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ABSTRACT

During the year May, 1972 to April, 1973 our laboratory carried on investigations in four separate areas. By analyzing the pre-ribosomal ribonuclear protein particles in the nucleolus and resting and phytohemagglutinin (PHA) stimulated lymphocytes we were able to show that protein synthesis is necessary for the increased production of ribonuclear protein precursor particles in the nucleolus in response to PHA.

We continued our analysis into the nuclear activation of lymphocytes by extracting the polynucleotide particles synthesized by isolated nuclei in response to PHA.

We reconfirmed some of our previous observations regarding the delayed response of chronic lymphocytic leukemia (CLL) lymphocytes to PHA. Our new studies established that a delayed response could only be seen with the leukemia cells and other unresponsive cells would not behave in this fashion nor could they induce a delayed reaction in a diluted population of normal cells.

Finally we completed a project on monocyte metabolism demonstrating that phagocytosis increases ribosome synthesis in the nuclear monocytes within 1-2 hours of exposing these cells to latex particles. Furthermore, similar phenomena could be reproduced by exposing monocytes to an activating factor isolated from the supernatants of stimulated lymphocytes.
During the year May, 1972 to April, 1973 our laboratory has been engaged in four separate projects:

A. **Preribosomal Ribonucleoprotein Particles (RNP's) and the Control of Ribosome Assembly.**

We were able to refine our technique for isolation of RNP particles by treating isolated lymphocyte nuclei with desoxyribonuclease in high salt buffer in the presence of Ficoll. This allowed us to deal with pure nucleoli in extracting RNP particles from these structures without concern over contaminating nuclear protein particles not ribosomal in origin. Although the extra manipulation cut down our yield, the characterization of these RNP particles in sucrose gradients were not significantly different from previous results obtained by extracting particles from whole nuclei. However, the additional refinement enabled us to interpret the data with a little more confidence.

When intact resting lymphocytes were incubated with tritiated uridine for one hour before extraction and isolation of RNP particles, the pattern of labeling on sucrose gradients revealed a single peak of tritium label sedimenting at 80S. There was little evidence of processing to the more mature 60S particles during this labeling interval. It will be recalled that nascent preribosomal RNA (45S RNA) is quickly incorporated into 80S ribonucleoprotein particles while still in the nucleolus. Eventually these particles are cleaved into a heavier than 60S particle and approximately 30S particle, the
latter being exported immediately to the cytoplasm while the former remains in the nucleolus to be further cleaved to a 60S particle before itself being exported to the nucleus and joined with the 30S particle to form an intact 73S cytoplasmic ribosome. In nucleolar preparations, uncontaminated by cytoplasm, there are little or no 30S particles detected at any given time. Still, it is significant to note that in resting lymphocytes the one-hour interval was insufficient for any other newly labeled 80S particles to be cleaved and converted into the more mature 60S units.

We made an effort to establish the kinetics of 80S RNP processing to 60S products by following a one-hour exposure to tritiated uridine (pulse) by a 1-3 hour further incubation in the presence of an excess of unlabeled uridine and actinomycin-D (pulse-chase). The results of this experiment were not entirely reliable because of the problems in uniform labeling of the UTP precursor pool during a one hour interval. However, we were able to "chase" most of the label from the 80S peak sedimenting at 60S. This confirmed the precursor-product relationship of the RNP particles.

When the intact resting cells were incubated for the one hour interval in tritiated leucine (in a leucine-free medium), labeling was found exclusively in the 60S peak. We therefore concluded that most, if not all, of the newly labeled protein was utilized for the formation of mature 60S particles. Clearly the protein used for the nascent 80S particles was drawn from a pre-existing pool. This finding lent more credence to our previous
theory that ribosomal protein synthesis played some regulatory role in the post-transcriptional maturation of nascent ribonuclear proteins. Thus, incubating the cells in puromycin to inhibit all protein synthesis, completely inhibited the appearance of nascent uridine labeled 60S particles while the appearance of uridine labeled 80S particles was virtually unaffected.

The resting lymphocytes were then exposed to phytohemagglutinin (PHA) for a short 1-2 hour incubation period. It will be recalled that during this interval we have already demonstrated increased ribosomal RNA (rRNA) transcription and increasingly efficient processing of the nascent RNA into the mature 28S and 18S species found in the cytoplasm. No morphologic changes could be seen at this time. When RNP particles were isolated from the cells incubated with PHA for two hours, a 2-3 fold increase in the degree of uridine labeling of the 80S particles was observed. Furthermore, there seemed to be some evidence that more 60S particles were processed in these stimulated cells than in resting cells during a comparable interval. However, because of the uncertainties in precursor pool labeling, we were unable to firmly conclude that this phenomenon represented a more efficient degree of processing of the precursor particles in the presence of PHA.

The effect of PHA treatment on the incorporation of leucine was also seen in two hours. As in the resting cells, the increased incorporation was found exclusively in the 60S particles. There was no evidence of increased labeling of the nascent 80S particles at this time. The 2-3 fold increase in the rate of leucine labeling
in the 60S particles occurred many hours before the earliest detectable increase in overall protein synthesis measured in whole cells after PHA treatment. Therefore, this stimulation of protein synthesis within two hours of the initial PHA exposure indicates a unique phenomenon occurring in the mature RNP particles and does not appear to reflect an overall increase in protein synthesis by the whole cell. This early period of PHA stimulation marks the first detectable increases in RNA synthesis suggesting that the 60S particles contained additional proteins coded by pre-existing RNA messages. Such a phenomenon was predicted in our discussions presented in the project proposal for this contract one year ago.

From these experiments we have tentatively concluded that new ribosomal protein plays some role in the initiation of efficient ribosome processing in a stimulated cell. We are suggesting that this phenomenon bears some relationship to the post-transcriptional control of ribosome synthesis. In more recent experiments we have shown that puromycin almost completely inhibits the effect of PHA on the appearance of increased label in the RNP particles. The same dose has no effect on the uridine labeling of resting cells. Thus the pattern of labeling seen in puromycin-treated PHA stimulated cells is indistinguishable from that of the resting cell. Current experiments are designed to distinguish between an effect of puromycin on the steps regulating transcription (ribonuclease and chromatin activity) from post-transcriptional controls. It is entirely possible that the events immediately following exposure
to PHA depend upon the synthesis of new enzyme necessary for the increased RNA transcription.

This material has been published and included in the Appendices to this report as Appendix A and B. A more refined version was presented at the December, 1972 meetings of the American Society of Hematology in Hollywood, Florida.

B. Direct Nuclear Activation of Lymphocytes.

In the previous report, we outlined our successful attempts to stimulate isolated nuclei with PHA. We showed that after a one hour incubation of isolated intact lymphocyte nuclei with PHA there was a doubling in the rate of DNA-directed RNA synthesis. Simultaneously we detected a doubling in the amount of actinomycin-D binding to chromatin suggesting the availability of additional chromatin for transcription. This was also accompanied by a similar rise in histone acetylation. The latter two parameters reflected increased availability of metabolically active coumadien while we had no direct evidence for changes in DNA directed RNA polymerase activity. This information has appeared in publication and the reprint is included as Appendix C.

During the past nine months we have been attempting to confirm the significance of the direct nuclear activation. The previous studies gave no indication of a role in this phenomenon in the activation of intact cells. However, we have recently been able to isolate the RNA molecules synthesized by nuclei in a cell-free system. These molecules were characterized by sedimentation
across sucrose gradients. During a one-hour incubation of resting lymphocyte nuclei in tritiated nucleotide triphosphates in equimolar amounts resulted in the labeling of ribonucleotide molecules sedimenting in a polydisperse band over the heavier regions of a 5-20% sucrose gradient. If the intact cells were exposed to PHA for two hours before isolation of the nuclei for further incubation in a cell-free system with labeled nucleotide triphosphate, this resulted in a 3-fold increase in the appearance of labeled ribonucleic acid molecules sedimenting mostly at 20S. When nuclei from resting cells were incubated in the cell-free system with labeled nucleotide triphosphates in the presence of PHA, a similar increase of labeling was observed to occur in the 20S region of the gradient. Therefore the newly synthesized RNA species resulting from direct activation of nuclei closely resembled those observed in nuclei isolated from PHA-stimulated, intact cells. This served as more compelling evidence that the direct activation of lymphocyte nuclei reflected a phenomenon which might transpire during the whole cell stimulation by the mitogen. It should be emphasized that these RNA molecules do not represent any recognizable functional species as the cell-free system does not support the synthesis of intact 45S rRNA precursor. However, these nucleotide "fragments" sedimenting at 20S represented a reproducible phenomenon occurring in both systems as the result of PHA activation.

We were also concerned that the direct effect of PHA on lymphocyte nuclei might represent some physical phenomenon, unrelated to the cell's ultimate ability to enlarge and divide in
response to PHA. However, we could show that chronic lymphocytic leukemia (CLL) lymphocytes show a greatly diminished delayed response to PHA and therefore represented a unique opportunity to assess the role of the direct activation in the ultimate process of cell growth. Thus, actinomycin-D binding and histone acetylation was stimulated to a significantly lesser extent in the nuclei of CLL lymphocytes exposed to PHA when these parameters were compared to the effect of PHA on the nuclei of normal lymphocytes. Furthermore, we examined blast cells removed from the circulation of patients with acute lymphoblastic leukemia (ALL). Nuclei for these cells bound 10 times as much label from actinomycin-D as the unstimulated nuclei from resting lymphocytes. Presumably this represented the heightened transcriptional activity in the nuclei of the more metabolically active blast cells. The rate of histone acetylation in these blast cell nuclei was similar to the nuclei isolated from whole lymphocytes. When nuclei of ALL blast cells were exposed to PHA there was no effect on either the rate of actinomycin-D binding or the rate of histone acetylation. Thus, the effect of PHA on isolated normal nuclei took on more physiologic significance as nuclei from functionally defective cells showed a decreased ability to respond to PHA and the nuclei of metabolically heightened blast cells could not further be stimulated in this system. Actinomycin-D binding presumably reflects the availability of metabolically active chromatin for RNA synthesis. This was at minimal levels in the nuclei from resting cells and CLL cells. It
was heightened in the nuclei of freshly isolated ALL blasts, in the nuclei of PHA stimulated cells and in the nuclei of resting cells subsequently treated with PHA. Histone acetylation on the other hand probably reflects alterations in chromatin as additional segments are made available for RNA synthesis. Increased histone acetylation was seen to occur only in those cells responding to PHA. Nuclei from the more metabolically active ALL blasts were not altered by this brief exposure to PHA.

C. Functional Characterization of Circulating Lymphocytes

Problems related to the identification of the nature of CLL lymphocytes were outlined in last year's project proposal. We had previously concluded that CLL lymphocytes were defective in that they showed a delayed and diminished response to PHA. Furthermore, this phenomenon could be detected in the very early phases of the disease showing a monoclonal pattern in that there was a single wave of reactivity following PHA exposure. This was true in advanced as well as early cases. The finding of monoclonal immunoglobulin determinants on the surface of CLL lymphocytes confirmed the restricted nature of the cell population but it also suggested that the cells involved were B-cells, unable to respond to PHA rather than defective T-cells. This would imply that delayed reacting cells reported in our previous publications might not represent CLL cells at all but a population of non-leukemic lymphocytes growing through the much larger population of inactive monoclonal B cells. Earlier experiments did argue against these conclusions as mixtures of CLL
lymphocytes and normal lymphocytes failed to reproduce the phenomenon of delayed reaction. Furthermore, pulse-chase studies done with autoradiographs demonstrated that only lymphocytes from CLL patients showed initiation of growth after 72 hours of incubation with PHA.

During the past year we have accumulated more data to indirectly support our original conclusions. Thus, analysis of the growth response to pokeweed mitogen (PWM) was indistinguishable from normal in CLL lymphocytes which showed a clear-cut delayed and diminished response to PHA. Kinetic studies of the growth response demonstrated that both normal and CLL cells mounted a peak response at approximately 120 hours after exposure to the PWM. Since PWM is well known to stimulate both T and B cells, it would be difficult to understand how CLL patients could harbor virtually no T cells and unreactive B cells.

We attempted to mimic a situation in the circulation of CLL patients where a small population of normal T cells could be retarded by a dilution in a much larger population of unreactive cells. This was accomplished by treating a fraction of isolated normal circulating lymphocytes with mitomycin C to prevent any growth in response to PHA. These treated cells were then mixed with varying proportions of autologous normal cells. The mixed treated and untreated populations were exposed to PHA and the kinetics of growth assessed. The peak growth response in all these cases was mounted at 72 hours. No delayed reaction was observed.
Thus, we still regard the delayed response unique to CLL lymphocytes. We fell that this represents a fundamental defect in the growth potential of these cells to certain mitogens. With this frame of reference assignment of a T or B nature to these cells adds very little insight as the disease most likely represents a more fundamental growth abnormality where the abnormal immunoglobulins and the defective growth response are both reflections of an underlying neoplastic transformation. This material is being prepared for publication.

We are still attempting to come to grips with the nature of the PHA reactive cell in the circulation of patients with ALL. Autoradiographic pulse-chase studies suggest that at least some of the metabolically and morphologically distinguishable blast cells can respond to PHA. The statistics involved in proving this point have become quite confusing and we are unwilling to release data at the present time. The most intriguing experiments involve lymphocyte populations containing 70-80% blasts where the PHA response is normal in both magnitude and kinetics. Furthermore, the leukemic blast cell can be distinguished morphologically from a "PHA blast cell." This type of observation clearly involves pitfalls. None of this information will be presented for publication until we are more confident of the data.

We have extended our functional characterization of lymphocytes to two unique diseases in an attempt to demonstrate the potential value of this approach in understanding the nature of neoplastic cells. One case involves a 30 year old woman with congenital
hypogammaglobulinemia and lymphocytosis of the blood, marrow, liver and spleen since early childhood. This patient was found to have obvious hypoplasia of all peripheral and central lymph nodes and never bore any recognizable tonsilar tissue. A splenectomy was performed in 1965 revealing a spleen pathologically identified as lymphosarcoma. Her basic disease has been rather progressive immunologic deficiency with sin-bronchial pulmonary infections and bronchiectasis. Aside from the pancytopenia accompanying the replacement of marrow with small lymphocytes she has had no major difficulties directly ascribed to the lymphoid neoplasia. Still, from our understanding of lymphoid malignancies, a patient showing myelophthisis by small lymphocytes together with infiltration of liver and spleen with destruction of architecture must be considered to have lymphosarcomatosis. This would not be CLL as the patient is young and there is no peripheral lymphadenopathy. Partial characterization of the circulating cells in this patient revealed a normal reaction to PHA and PWM in both magnitude and kinetics. Furthermore, unlike CLL, circulating lymphocytes from this patient showed the normal number of immunoglobulin bearing cells without any evidence of monoclonal IgM contaminant. Fluorescent immunoglobulin analysis on these cells were performed by Dr. Fred Segal at the Rockefeller Institute.

Despite the functional abnormality of the circulating lymphocyte when analyzed in vitro, this patient was severely impaired in her skin sensitivity and the humoral antibody response. Therefore, the state of immunologic deficiency was not reflected in a
neoplastic or even functionally defective population of circulating lymphocytes. The significance of this intriguing phenomenon is unknown but it is clear that we must reassess our understanding of the functional nature of neoplastic cells and that in vitro reactivity or surface characteristics are insufficient to predict the ultimate immunologic capacity.

We also had the opportunity to study a patient with Sezary syndrome, an unusual disease manifesting marked peripheral lymphocytosis with little or no marrow involvement but diffuse infiltration of skin and internal lymphoid organs with atypical lymphocytes. While the circulating cells had the morphologic appearance of atypical lymphocytes, functional analysis revealed the following results: (a) there was no reaction to PHA, PWM or concanavalin-A; (b) approximately 60% of the circulating cells ingested latex particles; (c) nearly all of the cells bore complement receptors as well as immunoglobulin receptors; (d) there was no immunoglobulin determinants on the surface. These characteristics identify the cells as monocytes or reticuloendothelial cells and not lymphocytes. Surface analyses were performed by Dr. Steven Douglas of Mount Sinai School of Medicine and Dr. Fred Segal of the Rockefeller Institute. After electronmicroscopy examinations are completed this case study will be prepared for publication. We have concluded that the functional analysis allows us to diagnose as a malignant reticulosis and not a lymphoid process. Obviously this type of characterization can add greatly to our understanding of a wide variety of lymphoreticular malignancies.
D. Monocyte Function and Monocyte/Lymphocyte Interaction

The portion of this project performed by Dr. Manfred Schmidt who was in our laboratory as a Visiting Fellow was completed as of July, 1972. While he was in the laboratory, we succeeded in isolating intact monocyte nuclei from which nascent RNA could be extracted and characterized by sucrose density sedimentation. We found it impossible to isolate and identify nascent RNA species from whole monocytes with any reliability. This was presumably caused by contamination by cytoplasmic ribonucleases contained in such abundance in the cytoplasm of monocytes. Dr. Schmidt isolated circulating monocytes from normal individuals by albumin gradients by adherence to plastic vessels. RNA was isolated from nuclei using an adaptation of the technique worked out for circulating lymphocytes. In the adaptation we added rRNA from the cytoplasm of normal rats as a competitive inhibitor of ribonuclease. Furthermore, the extraction buffer contained 2% polyvinyl sulfate as an additional ribonuclease inhibitor. Using this technique we were able to demonstrate stimulation in rRNA precursor synthesis in monocytes following exposure of the intact cell to latex particles. Activation of RNA synthesis was never before detected in such a phagocytic system. This material has been accepted for publication and will appear in Biochemica Biophysica Acta and is included in this report as Appendix D.

Dr. Schmidt was further able to produce a similar stimulation of rRNA precursor synthesis in monocytes treated with a humoral factor isolated from concanavalin-A stimulated lymphocytes. The
conconavalin-A system was used for stimulation as culture supernatants could be isolated, concentrated and purified on sephadex columns thereby excluding any possible contamination with concanavalin-A. The studies clearly demonstrated that the macrophage activating factor could be isolated from lymphocytes and that the activating factor operated directly on monocyte RNA synthesis. In addition, Schmidt observed simultaneous alterations in monocyte function reflected by increased phagocytic activity and ruffling of monocyte surface membranes indicating that the RNA stimulating factor appeared to be also capable of inducing increased functional activity of these cells. This information will appear in Cellular Immunology, 1973. A pre-print marked Appendix E is included in the appendeces.

The professional personnel conducting the experiments described in this progress report were Dr. Arnold D. Rubin, Dr. Edward Schultz, and Dr. Manfred Schmidt.
APPENDIX


