University of Colorado Health Sciences Center

School of Pharmacy
Molecular Toxicology and Environmental Health Sciences Program

Campus Box C235
4200 East Ninth Avenue
Denver, Colorado 80262
(303) 270-7170, (303) 270-7072

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Edmond H. Vernot, Ph.D.
American Petroleum Institute.
1220 L Street Northwest
Washington, D.C. 20005

Dear Dr. Vernot:

We are writing to provide an interim report on our progress on the Benzene Leukemia Project. We have experienced considerable success in achieving our goals as set out at our last meeting. These are described in general terms below. A detailed discussion of individual experiments and supporting data will be provided at our meeting November 8th.

As you know, much of what we have proposed to do with respect to characterizing the effects of benzene and its metabolites on stem cell regulation and cell-specific metabolism is dependent on the successful enrichment and purification of standardized populations of bone marrow pluripotential stem cells (PSC) and committed hematopoietic progenitor cells (HPC).

The strategy that we adopted in order to achieve this goal was to separate these populations based on differences in the expression of specific surface antigens among different populations of bone marrow cells. Since the routine separation and study of these cell populations require a continuing source of specific antibodies, much of the first year of the project was dedicated to obtaining cell lines and producing and purifying the necessary antibody reagents. As of April of this year, our initial purification and characterization of these reagents was complete, and initial labeling experiments had been performed on unfractionated murine bone marrow, resulting in the identification of PSC ($Thy\text{-}1^{lo};Lin^{-};Sca\text{-}1^{+}$) and HPC ($Thy\text{-}1^{lo};Lin^{-};Sca\text{-}1^{-}$) populations.

The separation of small numbers of highly purified PSC and HPC can be achieved by the use of flow cytometry to sort the PSC/HPC populations. This methodology can provide small numbers of highly purified cells for cloning and bone marrow reconstitution (transplant) experiments. However, much larger numbers of cells are required for many of the biochemical and in vitro functional experiments that are outlined in the proposal. We, therefore, conducted a series of experiments to determine the feasibility of obtaining enriched populations of PSC and HPC in preparative numbers, comparing a variety of different methodologies including: immunomagnetic bead separation, panning, and affinity chromatography. Immunomagnetic bead separation is currently in wide use for the purging of bone marrow for autologous transplantation in man. In this application, antibody-labeled magnetic beads are used to remove or purge tumor cells from whole bone marrow. Initial efforts in our laboratory revealed the technique capable of purifying cells. However, it proved cumbersome if not impossible to completely remove the beads from purified cell populations. Since PSC and HPC cells are regulated via surface-surface and surface-molecule interactions, we concluded that the presence of beads might interfere with future experiments and that the technique was unsuitable for our purposes.

Another approach we investigated was the use of antibody- panning, in which specific antibodies are affixed to culture plates. This strategy is based on the selective removal of cells bearing specific lineage antigens on their surface followed by the positive selection of Sca-1+ cells, which include the PSC population. Flow cytometric analysis of panned cells revealed a 2 fold removal of Lin+ cells based on surface marker expression; however, clonigenic analysis in methyl cellulose culture revealed a 5.5 fold enrichment of colony-forming cells using defined growth factors. This suggests a 5-6 fold enrichment of HPC over whole bone marrow, which may be useful for some metabolism experiments. However, far greater purification is necessary for many of experiments described in the specific aims of the proposal.

In order to achieve better purification, lineage positive cells were removed from mouse bone marrow by affinity chromatography using protein A, protein G, and anti-kappa columns. The percent positive cells after each purification was 20.93%, 1.30%, and 2.09% respectively, while the starting sample of bone marrow was 41.45%

positive for the lineage antigens. In a further experiment, lineage positive cells were purified using affinity chromatography (anti-kappa, followed by elution of the positive cells with excess rat IgG. The original cells contained 67.81% lineage positive cells and the affinity purified cells contained 94.95% positive cells. These results demonstrate that we can obtain large numbers of cells (20 - 100 million cells) at purities approaching 98% for either lineage positive or lineage negative cell populations. While flow cytometric sorting or limiting dilution techniques will allow us to obtain virtual 100% purification of individual cell populations for clonigenic studies, these preparative procedures should prove adequate for the preparation of defined populations for metabolic studies.

Initial experiments to validate the reconstituting ability of PSC and to characterize optimal growth factor conditions for the culture of murine PSC and HPC are in progress at the present time. A number of different approaches have been described for the enrichment of human PSC and HPC populations. Based on our successful experience with affinity chromatography for the isolation and purification of mouse cells, we have chosen to develop a similar approach for the purification of human cells, based on initial enrichment of cells bearing the CD-34 surface marker. These experiments will be described in greater detail at our November meeting. Based on the interest expressed in our pursuit of the human culture model, we have dedicated a full time person to this subproject.

In Dr. Ross's laboratory, training of personnel in metabolic techniques (enzyme assays, tissue culture and cell handling, HPLC and radiolabel binding assays) has been completed in preparation for the availability of purified cell populations from Dr. Irons' laboratory.

Initially, these efforts have focused on activation and cellular defense systems in mouse bone marrow cells which influence the binding of electrophilic metabolites such as quinones to cellular nucleophiles in specific cells. The measurement of enzyme activities represent the first step in metabolic typing of defined cell populations, which will be discussed further, together with work in human systems, at the forthcoming meeting. We now can routinely measure total peroxidase, DT-diaphorase, glutathione-S-transferase and cellular GSH/GSSG in whole bone marrow or isolated cell populations. Under certain conditions, benzoquinones may

generate an oxidative stress in cellular systems and enzymatic defense systems against oxidizing species, including GSH peroxidase and superoxide dismutase; which can also be measured.

Techniques to measure the effects of benzene metabolites on eicosanoid production in different cell types are currently under development. At present, HPLC techniques have been set up to measure PGE_2, PGD_2, PGF_{2a}, TxB_2 and 6-keto PGF_{1a}. We are attempting to optimize recovery of eicosanoids from macrophage systems and are currently evaluating solid phase extraction systems. In addition, we are investigating the optimal methodology for incorporation of radiolabeled arachidonate into cells. Once these systems are developed, pilot experiments will be performed and quantification systems developed for leukotrienes.

Additional efforts are focused on verifying published methodologies for induction of differentiation in HL-60 cells. These human leukemia cells can be induced to differentiate either to macrophages or granulocytes via specific stimuli, and we will utilize this system as one way to examine metabolism as a function of differentiation.

The final specific aim of the metabolism portion the project is to compare metabolic events in human and mouse bone marrow and hepatic systems. Comparison of bone marrow metabolism is underway. While hepatic metabolism is not a major emphasis, we are improving our mouse hepatocyte isolation techniques and setting up collaborations to develop human hepatocyte systems.

In conclusion, we have been successful in addressing purification and methodologic issues critical to our future progress on the project and are now in a position to go forward with in vitro murine studies outlined in the research proposal. We are proceeding with development of the human bone marrow purification and culture system and in vivo studies to validate our purification schemes in the mouse. We look forward to our meeting on November 8th.

Very truly yours,

Richard D. Irons

David Ross

Signed in their absence
RDI:vk