PROGRESS REPORT

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PROGRESS REPORT

1. Continued Evaluation of ^{14}C -Labelled Carboxyethyl Ester 2-Carboxy Nethyl Ester of Arachidonic Acid (^{14}C -CME-AA)

As background, in fiscal year 81 we began a series of investigations designed to evaluate carbon-11 labeled arachidonic acid (AA) analogs hoping to study cardiac prostaglandin metabolism using positron emission tomography. A method had been developed at BNL (by Lade and Wolf) that produced ^{11}C -labelled-2 carboxy methyl ester of AA (CME-AA). Since the CME-AA molecule retains the highly reactive portion of AA, we had prepared ^{14}C -CME-AA to undertake metabolic studies with the carbon-14 analog ultimately aiming at a study of the carbon-14 analog in a myocardial infarction canine model in collaboration with our cardiology research laboratory. Initially (1980-81) we used ^{14}C -CME-AA with human platelets utilizing a method described by Dusting et al. (Progress in Cardiovascular Diseases, Vol. XXI, 405, 1979, Grune and Stratton, Inc.), but found that only approximately 17% of the carbon-14 analog was converted into prostaglandin-like substances compared to 97% of tritiated arachidonic acid (^3HAA) used as a control. We felt it was possible, however, that the chromatographic separation used may have obscured the biologically active compounds, since there was relatively poor separation between AA and the metabolites produced. Accordingly, we utilized a different chromatographic separation described by Alan et al. (Prostaglandins and Medicine, 4:227-237, 1981) utilizing HPLC to separate the metabolic products. With this technique, however, we still could not

establish significant metabolic activity of the ^{14}C -CME-AA whereas the control ^3HAA again showed significant metabolic activity.

In addition, we did experiments utilizing myocardial ischemia following the method of Kramer et al. (J. Pharm. and Exp. Therapeutics, 199:611, 1976). In brief, the protocol for these experiments (described in detail in last year's renewal proposal) requires an open chest preparation of an anesthetized adult dog with exposure of the left anterior descending artery (LAD) with infusion of both control ^3HAA and experimentally ^{14}C -CME-AA at a rate of 0.13 ml/minute for about 10 minutes. After equilibrium, blood samples were obtained from the great cardiac vein to serve as a control for the ischemia subsequently produced.

Ischemia was next induced by a 10 minute ligature occlusion of the LAD. Immediately following release of the ligature, a second venous blood sample was obtained from the cardiac vein with a third sample obtained some 10 minutes following release of the ligature. All blood samples were centrifuged, the plasma separated, and HPLC analysis performed as described by Alan et al. (Op Cit). Again, the metabolic activity of the carbon-14 labelled AA analog was minimal when compared to the tritiated control. In short, utilizing three different test systems we were unable to show significant metabolic activity of ^{14}C -CME-AA. Congruent with these results, Dr. Robert Lade left BNL with the consequence that no immediate additional work on arachidonic acid analogs seemed likely from Dr. Wolf's group. Accordingly, we concluded that there was no reason to pursue this effort further.

2. The Effects of Drug Intervention on Cardiac Inflammatory Response Following Experimental Myocardial Infarction Using Indium-111 Labeled Autologous Leukocytes

We undertook a series of preliminary studies to evaluate the effect of a non-steroid anti-inflammatory agent (ibuprofen) on the extent of myocardial necrosis assessed biochemically and the extent of inflammatory response assessed by the imaging of Indium-111 leukocyte distribution within the myocardium. These studies were based upon our previously published results in dogs whereby we were able to image Indium leukocyte distribution within the infarct zone with maximal uptake in the lowest flow areas (Circulation 60:297, 1979). The canine plug embolus infarct technique previously utilized by our laboratory was employed (Circulation 53:422, 1976).

The initial series of experiments were not rewarding. First, a series of ten "semi-conditioned" animals were evaluated. In four of these animals an adequate infarct was not created although the left anterior descending coronary artery had been embolized effectively. This is a known event consistent with the large degree of collateral flow frequently noted in dogs as opposed to other experimental models. In an additional three animals there was substantial pneumonia present. This resulted in marked leukocyte uptake in the lung obscuring any leukocyte uptake within the myocardium. In the final three animals, although infarction was present, there appeared to be inadequate leukocyte accumulation. These latter data were in marked contrast to that noted in our previously reported results and suggested an altered biologic activity of these labeled cells.

An additional five "chronic dogs" were studied to obtain what appeared to be a more stable experimental preparation. Two of these five animals showed excellent leukocyte accumulation in the infarct. However three of the animals showed minimal to no leukocyte accumulation.

At the present time these experiments have been temporarily terminated while we evaluate the biological activity of the labeled leukocytes. It should be noted, in addition, that there has been a marked increase in the cost of chronic dogs creating significant budgetary problems for the continuation of these studies.

3. The Evaluation of ⁹⁷Ru-Oxine to Label Human Platelets in Autologous Plasma.

Unfortunately, BNL stopped preparing ruthenium-97, and this portion of the renewal proposal could never even begin.

4. The Specific In Vitro Radiolabeling of Human Neutrophils

Current procedures for labeling all types of blood cells with indium-111-oxine such as Platelets, RBCs, Lymphocytes, and PMNs (M. Thakur et al., Int. J. Appl. Radiat. Isotopes, 28:183, 1977, A Ferrant et al., J. Nucl. Med. 21:844, 1980, M. Thakur et al., J. Lab. Clin. Med. 89:217, 1977, G. Rannie et al., Clin. Exp. Immunol. 29:509, 1977, J. Ferluga et al., J. Clin. Lab. Immunol. 1:339, 1979) necessitate the isolation of the required type of cells in vitro because indium-111-oxine is a nonspecific labeling agent. The isolation and handling of delicate blood cells is likely to result in the loss of cell viability (Welch et al., J. Nucl. Med. 20:460, 1979).

A technique of labeling a desired cell type selectively in vitro in a whole blood sample not only has the potential of improving the physiological properties of the labeled cells, but also permits the procedure to be simplified substantially. Two years ago, as part of this DOE contract, we submitted a proposal for the development of, FMLP-TF-indium-111, as a potential radioactive agent for labeling human neutrophils in whole blood.

N-formyl-methionyl-leucyl-phenylalanine (FMLP) is a synthetic peptide that has been reported to be a potent chemotactic agent for neutrophils with ED_{50} of 7×10^{-11} M (H. Showell, J. Exp. Med., 143:1154, 1976, and J. Reticuloendothelial Soc., 25:139, 1979). Each neutrophil is reported to have between 2,000 and 120,000 binding sites for FMLP on its surface membrane (William et al., Proc. Natl. Acad. Sci. U.S.A., 74:1204, 1977, Niedel et al., J. Biol. Chem. 254:10700, 1979, Istueiz et al., Science, 200:554, 1978).

This synthetic peptide was covalently conjugated to transferrin (TF) with the retention of its chemotactic activity (Marasco et al., J. Immunol. 128:956, 1982) and since transferrin has a strong affinity for indium-111, the complex FMLP-TF-indium-111 became a potential radioactive agent for labeling human neutrophils in whole blood. Our initial work showed promise for this technique and we succeeded in making neutrophils labeled with FMLP-TF-indium-111 with an efficiency of 28% and have previously proposed the technique which will improve the labeling efficiency. The essence of F.Y. 1982 progress report in this area is detailed in the following preprint wherein we report the improvement of

labeling efficiency to 60% while systematically studying the effect of the following on the labeling efficiency of neutrophils:

1. Ratio of FMLP to receptor sites
2. The molar ratio of FMLP to transferrin
3. The molar concentration of FMLP in the incubation solution
4. Temperature of incubation.

A Potential Radioactive Agent for the Selective
Labeling of Human Neutrophils

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ABSTRACT

We have developed a bifunctional chelate that incorporates indium-111 and selectively labels neutrophils. N-formyl-methionyl-leucyl-phenylalanine (FMLP), a synthetic chemotactic attractant for neutrophils, was covalently conjugated to human transferrin (TF) using 1-ethyl-3-(dimethylamino-propyl) carbodiimide HCl as a coupling agent. Then indium-111 was bound to TF (2.4×10^2 Ci/m mole TF) at pH 7 and 37°C in the presence or absence of bicarbonate ions during a 30 minute incubation period.

Optimal conditions for labeling neutrophils with FMLP-TF-indium-111 consist of incubation in plasma at 22°C for 20 minutes; labeling efficiency is dependent upon the FMLP:TF molar ratios. When an aliquot of FMLP-TF-indium-111 containing 322 million FMLP molecules with FMLP:TF ratio of 8:1 was added to 220 million cells, 60% of the added indium-111 became cell associated. Factors that influence the preparation of FMLP-TF-indium-111 and those that affect the labeling of the cells are discussed. Our results indicate that FMLP-TF-indium-111 is a promising agent for the in vitro selective labeling of human neutrophils.

INTRODUCTION

Indium-111-oxine efficiently labels such blood cells as erythrocytes, platelets, lymphocytes, and polymorphonuclear leukocytes (PMN) (1-5). The labeling process is highly nonspecific and necessitates the isolation of the desired type of cells in vitro. To overcome this problem for PMN's, we have attempted to formulate an agent that will selectively label only the PMN's in whole blood.

Many synthetic peptides have been reported to be chemotactic agents for PMN's (6,7). Studies of the structure/activity relationship of these synthetic peptides (8,9) have shown that formyl-methionyl-leucyl-phenylalanine (FMLP) is one of the most active. The concentration of FMLP at which 50% of the maximal chemotactic response occurred (ED_{50}) was $7 \times 10^{-11}M$ (9).

Each neutrophil is reported to have on its surface membrane between 2,000 and 120,000 structurally specific receptor sites for these agents (10-12). Labeled with a suitable gamma emitting radionuclide, this oligopeptide should, in principle, provide a specific cell label.

The feasibility of halogenation of proteinaceous compounds leads to the possibility of using the gamma emitting I-123 ($t_{1/2}$; 13.3 hrs., ; 159 keV-83%) as a tracer. However, the halogenation of the phenylalanine residue has been shown to reduce the biological activity of the molecule (13). Furthermore, the weakness of the C-I bond leads to deiodination. The intracellular fate of iodine is also unknown.

FMLP can be labeled with a radioactive cation such as indium-111. However, the weak association between the cation and the amino group

would result in the immediate loss of indium-111 to plasma transferrin upon contact, thus preventing the labeling of neutrophils.

FMLP, covalently bound to a protein molecule retains its chemotactic activity (14). Binding FMLP to a protein molecule such as TF, which is known to have a strong affinity ($\log k$, 30) for indium-111 (2), would provide a bifunctional chelate that may be useful for selective labeling of neutrophils. In vivo studies are possible with indium-111 since its gamma emissions ($t_{1/2}$ -67.4 hrs, ; 173 keV - 89%; 247 keV - 94%) permit external detection. Based on these facts we investigated FMLP-TF-indium-111 as a prototype agent that may allow us to label specifically human neutrophils in whole blood essentially by a kit method. Here we report our preliminary results.

MATERIALS AND METHODS

Preparation of FMLP-TF.

Tritiated N-formylmethionyl-leucyl-phenylalanine (FML- ^3H P), specific activity of 56.9 Ci/millimole, was obtained from New England Nuclear. The compound was covalently bound to human transferrin* using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide HCl* as a coupling agent (14).

Briefly, a stock solution of FML- ^3H P of 1.0-2.0 Ci/mole was prepared in 25 μl of dimethyl sulfoxide. A known quantity of this solution was then added to a desired amount of TF dissolved in 0.9% saline, together with 200 mg of the coupling agent. The reactants were incubated for approximately one hour at 4°C in a total volume of 2-5

ml. Following the incubation period, the reaction mixture was dialyzed against 0.9% NaCl in order to eliminate the unbound FML- $[^3\text{H}]$ P. The completion of the reaction was further confirmed by the lack of $[^3\text{H}]$ radioactivity in the supernate of the trichloroacetic acid precipitate of an aliquot of the dialysate. Using the known TF concentration and assaying the $[^3\text{H}]$ radioactivity, we determined the FMLP:TF molar ratio, which varied between 1:1 to 22:1, depending upon the initial quantities chosen. We stored the FMLP-TF solution at 4°C and periodically checked the compound for stability using a sephadex G-25 mini gel filtration technique (15).

Preparation of FMLP-TF-indium-111. We obtained indium-111- Cl_3 (2mCi/ml) solution, pH 1-3 from Medi + physics. A suitable volume (5-100 μl) of the radioactivity was drawn and placed in a 12 x 75 mm polypropylene test tube⁺; 30-100 μl of 0.01 M sodium acetate buffer, pH 7.0, were added and mixed well. In some preparations, sodium bicarbonate was added to a final concentration of between 4.5×10^{-3} and 4.8×10^{-3} M. To this mixture, we added a calculated amount of FMLP-TF (1.53×10^{-10} to 2.5×10^{-9} moles) and incubated the test tube at 37°C for 30 minutes. We eliminated the unbound indium-111 radioactivity by passing the reaction solution over a mini gel column.

Separation and labeling of cells. For the preliminary investigation, we used human or canine leukocytes in plasma. Fifty milliliters of whole blood from healthy human volunteers or dogs, anticoagulated with 500 U.S.P. units of heparin, or 43 ml of whole blood with 7 ml of acid citrate dextrose (ACD) was allowed to sediment by gravity. In some

instances, the sedimentation process was aided by the addition of 2% methylcellulose to constitute a 3% solution with respect to the whole blood. The 18-24 ml of leukocyte-rich plasma (LRP) in the upper layer were separated, and an aliquot of erythrocyte lysed LRP was assayed in a Coulter counter for the leukocyte concentration. The LRP was centrifuged at 450 xg for 5 minutes and the cells were resuspended in 2 ml of platelet rich plasma; 70% of leukocytes were assumed to be PMN's. A calculated amount of FMLP-indium-111 was then added to cover a range of 1.0 FMLP molecule per 3 PMN's to 10,000 molecules per PMN. Cells were incubated for 20 minutes at room temperature, if not otherwise indicated, washed at least once with plasma, and labeling efficiency was determined.

We measured the nonspecific binding in duplicate control samples of either platelet rich plasma or predenatured PMN's (incubated at 50°C for 20 minutes). The amount of FMLP-TF-indium-111 retained by the control cells after washing under identical conditions to the test cells was considered to be the nonspecific binding.

Depending on the amount present in the sample, indium-111 was assayed either in a dose calibrator⁺⁺ or in a NaI(Tl) well type scintillation counter." The [³H] radioactivity was assayed in a liquid scintillation spectrometer.^

RESULTS

Complexing of indium-111 with TF. Table 1 demonstrates the effect on the TF-indium-111 complex formation of the pH of the reaction media and the presence or absence of bicarbonate, known to be essential for

TF-metal binding (16). The use of acetate buffer avoided the risk of formation of In-hydroxide species. In the presence of bicarbonate ions, binding displayed an optimum of $90.0\% \pm 0.7\%$ at pH 7.0. The incorporation of radioactivity at pH 6.5 and pH 8.0, was $33.0\% \pm 5.7\%$ and $77.5\% \pm 6.4\%$, respectively. In the absence of bicarbonate ions, the binding at pH 6.5 was as low as $9.5\% \pm 3.5\%$. At pH 7.0 and 8.0, however, the binding efficiencies were $70.0\% \pm 0.0\%$ and $80.5\% \pm 2.1\%$ respectively. The higher labeling efficiencies at these pH values may be attributed to the dissolution of atmospheric CO_2 . The binding of indium-111 to TF in plasma at pH 7.0 without added bicarbonate and regarded as control was also high ($83.5\% \pm 2.1\%$).

At constant pH 7.0, the formation of the indium-111-TF complex was essentially complete within 5 minutes in the presence or the absence of bicarbonate ions (Table 2). However, a $25\% \pm 5\%$ increase in the binding was observed in the presence of added bicarbonate ions. As a result of these studies, we chose the optimal condition of neutral pH (pH 7.0) and a 10 minute incubation period without added bicarbonate. These conditions were directly applicable to the FMLP-TF-indium-111 formation.

The mini gel column chromatographic technique, well suited for the purification of protein and ionic indium-111 in microquantities, also was used to determine the stability of the FMLP- ^3H P-TF molecule. The molecule retained its ability to bind with indium-111 for at least 2 months when stored under conditions described in Materials and Methods.

Cell labeling. Table 3 depicts the effect of two variables on the efficiency of labeling human neutrophils with FMLP-TF-indium-111. These

were (1) the number of FMLP molecules or the molarity and (2) the molar ratios of FMLP to TF. By tracing the indium-111 incorporated into cells, we were able to determine the number of FMLP molecules taken up per cell. In this study, the nonspecific cell labeling ($12.9\% \pm 1.4\%$) was estimated by using platelet rich plasma as a control.

A log x log plot of the number of FMLP molecules added per cell versus the number of FMLP molecules incorporated per cell had a linear relationship with a reliability factor of 99.0% (Fig. 1), regardless of the FMLP:TF molar ratio. Maximal incorporation of 60% of added radioactivity into cells occurred when one FMLP molecule was made available to 3.3 cells. Although the percent radioactivity incorporated into the cells decreased as more radioactivity was added, the absolute amount taken up by each cell increased in a nonsaturable manner throughout the range studied. In this range the maximal number of FMLP molecules incorporated per cell was 1.2×10^4 .

The incorporation of FMLP-TF-indium-111 by human neutrophils suggested that at 37°C there was a progressive loss of radioactivity from the cells. We used canine neutrophils to study the effect of time and temperature on the incorporation of radioactivity by cells (Table 4). After 10 minutes of incubation, there was $35.5\% \pm 2.0\%$ of the radioactivity associated with cells incubated at room temperature and $33.7\% \pm 0.2\%$ associated with those incubated at 37°C . However, at 37°C , the amount of radioactivity associated with the cells declined by about 30% in 50 minutes of incubation.

DISCUSSION

The selective PMN's labeling approach is based on the existence of a structurally specific receptor for synthetic peptide molecules on the PMN surface. Previous findings have strongly suggested that the receptor-ligand interaction is followed by the internalization of the molecules. PMN's having internalized one type of chemoattractant still retain the ability to attract another (20). This process is expected to allow the FMLP-TF-indium-111 labeled PMN's to retain their normal kinetics and ability to respond to inflammatory processes.

This technique is fast, simple, and potentially more physiologic. Our preliminary data suggest that this approach is feasible, and may lead to the selective labeling of human PMN's in an in vitro whole blood sample by a kit method.

The conjugation of FMLP to macromolecules does not alter its chemoattractant properties (12,14). The FMLP-TF molecule was stable for several weeks and was readily available for chelation with indium-111. Using the chemical procedures described, we have been able to achieve a specific activity of 2.4×10^2 Ci/millimole and 60% incorporation of radioactivity in human PMN's.

For a practical clinical study, a radioactive dose of about 350-500 μ Ci is desirable. A 500 μ Ci dose would contain 6.5×10^{12} atoms of indium-111. Assuming each transferrin molecule binds to two indium-111 atoms (17,18), a total of 3.3×10^{12} TF molecules would be needed to complex 500 μ Ci of indium-111. If the number of TF molecules were equal to that of the FMLP molecules, then 3.2×10^{12} receptor sites would be

required. Therefore the 500 μCi of indium-111 would need 3.2×10^{12} receptor sites that are approximately equivalent to 5.3×10^7 PMN's. These can be obtained from 25-35 ml of human whole blood.

To accomplish selective cell labeling by a kit method, a high specific activity (in the order of $9 \times 10^4 \text{ Ci}^{111}\text{In}/\text{millimole TF}$) and a greater than 90% radioactivity incorporation are necessary. A high labeling yield will reduce the amount of free radioactivity thus eliminating the need for centrifugations. Our current achievement of $2.4 \times 10^2 \text{ Ci}^{111}\text{In}/\text{millimole TF}$ and 60% labeling efficiency fall short of these levels. One way of improving the specific activity would be to eliminate cationic impurities, such as cadmium, zinc, and iron suspected to be present in the $^{111}\text{InCl}_3$ solutions, which would occupy the binding sites in TF.

Recent literature indicates that agents such as ascorbic acid stimulate phagocytosis (19). The addition of such an agent might enhance internalization of FMLP-TF-indium-111 and thereby increase the labeling efficiency.

The third approach would involve using a chemoattractant such as the recently developed N-Formyl-Nle-Leu-Phe-Nle-Tyr-Lys that has higher affinity for human PMN's (11).

The results of our feasibility study using FMLP-TF-indium-111 as a prototype for selectively labeling human PMN's in whole blood are encouraging; we feel they warrant further investigation directed toward improving yields and studying the intracellular fate of the

FMLP-TF-indium-111 molecule. If the problems described above can be solved, then a kit for rapid, selective PMN labeling in whole blood can be prepared.

* Sigma

+ Falcon

++ Capintec, CRC-5R

" Beckman, Gamma 8000

^ Searle Analytic 92

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TABLE 1. EFFECT OF BICARBONATE AND pH ON THE PERCENT INCORPORATION OF INDIUM-111 BY TRANSFERRIN

Reaction components	Test tube number			
	1 - 2	3 - 4	5 - 6	7 - 8
- $^{111}\text{InCl}_3$ (2 mCi/ml)	10 ul	10 ul	10 ul	10 ul
- Bicarbonate (2 mg/ml)	20 ul	-	-	-
- Plasma	-	-	40 ul	-
- TF (1 mg/ml)	40 ul	40 ul	-	-
- Acetate buffer (0.01 M)	30 ul	50 ul	50 ul	90 ul
<hr/>				
pH of the reaction media	1 - 2	3 - 4	5 - 6	7 - 8
6.5	33.0 $\pm$ 5.7%	9.5 $\pm$ 3.5%	-	0.0
7.0	90.0 $\pm$ 0.7%	70.0 $\pm$ 0.0%	83.5 $\pm$ 2.1%	0.0
8.0	77.5 $\pm$ 6.4%	80.5 $\pm$ 2.1%	-	0.0

- Notes: A. Complexing was done by incubating transferrin with $^{111}\text{InCl}_3$ at 37° C for 30 min. at different pH values.
 B. 10 ul $^{111}\text{InCl}_3$ = 20.0 $\pm$ 10.0 uCi = $2.6 \pm 1.3 \times 10^{11}$ In atoms.
 C. 40 ul TF = 2.7×10^{14} molecules.

TABLE 2. EFFECT OF BICARBONATE AND INCUBATION TIME ON THE PERCENT INCORPORATION OF INDIUM-111 BY TRANSFERRIN

Reaction components	Test tube number		
	1 - 2	3 - 4	5 - 6
- $^{111}\text{InCl}_3$ (2 mCi/ml)	5 μl	5 μl	5 μl
- TF (1 mg/ml)	40 μl	40 μl	-
- Bicarbonate (2 mg/ml)	20 μl	-	-
- Acetate buffer (0.01 M) (pH = 7.0)	40 μl	60 μl	100 μl
Incubation time (min.)	1 - 2	3 - 4	5 - 6
5	89.0 $\pm$ 0.0%	69.0 $\pm$ 0.0%	1.0%
15	81.0 $\pm$ 0.0%	69.0 $\pm$ 10.0%	1.0%
30	90.0 $\pm$ 0.7%	70.0 $\pm$ 0.0%	0.0%
60	83.0 $\pm$ 0.0%	66.5 $\pm$ 0.7%	3.0%

Notes: A. Complexing was done by incubating transferrin with $^{111}\text{InCl}_3$ at 37° C, pH = 7.0, and for different lengths of time.
 B. 5 μl In-111-Cl₃ = 12.0 $\pm$ 8.0 μCi = 1.7 $\pm$ 1.0 $\times 10^{11}$ In atoms.
 C. 40 μl TF = 2.7 $\times 10^{14}$ molecules.

TABLE 3. THE EFFECT OF VARYING, FMLP:TF MOLAR RATIOS, CONCENTRATION OF FMLP-TF-INDIUM-111, NEUTROPHIL BINDING SITES ON THE LABELING EFFICIENCY OF HUMAN NEUTROPHILS

Molar ratio FMLP:TF	FMLP mole- cules/cell added	FMLP molarity	No. cells	% of added radioactivity incorporated	Incorporated FMLP mole- cules/cell
22:1	3.4×10^5	9.7×10^{-8}	3.0×10^8	3.5	1.2×10^4
	9.7×10^4	1.7×10^{-8}	2.1×10^8	5.5	5.3×10^3
	2.8×10^4	9.7×10^{-10}	4.1×10^7	10.0	2.8×10^3
	2.4×10^4	8.2×10^{-10}	4.1×10^7	10.0	2.4×10^3
	9.7×10^3	1.7×10^{-9}	2.1×10^8	10.5	1.0×10^3
	2.8×10^3	3.4×10^{-10}	1.5×10^8	16.0	4.5×10^2
8:1	1.2	3.3×10^{-14}	3.3×10^7	28.0	3.3×10^{-1}
	3.0×10^{-1}	3.3×10^{-14}	1.3×10^8	60.0	2.0×10^{-1}
7:1	5.6×10^4	9.7×10^{-9}	2.1×10^8	6.0	3.3×10^3
	1.2×10^3	1.5×10^{-10}	1.5×10^8	11.7	1.4×10^2
	2.3×10^3	2.1×10^{-10}	1.5×10^8	16.5	3.8×10^2
	2.6×10^2	2.9×10^{-11}	1.4×10^8	14.0	3.6×10^1
2:1	3.2×10^4	5.6×10^{-10}	2.1×10^7	4.2	1.3×10^3
1:1	1.0×10^3	1.9×10^{-10}	2.3×10^8	3.3	3.3×10^1

Note: A. Cells were incubated with FMLP-TF-INDIUM-111 for 20 min. at room temp.

FIG. 1. FMLP incorporation per human PMN as a
function of increasing the number of FMLP
molecules added to the cell

-21e-

NUMBER of FMLP MOLECULES
INCORPORATED per CELL

TABLE 4. THE EFFECT OF TIME AND TEMPERATURE ON THE INCORPORATION
OF INDIUM-111 BY CANINE PMN'S

<u>% of added radioactivity (FMLP-TF-IN-111) incorporated by the cells</u>				
<u>Time (min)</u>	<u>Room temp</u>		<u>37 C</u>	
	<u>WBC</u>	<u>Control</u>	<u>WBC</u>	<u>Control</u>
10	35.5 $\pm$ 2.0	13.0 $\pm$ 0.2	33.7 $\pm$ 0.2	12.2 $\pm$ 0.2
20	33.2 $\pm$ 3.5	14.0 $\pm$ 2.2	24.1 $\pm$ 1.5	13.5 $\pm$ 1.6
30	31.2 $\pm$ 8.4	13.8 $\pm$ 2.0	21.4 $\pm$ 5.7	9.8 $\pm$ 3.1
50	28.4 $\pm$ 4.9	11.3 $\pm$ 4.5	23.8 $\pm$ 4.5	10.3 $\pm$ 1.4

- Notes: A. 3.4×10^7 PMN/ml plasma/tube
 B. 2 tubes per time x temp. combination
 C. 5.76×10^{11} FMLP molecules/tube 9.6×10^{-10} M
 D. Denatured PMN (3.1×10^7 PMN CELLS: 20 min. at 50°C)
 E. $3.8 \pm 0.2 \times 10^{-2}$ uCi indium-111/tube

In addition to the results detailed in the preprint, we have finished additional work detailed below.

The Purification of Indium-111-Chloride Solution

One possible way to increase the amount of radioactivity incorporated into the cells is by increasing the specific activity of FMLP-TF-indium-111 over the currently achieved 2.4×10^2 Ci-indium-111/millimole TF. In our last renewal proposal (Nov. 1, 1981-Oct. 31, 1982) we reported evidence for the presence of cationic impurities that may have resulted in the inefficient binding of indium-111 to TF. The cationic impurities are likely to be cadmium, zinc, and iron. All of these are known to compete for the available binding sites on TF. In 6 N HCl, these metal ions form anionic species which facilitate their separation from cationic indium on an anion exchange column. The purified $^{111}\text{InCl}_3$ will then be eluted in 6 N HCl. Below we report our efforts to purify $^{111}\text{InCl}_3$ to further increase labeling efficiency.

Materials and Methods

All glassware used were acid washed to remove all possible traces of metal ions. All reagents were analytical reagent grade. Bio-Rad AG 1x8, 100-200 mesh anion exchange resin was used. The resin was washed with 6 N HCl several times and then was packed in a disposable polyethylene column (0.5x2.5 cm) and further washed with 6 N HCl.

Indium-111-Cl₃ as supplied by Medi + physics (2.0 mCi/ml, 1.5 ml, pH 3.0-4.5) was dried under a stream of argon and redissolved in 0.1-0.3 ml of 6 N HCl. The indium-111-Cl₃ was then loaded on the column and further eluted with 6 N HCl. The eluate was evaporated under a slow stream of argon until dryness and then dissolved in 0.3M acetate buffer pH 7.4.

A decreasing amount of radioactivity was placed in four separate test tubes (Table 1). Acetate buffer was then added to eliminate the difference in volume. A fifth tube containing radioactivity without purification will serve as a control. Finally equal amounts of transferrin (1×10^{13} molecules) were finally added to the solutions and then incubated at 37°C for 30 min followed by gel filtration.

Results and Discussions

The following table summarizes the experimental set up and the results. Knowing the specific concentration of indium-111-Cl₃ in the stock solution prior to purification we were able to use varied amounts of purified indium-111-Cl₃ (43-474 uCi) and still relate it back to their original volume as supplied by the vendor (i.e. 25-440 ul).

The binding efficiency of varied amounts of purified indium-111 to a constant concentration of TF was no more than 2.0%. These data indicate that this purification process did not enhance binding of ¹¹¹In to TF.

Influence of the volume of indium-111 chloride solution and its purification on the incorporation of radioactivity into metal free transferrin

	<u>Test Tube No.</u>				
	1	2	3	4	5
<u>Reactants added</u>					
Nonpurified In-111-Cl ₃ (uCi)	----	---	---	---	69.0
Purified In-111-Cl ₃ (uCi)	474.0	262.0	119.0	43.0	--
(ul) Equivalent to commercial Stock sol.	440.0	220.0	100.0	60.0	25.0
(ul) Acetate Buffer pH. 7.4	---	100.0	150.0	--	200.0
<u>(ul)-TF (1x10⁶ molecules)</u>	<u>1.0</u>	<u>1.0</u>	<u>1.0</u>	<u>1.0</u>	<u>1.0</u>
Total Vol (ul)	201.0	201.0	201.0	201.0	226.0

INCUBATION AT 37 C, 30 MIN.

	<u>(Followed by)</u> <u>GEL FILTRATION</u>				
(uCi) bound to TF	5.0	4.0	1.6	0.2	59.0
% bound	1.0	1.8	0.8	---	85.0

In summary, we have been studying potential in vivo selective labeling of human neutrophils for the past two years. Our achievements are the following:

- A. The development of a radiochemical agent:
 - 1. The conjugation of FMLP to TF.
 - 2. The radiolabeling of FMLP-TF-indium-111.
 - 3. A specific radioactivity of 2.4×10^2 Ci/mole TF.

- B. The radiolabeling of neutrophils:
 - 1. Relationship between FMLP-TF and neutrophils.
 - 2. Effect of environmental factors surrounding the cells on the labeling efficiency.
 - 3. The ability to specifically label both canine and human neutrophils.
 - 4. A cell labeling efficiency of 60%.

- C. Defining problems that limit current application:
 - 1. The inability to increase the specific activity of FMLP-TF-indium-111 beyond 2.4×10^2 Ci/mole TF.
 - 2. Discovering that the radiolabeling of FMLP-TF was dependent on the volume of indium-111 solution rather than on the amount of radioactivity used.

Conclusion

In the past year, our ability to increase neutrophil labeling to an efficiency of 60% is encouraging. However, we still need to improve the specific activity of FMLP-TF-indium-111 beyond the 2.4×10^2 Ci/mmol TF. Improvement in specific activity will allow higher quantities of radioactivity to be incorporated into the cells. Once we produce neutrophils that contain high quantities of radioactivity, we can investigate the mechanisms by which FMLP-TF-indium-111 is incorporated into neutrophils. The better we understand these mechanisms, the more we can influence them to improve the labeling efficiency beyond 60%. In the renewal proposal we describe experiments to produce a macromolecule which we believe may provide a solution to the problems described in "C" above.

5. The Development of Short-Lived Indium-109 (T_{1/2}-4.9 Hrs.) Using the Brookhaven National Laboratory Cyclotron

The physical characteristics of short lived ^{109}In with a 203 keV gamma photon (74%) prompted us to investigate the development of this isotope in collaboration with BNL. The synthesis of this tracer would be particularly useful since the entire spectrum of cell labeling techniques could be immediately transferred from Indium-111 to Indium-109 with a substantial reduction in patient irradiation. Because of the larger doses that could be given, improved scintigraphs with Indium-109 oxine labeled blood cells would be possible for the investigation of cardiac inflammatory response, abscess detection, and animal and/or human pulmonary embolism detection. The observation by others (Lavender et al., Hammersmith Hospital, presented to the Fleischner Society in May,

1982, Vienna, Austria) that Indium-tropolone labeled leukocytes have a shorter residence time in the lung than do Indium-oxine labeled leukocytes might be of particular import with Indium-109 when used for the detection of myocardial disease.

The following production technique was studied at BNL in collaboration with Dr. R. Lambrecht in the BNL hot-atom chemistry group (Dr. Wolfe). The production method studied was the alpha particle bombardment of enriched silver-107 (99.5% pure, natural abundance - 51.3%). This was bombarded with the 46 MeV alpha particle beam from the BNL cyclotron. When this occurs, the desired $^{107}\text{Ag}(\alpha, 2n)$ occurs but in addition the (α, n) and $(\alpha, 3n)$ reactions also take place. This produces impurities such as Indium-110 and Indium-108 in addition to the desired Indium-109. Indium-110 in particular with half-life of 4.9 hours with unusable high energy gammas needs to be eliminated or minimized in the production scheme. The experiments performed consisted of construction of 13 thin silver-107 targets deposited on 0.002 inch thick aluminum foil and stacked together for bombardment with the 46 MeV incident energy alpha particle beam. A 0.005 inch thick copper foil placed in front of the stacked targets served as a beam monitor. Each target contained about 33 mg of silver per square centimeter.

Following bombardment, the 13 foils were analyzed by gamma spectroscopy to determine the optimal excitation function. These data are currently stored on a computer and will be analyzed by N. Lambrecht at BNL. At the time of this writing, this analysis has not yet been completed. We anticipate, however, that it will be finished by the end of the current contract year.

Appendix

Published abstracts or articles from the 1981-1982 contract period:

1. Thakur, M.L. Cell labeling: achievements, challenges, and prospects. J. Nucl. Med. 22: 1011, 1981.
2. Zoghbi, S.S., Thakur, M.L. and Gottschalk, A. Investigations into agents for improving cell labeling with positron and gamma emitting radionuclides. J. Label. Comp. Radiopharm. 18: 280-281, 1981 (abst.).
3. Zoghbi, S.S., Thakur, M.L. and Gottschalk, A. et al. Selective cell labeling: A potential radioactive agent for labeling of human neutrophils. J. Nucl. Med. 22: 32, 1981 (abst.).
4. Thakur, M.L., Walsh, L.J., Zaret, B.L. and Gottschalk, A. Effect of antiarrhythmic drugs on the chemotactic ability and nylon wool adherence of Indium-111 labeled human polymorphonuclear leukocytes. J. Nucl. Med. 23: 131, 1982.
5. Zoghbi, S.S., Sostman, H.D., Neumann, R. et al. The influence of heparin on the in vivo distribution of ¹¹¹In-platelets in experimental canine model. J. Nucl. Med. 22: 55, 1981 (abst.).

Articles in press:

1. Zoghbi, S.S., Thakur, M.L., Gottschalk, A. et al. A potential radioactive agent for the selective labeling of human neutrophils (to be submitted Int. J. Appl. Rad. & Isotopes).