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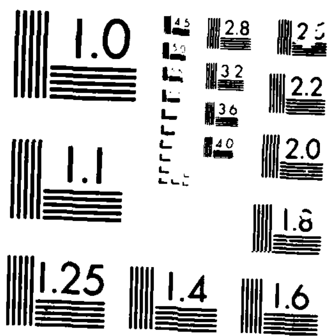
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SUMMARY

Liposomal-Encapsulated Stroma-Free Hemoglobin as a Potential Blood Substitute

Annual Progress Report

by

C. Anthony Hunt, Ph.D.

March 31, 1983

For the period April 1, 1982 through March 31, 1983

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DEFINITIONS AND ABBREVIATIONS

CH = Cholesterol

DPG = 2, 3-diphospholycerate

Hb = hemoglobin

LAIR = Letterman Army Institute for Research, San Francisco

LIPOSOME = phospholipid vesicle, with or without other added lipids.

met-Hb = methemoglobin

"n" = Hill number

NEOHEMOCYTE = the product resulting from encapsulation of SFH and other agents in liposomes; defined further as the product resulting from encapsulation of SFH at 18g%; where liposome size ranges from 0.05-1.0 μm; where the fraction of the particle displaced volume that is the SFH solution is about 95%, and where the encapsulated Hb has been concentrated by osmotic dehydration.

PA = phosphatidic acid

PC = phosphatidylcholine

RES = reticuloendothelial system

SFH = stroma-free hemoglobin. In this context SFH refers to the purified Hb suspensions provided to the P.I. by LAIR.

TC = alpha tocopherol

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SUMMARY AND ABSTRACT

This contract started as a feasibility study and has involved into a pilot development project. The operational hypothesis is that one can obtain a functional resucitative fluid by encapsulating Hb in physiologically compatible lipid vesicles. We have demonstrated the basic feasibility of the idea. Our objective now is to develop the neohemocyte idea further until we have a prototype product suitable for testing in large animals and man; and we propose studies to do this. One unique approach to minimize acute and chronic toxicity is to design and incorporate into the neohemocyte "microcapsule membrane" novel "masking lipids" that may limit neohemocyte interaction with tissues and dramatically retard recognition and clearance by the RES.

PROGRESS REPORT

IN VITRO STABILITY. Only a limited number of in vitro stabilty studies have been conducted to date. Membrane stability (retention of solutes) is not a problem. All indications are that shelf life will be goverened by Hb stability. We must identify the best approach to retard met-Hb formation. Fortunately our preliminary studies show that met-Hb formation is slower (by 50 to 200%) when it is in neohemocytes relative to being free Hb in solution, all other variables being equal.

IN VIVO PROTERTIES OF NEOHEMOCYTES. We have carried out transfusions in about three dozen rats, and given both rats and mice injections of neohemocytes. Our protocol for transfusing rats is the same as that used at LAIR. The objective of one study was to compare the survival times of rats completely transfused with an isoosmotic albumin solution, with 7g%SFH, with neohemocytes, and with a solution of SFH containing the same amount of Hb as the transfused neohemocyte suspension. In all cases we replaced at least of 97% of the blood volume. As expected, all rats transfused the albumin solution died within a few minutes of completing the transfusion. The average survival time of rats transfused with 7g% SFH was about 6.5 hrs. The neohemocyte suspension had an apparent "hematocrit" of 0.25; the Hb content of this suspension having 3.2g% (pure neohemocytes contained about 13g% Hb). We expected that if neohemocytes worked to retard the excretion of Hb then a transfusion of a neohemocyte suspension having 3.2g% Hb would give survival times equal to -or greater than - those resulting from a transfusion of 7g% Hb. Results were better than expected. In the most recent study 2 of 5 rats completely survived; the three rats that died had survival times of 16 to 25 hrs. In the control study where rats were transfused with 3.2g% SFH, the average survival time was only about 3.5 hrs. One would expect, in the absence of lethal acute toxicity, that as long as the total functional

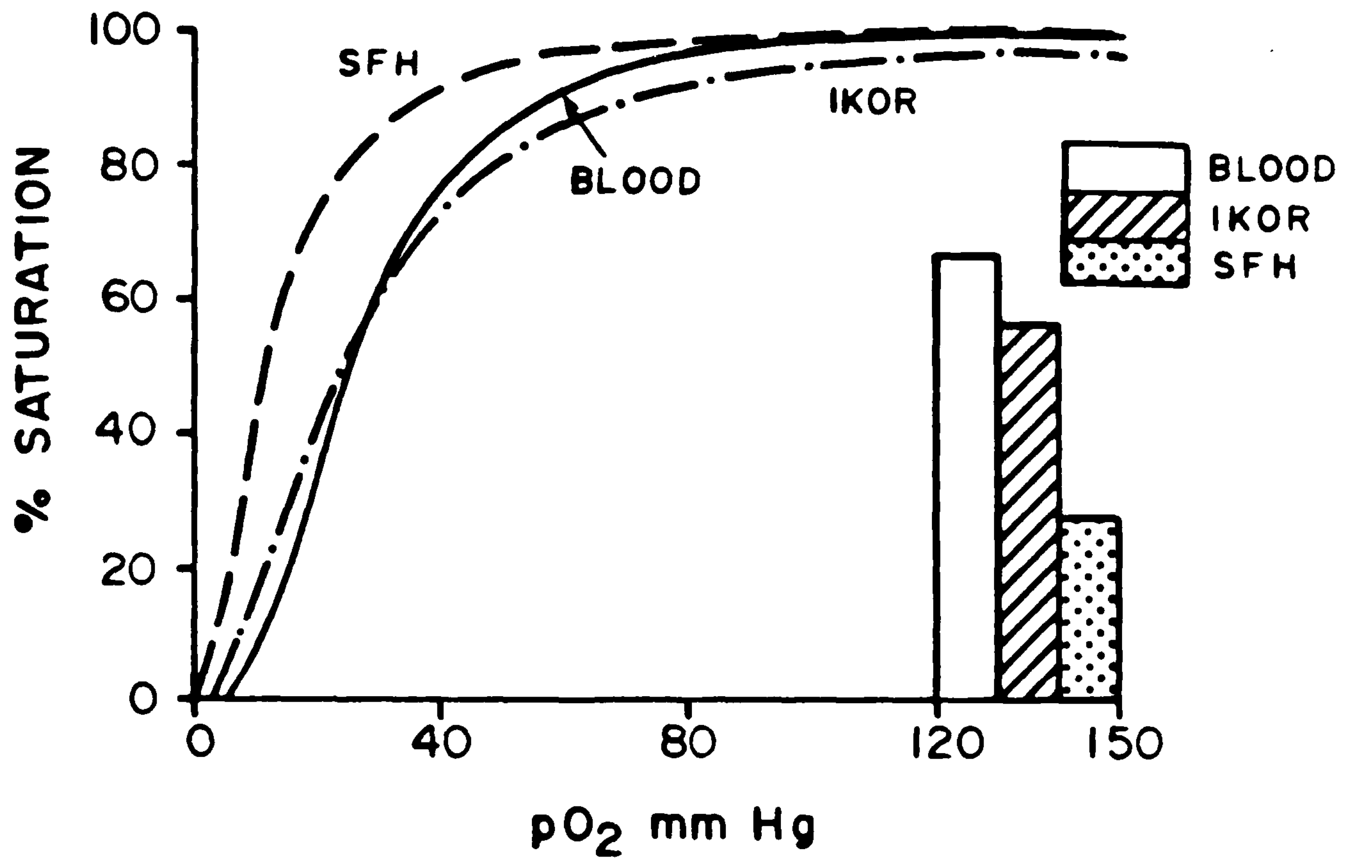


FIGURE 1.

The classical oxygen dissociation curves are shown for normal human blood, IKOR (i.e., a solution of neohemocytes) and 7g% SFH. The P_{50}'s are: blood, 24; IKOR, 23; and SFH, 17. The bar graph shows their relative ability to unload oxygen as the O_2 pressure is dropped from 100 to 20 mm Hg.

Hb, which would be from a combination of that remaining in circulating neohemocytes plus that from regenerated red cells, remained above the critical level, rats would completely survive the transfusion. Apparently this was the case for two of the rats transfused with neohemocytes.

Autopsy and histological examination of organs by Dr. Lau revealed some tissue damage in the rats transfused with neohemocytes. All tissue damage was consistent with hyperproteinemia. Hyperproteinemia would result if a significant fraction of neohemocytes lost their Hb shortly after transfusion (which occured to some extent in these preparations). The addition of free Hb to the 5g% albumin could have easily produced localized hyperporteinemia. So, in subsequent transfusions the albumin content of the neohemocyte suspending solution was reduced from 5 to 3g%. Blood was removed at death and in selected cases from surviving rats to determine the percent of transfused Hb remaining as neohemocytes in the circulation, and the percent that remained functional by O_2 binding (Fig. 2). About 40% of the transfused Hb was quickly lost, probably from lysis and binding to and uptake by tissues. The apparent half-life for neohemocytes remaining in the circulation after 12 hrs was between 24 and 72 hrs. The amount of met-Hb increased in the circulation following transfusion (Fig. 2) and the majority of this was inside circulating neohemocytes.

Clearly, the "functional half-life" of neohemocytes is the important figure. SFH is excreted too fast for met-Hb formation in vivo to be a significant problem. The rates, extent, fate and physiological consequences of met-Hb formation in surviving neohemocytes need further study. It will become necessary to identify methods to retard Hb oxidation in vivo.

ACUTE TOXICITY OF NEOHEMOCYTES. Thirteen experimental rats were given 50% transfusions of neohemocyte suspension (0.25, apparent hematocrit). There were seven untreated controls. Blood was sampled and analyzed before and after transfusion and at the time of sacrifice, either 1, 7 or 30 days post-transfusion. Organs from both experimental and control groups were combined, randomized, and processed for "blind" pathological examination. Blood samples were processed by SMA-12. Aspects of this study are only summarized here. Results of the SMA-12 are given in Figs. 3, 4 and 5. RBC hematocrit values are given in Fig. 6. Results of the tissue morphology are as follows.

Control Animals. Four animals exhibited extramedullary hematopoiesis of the spleen, two showed lymphoid hyperplasia of the spleen, and two had essentially normal tissues. This degree of ENH and LH was consistent with that often seen in normal animals as well as in animals affected with chronic, low grade anemic or hypoxic conditions.

One day post-transfusion. One (of 4) animals showed acute focal hepatic necrosis with possible toxic on hypoxic pathogenesis. All four displayed mild to moderate liver and spleen erythropathagocytosis suggesting that neohemocytes may have activated the RES. Lungs, kidneys and other tissues were normal.

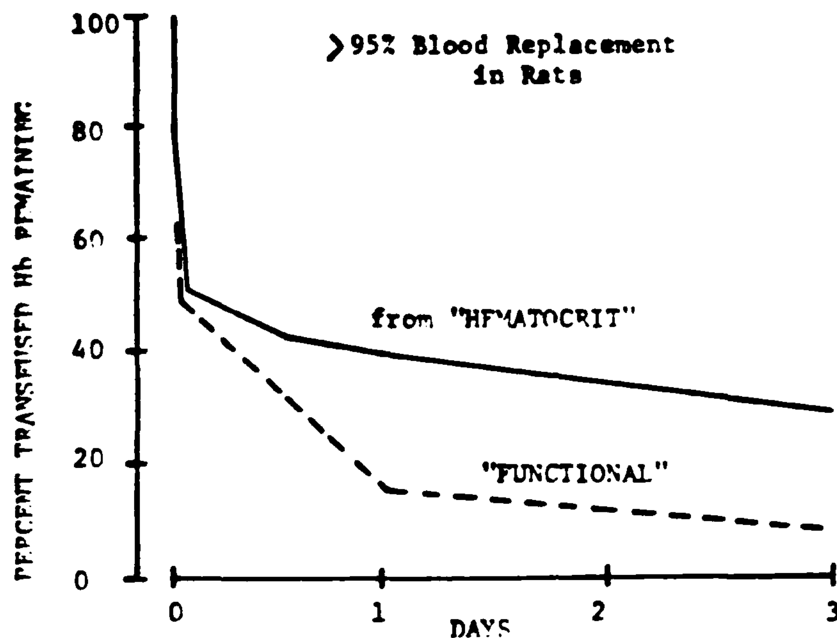
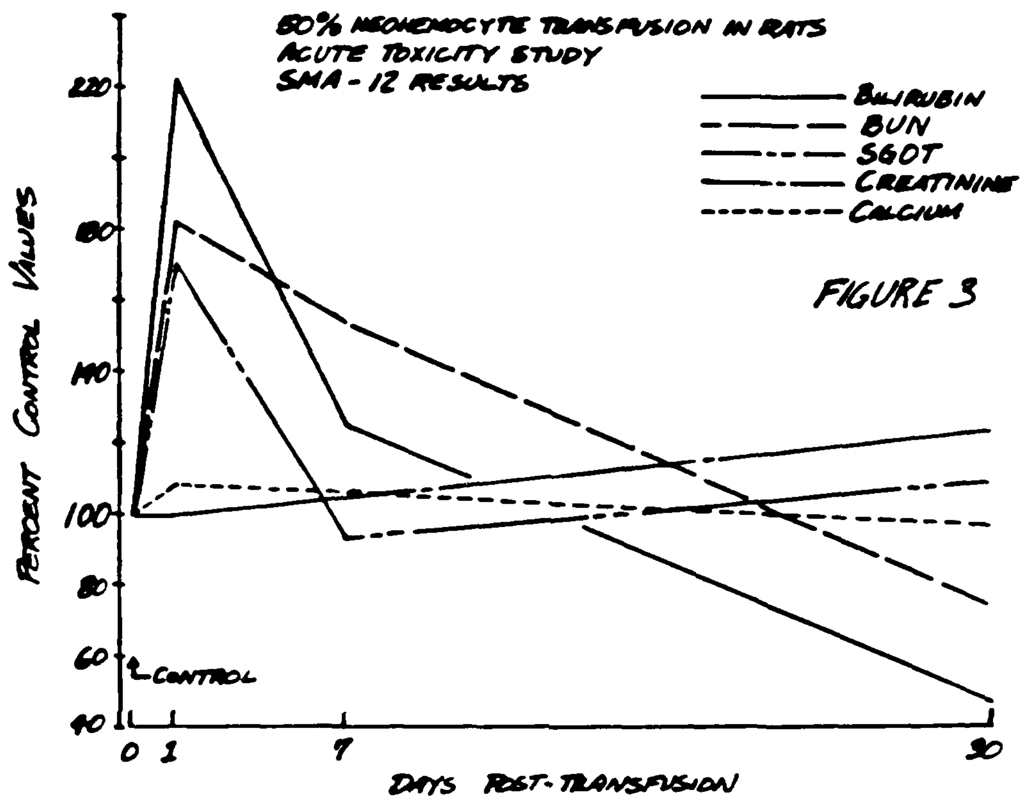
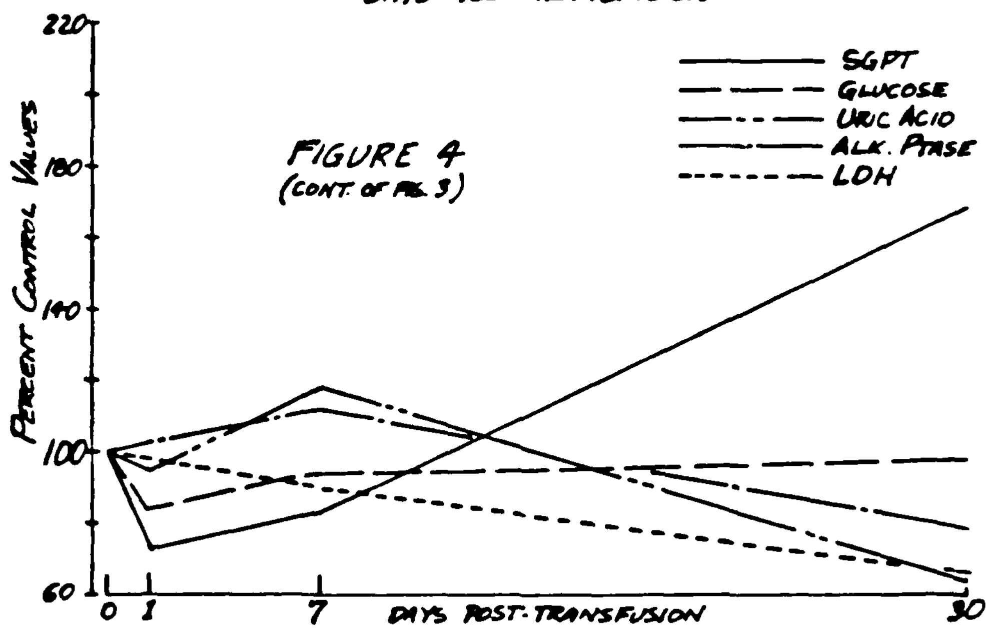


FIGURE 2.

The percentage of transfused Hb (as neohemocytes in the circulation) remaining in the circulation is plotted vs days after transfusion. The 100% figure was calculated from the size and number of partial transfusions, and may be somewhat overestimated. The amount of neohemocyte Hb was calculated in two ways. The solid curve was calculated from the observed neohemocyte 'hematocrit' at the time of death or sacrifice, and the known average Hb content of the transfused neohemocytes. The dashed curve was calculated from the observed neohemocyte 'hematocrit' and the measured O_2 binding capacity of the neohemocytes. The majority of the difference in the two curves is accounted for by formation of met-Hb.


80% NEOHEMOCYTE TRANSFUSION IN RATS
ACUTE TOXICITY STUDY
SMA - 12 RESULTS
BILIRUBIN
BUN
SGOT
CREATININE
CALCIUM
FIGURE 3
PERCENT CONTROL VALUES
220
180
140
100
80
60
40
CONTROL
0
1
7
30
DAYS POST-TRANSFUSION


SGPT
GLUCOSE
URIC ACID
ALK. PTASE
LDH
FIGURE 4
(CONT. OF FIG. 3)
PERCENT CONTROL VALUES
220
180
140
100
60
0
1
7
30
DAYS POST-TRANSFUSION

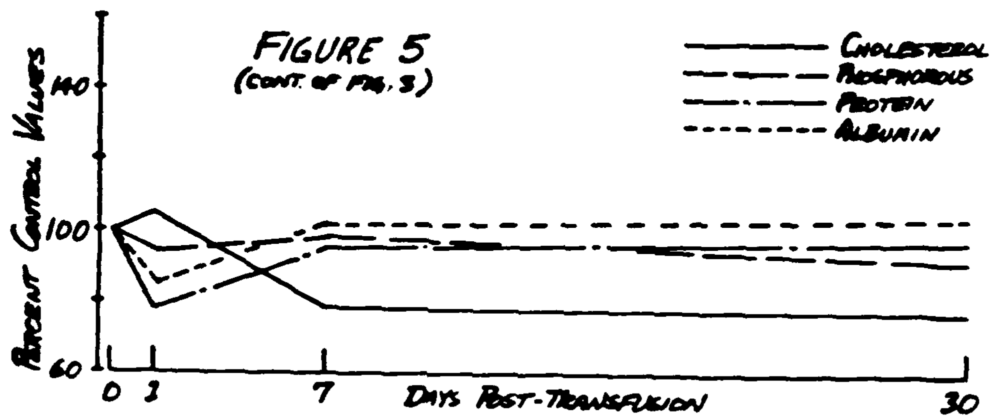
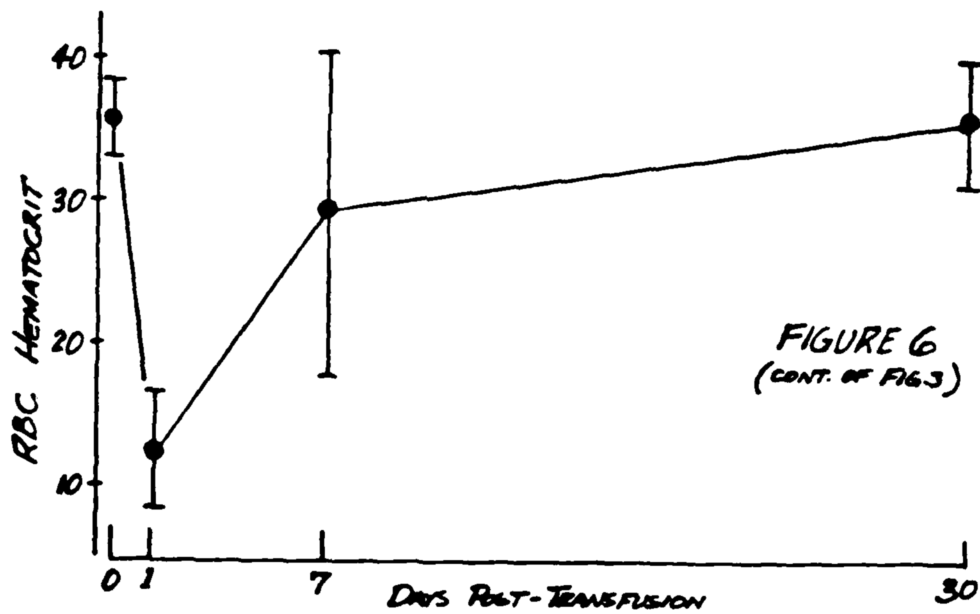


FIGURE 5
(CONT. OF FIG. 3)
CHOLESTEROL
PHOSPHOROUS
PROTEIN
ALBUMIN
PERCENT CONTROL VALUES
140
100
60
0
1
7
30
DAYS POST-TRANSFUSION


40
30
20
10
RBC HEMATOCRIT
FIGURE 6
(CONT. OF FIG.3)
0
1
7
30
DAYS POST-TRANSFUSION

Seven days post-transfusion. All four animals displayed marked extramedullory hematopiesis of the spleen, representing a possible mild antigenic stimulation and an anemic or hypoxic condition stimulating EMH. No significant erythropathagocytosis was seen in spleen or liver. Other organs were essentially normal. The conditions found after one day was apparently reversible.

Thirty days post-transfusion. Three out of five animals displayed mild EMH of the spleen; the other two showed moderate EMH of the spleen. Four animals showed moderate sheath hyperplasia possibly resulting from mild antigenic stimulation (similar to the condition found in some control animals). Clearly, by thirty days conditions found earlier have significantly reversed.

PHARMACOKINETIC STUDIES. We have completed two neohemocyte pharmacokinetic studies in 50% transfused rats. In both ^{14}C-inulin was encapsulated, along with Hb, as a marker (inulin has several advantages over labeled Hb). Since the results of both studies support the same conclusions, only one will be discussed here. Approximately 2ml of neohemocyte suspension was exchanged for 2ml of of blood in sets of 150g rats. There were three such exchanges in each case. The neohemocytes comprising the last exchange included ^{14}C-inulin. Beginning 15 min post-transfusion (after recover from anesthesia) blood samples were taken and all urine collected for a total of 8 hrs. Results are given in Fig. 7A. The blood level curve is bi-phasic with a terminal half-life of approximately 12.5 hrs. The initial portion of the curve has an apparent half-life of 1.6 hrs. Within the first 30 min there is evidence of some neohemocyte break-down, which is reflected in the urine data, Fig. 7B. The faster process may represent neohemocyte distribution, binding to tissues and uptake by some RE cells; the slower phase may represent post-equilibrium, overall neohemocyte clearance.

A current operational hypothesis is that neohemocyte half-life is dose dependent and that each remaining fraction of neohemocytes will have a correspondingly longer half-life.

It should be noted that in this study the amount of apparent neohemocyte breakdown shown in Figs. 2 and 7 is different. This may simply reflect differences in the degree of transfusion (if so it is difficult to explain), or it may be that the neohemocytes used in the pharmacokinetic study were somewhat better and more stable.

SCALE-UP OF NEOHEMOCYTE PREPARATION. Considerable effort has been invested in various aspects of scaling- up neohemocyte preparation to obtain batches large enough for rat trasnfusions. From the start until mid-1981 we produced only one ml batches of neohemocytes, which was adequate for the *in vitro* research objectives at the time. By the beginning of 1982 we had scaled-up to produce 6-8 ml batches; each batch required about 30 hrs over six days. By June, 1982 we had doubled our production capabilities. We have successfully scaled up again, and can now prepare in 3 days a sufficient volume of 0.25 apparent "hemotocrit" neohemocyte suspension to completely transfuse 2 160g rats. To transfuse several rats or one dog a month we must scale up further to the 1,000 ml/batch level.

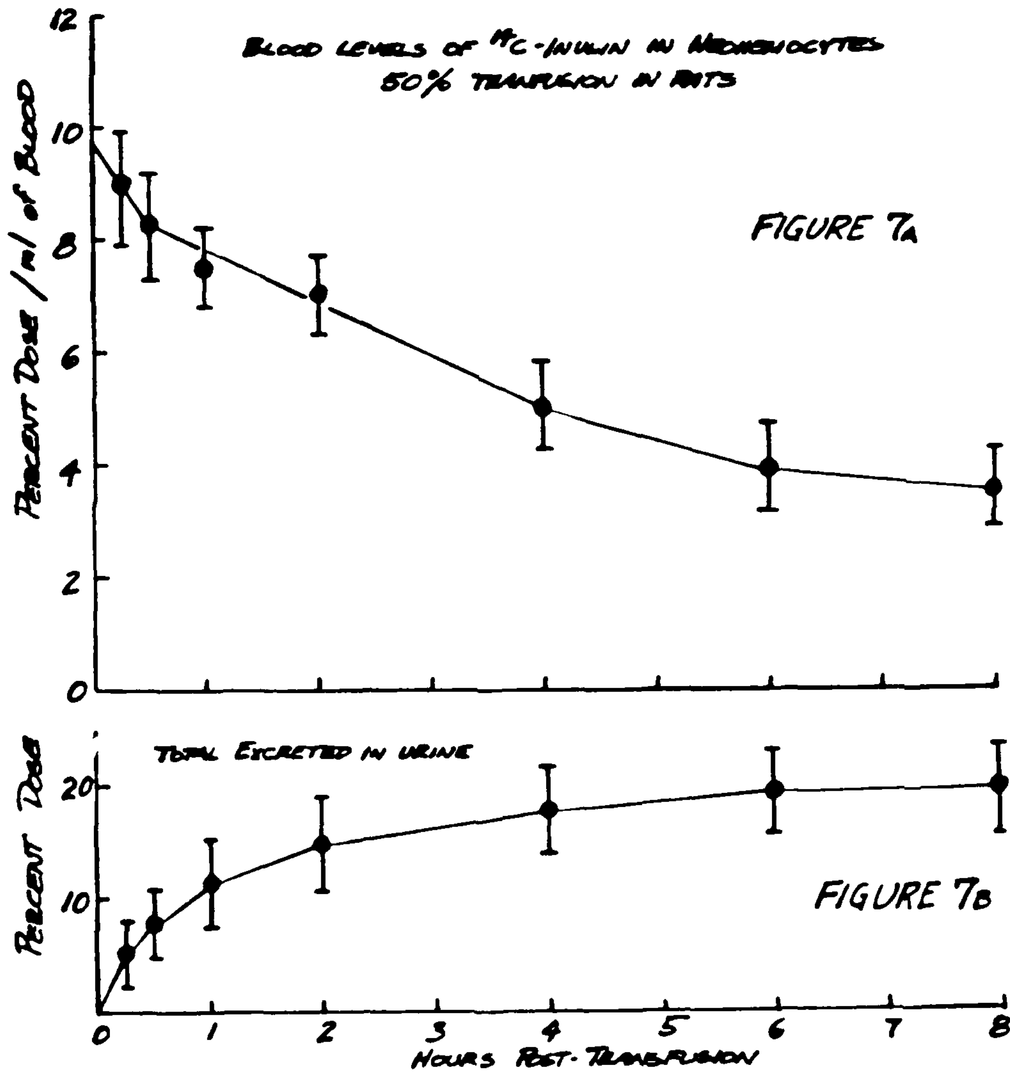


FIGURE 7A

FIGURE 7B

MASKING LIPIDS. The masking lipid concept evolved beginning in 1980 out of our desire to decrease further the interaction of neohemocytes with tissues, and retard or even eliminate RES clearance. The ideal masking lipid, when incorporated into the membrane, would act as a neohemocyte "cloaking device," reducing tissue binding, reducing RES clearance, increasing in vivo physical stability, and increasing the circulation half-life of neohemocytes. Our patent (which is in progress) includes data on eighteen "masked" neohemocyte formulations using synthetic lipis with one of four different carbohydrate head groups. As an example, our currently preferred masking lipid is a covalent adduct of phosphotidylethanolamine (PE) with raffinaldehyde. Raffinaldehyde is an enzymatic oxidation product of raffinose, which is a trisaccaride composed of galactose attached to sucrose; the galactose end is covalently attached to the free amine of PE. Because of the inert nature of sucrose it was postulated that when exposed on the neohemocyte surface it would be an effective "mask." Indeed this does appear to be the case; neohemocytes containing PE-raffinose have a minimum of a 100% increase in vascular retention, relative to the "unmnasked" controls.

The masking concept is totally new. We expect that better masking lipids can be developed, and that their incorporation into neohemocytes will yield a clearly superior product. To date, however, only trace amounts of "masked" neohemocytes have been injected into animals; they have not been used in transfusion studies. The synthesis and purification needs refinement before the amounts needed for transfusion-size batches can be prepared.

HYPOTHESIS

This project is based on several working hypotheses that are not testable by a single experiment, but rather will require the systematic accumulation of evidence. We have developed new and novel procedures such that Hb and other solutes can be encapsulated in lipid vesicles without significant Hb denaturation or met-Hb formation; an added benefit is an actual improvement in O_2 binding properties relative to those observed in simple solutions.

Our first working hypothesis is that neohemocytes are prototypal artificial red blood cells, and that when suspended in an appropriate plasma expander solution will produce a resusitative fluid superior to any currently under development for military field use or any other use. Storage and shelf-life will be important considerations. Our long range goal is to develop neohemocytes that can be lyophilized and stock-piled, and that will be for use following reconstitution in the field. Second, we propose a unique and novel hypothesis: that the external surface of neohemocytes can be optimized by adjusting lipid composition, and can be modified to improve in vivo neohemocyte function by inclusion of synthetic, phramacologically inert, biodegradable "masking" lipids. As a result the "masked" neohemocytes will interact less with tissues and are expected to be more invisible to the RES so that they are more slowly cleared and do not produce any significant RES blockaid.

A final working hypothesis is that properly designed neohemocytes will be non-antigenic and relatively non-toxic when used acutely.

OBJECTIVES

In order to reach that point where neohemocytes look attractive enough to warrant an expanded development effort leading to a product that is potentially acceptable to the Army, and that is suitable for further study in man, a substantial, expanded body of knowledge must be collected. This information is represented, in part, in the following objectives. At our current level of effort it will take several years to reach these objectives. They are listed in the order we now plan to pursue them, but we will remain flexible so as to respond to new developments.

1. Pursue methods and techniques to further optimize neohemocytes: A. Optimize membrane composition; B. Optimize Hb encapsulation, and nature and concentration of coencapsulated solutes; C. Optimize amount needed in membrane and the structure of masking lipids.
2. Increase neohemocyte production capacity.
3. Specifiy and implement quality asurance guidelines for neohemocytes.
4. Evaluate the temporal aspects of the observed acute toxicity in transfused rats. Pathology studies of transfused rats terminated at various times after their transfusion show indications of some limited toxicity. Determine the range of these acute effects and the time-course for return to normal status.
5. Determine the pharmacokinetic properties of neohemocytes in rats.
6. Evaluate the degree to which neohemocytes modify or reduce RES function.
7. Establish the nature and magnitude of neohemocyte effects on blood clotting. Using various levels of neohemocyte administration to animals, quantitate clotting time.
8. Identify the primary variables governing shelf life and identify strategies for its general improvement.
9. Evaluate the effects of lyophilization on neohemocyte properties. Identify ways that neohemocytes can be lyophilized, stored, and then reconstituted without being significantly damaged.
10. Establish if antibodies to neohemocytes occur following acute and repeated administration.

METHODS AND PROCEDURES

Approach to Objective One. An attempted optimization will be labelled "positive" if it significantly immproves one of the following: (a) P_{50} (b) "n" (c) lipid to encapsulated Hb ratio, (d) relative fraction of Hb-rich to Hb-poor neohemocytes, (e)

viscosity at 50% 'hematocrit,' (f) fraction remaining in the circulation of mice two hours after a 0.2 ml injection of neohemocytes, and (g) the fraction of neohemocyte-associated Hb that is met-Hb in (f), and finally (h) shelf life.

The next three efforts to optimize neohemocytes through membrane modification will be to (i) substitute 33 and 66% of the egg PC with a heterogenous to avoid a detectable phase transition temperature mixture of hydrogenated soy PC; (ii) decrease PA content from 10 to 3 mol%; (iii) replace 33 and 66% of the PC with a masking lipid.

Keeping the molar ratio of DPG to Hb at 1.6 seems reasonable; P_{50} and "n" values have not significantly improved at a ratio of 3.3. Improved neohemocyte function might result from inclusion of other solutes, especially one or more antioxidents. However, a top priority is to keep neohemocytes as simple as possible. It is important in this context not to confuse optimization efforts aimed at making neohemocytes a better, more efficacious, more generally useful product with efforts designed to get new information, new insights or explore the limits of the concept. From a practical point several antioxidents and/or reducing agents should be included to retard met-Hb formation; we have not yet selected our top three or four candiates; we are in the midst of literature research. From a more scientific perspective we want to see if carbonic anhydase and met-Hb reductase can be coencapsulated without adversely affecting other neohemocyte properties or the function of these proteins. Relative to other objectives these have rather low priority.

We expect that when vascular retention, tissue binding or RES uptake is plotted vs the mol-fraction of a specific lipid component we will see a classical S-shaped curve; going from 50 to 75 mol % masking lipid may yield only a fraction of improvement of going from 25 to 50%. Optimization studies, outlined above, with different types and levels of masking lipid will be aimed at getting a crude idea about the shape of such curves. We have done very little in this area over the past year. When we do begin such studies we will first evaluate those masking lipids already synthesized.

Approach to Objective Two. At our current scale of production it takes about 3 to 4.5 days (one person working full time) to produce enough material for a 97% transfusion of two rats. We will explore several procedural changes that should reduce the "hands-on" time of production, and which will allow easy scale-up to the 1,000 ml batch level.

Scale up is primarily an organizational and logistic problem. When we first made the jump from 1 ml to 10 ml batches we simply prepared ten 1 ml batches. Now, at the 100 ml stage, we prepare five 20 ml batches. A time consuming step is the removal of unencapsulated Hb after neohemocyte formation. This is now done chromatographically. We will try diafiltration and density-adjusted centrifugation.

Approach to Objective Three. Currently we are careful to follow the

exact same protocol each time. New control measures must be introduced. These measures will be used as a guide for further systematic, improvements.

At the end of a production sequence aliquots are evaluated. We determine P_{50}, "n," met-Hb level, fraction of Hb entrapped, and ratio of Hb to coencapsulated aqueous space marker, ^{14}C-sucrose. There is variability in these results. We do not know either the extent to which this variability can be reduced and controlled, or which aspects or steps in the procedure are responsible for the variability. Nor do we know how this variability relates to *in vivo* function. Several additional measurements are made periodically: ratio of Hb-rich to Hb-poor neohemocytes, neohemocyte density, osmolarity and pH. This information, will be the basis for future quality assurance guidelines.

One important aspect of quality assurance will be sterility. We plate aliquots of each final neohemocyte preparation. Sometimes it is sterile, sometimes it is not. There is not enough room to carry out the entire preparation sequence on our small clean bench. A sterile, pyrogen-free product will be essential. We have not prepared such a product, but we can. We must demonstrate this in the near future.

Approach to Objective Four. To establish the degree and speed of recovery of transfused rats from the acute toxic effects of neohemocytes we will follow the same protocol used for the 50% transfusions, acute toxicity study described above. We will evaluate recovery 1, 7 and 30 days following (1) a complete transfusion; (2) a 33% transfusion; and (3) the second of two 50% transfusions spaced 10 days apart. Livers, hearts, kidneys, lungs and spleens from experimental and sham transfused rats will undergo blind histological examination by Dr. Lau on a recharge basis. Typical sections will be recorded and qualitatively graded.

Approach to Objective Five. We have measured blood levels of neohemocytes at several times following administration to mice, and after transfusions in rats. Indications are that intravascular retention and stability are dose dependent. Preliminary pharmacokinetic studies in rats have been completed. More detailed phramacokinetics at several doses are critically needed to establish how sensitive half-life and stability are to neohemocyte dose (or transfusion level), and subsequently to attempt their modulation.

Pharmacokinetic data from the recent studies was obtained using ^{14}C-inulin as a marker. Additional studies are needed using ^{51}Cr-Hb and/or ^{121}I-Hb, and at both higher and lower transfusion levels. As neohemocytes evolve we can use pharmacokinetic studies to assess how disposition, neohemocyte stability, met-Hb formation, etc. changes as a result. Again, we assume the information obtained will aid in optimization efforts.

Approach to Objective Six. Large amounts of some particulates, e.g. Fluosol-DA, can reduce and even block RES function. We have not yet identified a reduction in RES function following administration of neohemocytes. We need to employ more rigorous tests, e.g. changes in endotoxin LD_{50}, to evaluate the impact of the larger, transfusion doses. Studies to date indicate that if the current, "unmasked" neohemocytes do cause some RES blockaid, then it most likely is relatively weak, much less than what occurs with Fluosol-DA (of course, we have attempted to optimize composition in this direction). Indications are that extracellular binding to tissues is the primary cause of blood clearance. Based on discussions with several RES experts we will test RES blockaid using endotoxin, rather than the more common live bacterial protocols. A reduction in the endotoxin LD_{50} following transfusion will indicate a measure of RES blockaid. We actually would prefer to evaluate RES blockaid in mice. Although we can not transfuse mice, we can give a series of i.v. injections of concentrated neohemocytes over a 4 to 6 hr period followed later by the endotoxin challenge (time between last dose and challenge remains to be determined). We have estimated that the cost difference between a RES blockaid study in rats and one in mice will be about a factor of 14. In addition to albumin in saline, we will use Fluosol-DA as a control for these studies.

Approach to Objective Seven. Evaluations of the effects of neohemocytes on blood clotting will be part of the later pharmacokinetic studies. Studies outlined above will be carried out following the protocols currently in use at LAIR for evaluating the *in vivo* properties of polyhemoglobins.

Approach to Objective Eight. Clumping, vesicle fusion and membrane stability do not appear to be major determinants of shelf life; however, met-Hb formation does. The time-course of met-Hb formation both *in vivo* and *in vitro* needs to be quantified and then modulated. We have our first long-term, variable temperature shelf life study of neohemocytes under way. Preliminary results indicate a refrigerated shelf-life in excess of 60 days. This and future shelf life studies will use the efficient fractional factorial, centered block design with three levels of each of two independent variables (e.g. membrane composition, temperature, pO_2, antioxidant level, etc.). Neohemocyte samples are stored in in sealed tubes in the dark at the specified temperature. Because decreases in optimum properties will likely follow either zero order or autoxidation kinetics, sampling frequency must be judged based on results from the last sample analyzed.

These studies will address several different questions. Depending on the results obtained from the above studies, subsequent studies will be appropriately redesigned. The questions of interest are: (a) Is the change in O_2 binding properties consistent with autoxidation? (b) Is there a log-linear Arrhenius plot between 4 and 37°? (c) Which helps most, membrane or aqueous phase antioxidents? (d) Within each antioxident class, which one works best? (e) Does the number of times

a Hb batch is reduced (to reduce met-Hb levels; we do this with most Hb batches) increase the rate at which met-Hb is subsequently formed? (f) Can stability be increased by reducing the ratio of the volumes of the suspending phase to the neohemocyte phase (we can reduce this ratio to about 0.06)? (g) Does albumin in the suspending phase affect stbility? The basic protocol will consist of three-level, two-variable stability studies.

Finally, (h) how is the viscosity and neological propertie of neohemocytes suspensions related to the above?

Approach to Objective Nine. Neohemocytes would be more attractive to the Army if they could be lyophilized and subsequently reconstituted. This would make stock-piling more reasonable. We do not plan to study lyophilization soon, but never-the-less, expect progress in this area. We know that several pharmaceutical companies have research programs to develop liposome dosage forms. As part of such programs substantial effort is being invested in perfecting liposome lyophilization techniques. We feel we can constibue little to this effort at this time. Once procedures are perfected, it should be possible to apply their procedures to neohemocytes. We are being kept informed of progress at E.I. Lilly, Merck, Cooper Biomedical, and Syntex.

Professor Puisieux at the University of Paris is also working on the lyophilization of liposomes. One of the investigators on this project from his group, Pierre-Andre Poly, is working as a post-doc in our lab for the next year. We may send some neohemocytes to Professor Puisieux for inclusion in his lyophilization studies; this will not result in any charges to the contract.

Approach to Objective Ten. Other investigators have either been unable to either been unable to produce antibodies or have found it extremely difficult to produce antibodies to similar vesicles having protein-free lipid membranes.

We have few skills in immunobiochemistry. However, it will be be important to establish the degree and nature of any antibody formation in response to neohemocytes. Our aim is to find a collaborating laboratory here at UCSF that is interested in this problem, and willing to collaborate.

FACILITIES

The University of California, San Francisco is the only health sciences cmpus of the University system. We have an exceptional staff and resourses for research and teaching activites. The School of Pharmacy contains most of the chemistry faculty on campus and the bulk of the large analytical instrumentation. The laboratories of the P.I. are equipped with the usual chromatographic, spectroscopic, and liposome related equipment needed for research scale work in this area. Additional equipment will be needed to meet the demands of sacle up. A complete animal care facility is maintained on campus.

END

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