PROGRESS REPORT

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University of Hawaii School of Medicine

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A. Introduction

A number of problems have been solved and several changes have occurred which can broadly be classified as "administrative". One major change has been the appointment of a chairman in Pharmacology who has given strong support and encouragement to the development of this program. Similarly, the awareness and support of the University and the community has become more evident producing a marked improvement in the environment in which the program functions.

Space has been expanded. A separate building adjacent to the other laboratories on the Leahi campus grounds has been turned over for occupancy. The building consists of a general laboratory area (25' x 30') and a separate air-conditioned room (12' x 20') for isolated handling of animals and special procedures. A large hood is in transit (delayed by trucker strike) and will be installed in this building along with appropriate filtration to meet requirements of safety. Two other hoods are being provided by other funds (University), one in delayed shipment and one to be ordered July 1.

Licensure proceedings were initiated and the applicant has created a radiological safety committee at Leahi Hospital which has, in turn obtained an interim license for isotope use. Although a limited amount of isotope use has occurred, safety requirements still delay the full use of aerosolized radioactive material. However, this will soon be remedied (before the beginning of the contract year). At the same time, the applicant has written and provided an application for a broad license for the University around a number of political obstacles. Although it may seem not to be "progress" directly relatable to the present program, this has been a major advance in the promotion of safe and effective use of isotopes in this Institution. The applicant is now a member of the University Radiological Health Committee and will probably assume chairmanship about July 1.

Despite a few residual problems, it is considered that the most difficult part of the initiation of the program is now in the past and it is felt that the group has reached a point where a reasonable degree of productivity of quality research can be expected.

B. Summary of Progress in Current year (69-70)

1. Locus of Deposition and Redistribution of Particles
   a. Particle size data from rats and dogs exposed to Pu\(^{239}\)O\(_2\) from dogs exposed to Pu\(^{238}\)O\(_2\) and U\(^{238}\)O\(_2\) are virtually complete. Manuscripts are in preparation.
   b. Several exposures of rats to metallic oxides have been carried out. For example, exposures of rats for 6 and 12 hours to Cr\(_2\)O\(_3\) were followed by periodic sacrifice, washing of the lungs of some rats to obtain "free cells" for measure of the extent of cellular response and involvement in phagocytosis of the inhaled population. In the six-hour exposure the phagocytic index of the pulmonary cell washes were measured up to 36 hours post-exposure. Sample results are shown in Figure 1. The data on the particle number distribution have
not yet been completed from this study but the pattern of the phagocytic index is similar to the measure of mean or median particle content of cells for four-hour exposures to Cr\textsubscript{2}O\textsubscript{3} and Fe\textsubscript{2}O\textsubscript{3}. Data for the iron study are presented in Figure 2.

c. A series of 12-hour exposures was arranged to provide a larger initial load. For these exposures, no early pattern of time has been carried out - rather a few points of time from one to fifteen days after termination of the exposure. No consistent changes in phagocytic index have been seen, which is not surprising as judged by previous data. However, the relative degree of phagocytic activity of the cells can be judged by a rough distribution of particle number concentration. Table 1 presents some exemplary data.

<table>
<thead>
<tr>
<th>Time after exposure</th>
<th>Percent cells &lt; 10 particles</th>
<th>Percent cells &gt; 10 particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>13.9</td>
<td>46.6</td>
</tr>
<tr>
<td>5 days</td>
<td>17.5</td>
<td>49.1</td>
</tr>
<tr>
<td>12 days</td>
<td>31.1</td>
<td>31.5</td>
</tr>
<tr>
<td>15 days</td>
<td>28.4</td>
<td>34.4</td>
</tr>
</tbody>
</table>

From these and other data, a number of points are becoming clear. Among these: - it appears that there is a peak phagocytic response at some hours (4-8) after the termination of exposure, that the phagocytic index returns to immediate post-exposure levels within 24 to 48 hours and a significant phagocytic index may be retained for weeks with continued active phagocytosis of large numbers of particles.

Similar exposures have also been used for other purposes. Some of these data and others summarized below will be presented at the 10th Annual Hanford Biology Symposium (1).

2. Pulmonary Cells, Cytodynamics

a. Cell counts at periods of time after exposure represent one measure of the alveolar cell response to dust loads just as the cell turnover rate has been an index of cellular proliferation after inhalation exposures or treatment with "stimulants". (2,3). Prior experience with cell counts (Progress Report 68-69) indicated that this was an inconsistent measure. Using higher concentrations of aerosol, longer exposures, various collection techniques and larger numbers of animals does not reduce inconsistency sufficiently to make the technique useful.

b. Use of several immunological agents were found to stimulate an alveolar cell proliferative response. However, respiratory difficulty (with occasional death) accompanied doses which were
necessary to produce a marked and obvious change of cell number. Furthermore, the microscopic appearance of the cells included a high degree of vacuolization, loss of cellular membrane definition and other evidence of cytotoxicity. Pertussis and rodent antimalarial preparations appeared slightly less toxic for the response produced. Lower doses may produce an appropriately "primed" animal without evidence of toxicity or chemical entities which enhance lipid synthesis (as opposed to immunologic agents) may have more potential.

c. Among the histochemical techniques applied to alveolar cell populations and to lung sections of both control animals and those exposed to metallic oxides, early data on succinic dehydrogenase is available. Following as little as four hours' exposure to a concentration of about $3 \times 10^5$ particles/liter of air (0.1 μ "monodisperse") changes in SDH can be detected. The most interesting observations are 1) a progression of increased intensity of SDH stain at one day in alveolar walls, and a three-day peak in mobile macrophages and 2) a still more intense staining reaction at 7-9 days after exposure accompanied by a reduction in the macrophage content of SDH.

d. Early changes in some other enzymes have been sought on a more quantitative basis. For example, lactic dehydrogenase has been measured at various times after exposure to Cr₂O₃ both in terms of the total enzyme activity and the relative concentrations of the five common isozymal bands. An example of some of these data is shown in Figure 3. Although all LDH bands change during this period, the return of the total activity is accounted for, not by shifts within the spectrum, but in increase in bands IV and V with the suggestion of a lag period between the two bands. Similar data have been obtained for a 12-hour exposure and with different times of sampling. No changes in catalase activity have been found. As an aside, some of the work from the latter exposure are being redone as it has been discovered that procedures published in the literature may lead to erroneous conclusions.

3. Alveolar macrophage

a. A major effort was expended in the search for a means of understanding the origin, development and activation of alveolar macrophages using immunologic techniques. Antigen was prepared as follows. Lung washes from rats were made with cold saline containing 0.2% EDTA. The cells were washed, sonicated and centrifuged to separate "soluble fraction" from membrane fraction.

Each fraction was injected into a rabbit in doses of 0.25 ml (in Freund's adjuvant) in right and left flanks and intraperitoneally. Injections were made once a week for three weeks with an additional injection 10 days later. Antiserum was collected one week after the final injection.

The antisera were plated on agar against five different sources of antigen. Antiserum created from injected "membrane fraction" showed a
"reaction of identity" when plated against lung mince, alveolar macrophages and peritoneal macrophages, with no crossing over of the reaction lines. With the "soluble fraction" of the lung wash, however, the response was different. There was evidence of three separate antigen-antibody reactions as shown diagramatically below.

1 and 4 - lung mince
2 and 5 - lung cell wash
3 and 6 - peritoneal wash

There was no remarkable response when the fraction was tested against bone marrow or liver preparations.

From an animal receiving only the "membrane fraction" antiserum from lung wash injections were treated with cold saturated (NH₄)₂SO₄ to precipitate globulins, the protein was conjugated with FITC (Fluorescein isothiocyanate). The conjugate was then applied to fresh-frozen sections of lung and liver as well as to smears of bone marrow and peritoneal cells. As might be expected from the plating studies, distribution of fluorescence was wide. Among the observations made thus far were the following:

a) peritoneal macrophages showed a nuclear reaction while bone marrow cells showed only a cytoplasmic reaction
b) Liver cells of several types were "labelled" in both nucleus and cytoplasm.
c) in lung elements of alveolar wall showed reaction as did alveolar macrophages. "Soluble fraction" antiserum was to have been used and would have been of greater interest but was aborted for technical reasons.

3. Phagocytosis
   a. Peritoneal Cells

Although most in vitro work has concerned itself with lung, some additional work was completed using peritoneal exudates. The influence of Ca and Mg ions on phagocytosis of UO₂ particles (Figure 4) in part confirmed a previous finding, viz., that there is a specificity to Ca in the enhancement of phagocytosis rather than the non-specific effect of a divalent cation. However, the pattern of the
curve is somewhat different than that found for other particles \(4,5\) although there is an approximately peak effect around the \(10^{-2}\) M Ca ion concentration.

Obversely, \(UO_2\) ion which was previously demonstrated to have a time and concentration dependent effect on the phagocytosis of \(Cr_2O_3\) particles was also extended to include another variable previously reported (Previous Progress Report) viz., the ratio of particles to phagocytic cells to promote optimal phagocytic response. Figure 5 illustrates at ratios of approximately 2, 5 and 8.5 that there is increasing response in control populations and \(10^{-10}\) M \(UO_2\) ion as the ratio increases and there is a consistent enhancement at \(10^{-7}\) M \(UO_2\) ion and equivocal responses at \(10^{-4}\) M. It may be noted that the relative increase in phagocytic index at \(10^{-8}\) M varies but the absolute increase is approximately the same in each case.

For comparison with anticipated metabolic examination of alveolar cells during the phagocytic process other variables have been examined. A few interesting data have been obtained but two prosaic bits are reported here to illustrate problems both with our studies and those reported in the literature. Virtually all studies in the literature attempt to arrive at cell concentrations greater than \(0.5 - 1.0\) million per ml of incubation medium. As an example of one potentially serious pitfall, Figure 6 is presented showing the rate of glucose utilization as a function of cell concentration. Obviously the cell concentration will markedly influence the metabolic parameters measured. As another small point, the chance collision postulated as one determinant in such studies \(5\) suggests that some measure of "contact potential" should be supplied with experimental method. Figure 7 shows a phenomenon found for both peritoneal and alveolar cell populations.

b. Alveolar cells

The response of alveolar cells to \(UO_2\) ion in a \(Cr_2O_3\) system roughly parallels that found with peritoneal cells (Previous Progress Report). Using \(Fe_2O_3\) particles, the pattern was somewhat different. In Figure 8, data from one such experiment are presented and illustrate a progressive rise in phagocytic index throughout the range of \(UO_2\) ion concentration from \(10^{-9}\) M to \(10^{-4}\) M with no additional increase at higher concentrations (but no depression either).

Studies with compounds which may alter surface charge characteristics have continued. A number of materials have been used at molecular weight equivalents of an excess of 10,000. An example of some of the data is given in Figure 9. The interaction of polylysine is more pronounced and dramatic for \(Cr_2O_3\) particles with these alveolar cells than that which was observed with peritoneal macrophages (Previous Progress Report). Data from these and other studies with alveolar macrophages will soon be readied for submission for publication.
4. Pulmonary Effects

The chief interest in this area has settled into two main pathways. One of these is the pattern of changing enzyme constitution from a viewpoint of the alveolar response in its role in clearance but also with respect to these as early changes in a developing toxic response. These efforts have been alluded to in a previous section of this report. A second interest lies in the interaction of the chemical changes which may be expected and the radiologically induced changes with special attention to whether these are only quantitatively related or whether there are qualitative differences in effects. To this end, exploration of some aspects of the biochemistry of the lung have been initiated and some consideration has been given to the mutual influence of metallic oxides (chemical or radioactive) and other impinging toxic chemicals.

C. Publications

References made to personal publications and pending publications supported in whole or in part by this contract are listed below.


Other publications not directly related to the project:


D. Anticipated Progress in Next Contract Year.

Major directions of the research program are indicated in the renewal proposal and points of emphasis are suggested both in the proposal and in the outline of progress given above. The following is a summary of some of the accomplishments to be expected in the next working year.

1. All in vivo alpha emitter studies can be expected to reach print. In vitro work with peritoneal macrophages will have appeared in print or be in press and additional work on mast cell-particle interaction should be in manuscript form.

2. A program of inhalation of radioactive and stable materials will be well along and have superimposed on it the comparison of clearance rates and alveolar cell responses between two otherwise identical materials, the effect of pre-exposure (sub-chronic) on clearance and trials of cell proliferants on the clearance pattern.

3. Purification of alveolar cell antibody and more precise localization of immunologic and tissue reactivity will be carried out.

4. In vitro studies of alveolar cells will be extended to other particle species including some rare earths and transuranics to examine the influence of particle species and radiation on the phagocytic process.

5. Histochemical and isozymal patterns will be extended to include esterase, catalase, carboxypeptidase and perhaps ATPase related on the one hand to the activities of these materials in the cellular role in clearance and, on the other hand, to initial effects of the inhaled materials.

6. Initial exploration will be carried out in the general area of lung "biochemistry" in which we will focus some attention on the synergistic potential of other environmental substances on the behavior of inhaled particulate material.
Figure 2

PARTICLES/CELL

PERCENT OF 4 HR. EXPOSURE VALUE

140

100

60

EXP.

POST-EXPOSURE HOURS

48
FIGURE 3

TOTAL ACTIVITY

CONTROL TOTAL ACTIVITY ± S.E

BAND IV

BAND III

Total LDH Activity (mU/mg Protein)

LDH Activity (mU/mg Protein)

Hours after CaO Exposure (6 hours)
Figure 4

- PHAGOCYTIC INDEX

- LOG-MOLAR CONC. Ca⁺⁺ OR Mg⁺⁺

[CELL] = 0.9 x 10⁶/cc
[UCO₂] = 5.0 x 10⁶/cc
20 min. 37°C
Figure 5

[CELL] = 2.0 \times 10^6/\text{cc}
[Cr_2O_3] = 17.3 \times 10^6/\text{cc}
20 \text{ min. } 37^\circ \text{C}

[CELL] = 2.2 \times 10^6/\text{cc}
[Cr_2O_3] = 11.5 \times 10^6/\text{cc}

[CELL] = 2.8 \times 10^6/\text{cc}
[Cr_2O_3] = 6.3 \times 10^6/\text{cc}

LOG-MOLAR CONC. UO_2^{++}
Figure 7

Phasocptic Index

Incubator Shaker Speed

90 120 160 200

$\text{Cu}_2\text{O}_3$

$\text{Fe}_2\text{O}_3$
ALVEOLAR CELLS

PHAGOCYTIC INDEX

CELL CONC. = 0.815 x 10^6/cc.
Fe_2O_3 = 6.75 x 10^6/cc.
20 MIN. 37°C
10^-4 M Ca^{+2}