy to induce pseudopregnancies, and histochemical analysis was done of uterine tissue to detect the presence of NK cells. The cells in the uterus were stained with hematoxylin, or viewed under an electron microscope. Using electron microscopy, uNK cells that originated from mouse bone marrow cells showed cap regions within the cell, whereas uNK cells from rat bone marrow cells showed regions of myelin. These features are unique to the species, and were used to differentiate between mouse and rat uNK cells. The studies proved that there was migration of the reconstituted bone marrow cells to the uterus in the irradiated and reconstituted mice, as indicated by the detection of mouse or rat uNK cells in the deciduas at day 14 of pregnancy (Peel et al., 1983).

However, there also has been evidence by Parr and coworkers suggesting that uNK cells originate from a local pool of natural killer cells that express a cell surface marker LGL-1. They were able to successfully identify these uNK cells before mating their experimental mice. They used an antibody against the NK1.1 cell surface marker, and a secondary antibody

conjugated with a fluorescent molecule. After mating the mice, they depleted peripheral NK cells from pregnant mice by using antibodies against either the LGL-1 marker or the asialo GMI marker found on all mouse NK cells. This depletion was done to see if it would affect the number of uNK cells found in the uterus. They then used double immunostaining to prove that the NK cells expressing the LGL-1 marker were actually proliferating into uNK cells that later on expressed perforin. Depletion of the NK cells in the circulating blood did not affect the number of uNK cells. They, therefore, concluded that uNK cells do not originate from bone marrow cell precursors (Parr et al., 1990). However, in these studies depletion of NK cells did not result in a total elimination of the cells from the spleen, nor was the bone marrow examined directly. It is possible that the remaining splenic NK cells were able to repopulate the uterus.

Purpose of study

The previously described studies have demonstrated that uNK cells play a role in preeclampsia. Since the mouse reproductive system is similar to the human reproductive system, and since mice without uNK cells have symptoms similar to those of preeclamptic women, our study goal is to further investigate the role of these cells in pregnancy. However, this project

focuses on the initial stage of determining the migration of uNK cells from the periphery (from bone marrow reconstitution) to the uterus through histochemical analysis of collected organs.

In all the previous engraftment studies done using the above mentioned models, the mice were irradiated or treated with 5FU presumably to destroy any immune cells that could potentially reject the engrafted bone marrow cells. The purpose of our study is to follow engraftment patterns of the uNK cells in the Rag2$^-$, common gamma γc^- double knockout (RAG2$^-$ /γc) mice. Since these mice lack B cells, T cells and NK cells, the current studies were done without irradiating this model with the belief that bone marrow engraftment should be successful. Our goal was to determine if engraftment would be successful in this mouse model, so that it can be used in subsequent studies without prior irradiation.

In previous studies from this laboratory immunodeficient mice were injected with bone marrow cells from normal donors 3 weeks prior to mating. The immunodeficient engrafted mice and normal C57Bl6 and Balb/c mice were mated, and at day 10 of pregnancy, they were sacrificed. Spleens and implants were harvested, processed, and embedded. Sera obtained from these mice were tested by ELISA to determine the presence of antibodies.

IgG and IgM were detected in most mice, suggesting successful engraftment (Toth, 2002).

In the current studies, the previously harvested tissues were examined using histochemical methods and immunohistochemistry to detect the presence or absence of NK cells in the spleen or implants from the engrafted RAG2$^-$/γc$^-$ mice.

MATERIALS:

Sodium chloride was obtained from Amresco (Solon, OH). Rat link (biotinylated rabbit anti-rat immunoglobulin), peroxidase-conjugated streptavidin, and the diaminobenzidine (DAB) substrate pack (chromogen, substrate buffer, hydrogen peroxide) were obtained from Biogenex (San Ramon, CA). Anhydrous potassium phosphate dibasic was obtained from Calbiochem-Behring Corp (La Jolla, CA). Formalin, Gill's Hematoxylin, and alcoholic eosin solution were obtained from Fisher Chemical Company (Fair Lawn, NJ). Sodium citrate was obtained from Mallinckrodt (Paris, KY). Ethanol and methanol were obtained from Pharmco Products, Inc (Brookfield, CT). Protein blocking agent was obtained from Immunon Thermo Shandon (Pittsburgh, PA). Bovine serum albumin, biotinylated dolichos biflorus agglutinin lectin, N-acetyl-D-galactosamine, anhydrous

potassium phosphate monobasic, anhydrous sodium phosphate monobasic, anhydrous sodium phosphate dibasic, periodic acid, and Schiff's reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide was obtained from SuperValu, Inc. (Edon Prairie, MN). Purified Formula R paraffin wax with select polymers (mp 56-57°C) and Supermount were obtained from Surgipath Medical Industries Inc. (Richmond, IL). Xylene was obtained from VWR Scientific (San Francisco, CA).

METHODS:

Hematoxylin and Eosin Staining for viewing basic tissue morphology

Tissue section slides were placed in a heating chamber at 65°C for 10 minutes to melt the paraffin. They were then quickly transferred to xylene while hot, and washed with xylene 3 times for 5 minutes each. The slides were hydrated by immersion in decreasing concentrations of ethanol (100%, 100%, 95%, 95% and 70%) for 1 minute each (Carson, F. 1997). The slides were then rinsed in distilled water for 5 minutes, with stirring. The slides were stained with Gill's hematoxylin for 1 minute, then rinsed with warm tap water until the water in the container ran clear. The slides were rinsed with distilled water for 5 minutes, placed in eosin for 15 seconds, and then taken back through the graded alcohols (70%, 95%, 95%, 100%). The slides

were then cleared in xylene for 1-2 minutes, and mounted with coverslips using xylene based Supermount.

Lectin Immunohistochemistry Protocol for detection of uNK cells

Tissue section slides were deparaffinized for 15 minutes in a heating chamber at 65°C. The slides were rehydrated through three washes of xylene (7 minutes each), and graded ethanol (100%, 100%, 95%, 95%, 70%) for a minute each before being washed in tap water for 2 minutes, and in distilled water for 3 minutes. The slides were then washed in 0.05M PBS (pH 7.4) for 10 minutes, with stirring. Sections were incubated with 60 µl of 3% H_2O_2 for 15 minutes, and then washed in 0.05M PBS for 10 minutes, with stirring (Paffaro, 2003). Some slides were pretreated with sodium citrate buffer (10 mM, pH 6.0), which retrieves antigens masked by formalin. The treatment was carried out by placing the slides in a coplin jar and boiling them in a microwave. The slides were then boiled for 5 minutes in the buffer. More buffer was added to keep the sections covered and they were boiled again for another 5 minutes. The sections were then cooled to 37°C on the bench. The sections serving as positive controls were incubated with biotinylated dolichos biflorus agglutinin lectin (DBA) diluted in 0.05M PBS containing 1% BSA, pH 6.8. DBA binds to N-acetyl-D-galactosamine on the surface of natural killer cells and some other cells. Negative control sections were

incubated with DBA lectin that had been preincubated with N-acetyl-D-galactosamine in the same buffer for an hour prior to use. Following an overnight incubation, the sections were washed with 0.05M PBS for 10 minutes with stirring and then incubated with streptavidin-conjugated peroxidase for 20 minutes. The sections were washed again in PBS as before, and each section incubated with 60μL of the colorimetric substrate diaminobenzidine solution (Biogenex liquid DBA substrate pack)for varying times, depending on the section and staining intensity. Staining was stopped when dark brown stained cells were visible under the 10X objective lens (2-30 minutes). All slides were washed in distilled water for 5 minutes, counterstained with Gill's hematoxylin for 15-60 seconds, washed with warm tap water, taken through the graded alcohols for a minute in each (70%, 95%, 95%, 100%) and xylene for 2 minutes. They were finally cover-slipped with Supermount. The mount was left to dry overnight at room temperature.

Immunohistochemistry: Antibody protocol to detect white blood cells

Tissue sections were baked at 60°C for 20 minutes in a heating chamber to melt the paraffin. The sections were deparaffinized in xylene three times, for 10 minutes each. All slides were hydrated through a series of alcohols (100%, 100%, 95%, 95%, and 70%) for 1 minute each. They were rinsed in

distilled water for 5 minutes, then in potassium phosphate (0.01M K_2HPO_4, 0.13M NaCl) for 5 minutes. The sections were quenched in 3% H_2O_2 for 15 minutes in a humid chamber. The sections were then rinsed with 0.01M PBS, and washed for 10 minutes in a PBS bath, with stirring. Sodium citrate buffer (10mM, pH 6.0) was used for pretreatment of some slides. The sections were placed in a coplin jar, and covered with the buffer. The slides and buffer were brought to boil in a microwave, and then boiled for 5 minutes, making certain that buffer did not boil away below level of the sections. Additional buffer was added, and the buffer was boiled again for another 5 minutes. The coplin jar was then left at room temperature to cool to 37°C. The sections were then blocked in Immunon-Thermo Shandon Protein Blocking Agent for 20 minutes. The positive controls were incubated for an hour with the primary antibody anti-mouse CD45 diluted in PBS (Krasnow, 1996), while the negative controls were incubated for the same time with PBS. The sections were rinsed in PBS, and then washed in a PBS bath for 10 minutes, with stirring. They were incubated for 20 minutes in the secondary Ab, peroxidase conjugated anti-rat (Fab fragments) for 20 minutes before being washed in PBS. The slides were then incubated with 60µl Biogenex diaminobenzidine solution (chromogen, substrate buffer, and H_2O_2) for varying times depending on the staining intensity of each section

(2-30 minutes). They were washed in tap water for 5 minutes before being counterstained in Gill's hematoxylin for 45 seconds. The sections were washed again with warm tap water until the water ran clear. Finally, they were dehydrated through alcohols (70%, 95%, 95%, 100%), cleared with xylene twice for 2 minutes, and mounted with Supermount. They were left to dry overnight at room temperature.

Periodic Acid Schiff for detection of uNK cells

Tissue section slides were heated in a heating chamber at 60°C for 20 minutes. They were deparaffinized through 3 rounds of xylene for 5 minutes in each, and dehydrated through a series of ethanols (100%, 100%, 95%, 95%, and 70%) for 1 minute each. The tissue sections were then hydrated by washing them in distilled water for 5 minutes, and were oxidized in 0.5% periodic acid solution for 5 minutes at room temperature (Sigma Chemical Company). The sections were rinsed in distilled water twice, for 3 minutes and 2 minutes respectively, and then placed in Schiff reagent for 15 minutes at room temperature. All slides were then placed in lukewarm tap water and washed for 5 minutes. The slides were counterstained with Gill's hematoxylin for 45 seconds, washed in tap water for 5 minutes and then dehydrated back through the series of alcohols (70%, 95%, 95%, 100%) for

1 minute each. The tissue section slides were cleared in xylene for 2 minutes, and then cover-slipped using xylene based Supermount. They were left overnight to dry (Sigma Chemical Company). PAS stained cells in spleens were counted using oil immersion (100x lens), and 8 fields of view were examined per section.

Follicle counts

Follicles were counted in spleen sections of positive control C57Bl6 mice, as well as spleen sections from the bone marrow transplanted RAG2-/γc^- mice. Follicles were counted in one 4x field of view per tissue section.

RESULTS

The purpose of this study was to determine if there was migration of white blood cells into the spleen or uterus following intravenous injection of bone marrow cells from normal mice into RAG2$^-$/γc^- mice. In the study, two untreated female Balb/c mice (FM1 and FM2) and 2 C57/Bl6 mice (FM 4 and FM16) were used as positive controls (Table 1). One male RAG2$^-$/γc^- mouse (ML9) and 3 female RAG2$^-$/γc^- were used as negative controls. In addition, 14 female RAG2$^-$/γc^- mice were injected with donor bone marrow

Table 1: Mice Used in Project

a) Control Mice

Mouse	Species
B1-B12	Balb/c
FM1	Balb/c
FM2	Balb/c
FM4	C57Bl6
FM16	C57Bl6
FM19	$RAG2^{-}/\gamma c^{-}$
FM21	$RAG2^{-}/\gamma c^{-}$
FM22	$RAG2^{-}/\gamma c^{-}$
ML9	$RAG2^{-}/\gamma c^{-}$

b) Experimental Mice

Date	Mouse	Injected Species	Amount	Days till pregnant	Treatment of cells
	FM3	Balb/c	$2x10^6$	3d	-
	FM5	Balb/c	$2x10^6$	24d	-
	FM6	Balb/c	$2x10^6$	24d	-
6/3/02	FM7	Balb/c	$2x10^6$	24d	-
"	FM8	Balb/c	$2x10^6$	24d	-
6/11/02	FM9	Balb/c	$2x10^6$	24d	αThy1.2[a]
"	FM10	Balb/c	$2x10^6$	26d	αThy1.2
"	FM11	Balb/c	$2x10^6$	26d	αThy1.2
"	FM12	Balb/c	$2x10^6$	30d	αThy1.2
6/21/02	FM13	C57Bl6	$2x10^7$	23d	-
"	FM14	C57Bl6	$2x10^7$	23d	-
"	FM15	C57Bl6	$2x10^7$	25d	-
12/16/02	FM17	C57Bl6	$4x10^6$	24d	-
"	FM18	C57Bl6	$4x10^6$	22d	-

[a] Anti Thy1.2 antibody (targets CD90 on mouse thymocytes and mature T cells)

cells 3-30 days prior to mating (mean = 24 days). The mice were injected with either Balb/c bone marrow cells, Balb/c bone marrow cells treated with Anti-Thy1.2 (to remove mature T cells), or with C57BL6 bone marrow cells (Table 1). At day 10.5 of pregnancy, the mice were anaesthetized, and the organs collected and paraffin embedded for tests to determine the presence of natural killer (NK) cells (Toth N, 2002).

The sectioned spleen and implant tissues were stained using DBA lectin, anti-CD45, anti-Dx5 antibody, or periodic acid schiff staining (PAS) to determine the presence of white blood cells (WBC) or uterine Natural Killer (uNK) cells. DBA binds to N-acetyl-D-galactosamine on the surface of natural killer cells, while anti-CD45 stains detects the antigen CD45 on the surface of WBC. Anti-Dx5 is an antibody that stains NK cells by binding to CD49b on their cell surface, while PAS reacts with glycogen present in natural killer cell granules, and stains the granules red. Tables 2 & 3 show the staining that was performed on each tissue section, with H&E performed on the first section of each organ to determine cell morphology and thickness of sections. The table also provides a summary of results from follicle counts performed on spleen sections. Follicles are concentric rings of lymphocytes formed in the spleen during an immune response.

Table 2: Spleen and Implant General Spreadsheet

Mouse	Identity	Block	Sectioned Slides	Staining	Follicle Count[d]
FM #1	Control Balbc	Spleen	5 slides	H&E[e] (1)[g]	0
FM #2	Control Balbc	Spleen	5 slides	H&E (1), PAS[f] (3)	40
FM #3	Balbc →RAG2^{-}/γc^{-} [a]	Spleen	1 slide	H&E (1)	23
		Implant A	5 slides	H&E (1)	
FM #4	Control C57Bl6	Spleen	5 slides	H&E (1), PAS (3)	22
FM #5	Balbc →RAG2^{-}/γc^{-}	Spleen	10 slides	PAS	0
		Implant A	5 slides	H&E (1)	
FM #6	Balbc →RAG2^{-}/γc^{-}	Spleen	5 slides	PAS	0
FM #7	Balbc →RAG2^{-}/γc^{-}	Spleen	8 slides	H&E (1), PAS	0
		Implant A	4 slides	H&E (1)	
FM #8	Balbc →RAG2^{-}/γc^{-}	Spleen	5 slides	PAS (3)	0
		Implant A	4 slides	H&E (1)	
FM #9	Balbc (T) →RAG2^{-}/γc^{-} [b]	Spleen	5 slides	PAS (4)	0
		Implant A	5 slides	H&E (1)	
FM #10	Balbc (T) →RAG2^{-}/γc^{-}	Spleen	5 slides	PAS (3)	0
		Implant A	5 slides	H&E (1)	
FM #11	Balbc (T) →RAG2^{-}/γc^{-}	Spleen	4 slides	H&E (1), PAS (3)	0
		Implant A	5 slides	H&E (1)	
FM #12	Balbc (T) →RAG2^{-}/γc^{-}	Spleen	5 slides	PAS (3)	0
FM #14	C57Bl6 → RAG2^{-}/γc^{-} [c]	Spleen	5 slides	PAS (3)	0
		Implant A	5 slides	H&E (1)	
FM #15	C57Bl6 → RAG2^{-}/γc^{-}	Spleen	5 slides	PAS (3)	0
FM #16	Control C57Bl6	Spleen	5 slides	H&E (1), PAS (3)	28
FM #17	C57Bl6 → RAG2^{-}/γc^{-}	Spleen	5 slides	H&E (1), PAS (3)	0
FM #18	C57Bl6 → RAG2^{-}/γc^{-}	Spleen	5 slides	H&E (1), PAS (3)	0
		Implant	5 slides	H&E (1)	
FM #19	RAG2^{-}/γc^{-}	Spleen	5 slides	H&E (1)	0

Mouse	Identity	Block	Sectioned Slides	Staining	Follicle Count
FM #21	$RAG2^{-}/\gamma c^{-}$	Spleen	5 slides	H&E (1), Lectin (2), PAS (5)	0
		Implant A	5 slides	H&E (1), Lectin (2,3)	
FM #22	$RAG2^{-}/\gamma c^{-}$	Spleen	5 slides	H&E (1), PAS (5)	0

[a] $RAG2^{-}/\gamma c^{-}$ mouse engrafted with bone marrow cells from Balbc mouse
[b] $RAG2^{-}/\gamma c^{-}$ mouse engrafted with bone marrow cells from Balbc mouse pretreated with anti-Thy1.2
[c] $RAG2^{-}/\gamma c^{-}$ mouse engrafted with bone marrow cells from C57Bl6 mouse
[d] Number of follicles counted per 10X field of view
[e] H&E = Hematoxylin and Eosin staining
[f] PAS = Periodic Acid Schiff staining
[g] Numbers 1-5 = Designated number of tissue section per organ

Table 3: General Staining Spreadsheet

Date of Staining	Mouse	Type of Staining/dilution	Results/Number of Positive cells
6/10/04	C57Bl6-4 Spleen	+: DBA[a]	0
7/7/04	C57Bl6-4 Spleen	+: DBA	0
6/10/04	NK Male 1 Spleen	+: DBA	0
6/1/04	Male 4 Spleen	+: DBA	0
"	FM22 Spleen	-: DBA + NADG[b]	0
6/21/04	ME9.5	+: DBA	0
"	ME9.5	-: DBA + NADG	0
6/8/04	NK FM8 Implant D	+: DBA	0
"	FM9 Implant A	+: DBA	0
"	FM9 Implant A	-: DBA + NADG	0
6/21/04	C57Bl6-5 Spleen A	+: αCD45[c] (1:100)	0
"	C57Bl6-5 Spleen A	-: Mouse IgG serum (1:1000)	0
6/23/04	C57Bl6-5 Spleen B	+: αCD45 (1:100)	0
6/25/04	C57Bl6-4 Spleen	+: αCD45 (1:100)	0
7/2/04	C57Bl6-4 Spleen	+: αCD45 (1:100)	2[f]
6/15/04	C57Bl6-5 Kidney B	+: αCD45 (1:100)	2[f]
"	Male 4 Kidney A	+: αCD45 (1:100)	0
"	Male 4 Kidney A	-: PBS[d]	0
"	FM19 Kidney	+: αCD45 (1:100)	0
"	FM19 Kidney	-: mouse serum	0
6/24/04	ME 9.5	+: αCD45 (1:100)	0
"	ME 9.5	-: PBS	0
6/28/04	ME8.5 10 (3)	+: αCD45 (1:100)	0
6/30/04	NK Male #1	+: αCD45 (1:100)	0
7/7/04	C57Bl6-4 Spleen	+: Dx5[e] (1:200)	0
"	C57Bl6-4 Spleen	+: Dx5 (1:1000)	0
"	C57Bl6-4 Spleen	-: PBS	0

[a] DBA = Dolichos Biflorus Agglutinin
[b] NADG = N-acetyl-D-Galactosamine
[c] αCD45 = anti-CD45 antibody
[d] PBS = Phosphate Buffered Saline
[e] Dx5 = Pan NK antibody
[f] Staining of cells (2) was faint. DAB should have been left on tissue sections longer

In an attempt to determine the optimal antibody concentrations to detect WBC in different organs as well as to determine the optimal staining times for both the antibody (αCD45 and Dx5) and lectin stains, different concentrations and staining times were tested. Although several adjustments were made to the general protocol for antibody staining, no WBC cells were detected in most spleen or kidney sections of test mice (Table 3). However, one spleen section stained with anti-CD45 (1:100 in PBS) showed two faintly positive cells (Table 3). A control kidney section also yielded two faintly stained cells using an antibody dilution of 1:100 (Table 3). The rest of the control sections stained with the anti-CD45 antibody did not yield positive results. Dx5 (Pan NK antibody) was tested in three spleen sections using two different concentrations (1:200 and 1:1000) to determine if it would successfully detect the presence of NK cells in these tissues. These sections were all from the same C57Bl6 mouse, with two of them serving as positive controls while one was used as a negative control (no primary antibody) (Table 3, 7/7/04). However, this stain also failed to demonstrate any positive cells (Table 3). All known antibody companies were contacted to try find a better antibody. No known antibodies were found that would identify mouse white blood cells in paraffin embedded tissues (Appendix A).

A lectin, dolichos biflorus agglutinin (DBA) was next used to determine the number of uNK cells in spleen sections (Table 4) and a few implant (day 10 fetus) sections (Table 3). Table 3 shows the tissue sections that were used in trials to determine if any adjustments were necessary to the protocol. Nine tissue sections with either DBA or DBA preincubated with NADG (negative control) showed non-specific staining around the tissue sections, and therefore could not provide any useful insight into the migration of the NK cells (data not shown). Some of the adjustments made to the protocol included using new H_2O_2 to try and inactivate endogenous peroxidases within the tissue and prevent non-specific staining. These sections were also pretreated with citrate buffer to remove excess formalin and unmask more antigens on the tissue surfaces, but the changes did not improve the results (data not shown). All engrafted mice and RAG2$^-$/γc$^-$ mice were negative for NK cells in both spleens and implants using the lectin stain (Table 3 & 4). However, two C57Bl6 control mice and a Balb/c (FM1) mouse showed 2-3 positive cells per spleen section (Table 4). Figure 1 shows a positive NK cell in a C57Bl6 mouse spleen section, whereas none of the spleen and implant sections showed positive DBA staining. Figure 2 shows an example of a negative control RAG2$^-$/γc$^-$ mouse (FM22: not engrafted) without any DBA lectin positive cells, while Figure 3 shows an

Table 4: DBA Lectin Stained Spleen Sections

Date of Staining	Mouse Strain	BM injected	Treatment α-Thy1.2	Results[a]
7/15/04-7/25/04	C57Bl6-5 Spleen A	-	-	0 (Average)
7/21/04	C57Bl6-5 Spleen A	-	-	2
7/27/04	C57Bl6-5 Spleen A	-	-	3
7/15/04	FM1 Spleen (Balb/c)	-	-	2[c]
"	FM2 Spleen (Balb/c)	-	-	0
7/23/04	FM3 Spleen	Balbc	-	0
"	FM4 Spleen (C57Bl6)	-	-	0
"	FM5 Spleen	Balbc	-	0
"	FM6 Spleen	Balbc	-	0
"	FM7 Spleen	Balbc	-	0
7/19/04	FM8 Spleen	Balbc	-	0
"	FM9 Spleen	Balbc	αThy1.2[b]	0
"	FM10 Spleen	Balbc	αThy1.2	0
"	FM11 Spleen	Balbc	αThy1.2	0
"	FM12 Spleen	Balbc	αThy1.2	0
"	FM14 Spleen	C57Bl6	-	0
7/21/04	FM15 Spleen	C57Bl6	-	0
"	FM16 Spleen (C57Bl6)	-	-	0
"	FM17 Spleen	C57Bl6	-	0
"	FM18 Spleen	C57Bl6	-	0
"	FM19 Spleen	-	-	0
"	FM20 Spleen	-	-	0
7/23/04	FM21 Spleen	-	-	0
"	FM22 Spleen	-	-	0
7/15/04-7/27/04	C57Bl6-5 Spleen A (-control: DBA + NADG)	-	-	0

[a] # of cells/40x field of view

[b] The test mice received bone marrow cells pretreated with anti-Thy1.2 to deplete any mature T cells

[c] Staining was faint, more time was required for DAB staining

Fig. 1 DBA lectin positive cell from C57Bl6 spleen section

The sectioned C57Bl6 spleen tissue was stained with DBA lectin. The tissue section was counterstained with hematoxylin and eosin. Positive cells were detected by their brown color seen against the purplish-pink background, and uNK cells were counted in a 40x field of view. The entire tissue section was observed for positive cells, and only one cell showed strong staining.

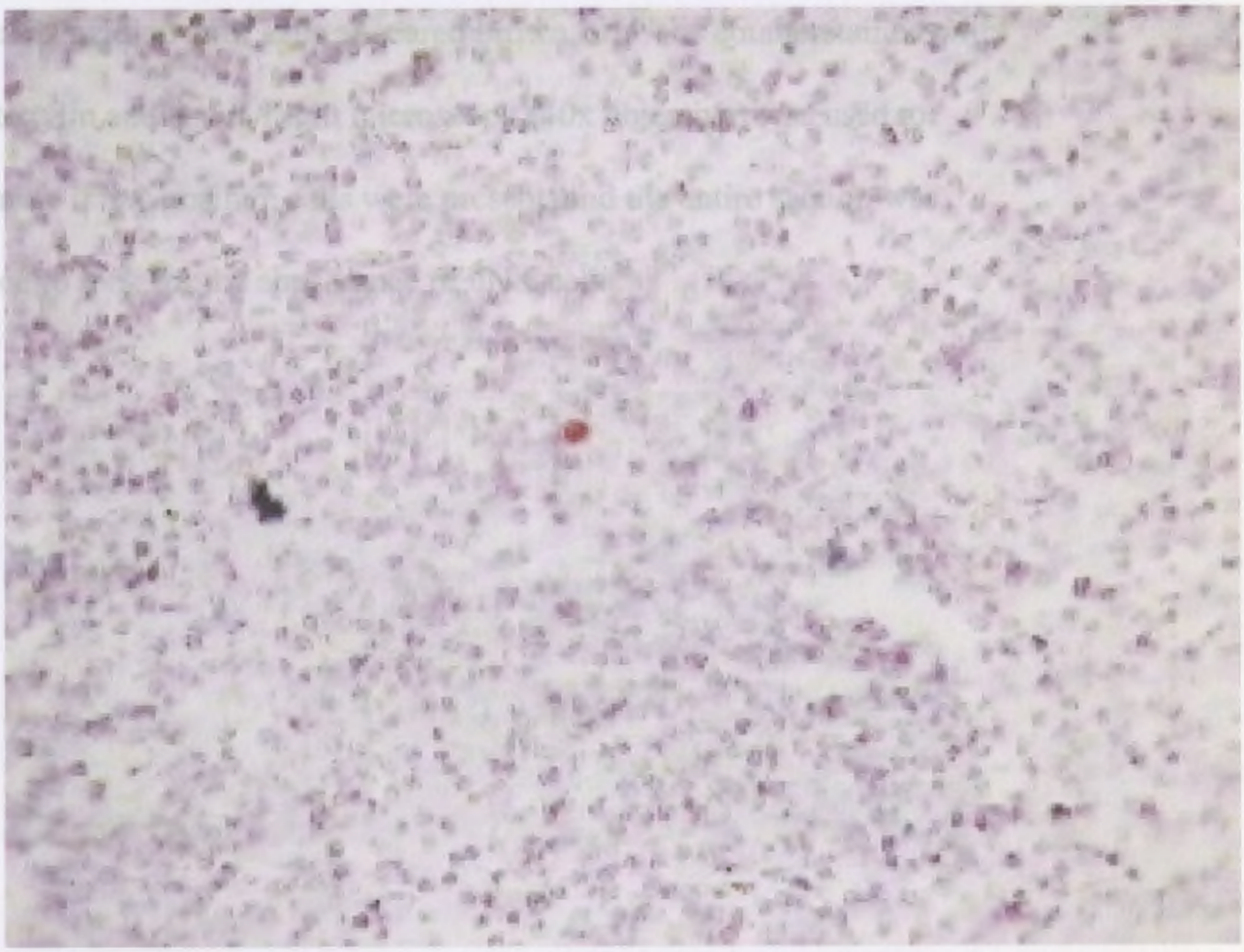


Fig. 2 RAG2^{-}/γc^{-} (FM19) spleen negative control for DBA staining

FM19 served as a negative RAG2^{-}/γc^{-} control, and the spleen section was stained with DBA lectin. It was then stained with Biogenex diaminobenzidine until cells appeared brown, and was counterstained with hematoxylin and eosin. Light microscopy (40x objective) was used to determine if any positive cells were present, and the entire section was scanned to look for the appearance of uNK cells.

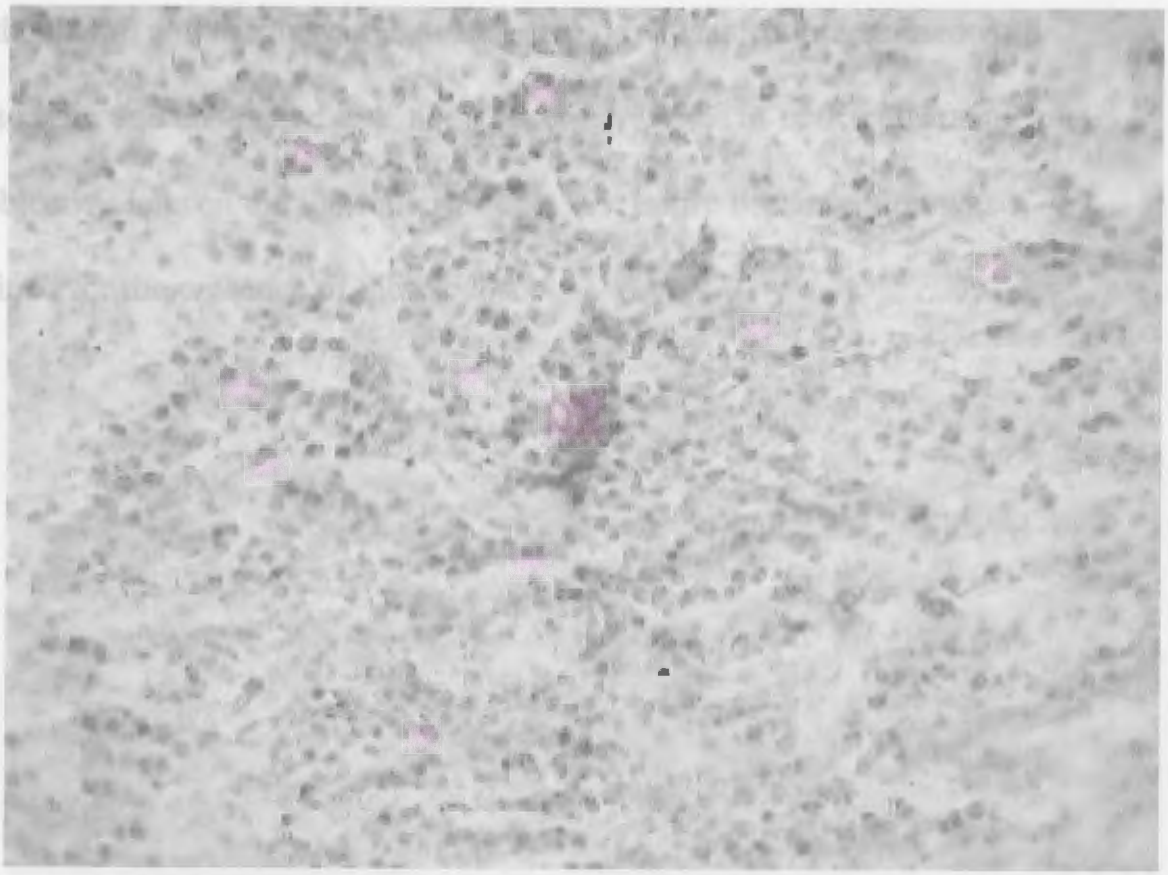
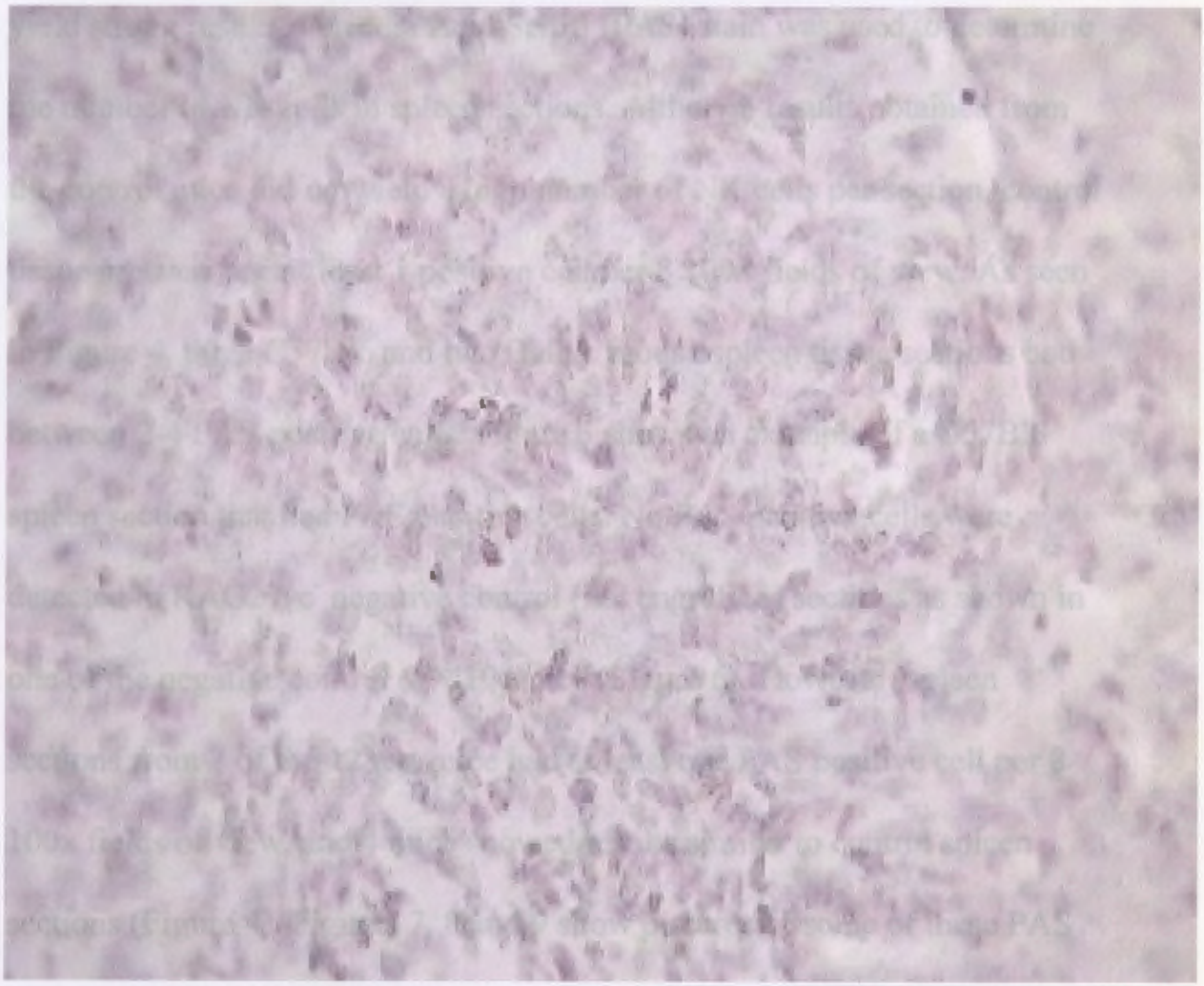


Fig. 3 DBA lectin stained FM9 Implant

To determine the number of uNK cells in some implant sections, they were stained with DBA lectin. A tissue section from an FM9 implant was stained with the lectin, and then with diaminobenzidine. It was counterstained with hematoxylin and eosin, and examined for the presence of uNK cells using a 40x objective lens on the light microscope. The entire tissue section was examined for the presence of these cells.



implant from an engrafted RAG2$^-$/γc$^-$ mouse that tested negative for DBA cells (FM9, implant A).

Because the staining results for control mice using DBA lectin did not yield strong results, Periodic Acid Schiff (PAS) stain was used to determine the number of NK cells in spleen sections. Although results obtained from the control mice did not yield a high number of NK cells per section, control tissue sections had at least 1 positive cell per 8 100x fields of view. As seen in Figure 4, three C57Bl6 and two Balb/c mouse spleen tissue sections had between 2-4 PAS-positive cells. Figure 5 shows an example of a C57Bl6 spleen section that had PAS positive cells. No PAS positive cells were detected in RAG2$^-$/γc$^-$ negative control (not engrafted) sections as shown in one of the negative control (FM19) mice (Figure 6). However, spleen sections from 7 of the 12 test mice had at least one PAS positive cell per 8 100x fields of view, and 4 mice showed results similar to control spleen sections (Figure 4). Figures 7, 8 and 9 show pictures of some of these PAS positive cells in spleen sections from FM6, FM10 and FM11.

Follicles are concentric rings of lymphocytes known to develop in the spleen during an immune response and in these studies their presence in our test mice would have indicated that the RAG2$^-$/γc$^-$ had been successfully

engrafted. To determine the number of follicles in spleen sections, a complete 10x field of view was examined and follicles counted. Spleen

Fig. 4 Periodic Acid Schiff stained cells in spleen sections

Spleen sections from all mice (controls and test mice) were stained using periodic acid Schiff to determine the number of uNK cells per 8 fields of view. The sections were counterstained with hematoxylin, and positive cells were identified by the presence of granules within the cells. All sections were viewed using light microscopy under oil immersion (100x). Data from all negative control sections (RAG2$^-$/γc$^-$ mice) was averaged and recorded as a single number.

Figure 4: Spleen PAS stained cells

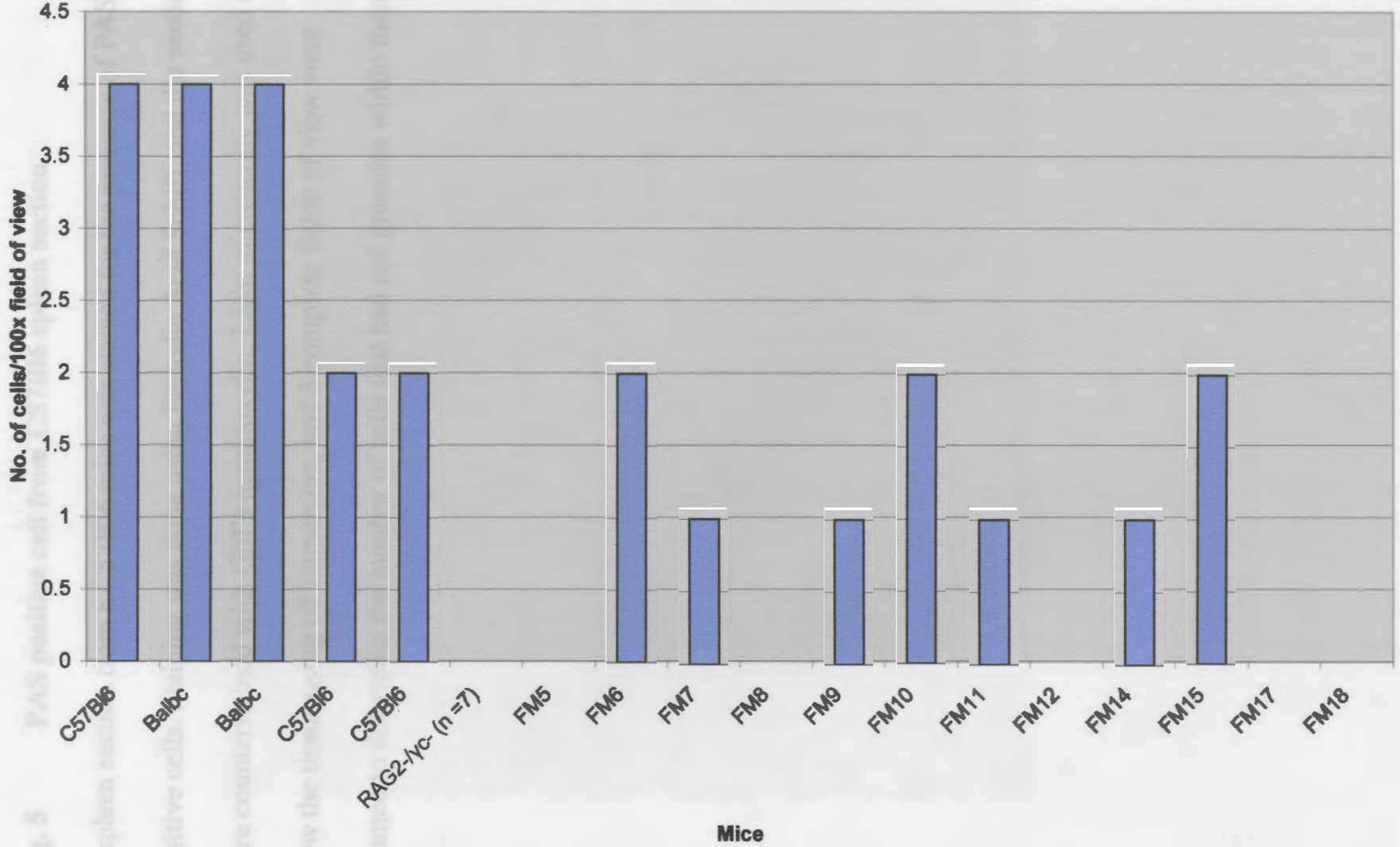


Fig. 5 PAS positive cell from C57Bl6 spleen section

A spleen section from a C57Bl6 mice was stained for the presence of PAS positive cells. Staining was done using Periodic Acid Schiff, and the sections were counterstained with Gill's hematoxylin. Light microscopy was used to view the tissue using oil immersion, and 8 complete fields of view were counted to determine the number of cells that had red granules within them.

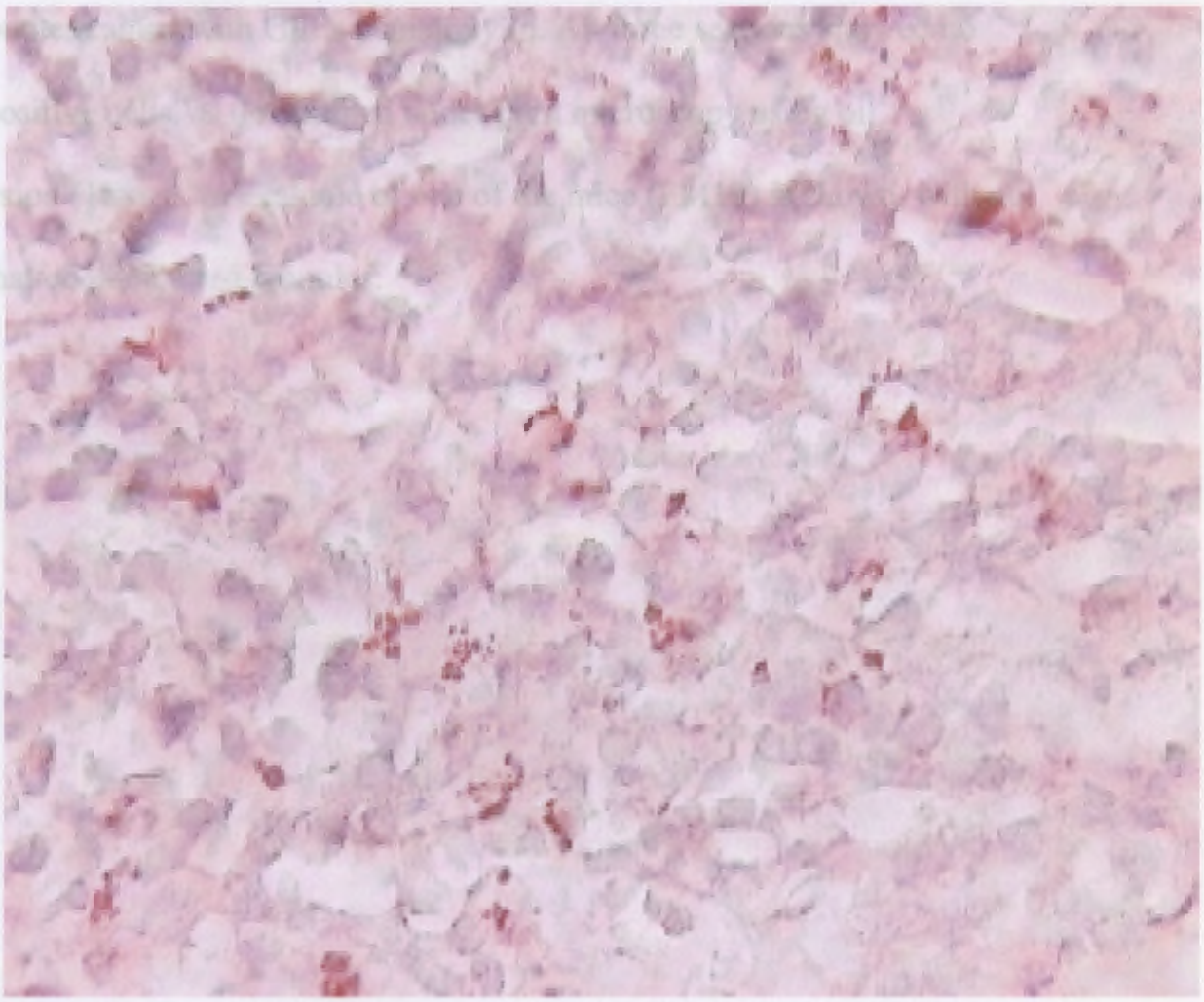


Fig. 6 RAG2^{-}/γc^{-} (FM19) spleen negative control for PAS staining

Three mice (FM19, FM21 and FM22) were used as negative controls. A spleen section from each of the mice was stained using Periodic Acid Schiff, and counterstained with Gill's hematoxylin. All three sections showed no PAS positive cells, as determined under light microscopy using oil immersion (100x). An example of one of the mice (FM19) is shown, with no cells that contained red granules.

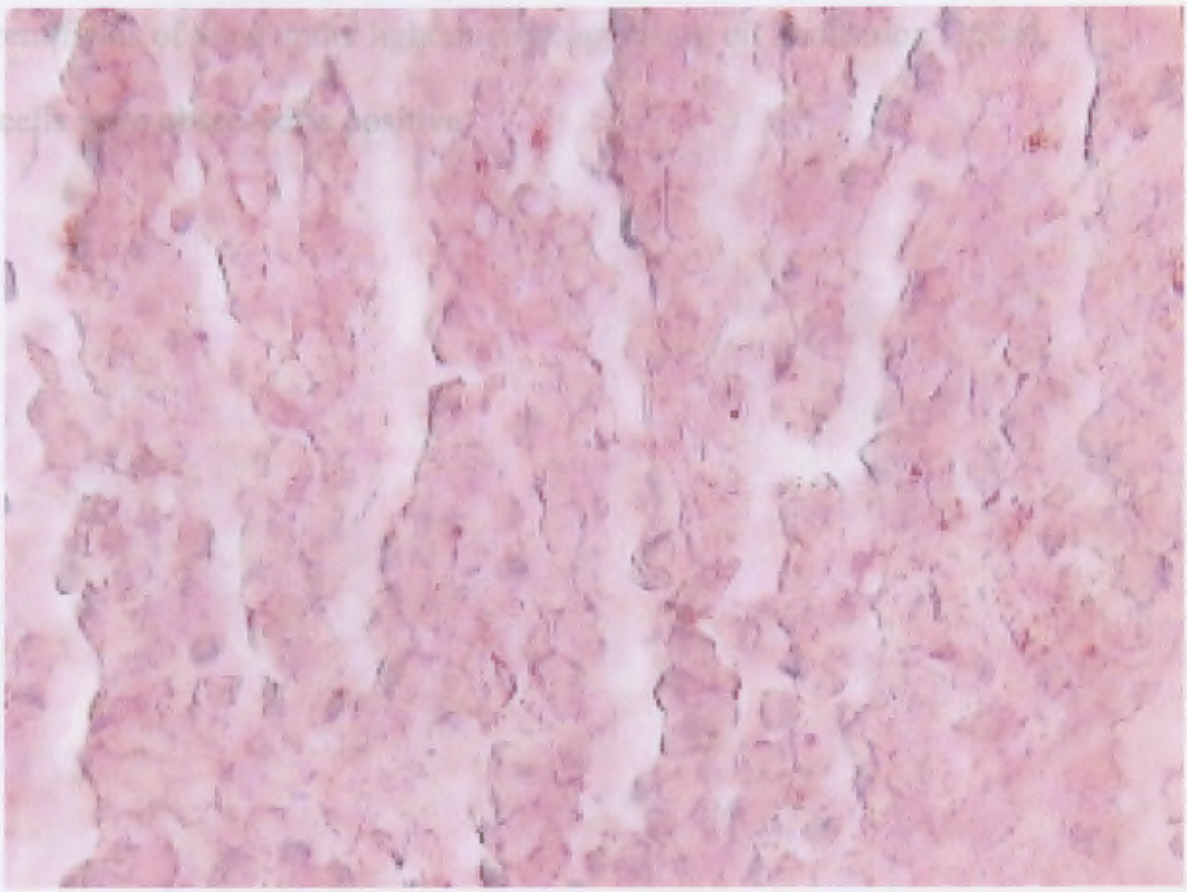


Fig. 7 PAS positive cells from a FM6 spleen section

A spleen section from FM6 was stained with PAS, and counterstained with Gill's hematoxylin. Cells with red granules (positive) were counted in 8 different fields of view under light microscopy using oil immersion (100x). Two cells were observed as positive.

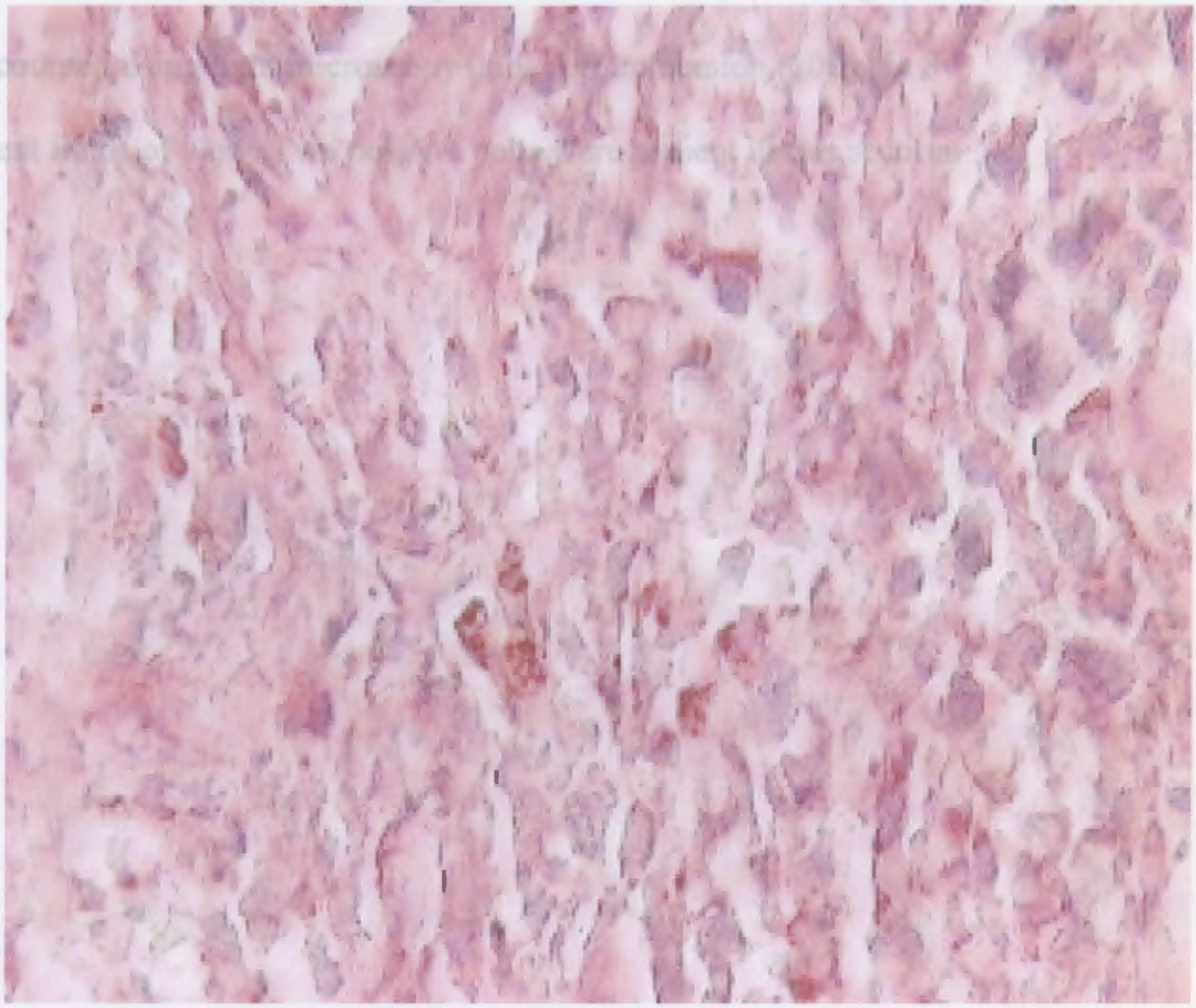


Fig. 8 PAS positive cells from a FM10 spleen section

A spleen section from FM10 was stained with Periodic Acid Schiff and counterstained with Gill's hematoxylin. Positive cells (with red granules) were counted using light microscopy under oil immersion (100x) in 8 different fields of view. Two positive cells were present in this section.

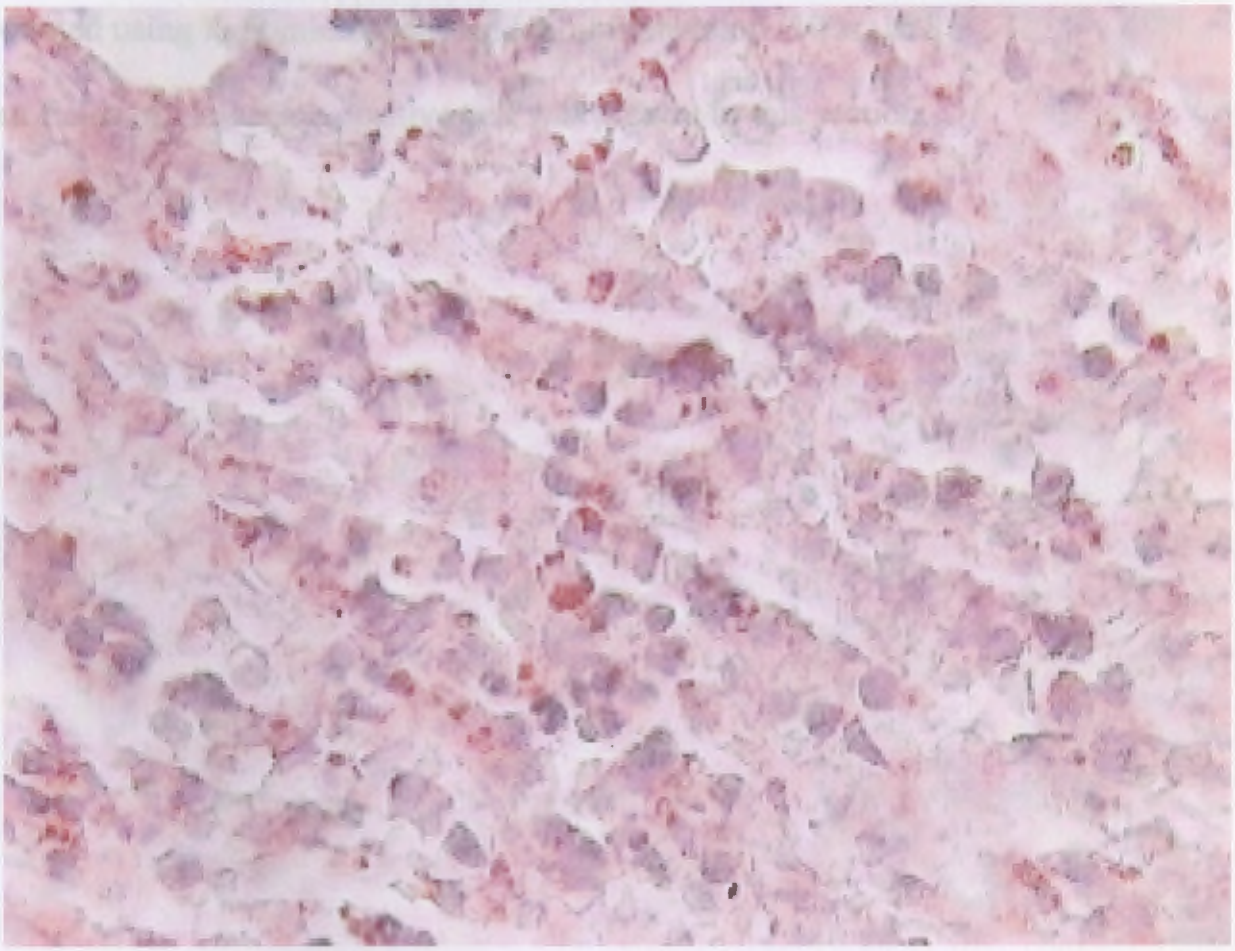
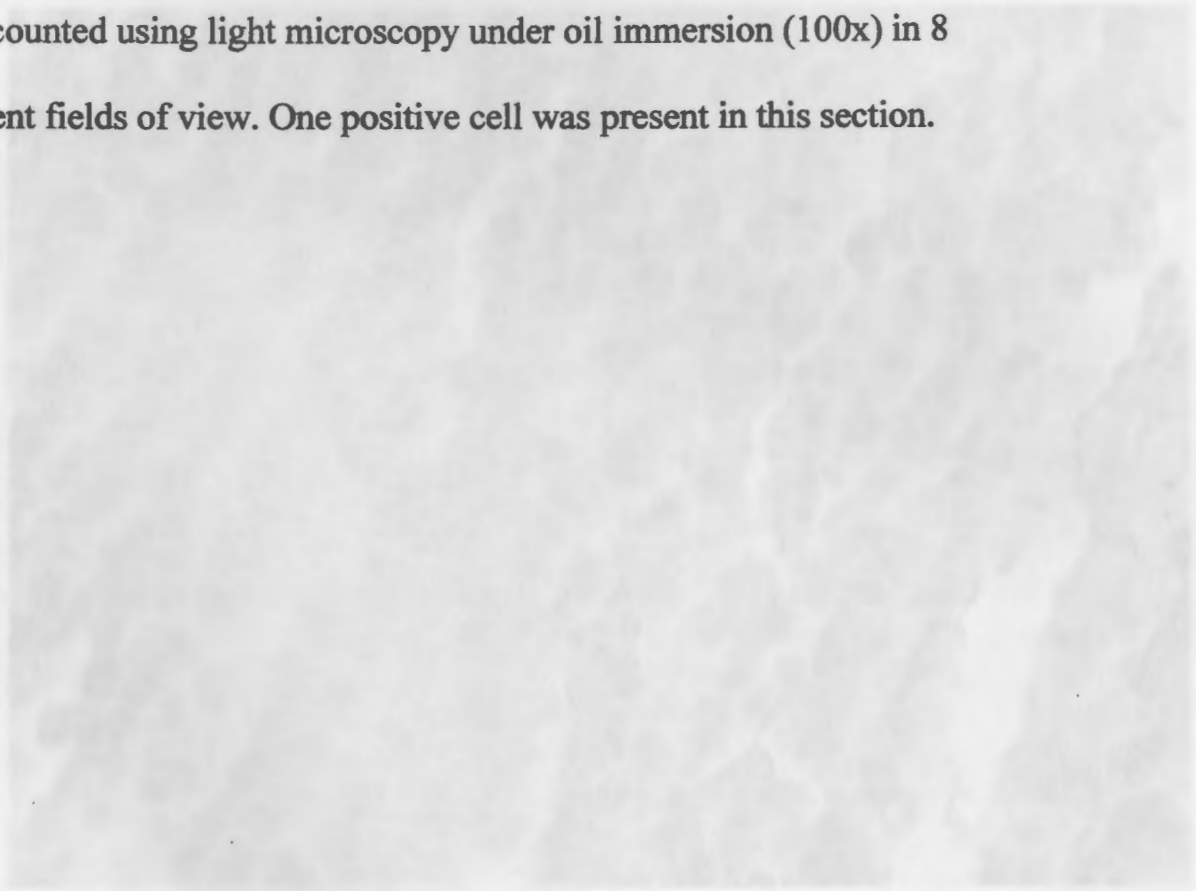
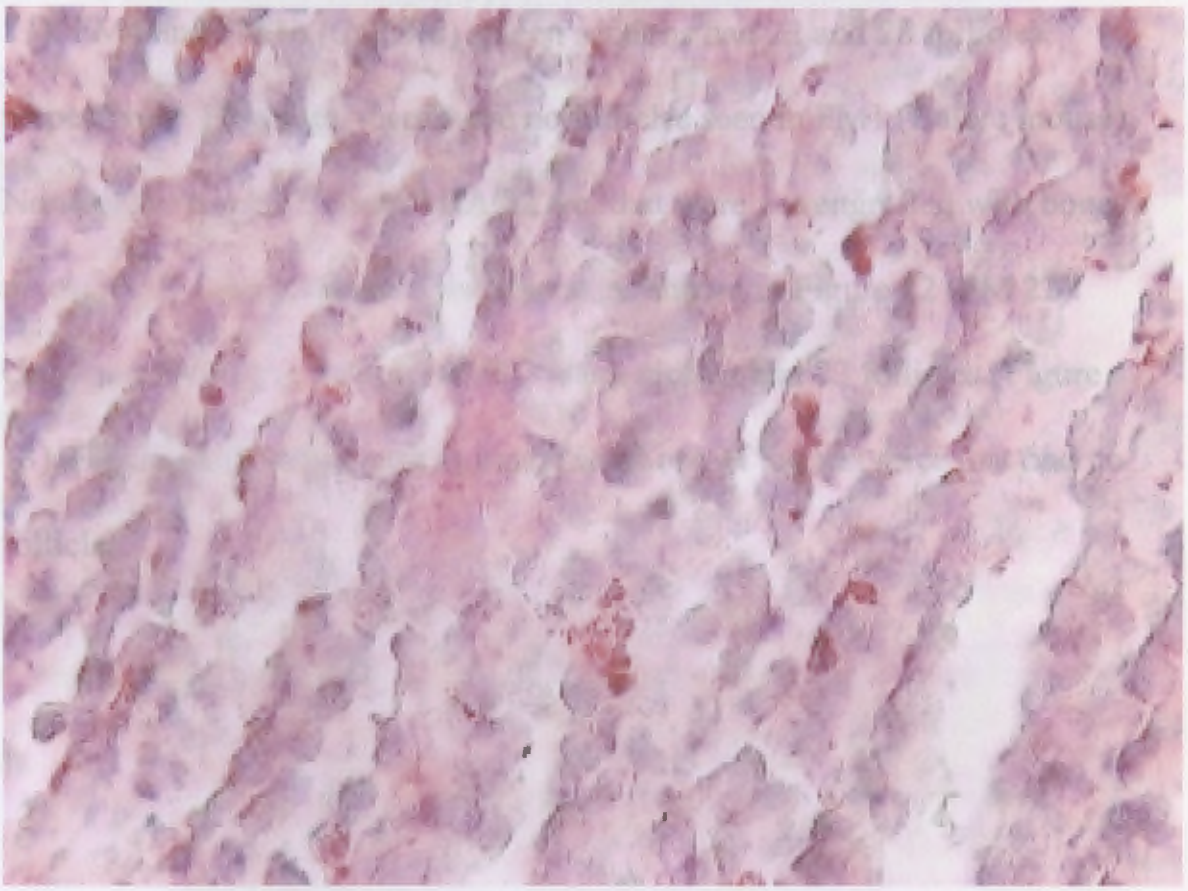


Fig 9 PAS positive cell from a FM11 spleen sections

A spleen section from FM11 was stained with Periodic Acid Schiff and counterstained with Gill's hematoxylin. Positive cells (with red granules) were counted using light microscopy under oil immersion (100x) in 8 different fields of view. One positive cell was present in this section.





sections from most positive control mice were demonstrated to have follicles (Table 2 & Figure 10). A spleen section from a C57Bl6 mouse had 21 follicles while a Balb/c (FM2) mouse spleen section had 40 follicles (Figure 11). FM4 and FM16 (C57Bl6) spleen sections had 22 and 28 follicles, respectively. However, there were no follicles seen in FM1 (Balb/c mouse). None of the negative control RAG2^{-}/γc^{-} that were not engrafted with bone marrow cells had follicles in the spleen, as shown in Figure 12 (FM 22). Only one RAG2^{-}/γc^{-} mouse (FM3) spleen section had 23 follicles (Figure 13). Figure 14 (FM7) shows an example of one of the test mice that had no follicles.

Fig 10 Number of follicles in spleen sections of control and test mice

To determine engraftment success in the RAG2$^-$/γc$^-$ test mice, their spleen sections were examined for the presence of follicles. The sections were stained with hematoxylin and eosin, and were viewed under light microscopy using a 10x objective lens. One complete field of view was used to count the number of follicles (concentric rings of lymphocytes). Results were recorded in the graph. One BalbC mouse (FM1) tested negative for follicles, but all other positive control mice had follicles in their spleens. Data from all negative control non-engrafted RAG2$^-$/γc$^-$ mice was averaged and recorded as one value. FM3 is the only engrafted RAG2$^-$/γc$^-$ test mouse that showed follicles, whereas the rest of the test mice were all negative for follicles.

Figure 10: Follicle count

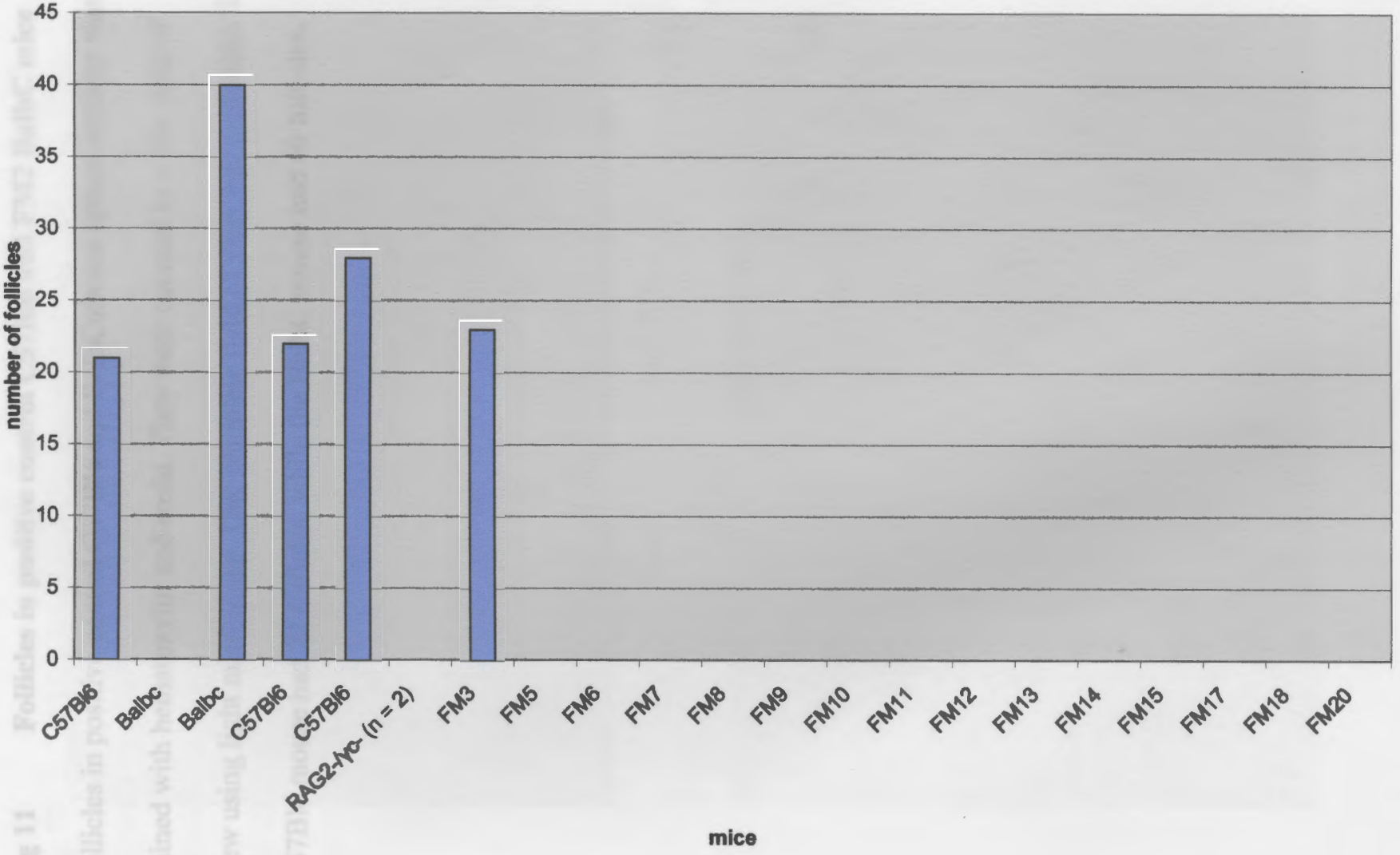


Fig 11 Follicles in positive control C57Bl6 and FM2 BalbC mice

Follicles in positive control C57Bl6 and BalbC mouse spleen sections were stained with hematoxylin and eosin. They were counted in a 10x field of view using light microscopy. One complete field of view was examined. The C57Bl6 mouse had 22 follicles, while the BalbC mouse had 40 follicles.

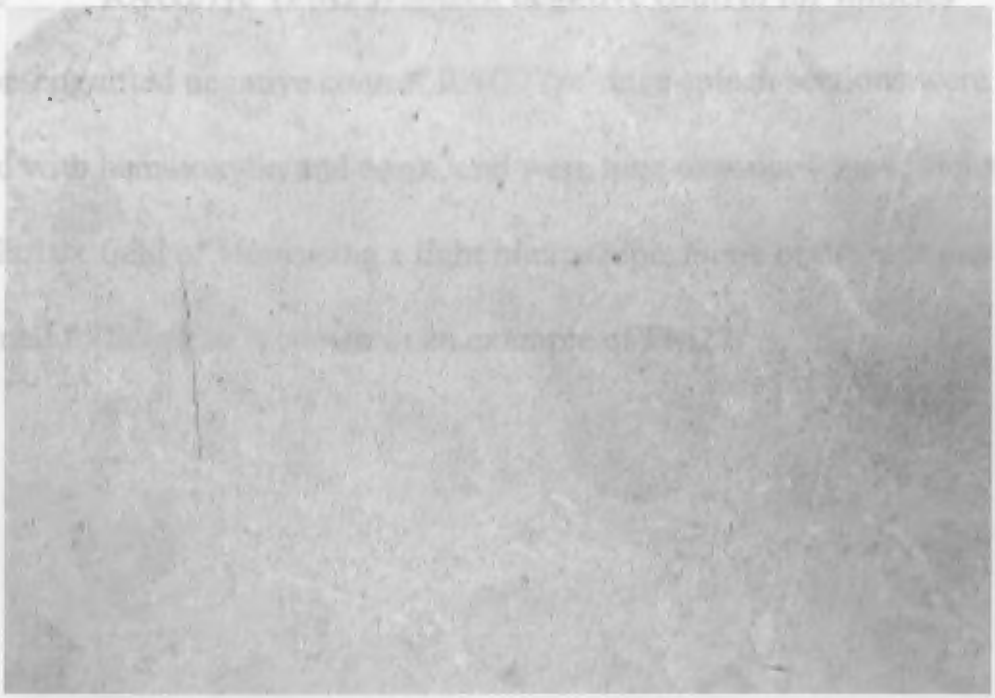


Fig 12 RAG2^{-}/γc^{-} (FM22) spleen negative control for follicles

All non-engrafted negative control RAG2^{-}/γc^{-} mice spleen sections were stained with hematoxylin and eosin, and were then examined for follicles in a single 10x field of view using a light microscope. None of the sections contained follicles, as is shown in an example of FM22.

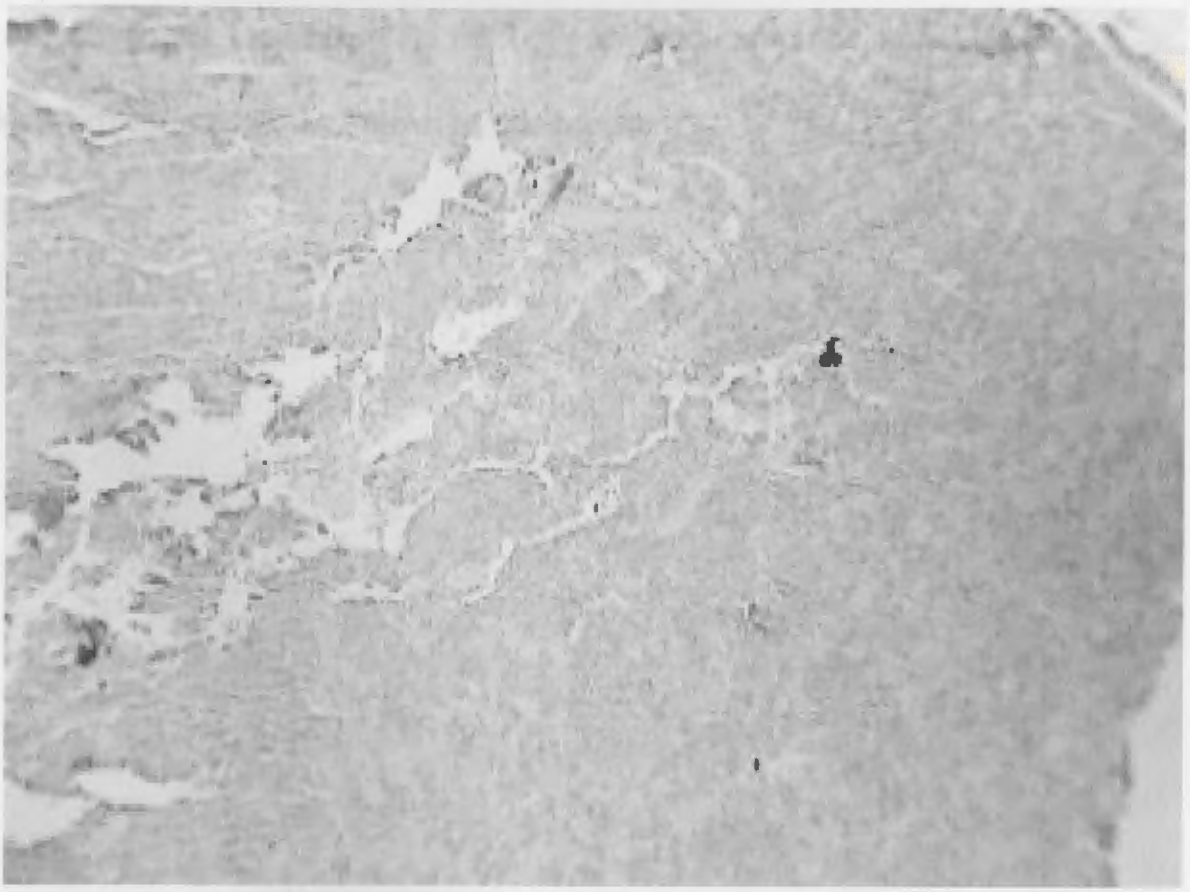


Fig. 13 Follicles in an engrafted RAG2^{-}/γc^{-} (FM3) test mouse

As with other spleen sections, the spleen section from FM3 was stained with hematoxylin and eosin, and then examined under the light microscope using a 10x objective lens. One complete field of view was examined, and 23 follicles were counted in the spleen of this mouse.

Fig. 14 Follicles in a FM7 RAG2^{-}/γc^{-} mouse

All (except FM3) of the engrafted RAG2^{-}/γc^{-} test mouse sections did not show follicles in their spleens. The sections were stained with hematoxylin and eosin, and examined using a light microscope (10x objective lens). One complete field of view was examined, and FM7 shows an example of a spleen section with no follicles.

Discussion

The purpose of our study was to investigate the survival and engraftment success of bone marrow (BM) cells from normal mice injected into an immunodeficient mouse model, RAG2$^-$/γc$^-$. RAG2$^-$/γc$^-$ mice were produced by mating mice with a mutation in the cytokine receptor common gamma chain (γc$^-$) and those with a mutation in the RAG2 gene. They, therefore, lack B cells, T cells and NK cells (Sharara et al., 1997). Bone marrow transplantation has been demonstrated to reconstitute uterine natural killer (uNK) cells in some mouse models used in previous studies. However, most of the previously tested models were not immunodeficient and therefore had to be treated to remove all white blood cells prior to reconstitution with BM cells from normal mice.

Several groups of researchers have used SCID mice (lacking B and T cells) to investigate the success of engraftment of human peripheral blood mononuclear cells (PBMC) in an attempt to gain insight into transplantation models for use in diseases such as cancer. Ramirez and coworkers investigated the success of bone marrow transplantation of human bone marrow cells into non-obese diabetic (NOD/SCID) mice lacking B and T lymphocytes, using either T cell depleted or enriched progenitor cells. These cells were injected into the mice after they had been irradiated using a

Cesium source. The human bone marrow cells were injected intravenously, and the mice were sacrificed 1-2 months after transplantation. Flow cytometry was performed to determine engraftment and cell survival success using fluorescent anti-CD45 labelled cell suspensions obtained from the spleen, bone marrow and thymus. All surviving engrafted mice showed significant numbers of human BM cells. The antibodies used in the flow cytometry experiments did not bind to mouse cells, therefore all counted cells were human cells engrafted from human bone marrow cells. The investigators, therefore, concluded that human cells could successfully be engrafted into this mouse model (Ramirez et al., 1998).

Another study investigating the effect of irradiating recipient SCID mice before they were transplanted with human PBMC (Cavacini et al., 1992) showed that immunosuppression by irradiation is necessary for successful transplantation. They compared engraftment success between mice that were irradiated 3 days prior to engraftment with mice that were not irradiated. Twenty eight days post-injection, the mice were sacrificed and spleen cell suspensions were examined by flow cytometry using fluorescent anti-CD45. An ELISA was also done to detect IgG levels in the serum. More IgG (10-fold increase) was detected in irradiated mice compared to non-irradiated mice (Cavacini et al., 1992). This study proved that irradiation of

SCID mice was important to increase the success of human bone marrow cell engraftment.

Other studies using human-mouse chimera (organism composed of genetically different tissue types).have focused on improving engraftment success by down-regulating the innate system (macrophages and natural killer cells) which is active in these SCID mouse models. In an attempt to improve success of peripheral blood mononuclear cell engraftment in C.B.-17-SCID mice, Shibata and coworkers used a monoclonal antibody (R4-6A2) against IFN-γ. Neutralizing IFN-γ in these mice was done to downregulate activation of macrophages, as well as to decrease activation of T and NK cells. Mice received this treatment at various times between 1 day before and up to 14 days after injection with PBMC. Flow cytometry (of peritoneal cavity cells and splenocytes) using anti-CD45 to determine white blood cells was performed, as well as ELISA tests (from sera) to determine antibody levels. Results indicated that the mice receiving pretreatment with R4-6A2 had higher engraftment success, and showed higher levels of IgG and IgM when compared to controls (Shibata et al., 1998). Again, this study showed the necessity of pretreating mice before engraftment to ensure complete depletion or downregulation of cells of the innate immune system.

In a similar study done by Tournoy and coworkers using NOD-SCID mice for human PBMC engraftment, some mice were treated with an antibody TM-β1 to block activity of the mouse IL-2 receptor β chain. This treatment would result in a decrease in NK cell levels. Flow cytometry analysis (using different antibodies against human cell surface antigens) and ELISA tests were done to determine numbers of human cells in spleens. Results show that engraftment was more successful in mice that had received this pretreatment, as noted by the higher levels of cells. This indicated that pretreatment of mice to downregulate the innate immune system results in a higher engraftment success (Tournoy, 2000).

Peel and coworkers (Peel, et al., 1983) used bone marrow engraftement of Porter (random outbred colony) mice to investigate the origin of uNK cells. The mice were irradiated (day 1) before they were injected with bone marrow cells from either Porter mice or Wistar rats on day 2. Some mice also received colchicine (for chromosome spreads) 3 hours before they were sacrificed. Uteri were collected at days 8 and 9 of pregnancy, processed and embedded in glycol methacrylate. This chemical is used to embed tissue sections with the advantage of preserving the structure of the nuclei of cells. Sections of 1-2μm were cut, and stained with PAS. The chromosome spreads to determine the species origin of the

engrafted bone marrow cells in the mice was a confirmation that these cells had migrated to the uterus after transplantation. Their results showed that the uNK cells did originate from the bone marrow since they were detected in the uterus at days 13 and 14 of pregnancy.

In a study done by Guimond and coworkers (Guimond et al., 1998) to investigate the role of uNK cells during pregnancy using tgE26 mice, some bone marrow cells from positive control donor mice were pretreated with two rounds of anti-Thy-1 antibody before they were injected into the immunodeficient mice. Other mice in that study received untreated bone marrow cells. The pretreatment was done to deplete any mature T cells that could possibly be in the cell suspension and may cause graft versus host disease in which some of those mature cells could possibly attack host cells since they are recognized as foreign. At day 14, the number of uNK cells from the two groups was not significantly different, although the mice that had received pretreatment with the antibody showed a higher number of these cells in the spleen. In our study, some of the bone marrow cells were also pretreated with anti-Thy1.2 antibody to determine if there was a significant effect on survival rate and reconstitution success. We saw no difference in results when comparing these two populations of mice.

Previous studies from our laboratory (Toth, 2002) demonstrated, using ELISA tests to detect the antibodies IgM and IgG in the serum, that the immunodeficient RAG2$^-$/γc$^-$ mice had somewhat been successfully engrafted with the bone marrow cells from normal mice. These tests performed to detect IgG and IgM showed that most of the mice (FM6, 10, 11, 12, 14 and 15) had antibodies in their serum. This suggests that there were B cells making these antibodies. However, two of the mice (FM9 and 13) did not have positive ELISA results, showing that the injection may have been unsuccessful due to bone marrow cells failing to get into the veins (Toth, 2002). IgG has a longer half life of 7-23 days compared to IgM whose half life is about 5 days. Since mating was done 3 weeks after engraftment, the mice would be expected to show little to no levels of IgM, whereas they might still have detectable amounts of IgG even if the cells producing antibody were no longer viable. From the test mice, the levels of IgM detected by ELISA were between 0 and 1.6nm (absorbance). Of the 4 test mice that received anti-Thy-1 pretreated bone marrow cells, only one (FM9) tested negative for IgM, whereas the rest had IgM levels similar to test mice that had received untreated BM cells (Toth, 2002). Since both groups of mice produced antibody after engraftment, it suggests that the antibody treatment may not be necessary in this mouse model.

Goldman and coworkers published a study similar to ours investigating the success of engrafting human peripheral blood mononuclear cells into the RAG2$^-$/γc$^-$ mouse model. In these studies, they tested the effect of prior irradiation in the mice. Results were determined by counting the number of white blood cells (WBC) present in single cell suspensions from the spleen between the irradiated and the untreated groups. The white blood cells were detected through flow cytometry using fluorescent antibodies against the human CD45 antigen. Results proved that irradiation of the mice with cesium prior to intraperitoneal injection of human peripheral blood mononuclear cells did not make a difference in the success of engraftment. The same study also showed that mice engrafted with $5x10^6$ cells survived for the longest period after post-injection (Goldman, et al., 1998).

The mated RAG2$^-$/γc$^-$ mice used in our study were anaesthetized 10 days after the appearance of a copulation plug. Organs collected from the control and test mice, were processed in formalin, embedded in paraffin and sectioned to a thickness of about 4-5um. This is consistent with the methods used in most previous studies done to detect uNK cells. A study done by Kusakabe et al. investigating the mechanism of uNK cell death in murine placenta during the course of pregnancy in mice showed that these cells were most abundant in the uterus at day 11 of pregnancy (Kusakabe et al.,

1999). This confirms that our spleen and implant sections from the test mice should have had the maximum number of uNK cells at this time.

Staining of sectioned spleens and implants was done to determine if there was successful migration of the uterine Natural Killer (uNK) cells using lectin. A study done by Paffaro and coworkers (Paffaro et al., 1999) described the method of using DBA lectin to stain uNK cells by targeting the N-acetyl-D-glucosamine (NADG) on their cell surface. In our study, the spleen sections were stained with DBA lectin and uNK cells were counted in a 40x field of view. All test mice were negative for uNK cells, while 3 control mice had 2 to 3 positive cells per 40x field of view. All the negative controls that were incubated with DBA lectin pre-incubated with purified NADG stained negative as expected. This, therefore, proves that the DBA lectin stain was not binding non-specifically to cells. Although our results suggest that none of our immunodeficient mice had successful uNK cell migration after they were engrafted with bone marrow cells from normal mice, the fact that the positive control sections from C57Bl6 mice showed staining for only a very few cells could possibly suggest that the stain did not work well in the paraffin embedded tissue sections.

Anti-CD45 antibody was used to detect white blood cells in some spleens and implants. This antibody from rat serum did not yield positive

results for positive control tissue sections; neither did it produce positive results for our test mice. It was, therefore, concluded that the antibody was not successfully binding to the CD45 ligand on these cells since positive control mice (C57Bl6) are expected to have high quantities of white blood cells in their spleens. Currently, no antibody can be found that works in mouse tissue sections processed in formalin and embedded in paraffin. As discussed, most laboratories have used flow cytometry to determine the number of white blood cells using fluorescent antibodies against the CD45 cell surface antigen (Goldman, et al., 1998). As we do not have a flow cytometer, we could not use this method to detect white blood cells in the various organs.

Some previous studies have detected the presence of uNK cells in various organs by using Periodic Acid Schiff stain to target these cells. The stain works by staining glycogen in the granules red. uNK cells are distinguished from the rest of the cells with glycogen by their high granularity. Studies by Peel et al (1989), Guimond et al. (1998) and Kusakabe et al (1999) all successfully detected the presence of uNK cells in implant sections (metrial gland) using this stain (as previously discussed). Our staining results of spleens with PAS yielded a few positive cells in almost all 12 of the test mice. Seven of them had at least one positive cell,

while 4 of them had levels similar to control mice. However, as with the DBA stain, the results obtained from positive control sections were not strong since only low numbers of cells were detected in the spleens.

A number of spleen sections from control mice indicated the presence of follicles (concentric rings of lymphocytes that form during an immune response) in a 10x field of view. However, only one spleen section from engrafted RAG2$^-$/γc$^-$ (FM3) mice showed a significantly high (23) number of follicles. However, it could be possible that the block with the spleen was mislabeled, and that a positive control spleen was embedded on the cassette. Because all other test mice were negative for follicles, they may have been engrafted but not surviving in the spleen for 4 weeks.

Because of the successful engraftment results obtained by Goldman and coworkers in the RAG2$^-$/γc$^-$ mice that they did not irradiate, we speculated that engraftment in our model should be successful. In our RAG2$^-$/γc$^-$ mouse model, we hypothesized that this irradiation was not necessary since these mice not only lack B cells and T cells but are also deficient in NK cells. This is of importance since NK cells secrete IFN-γ, which would in turn activate macrophages that would elicit an immune response against the engrafted bone marrow cells (host versus graft disease). In a previous study by our laboratory, Dr. Fagan tried to reconstitute these

mice with human peripheral blood mononuclear cells without irradiating them. Results obtained from the study were poor, showing little to no engraftment (unpublished results). With the possibility that engraftment and survival of these cells in the mice was poor due to species specificity, the present study tested the engraftment of bone marrow cells from C57Bl6 or Balb/c mice into these RAG2^{-}/γc^{-} mice. However, as discussed, results obtained from the lectin and antibody staining were poor, and there were no uNK cells detected in the spleen sections stained with PAS. These results suggest that for future studies on the mechanism action of uNK cells may be more effective in this mouse model if the mice are pretreated with irradiation.

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Appendix A: Antibody Search Spreadsheet

Company Name	Telephone Number
Accurate Chemical and Scientific Corporation	1-800-645-6264
Advanced Magnetics	1-800-343-1346
Ambion	1-800-343-1397
Amresco	1-800-448-4442
Babco	415-222-4940
BBI International	1-800-227-6666
Biomeda	1-800-341-8787
Bionetics Research	1-301-258-5200
Biosource	1-800-242-0607
The Binding Site Industry	1-800-633-4484
Calbiochem	1-800-845-9256
Cellgro	1-800-235-5476
Cell Pro Inc	1-206-485-7644
Clontech	1-800-662-2566
Collaborative Research Inc	1-800-343-2035
Dako Corporation	1-800-424-0021
Denville Scientific Inc	1-201-328-0822
Diatome US	1-215-646-1478
Dynal	1-516-529-0039
Gould Electronics	1-877-232-8995
Insight Media	1-212-721-6316
Life Sciences	1-201-256-1699
Mediatech Inc	1-800-388-8355
Oncogene Research Products	1-800-828-4871
Peninsula Laboratories	1-800-922-1516
Promega Life Science	1-800-356-9526
R&D Systems	1-800-343-7475
RBI	1-800-736-3690
Research Antibodies	1-800-457-3800
Research Products International	1-800-323-9814
Rockland Immunochemical Antibodies	1-800-656-7625
Serotech	1-800-265-7376
Sigma Chemical Company	1-800-848-7791
Signet Laboratories	1-800-223-0769
Southern Biotech Associates	1-205-945-1774
Stem Cell Techs	1-800-667-0322
Surgipath	1-800-225-3035

Company Name	Telephone Number
Taconic	1-888-822-6642
Vector Laboratories	1-650-697-3600
Wako	1-800-992-9256
Ward's	1-800-962-2660
Zymed	1-800-847-4494