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Sickle cell disease: animal models and gene therapy

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SICKLE CELL DISEASE:
ANIMAL MODELS AND GENE THERAPY

A THESIS

Presented to the

Honors College at Southern University
Baton Rouge, Louisiana

In Partial Fulfillment of the Requirements for the
Honors College Degree

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by

Francesca M. Mellieon

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CERTIFICATE OF APPROVAL

HONORS THESIS

This is to certify that the Honors Thesis of
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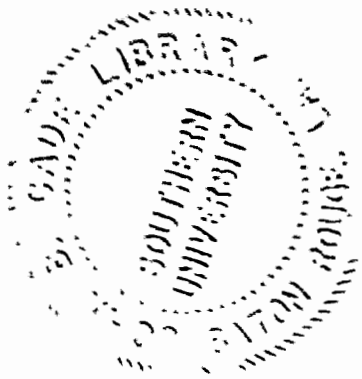
SICKLE CELL DISEASE: ANIMAL MODELS AND GENE THERAPY



An Abstract of a Thesis

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Sickle cell disease is a worldwide health problem in countries ranging from its origin, Africa, to the United States of America. The understanding of sickle cell disease dates back to Zimbabwe in the 1940's. Sickle cell trait is the term used to describe healthy unaffected carriers (heterozygous) of the genetic condition known as Sickle Cell Disease. It is estimated that there are about 100,000 genes in the human body and each of them codes for a different trait. About 5,000 of these genes are known to carry mutations serious enough to cause genetic diseases. This research deals with sickle cell mice models. These mice have many different traits and genes that are under research in order to manipulate their genes to create a model useful for testing treatments for sickle cell disease. The primary goal of this lab research is to perform bone marrow transplants to determine whether or not marrow cells from the models under development are histocompatible with standard recipients. The ultimate goal of this lab research as well as other researchers is to be able to complete gene therapy successfully in mouse models using approaches directly applicable with humans.

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CHAPTER I
BACKGROUND OF THE STUDY

INTRODUCTION

Sickle cell disease is a worldwide health problem in countries ranging from its origin, Africa, to the United States of America. The understanding of sickle cell disease dates back to Zimbabwe in the 1940's. A British physician was studying blood from malaria patients; some patients who demonstrated the sickle cell trait had fewer malaria parasites than those without the sickle cell trait. Upon publication of these findings, a physician in Zaire began more intense research. This in turn led to the hypothesis in 1954 that people with the trait did not contract malaria as often as those lacking the sickle trait, and if they did, the disease was less severe (1).

Sickle cell trait is the term used to describe healthy unaffected carriers (heterozygous) of the genetic condition known as Sickle Cell Disease. It is estimated that there are about 100,000 genes in the human body and each of them codes for a different trait. About 5,000 of these genes are known to carry mutations serious enough to cause genetic diseases. There are two copies of every autosomal gene and the normal copy usually prevents expression of the disease. In this case, the defect is said to be recessive; one must inherit two copies of the mutant gene for it to cause trouble. If there is only one copy of the recessive gene, it lies waiting to be passed along to the next generation. In other diseases said to be dominant, the presence of only one defective gene will cause the genetic disorder. In dealing with inheritance and recessive genes, if both parents carry the recessive, there is a 25% chance a child will get two bad copies, and the disease. In the dominant

forms of the gene, anyone possessing a single bad gene has the condition, and there is a 50-50 chance of passing it to his or her offspring (2).

Sickle cell disease begins with the presence of a point mutation in DNA that encodes the beta globin of sickling hemoglobin. Some of the abnormal hemoglobin manifestations include: sickle cell formation seen on a peripheral blood smear, the presence of microcytosis observed in red cell morphology or stated on the laboratory report, target cells, Heinz body formation, an increased red blood cell mass, and formation of methemoglobin in an individual (3). Sickling of the cells is due to the deoxygenation of the hemoglobin S. Once the cells have gone through deoxygenation, the red blood cells start to become elongated or develop a sickle shape (See Figure 8). Because the red cells lose deformability, they are not able to perform to the full capacity. Sickling of red blood cells results in blockage of blood vessels which causes pain and repeated episodes of organ dysfunction. The episodes of pain may result in tissue ischemia or the lack of blood flow. However, these episodes of ischemia rarely occur before six months of age because fetal hemoglobin that is present then reduces the sickling of cells. This fetal hemoglobin begins to decrease as the patient becomes older, and adult hemoglobin predominates; if it were possible to keep the levels of fetal hemoglobin at about 25%, then the patients' red blood cells would not begin to sickle.

The goal of maintaining the level of fetal hemoglobin is one of the many tasks that researchers are attempting to achieve. There are various treatments for sickle cell disease patients, both short term and long term; however, most drugs as well as treatments have great benefits but possible risks and long term side effects as well. Some of the drug treatments used are 1) vanillin which is a chemical that interacts with hemoglobin and has

been shown to prevent sickling is still being investigated in clinical settings, 2) hydroxyurea causes fetal hemoglobin levels to rise in some patients, preventing crises, but it is cytotoxic, 3) cetedil and clotrimazole are drugs that make red blood cells more flexible and less likely to sickle, 4) 5-Azacytidine was used in the 1980s, but was stopped because it was shown to cause cancer in laboratory rodents, 5) butyric acid is reported to delay the turning off of the fetal hemoglobin gene; this drug is still being tested on humans but has not been approved for clinical use, 6) erythropoietin (EPO) is a naturally occurring hormone that promotes the production of red blood cells in the body; it is hoped that if administered in large doses the body will produce more red blood cells and more fetal hemoglobin, 7) bone marrow transplants are available in the US on a limited basis; for this to be considered a suitable donor must be found but there are many complications including: death, histoincompatibility or the rejection of the donor marrow cells, and dependence on immunosuppressant drugs (4).

As previously mentioned, the disease and its treatment are great obstacles to deal with in one's life. In Africa alone, there are approximately 200,000 infants born with Hb SS (2). An estimated 60,000 Americans suffer from the disease, and about 2.5 million have the sickle cell trait (5). Therefore, Hb SS is a major health problem in the world. While most people with SCD do live in Africa, the disease is a significant health problem elsewhere as well.

The need for more effective treatments and cures inspires a variety of approaches to laboratory research. Our own research deals with sickle cell mice models. These mice have many different traits and genes that are under research in order to manipulate their genes to

create a model useful for testing treatments for sickle cell disease. The first progress in this aspect of research was made by Ryan *et al.* and Greaves *et al.* who produced transgenic mice that made about 50% human sickle hemoglobin and 50% mouse hemoglobin. Consequently, the models resembled sickle cell trait, not the disease. Later, more other mouse models were produced but none has been suitable for testing gene therapy (6).

Statement of the Problem

The primary goal of this lab research is to perform bone marrow transplants to determine whether or not marrow cells from the models under development are histocompatible with standard recipients. The current status of gene transfer experiments indicates that it is possible to provide 1) safe and efficient retroviral packaging lines for gene transfer and 2) vectors containing the human beta-globin genes and selectable marker genes which can be transmitted into erythroid (red) cells and are appropriately expressed (7). Remaining challenges include the development of vectors that drive high-level globin expression over an extended period. The ultimate goal of this lab research as well as other researchers is to be able to complete gene therapy successfully in mouse models using approaches directly applicable with humans. The breakthrough of gene therapy will be a benefit to the entire world of medicine. It is possible that gene therapy will eventually be used to prevent or cure diseases that now kill or disable thousands of Americans. It also has the potential to revolutionize health care by enabling more people to remain productive members of society and by eliminating or reducing the need for costly medications and other treatments that ameliorate symptoms but do not cure disease (8).

Definition of Terms

1. heterozygous – Having two different alleles (one dominant, one recessive) of a gene pair (11)

2. autosomal gene – independent of sex and is expressed in a person with a single copy of the diseased gene. (11)

3. recessive – Refers to an allele of a gene that is expressed when the dominant allele is not present. An allele expressed only in homozygous form, when the dominant allele is absent. (11)

4. dominant – The property of one of a pair of alleles that suppresses the expression of the other member of the pair in heterozygotes. (11)

5. point mutation – mutation due to intramolecular gene reorganization (as by substitution, addition, or deletion of a nucleotide) (12)

6. Hb SS – the allele for sickle cell disease

CHAPTER II
REVIEW OF LITERATURE

Gene transfer. A potential approach to gene therapy for sickle cell disease. Bank A, Marowitz D, Lerner N *Sci.* 565,37 (1989) Abstract from Pub Med Query.

The current status of gene transfer experiments indicates that it is possible to provide 1 safe and efficient retroviral packaging lines for gene transfer; and 2 vectors containing the human beta-globin genes and selectable marker genes which can be transmitted into erythroid cells and are appropriately expressed. The long term goal of human gene therapy for sickle cell disease consists of constructing optimally safe and efficient retroviral packaging lines as well as retroviral vectors containing the human beta-globin gene and selectable markers such as the neoR gene. There are many alternative approaches to gene therapy. The direct correction of the defect in the beta-globin gene by site specific recombination of the defective gene with incoming normal gene sequences is one alternative approach to gene therapy. Yet another alternative is gene correction at a low frequency; however this is only an experiment because with current technology it is not fully achievable.

Simplified Typing of Mouse Hemoglobin(Hbb) Phenotypes Using Cystamine J. Barry Whitney, *Biomedical Genetics.* 16, 667 (1977).

Cellulose acetate electrophoresis of mouse hemoglobins treated with cystamine creates a very rapid and distinct separation of all combinations of mouse hemoglobin bands produced by all homozygous and heterozygous combinations. The purpose of the cystamine is to enhance the *Hbb* as a marker. The results of this experiment provides a

basis of comparison for the electrophoresis that was performed on the sickle mice. It was found that the diffuse, single and heterozygote phenotypes are obvious within 5 minutes of the start of electrophoresis.

Knockout-Transgenic Mouse Model of Sickle Cell Disease
T. M. Ryan, D. J. Ciavatta. T.M.Townes,, *Science 278,* 823 (1997)

Transgenic mice that expressed human sickle were mated with mice having knockout mutations of the mouse alpha and beta-globin genes. Surprisingly, animals were produced synthesizing only human hemoglobin in adult red blood cells. This means that this mouse model will mimic the characteristics of hemoglobin in humans. As a result the mice were able to develop severe hemolytic anemia. "Individual spleens in these animals ranged from 7 to 20 times the mass of normal nonanemic mouse spleens." Although there was severe anemia in 90% of the mice, they survived for 2 to 9 months and both the male and female were fertile.

CHAPTER III
MATERIALS AND METHODS

The primary materials used for this experiment were sickle mice or *Mus musculus.* The methods for the experiment include bone marrow transplant, timed embryonic isolation, cystamine treatment and cellulose acetate and quantifiying hemoglobin bands.

The sickle mice, *Mus musculus*, are produced in breeding colonies at the Medical College of Georgia. The background strains of mice are C57BL/6 and 129/Ola; some mice have hemoglobin variations including transgenic human alpha, alpha thalassemia, transgenic human beta, no mouse alpha, and single and diffuse mouse beta globin alleles.

Bone Marrow Transplant

In order to test histocompatibility by bone marrow transplants, donors and recipients have to be selected for their genetic types. For example, a C57BL/6 "California" alpha thalassemic, transgenic human alpha; a C57BL/6 Transgenic alpha; and a 129/Ola/sickle were chosen for the donors, and seven recipients were used. Once both the donors and recipients were chosen, radiation was given to ablate the recipients' own hemopoietic systems: the radiation was given in order to make space for the new bone marrow. Each mouse was exposed to various amounts of radiation ranging from 200 Rads to 750 Rads. In this first case, all the mice received 750 Rads. The following day, the transplant procedure took place. The donor mice were sacrificed and submerged in betadine in order to get rid of any viruses or other germs. The donor was dissected and femur retrieved to provide bone marrow stem cells. The bone marrow was extracted and diluted with saline solution. Before the injection procedures take place, the cells from each donor were counted. The

device used to count cells is a hemacytometer, which is used for making red and white blood cell counts as well as platelet counts. Once each donor's cells have been counted, it is then determined how much each recipient will receive. Recipients are now injected with the bone marrow from the donor mice. The injection may done by anesthetizing the mice or allowing them to be conscious. If anesthetizing is chosen, the mouse must be injected intraperitoneally with 64.8mg/ml veterinary pentobarbital, a dose calculated according to his weight in grams. The marrow injections are done behind the eyes of the mice because there is a blood pocket, retrorbital sinus, directly behind the eye that makes the procedure less complicated. Upon the completion of the injections the recipients are returned to their original settings and are tested in two to three weeks in order to determine the degree of engraftment and the histocompatibility of the cells.

Timed Embryonic Isolation

The embryonic isolation procedure is performed when the embryos are between 14½ and 15 ½ days of gestation. First, blood is taken from both parents in order to compare with samples from the offspring. Next, the mother is sacrificed and the embryos are taken. Once the mother is sacrificed, the uterus is removed and one sees the embryos are lined against its walls (Fig. 1). Each embryo is separated and placed into its own petri dish containing a saline solution where the blood is allowed to flow into the dish. After each has bled to its full capacity, the blood suspensions are placed into centrifuge tubes. The suspension is centrifuged in order to cause the red cells to go to the bottom. The red cells are drawn into microcapillary tubes that are centrifuged in order to separate the red blood cells, the white blood cells and the saline. The cells are subjected to cystamine treatment

and cellulose acetate electrophoresis, which will be discussed in the proceeding paragraphs.

The embryos are measured and weighed. Next, the embryos are placed on a small petri dish to display their relative sizes (Fig. 2). Blood suspensions are also used to determine which cells will sickle. The blood is diluted with a metabisulfite solution and NaCl then deposited onto a slide sealed with petrolatum. The metabisulfite is added in order to deoxygenate the blood and cause the cells to sickle.

Cystamine Treatment and Cellulose Acetate

To determine the degree of engraftment and the histocompatibilty, blood had to be retrieved from each recipient mouse. The blood was collected in microhematocrit tubes and centrifuged. The centrifugation of the tubes separates the red blood cells from the white blood cells and the plasma. Upon the completion of centrifugation the hematocrit tubes are cut and 10 microliters of packed cells are lysed with 100 microliters of a cystamine solution. Cystamine modification before cellulose acetate electrophoresis allows good separation of each of the hemoglobin bands produced by homozygotes and heterozygotes with combinations of *Hbb* alleles (9). The cystamine solution contains 112.6 mg Cystamine dihydrochloride, 0.1 ml dithiothreitol (DTE) at 15.43 mg/ml in DW, 0.9 ml distilled water (DW) and 0.5 ml of 1:10 concentrated ammonia. The lysate that is a combination of the cystamine and blood from each mouse is now loaded onto a Cellulose Acetate card that is taken from the buffer. There are eight lanes per cellulose acetate card and there is one sample per lane. After the samples have been loaded on to the card, it is placed into an electrophoresis apparatus with the plastic side of the card facing upward and the cellulose

acetate facing downward. The card must run for about 30 to 32 minutes at 300V / 75mm in order to retrieve adequate results for quantification. After the electrophoresis is completed, each card is stained with Ponceau S, a protein stain. When 5 minutes have elapsed, the card is drained and put through a series of 5% acetic acid and methanol washes. The methanol extracts the water prior to the clear aid procedure. Clear Aid is composed of 70 ml methanol, 30 ml 100% acetic acid, and 4 ml clear aid, polyethylene glycol. The cards are washed with clear aid for 5-10 minutes. When the card is ready to be drained it is placed vertical on a paper towel in order for the cellulose acetate to clear.

Quantifying Hemoglobin Bands

To begin the use of the Shimadzu densitometer, it is necessary to use Microsoft Windows and the CSTURBO program. Before any procedures are allowed to start, all machines must be on--the computer and the CS9000. Once the computer is on and is set in the CSTURBO program, the use of the CS9000 begins. The card is placed squarely on the glass plate of the CS9000. The card may need to be taped in order to prevent moving during the scanning process and to insure an accurate scan of the card. The starting and finishing positions on the X-axis and Y-axis must be determined on the CS9000 to make sure that each of the eight lanes is scanned properly and accurately. Before the scanning process begins, it is necessary to set the parameters that were found on the CS9000. Also, all other settings are confirmed to be correct before starting the scanning process. Once that card has been scanned, the files are saved for future analysis. Each lane is displayed separately in the form of peaks, which represent the bands and their strengths. The peaks must be analyzed

and percent values must be determined in order to compare their strengths. For a better comparison, a spreadsheet program is used to give a more in depth comparison of the values and to create a chart or graph for a visual comparison.

CHAPTER IV

RESULTS

The types of bands on the cellulose acetate cards become evident during the cellulose acetate electrophoresis procedure. The analysis of a transplant experiment to test histocompatibilty is shown in Figure 3: some lanes have two bands, and other bands have three. Lane 1, representing a control heterozygote mouse, has three bands that have a ratio of 1:4:5; the first two bands represent the minor and major and mouse hemoglobin and the third band represents hemoglobin with Beta-single. Next, the second and third lanes represent 129 control mice and demonstrate only the major and minor bands, which have a ratio of 1:4. The last two lanes are the donor samples that demonstrate both major and minor mouse bands but the third band contains beta-human sickle. The fourth, fifth and sixth lanes are the strain 129 transplant recipients that received marrow from a 129 sickle donor. In these, there are two very distinct bands, which are major and minor, and the third band—though clearly present--is not as heavy as the other two bands. This shows that the bone marrow repopulation is already 50% complete and 50% of the cells have been replaced. The ratio of these bands is 1:4:0.5.

Figures 4 through 7 are examples of the quantification of each of the bands. Figure 4 is lane 1 that was previously described; it gives a visual aid to the ratio of 1:4:5. Figure 5 is lane 2 and clearly represents only the major and minor bands, which has a ratio of 1:4. Figure 6 is lane 6, recipient of the bone marrow, that demonstrates the major and minor bands, and a third band that is not as strong as the corresponding band in figure 7, showing the recipient has not been converted 100%. Figure 7 is lane 7, which is the actual donor; it

Figure 1. 15 ½ embryos are inside the uterus during embryonic isolation.

Figure 2. Embryos taken from the uterus after being weighed and measured. Embryos 3 and 7 have Sickle Cell Disease.

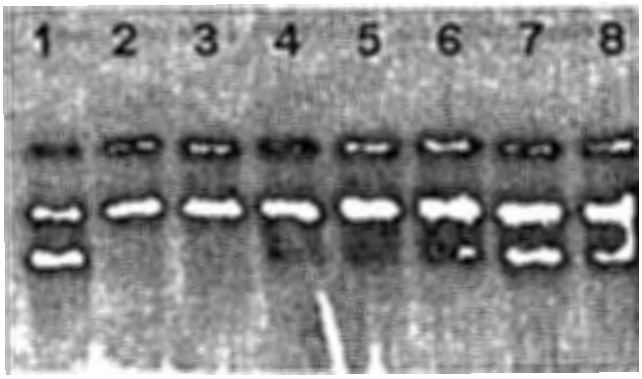


Figure 3. Cellulose acetate electrophoresis. Lane 1 from a mouse of type D/S or diffuse over single demonstrates from top to bottom alpha2 beta2 minor, alpha2 beta2 major, and alpha2 beta2 single. Lanes 2 and 3 are 129 controls that have only major and minor bands. Lanes 4, 5, and 6 are the recipients at 2 ½ weeks of repopulation. These demonstrate the minor, major and alpha2 beta2 human sickle. Lanes 7 and 8 are the donors that have minor, major and human sickle.

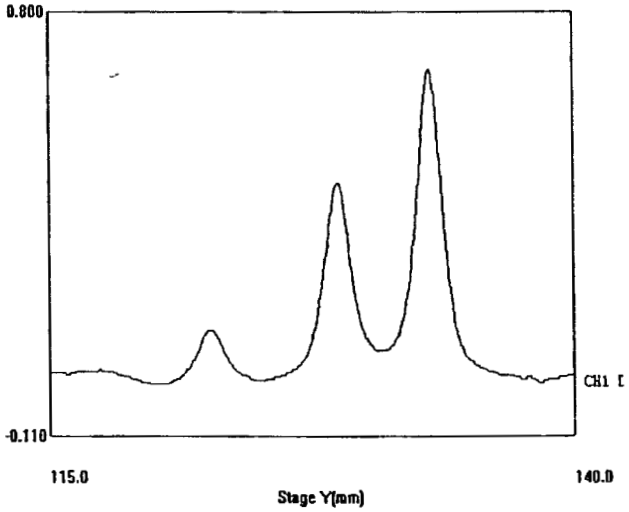


Figure 4. Cellulose Acetate Electrophoresis –analysis of a sample from a D/S heterozygous mouse. Quantification of lane 1 demonstrates three peaks—minor, major and single. Ratio is 1:4:5.

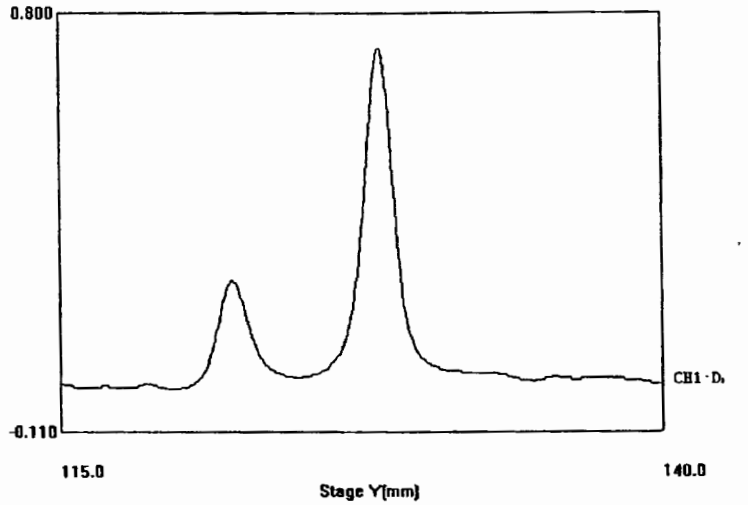


Figure 5. Quantification of lane 2, from the 129 control(recipient type) which has only two peaks—minor and major. The ratio is 1:4. This is the pattern that the recipient, shown in Figure 6, had prior to the transplant.

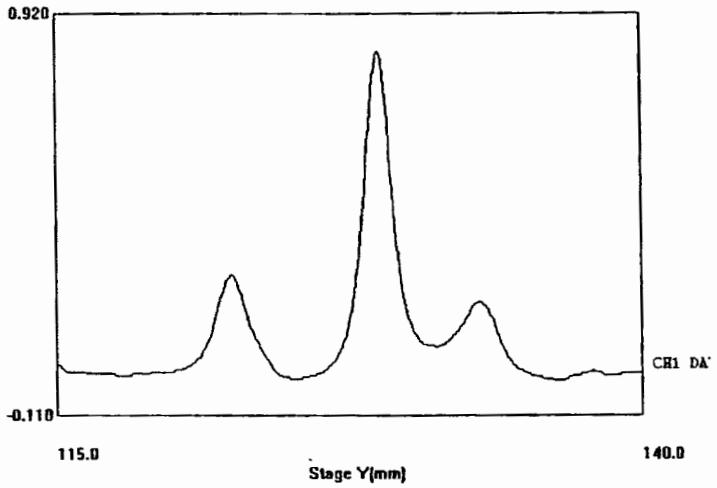


Figure 6 Quantification of lane 6, hemoglobin from the recipient that demonstrates 3 peaks—minor, major and sickle. The ratio is 1:4:0.5, and the quantity of the third peak demonstrates 50% repopulation—half donor/half recipient.

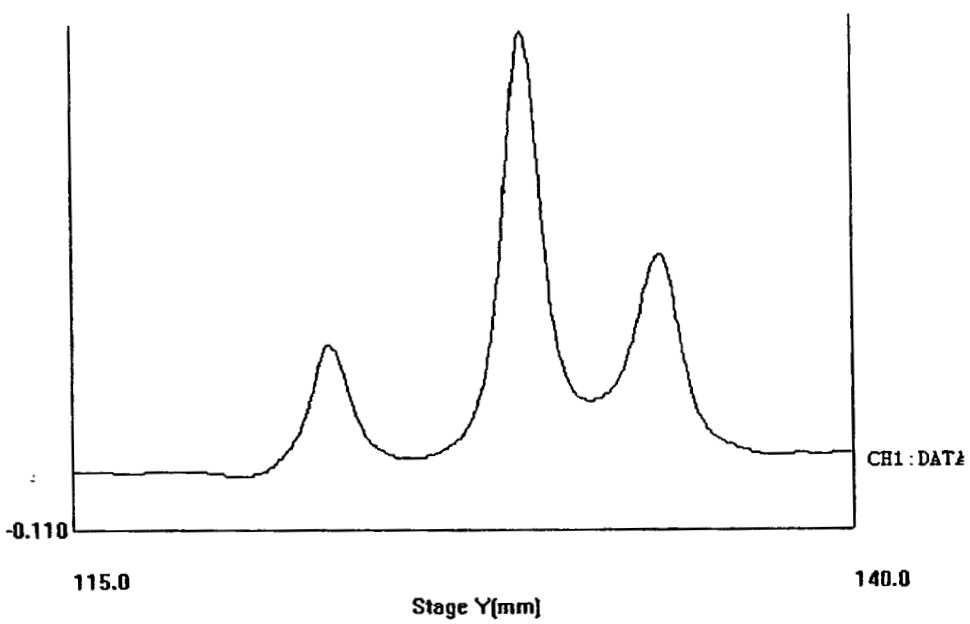


Figure 7. Quantification of lane 7, hemoglobin from the donor that demonstrates 3 peaks—minor, major, and sickle. The ratio is 1:4:1, the third peak is the goal for figure 6.

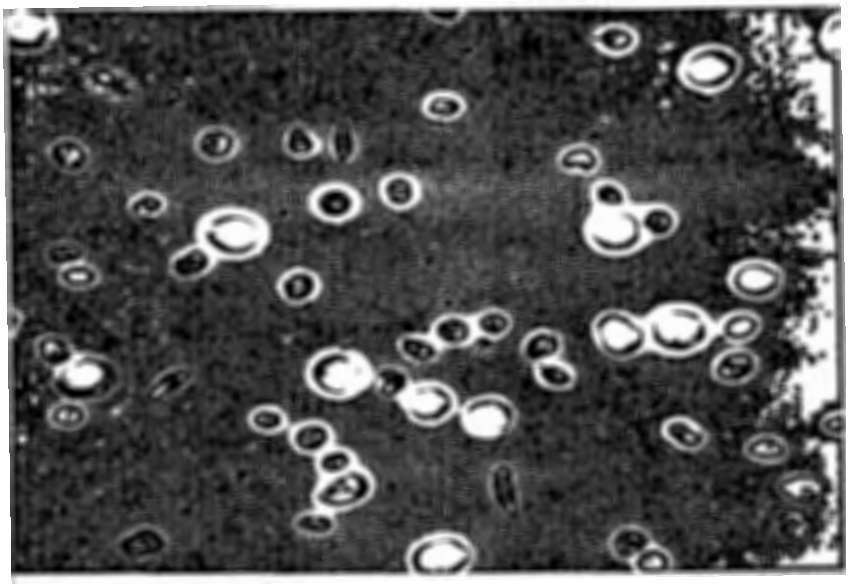


Figure 8. Embryo 3's cells were treated with metabisulfite that caused cells to deoxygenate and sickle. The sickled cells have a pointed shape unlike the other cells.

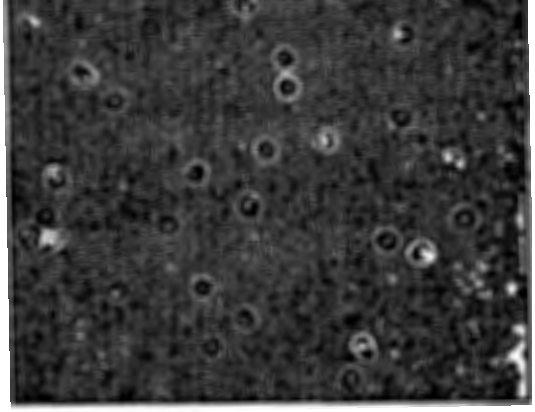


Figure 9. Embryo 5's cells treated with metabisulfite but cells did not sickle. This mouse was not homozygous sickle.

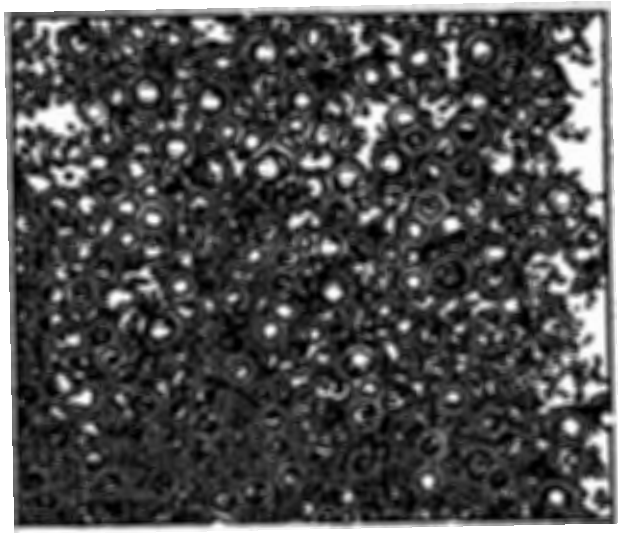


Figure 10. Embryo 4's cells were treated with metabisulfite but cells did not sickle. This mouse was not homozygous sickle.

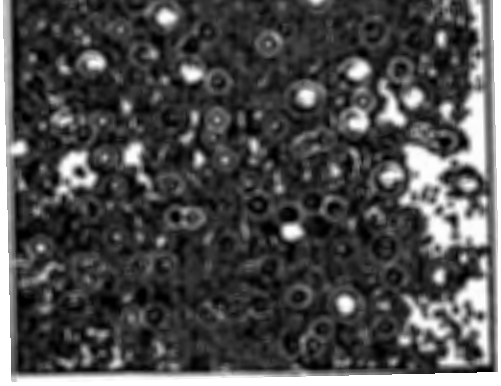


Figure 11. Embryo 1 is not sickle.

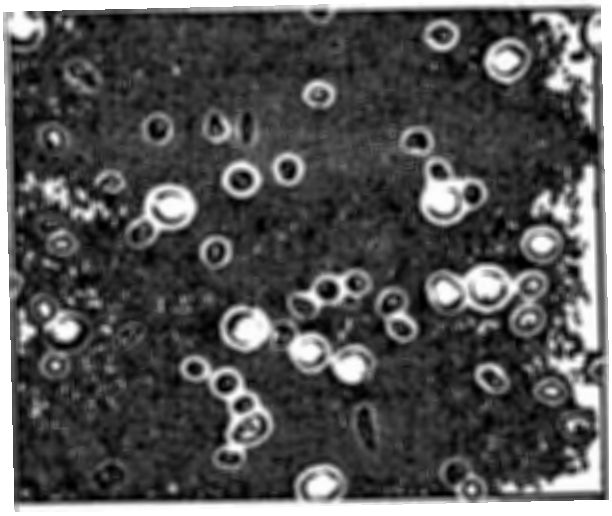


Figure 12. Embryo 3 is another area of the slide shown in figure 8.

Figure 13. Embryonic Cellulose Acetate Electrophoresis. These are the embryos from figures 9-12 that have been verified for sickle cell disease. The samples in lanes 2 and 6 are from Sickle Cell embryos 3 and 7 shown in figure 2.

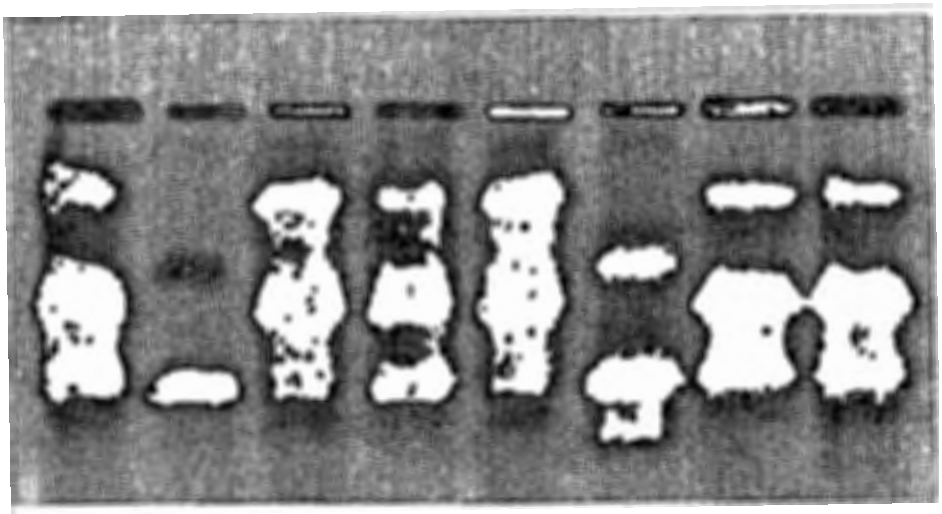
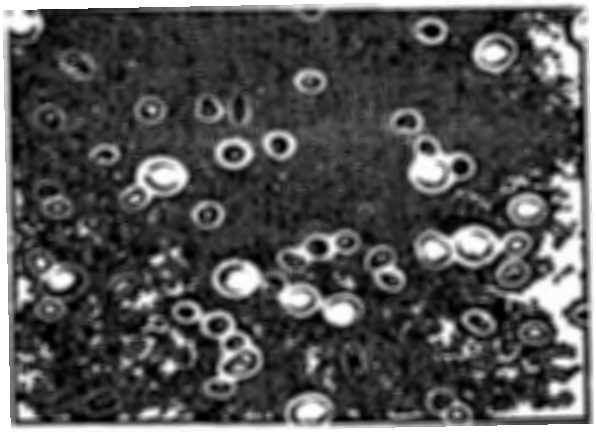
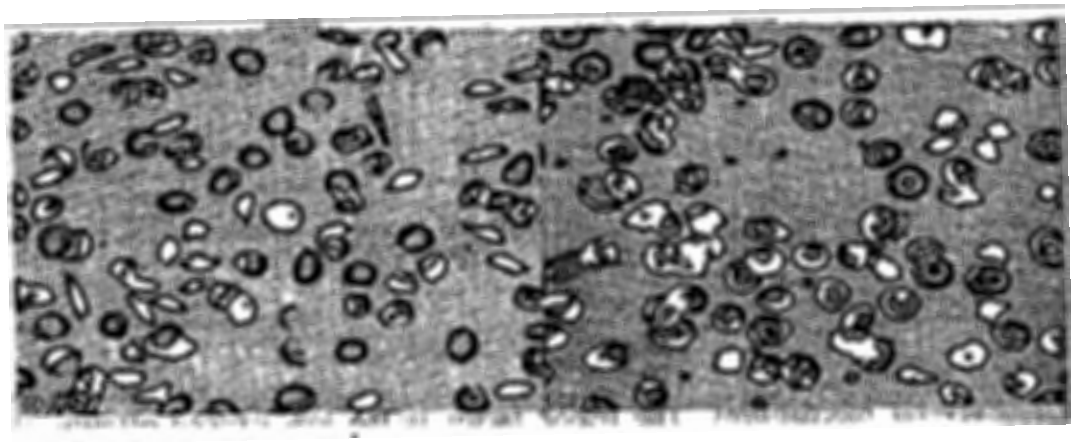


Figure 14 demonstrates sickle mouse embryo cells and Figure 15 demonstrates human sickle cells. What a resemblance!





CHAPTER V
DISCUSSION AND CONCLUSION

DISCUSSION

In the first bone marrow transplant experiment that used 750 Rads, seven mice were tested and only one mouse is still alive. The deaths of the other six may have been caused by many factors including the amount of radiation given, the amount of barbital, and stroke. The actual cause will never be known but the only way to have an idea is to retest more mice. The next time the procedure took place, the mice were given different doses of radiation—0 Rads, 250 Rads, 500 Rads and 750 Rads. These mice, even those receiving 750 Rads, have been found to be fine after both the radiation and bone marrow transplant.

In the one surviving recipient in experiment 1, the 50% repopulation of bone marrow cells is due to the time the mouse was tested. She was tested at 2½ weeks, which is about half of the time needed to repopulate her hemopoietic system completely. However, the test at this time does prove that the cells are histocompatible. Probably she will convert 100% by 6-8 weeks post-transplant.

Dealing with the embryonic isolation, it was found that some embryos bled less and were anemic. Cellulose Acetate Electrophoresis was also done to verify the sickle embryos. After the cellulose acetate electrophoresis, some embryos demonstrated a third band. This third band is embryonic hemoglobin that appears due to the age of the embryo (10). During embryo isolation, it was found that the sickle cells of mice and those of humans have a great resemblance (Fig. 13&14).

The use of the densitometer (the CS9000) is tremendously helpful because it quantifies the cards and gives an accurate ratio of the hemoglobin bands. For example, even

though there is an expected ratio for certain lanes, verification is still necessary. In other cases, the scanning procedures will give an account of a band that may have not been strong enough to determine but was able to be scanned. The use of the Microsoft Excel spreadsheet makes the ratios and comparisons more visual.

CONCLUSION

The overall goal of our research and other researchers is to find models that will mimic genetic diseases in humans. The process seems long and complicated, but when the results are complete, they will be outstanding. When researchers accomplish this goal, the cure and treatments for genetic diseases will be near at hand or much more feasible. The completion of this interesting yet limited research has an impact that this research must continue in order to get closer and closer to the goal of gene therapy.

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APPENDIX A

RECENT INFORMATION

Since the summer of 1998, many improvements and advancements have been made in sickle cell disease with the use of murine models. In December 1998, studies in transgenic mice have demonstrated that the normal gene order and spatial organization of the members of the human beta-globin gene family are required for appropriate development and stage restricted depression of the genes. A murine model has been generated that exclusively expresses HbS after transfer of a 240-kb betas yeast artificial chromosome; it has hemolytic anemia, 10% irreversibly sickled cells in their peripheral blood, reticulocytosis and other phenotypic features of SCD (13).

Most recently in the year 2000, it ahs been assessed in vivo the potential curative threshold of fetal hemoglobin in the SAD transgenic mouse model of sickle cell disease using mating with mice expressing the human fetal Agamma globin gene. The direct therapeutic effect of fetal hemoglobin on sickle cell disease has been established and demonstrated correction by increasing fetal hemoglobin to about 9-16% in this model (14). These studies emphasize the potential of SAD mouse models for gene therapy approaches.

VITA

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