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Progress Report on Grant N00014-91-J-1217

PRINCIPAL INVESTIGATOR: William T. Phillips, M.D.

GRANT TITLE: In Vivo Distribution of Liposome Encapsulated Hemoglobin Studied With Imaging Radiotracers

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RESEARCH OBJECTIVE: This project has as its objective the development of radiotracer imaging technology to follow the in vivo circulation and organ deposition of liposome encapsulated hemoglobin (LEH). LEH will be labeled with technetium-99m (^{99m}Tc) or indium-111 (^{111}In) and infused into small animals to monitor any in vivo differences between different LEH formulations. These studies will be correlated with any hematological and pathological changes associated with LEH treatment. Development of such non-invasive monitoring techniques may lead to significant cost effective manufacturing and formulation improvements, and ultimately a more efficacious LEH product. The development of this elegant labeling technique should make it possible to study the effect of various LEH modifications on biodistribution non-invasively in primates and humans.

PROGRESS: Our research progress for the period of April 1, 1993 to August 1, 1993 is covered in this report. We have completed a study to determine the changes in platelet biodistribution as a function of LEH administration. This study was proposed because of the evidence by Dr. Reuven Rabinovici that certain batches of LEH caused thrombocytopenia and increased production of Thromboxane B2. We decided to use an imaging protocol to try and follow the distribution of labeled platelets following an infusion of LEH. The labeling of platelets with indium-111 from both rats and rabbits has been set up in our laboratory. The animals were reinfused with autologous labeled platelets and monitored under a gamma camera. Following a ten minute equilibration period, an injection of LEH was given intravenously. The biodistribution of the labeled

platelets changed dramatically with the platelets leaving the blood pool and localizing in the lungs. We saw this effect in both the rabbit and rat studies. This effect was transient with the return of platelets to the blood pool occurring within 30 minutes. We also performed experiments in rats with 1) liposomes containing no hemoglobin but with the same lipid composition and at the same lipid concentration as the LEH used in this study and 2) free hemoglobin at the same concentration as determined for the LEH. The initial analysis of the results of this study showed that the liposomes containing no hemoglobin and the free hemoglobin showed no change in platelet distribution during the study. We have not determined the effect of the empty liposomes or free hemoglobin on platelet distribution in rabbits.

We have performed a pilot study to determine if our ^{99m}Tc labeled liposomes can be used as an imaging agent for the detection of atherosclerotic disease in collaboration with Dr. Bailey at our institution. Such an agent could be used not only to screen patients with the disease, but also to follow the efficacy of cholesterol lowering drugs in the treatment of atherosclerosis. We placed a balloon catheter in the abdominal aorta in Flemish Giant rabbits via the femoral artery. Once inflated the catheter was used to scrap the endothelial lining of the aorta. This procedure was performed using fluoroscopy to monitor the placement and extent of the lesion. Then the animals were injected via the ear artery with ^{99m}Tc labeled liposomes and imaged at various times under the gamma camera. We saw only a small amount of accumulation of the ^{99m}Tc liposomes at the lesion out to 24 hours post-injection. The aorta was removed and cut into 1 cm sections for radioactive counting. There was an increase in counts in the area of the lesion. This study looked primarily at the ability of the ^{99m}Tc liposomes to detect an acute lesion following balloon angioplasty. A better model yet to be tried is to allow the lesion to develop into an atherosclerotic plaque by keeping the animal on a high fat diet for 4-6 weeks prior to imaging. We would like to attempt this study in the near future.

We have begun a study to determine the efficacy of LEH using a positron emitting isotope of oxygen (^{15}O). To our knowledge, this study is the first attempt to actually quantitate and determine oxygen delivery to tissues. These studies are uniquely possible at our institution because of our newly operational cyclotron and positron emission tomography (PET) camera located at our Research Imaging Center and the previous experience of our group in both imaging and blood substitutes. The biodistribution studies using ^{99m}Tc have been very useful, but do not

provide any information about how efficient LEH is in delivering oxygen to the tissues in vivo. In initial experiments, we attempted to quantitatively determine the relative uptake of ^{15}O-O_2 from the lungs by LEH. The percent uptake by LEH was compared to the uptake of the remaining red blood cells following a 40% hypovolemic exchange. To provide an access for blood withdrawal, rats were catheterized via their femoral artery two days prior to the experiments. On the day of the experiment, the rats were anesthetized with pentobarbital and intubated with a converted 14 gauge intravenous catheter, to provide an access to the lungs for delivery of the ^{15}O-O_2. After a short stabilization period, the animals were bled at 1 ml/min and infused with LEH or free hemoglobin at 1 ml/min. At 15 minutes, 3 hours, and 24 hours after LEH or hemoglobin administration, the rats were given a bolus of ^{15}O-O_2. Since the half-life of ^{15}O is approximately 2 minutes, multiple time points can be obtained in the same rat. Blood samples were taken in capillary tubes over a two minute period, and separated into plasma, LEH, and red blood cell fractions by centrifugation. Each fraction was counted in a well counter. Following decay correction, the counts for each fraction were compared as a percentage of the total counts at a particular time point. These experiments showed that LEH and free hemoglobin could load and unload oxygen in the lungs. Also oxygen was removed from the LEH at a rate similar to the oxygen removal from the red blood cells. Our initial research shows that this technique for quantitatively determining oxygen uptake and delivery is feasible and has many applications for the development of blood substitutes. This technique applies not only to LEH, but can be used with other oxygen carriers such as cross-linked hemoglobin and perfluorochemical-based oxygen carriers. This technique allows direct comparisons of various oxygen carriers over an extended time period. We envision that the technique will become an important quality control test for individual batches of oxygen carriers. This technique could also prove invaluable for the development of improved blood substitutes.

WORK PLAN: During the next funding period, we will continue to use our ^{99m}Tc liposome labeling protocol to test LEH formulations as supplied by NRL or Vestar for their circulation properties and organ distribution. A LEH formulation being developed by Vestar which can be produced at a smaller more homogeneous size for sterile filtration has been modified for scale up. Biodistribution studies with these new LEH preparations

that contain recombinant human hemoglobin will be studied as soon as these preparations become available.

Although our ^{99m}Tc labeling procedure has provided valuable data concerning the biodistribution of LEH, it does not allow us to follow the ultimate metabolic fate of the hemoglobin. This information is very important for the safety of LEH as a blood substitute since we want a product which will be cleared from the body and produce few toxic side effects. To study this problem, we plan to label hemoglobin with ^{3}H and ^{14}C using a mild reductive methylation procedure. This mild labeling technique has been used to label the lysine residues of a number of proteins including hemoglobin without affecting the functionality of the protein. The hemoglobin will be supplied by NRL. Also the radiolabeled starting material used in the procedure is available from commercial sources. Once labeled, the hemoglobin will be used to make LEH. The labeled LEH will then be double labeled using the ^{99m}Tc liposome labeling protocol. This double labeled material will be injected into animals and imaged under the gamma camera. The animals will then be sacrificed for tissue biodistribution measurements. Samples of the tissues will be counted for both gamma activity as well as for ^{3}H or ^{14}C using liquid scintillation counting. This study will provide important information concerning the fate of both the hemoglobin and liposomal components of LEH.

Although we have completed our platelet distribution studies, we plan to correlate these findings with additional clinical tests, especially complement activation. We plan to collect serum from animals after each step in the biodistribution experiments and test them for complement activation using a commercially available Elisa assay kit. These experiments will be performed in conjunction with Dr. Alan Rudolph at the Naval Research Laboratory.

We plan to continue our ^{15}O-O_2 studies. We plan to study the effect of oxygen delivery in rats resuscitated with normal saline. Dr. Alan Rudolph will be sending us lyophilized LEH samples to test for its ability to load and unload oxygen. We plan to perform similar experiments as outlined in the progress section of this report. These studies will provide important information concerning this storage form of LEH.

INVENTIONS: A licensing agreement between Lipotek INC and The University of Texas Health Science Center at San Antonio/Department of the Navy is under negotiation.

PUBLICATIONS AND REPORTS: We have submitted a revised manuscript outlining our results using ^{99m}Tc labeled liposomes in infection imaging to Journal of Nuclear Medicine and are waiting acceptance of this article. We made an oral presentation entitled " Use of a Tc-99m liposome labeling procedure to monitor the circulation properties of liposome encapsulated hemoglobin in a rat shock model" and a poster presentation entitled " Evaluation of Tc-99m labeled liposomes as a tumor imaging and drug delivery agent" at the Society of Nuclear Medicine meeting held in Toronto, Canada on June 8-11, 1993. We are currently preparing a manuscript describing our results using ^{99m}Tc labeled liposomes in a tumor model. We plan to complete a manuscript describing the circulation kinetics and biodistribution of ^{99m}Tc labeled LEH in a rat hypovolemic model. We also plan to prepare manuscripts describing our platelet studies and ^{15}O-O_2 studies.

TRAINING ACTIVITIES: None