Cell Labeling: Achievements, Challenges, and Prospects

The potential value of the use of radiolabeled cellular blood elements for the noninvasive diagnosis and in vivo determination of cell kinetics has long been recognized. To facilitate such studies, scientists have investigated many gamma-emitting radionuclides with physical characteristics superior to those of Cr-51 and P-32, which were previously used as tracers for cellular blood elements. From their investigations in 1976, McAfee and Thakur concluded that In-111 chelates of oxine, acetylacetone, or tetraphenylporphyrin were promising agents (*1*). They also observed that In-111 acetylacetone incorporated with erythrocytes in higher quantities than it did with an equal number of leukocytes. However, separated blood cells, such as leukocytes, are frequently contaminated with erythrocytes, and since these may absorb a significant proportion of radioactivity, In-111 acetylacetone was not studied further. Indium-111 tetraphenylporphyrin was considered unacceptable for routine use because preparation is lengthy and inefficient. Studies of In-111 oxine revealed that the lipid solubility of the agent allowed the compound to diffuse passively through the cell membrane and thereby provided an efficient incorporation of radioactivity with each type of isolated blood cell as well as with tumor cells (*2–6*). The impetus generated by these findings has led to a growing number of investigations into exciting experimental and clinical applications (*7*).

There are, of course, limitations to the In-111 cell-labeling technique as well as challenges that scientists must meet to make the technique simpler, faster, and yet reliable. Let us first consider the limitations in the current technique.

The stability constants of In-111 oxine (log K = 11) are such that, once within the cell, In-111 dissociates from the compound, binds to cytoplasmic components, and thereby remains in association with cells in vivo (*2,4*). Oxine then diffuses out of the cell (*2,8*), but the low stability constants of In-111 oxine do not prevent the inherent problem of the formation of In-111 complex with plasma transferrin (log K = 30). This reaction impedes the incorporation of radioactivity into cells, and, consequently, plasma must be eliminated from the cell-suspending medium. Human platelets in particular, when suspended in nonplasma media such as normal saline, show detrimental effects as indicated by in vitro aggregation (36 ± 8%), which is lower than that of platelets labeled in autologous plasma (100%) (*9*). Citrated saline and a modified Tyrode's solution reportedly are better media than saline, yet the in vitro platelet aggregation remains only 30 ± 8% and 66 ± 15%, respectively, of that of platelets in plasma (*9,10*).

The half-life of In-111 (67 hr) allows investigators to carry out cell kinetic and localization studies without excessive radioactive decay, and the two gamma photons (173 keV 89% and 247 keV 94%) of the radionuclide permit adequate scintigraphic imaging. Auger electrons emitted by the radionuclide, however, deposit several ergs of energy and impart a high radiation dose to each labeled cell. For example, it has been calculated with certain assumptions, that when 10^8 neutrophils are labeled with 1 mCi In-111, each neutrophil receives an approximately 17.5 krad dose during the complete decay of In-111 (*11*). Despite the high radiation dose, studies have shown that the labeling of human neutrophils and platelets with many more times radioactivity than the standard dose (500 μCi per 10^8 neutrophils or 10^{10} platelets) does not alter the chemotactic ability and microbial capacity of neutrophils, nor does it affect the in vitro aggregation of labeled platelets (*9,12,13*).

Lymphocytes are sensitive to radiation, however, and conflicting results regarding the function of labeled lymphocytes have been generated. Some investigators have reported that In-111-labeled lymphocytes have normal distribution, recirculation, and the ability to accumulate in transplanted hearts and kidneys at the time of rejection (*14–16*). Others have observed that labeled lymphocytes (150 μCi/10^8 cells) have altered in vivo migration (*17*) and that the mitotic index was inversely correlated with In-111 radioactivity (Silberstein, EB; personal communication). Since T lymphocytes have a long life span and the ability to proliferate, the possible risk of neoplastic

transformation has been discussed, although only approximately 0.1% of the total circulating lymphocytes are labeled with In-111 and administered. In studies of patients treated by intrasynovial injection of Y-90 and Au-198 for rheumatoid arthritis of the knee joint, it was shown that an accumulation of radioactivity in the draining lymph nodes occurred and subjected recirculating lymphocytes to an intense irradiation indicated by the high proportion of chromosome aberrations (*5*). The procedure has not been associated with any serious consequences, but from this evidence, obviously, the smaller the radiation dose, the better the procedure.

In a normal preparation of 1 mCi In-111 oxine, 50 μg oxine are used, and the complex is dissolved in 50 μl ethanol. Therefore, when the entire dose is used in a normal labeling procedure, 10^8 neutrophils or lymphocytes, or approximately 10^{10} platelets, suspended in 4–5 ml, are exposed to 50 μg oxine and 50 μl ethanol. Extensive studies by several groups have revealed that neither oxine nor ethanol is toxic to cells until the chemical concentration is increased to greater than five times the standard amount (*9,12,13*).

More serious limitations occur because In-111 oxine is a nonspecific agent. It labels all cell types indiscriminately, necessitating separation of the desired type of cell from whole blood. Although the cell-separation procedures are not difficult, they are less than optimum, time-consuming, and if improperly performed, may have detrimental effects on the cells. Leukocytes are easy to obtain, but they invariably are contaminated with 5–25% erythrocytes and cannot be used for kinetic studies of a single cell type. Pure neutrophils, obtained by a gradient-separation technique followed by a hypotonic lysis of erythrocytes, have been shown to accumulate in the liver in higher quantities than those obtained similarly but injected without elimination of erythrocytes (*18*). The separation procedure for lymphocytes requires them to be exposed to a nonisotonic, nonphysiologic medium, and frequently they are highly contaminated with platelets. The centrifugation method used for platelet separation may eliminate the younger, denser, and physiologically more active platelets, but improper centrifugation may cause degranulation and result in reduced platelet aggregation (*9*).

The foregoing discussion indicates that there is a compelling need for (1) a radioactive agent that will label cells in plasma, (2) an agent that has physical characteristics similar to those of In-111 but that induces a smaller radiation dose to labeled cells, and (3) agents that will specifically label only one type of cell in whole blood and thus eliminate the need for cell separation. The aspiration for such refinements in cell-labeling techniques has fostered investigations of several new agents. Sinn and Silvester have prepared In-111 acetylacetone in an aqueous medium, reinvestigated its use as a cell tracer, and reported encouraging results (*19*). To avoid the use of ethanol, Goedemans prepared In-111 oxine in acetate TRIS buffer, and McAfee et al. demonstrated the use of water-soluble oxine sulfate (*20,21*). In his comparative evaluation, Goedemans reported that acetylacetone is 21 times less toxic than oxine. He observed, however, that to obtain labeling yields close to those of In-111 oxine, the acetylacetone concentration must be 120 times greater than that of oxine. For this reason, actual toxicity to cells from acetylacetone is no less than that from oxine (*20*).

The newcomer in this series of compounds is In-111 tropolone, described in this issue by Dewanjee, Rao, and Didisheim (*22*). Their thorough and methodical investigations into this water-soluble agent have led them to report that In-111 tropolone labels canine platelets suspended in citrated saline as well as in citrated plasma. Unfortunately, they also observed that the platelet labeling efficiency decreased as the plasma concentration in the medium increased. Platelets suspended in a medium containing 25% plasma, for example, incorporated only about 20% In-111 radioactivity (Fig. 2). Platelets labeled in citrated plasma also have reduced in vitro aggregation. For higher labeling efficiency and better platelet viability, therefore, the authors have recommended citrated saline as a labeling medium. In this medium, In-111 tropolone produces the highest labeling yield (80%) at pH 9 (Fig. 7) (*22*) compared with a pH 6.5 when In-111 oxine is used as a labeling compound (*10*). At alkaline pH (pH 9 and above), platelet permeability increases and degranulation probably occurs. Perhaps for this reason, only about 15% of the radioactivity is retained in platelets suspended in the medium at pH 11 (Fig. 7) (*22*). At the most commonly used pH, 6.5, In-111 tropolone produces approximately 55% labeling efficiency in ACD saline (Fig. 7) (*22*), compared with that of 73 ± 4% in the same medium and 86 ± 7% in modified Tyrode's solution with In-111 oxine (*9,10*).

The LD_{50} of tropolone administered to mice by intraperitoneal injection is 15–200 mg/kg body

weight compared with 88.8 mg/kg of oxine. The addition of tropolone to platelets in plasma dramatically reduces platelet aggregation (Fig. 8, top center) compared with that in plasma without added tropolone (Fig. 8-PRP-left) (*22*). (Figure 8 does not show the transmittance scale, but the comment is supported by the authors' statement in the text (*22*).) This effect of tropolone contrasts with that of oxine in plasma (*8*).

During the past few years, we have attempted to develop agents that will (1) replace In-111 and reduce radiation dose to cells, (2) enable us to use plasma as a labeling medium in order to preserve cell viability, and (3) permit selective cell labeling in whole blood by a kit method. The physical characteristics of Ru-97 ($t_{1/2}$ of 2.88 days, γ of 215 keV; 91%) are similar to those of In-111. Unlike In-111, however, ruthenium is not expected to bind to blood proteins at physiologic conditions and may permit cell labeling in plasma. Assuming similar subcellular localization, the radiation dose to each labeled cell from Ru-97 may be as little as $\frac{1}{3}$ that due to In-111 (*23*). Using commercially available, longer-lived, no-carrier-added Ru-103 as a tracer, we have prepared and evaluated ruthenium-labeled oxine and its analogs, Ru-tropolone, Ru-acetylacetone, and other ruthenium-labeled lipid soluble complexes (*23*, and unpublished data). Of these Ru-103 oxine incorporated the highest quantity (55 ± 4%) of radioactivity into human platelets suspended in plasma. Further attempts to enhance the yields, however, have not yet been successful.

To assess the feasibility of using positron-emitting Co-55 ($t_{1/2}$ = 18.5 hr) as a tracer for blood cells, investigations were carried out using the longer-lived Co-58 oxine (*23*). Although 22 ± 3% of the added Co-58 oxine became associated with platelets in plasma, only 14 ± 3% was retained after washing the cells. Similar low-labeling yields and losses of radioactivity after washing were also observed when cells were labeled with Tc-99m tropolone. In a comparative study of radioactive metal oxines, Dewanjee et al. have noted that cell labeling yields were In-111 $\gg$ Ga-67 $\gg$ Co-57 > Tc-99m > Cr-51 > Fe-59 (*24*). From these results it seems clear that of the potentially useful radionuclides, cell cytoplasmic components have the highest affinity for In-111. This affinity thereby makes In-111 a leading cell tracer despite its high radiation dose to labeled cells.

The attempts to label cells in plasma and neutrophils selectively in whole blood appear to be promising. We have developed a new In-111 agent that is also nonspecific but is less toxic, soluble in an aqueous medium, and allows cell labeling in plasma in high yields (unpublished data). When chelated with Ru-103 or with Tc-99m, this agent, however, failed to label cells as effectively as with In-111. Further evaluations with this new In-111 agent are currently in progress. Promising preliminary results have been obtained and may lead to a procedure that selectively labels human neutrophils in whole blood by a kit method (*25*). The technique involves the use of a synthetic chemotactic agent, formyl-methionyl-leucyl-phenylalanine, covalently bound to human transferrin, which, in turn is chelated with In-111. The entire molecule acts as a chemotactic factor and is recognized by structurally specific receptors on the neutrophil surface. The chemotactic factor-receptor interaction leads to the internalization of the agent and thereby allows the incorporation of radioactivity into cells. The labeled neutrophils are expected to retain their biologic responsiveness to other types of chemotactic stimuli (*26*). If successful, this new technique will lead to a significant advancement in cell labeling.

Until five years ago, owing to the lack of a suitable radioactive agent, scientists were unable to carry out noninvasive investigations using cellular blood elements. Today, fortunately, we have several potential agents, and the continuous reassessment and refinement of techniques will unquestionably lead to the development of many more. It is unlikely, however, that small differences in labeling efficiency or ease of preparation will govern the choice of labeling agents. Far more important will be the discovery of those that will be nontoxic to cells and will offer effective solutions to the fundamental problems in cell labeling.

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