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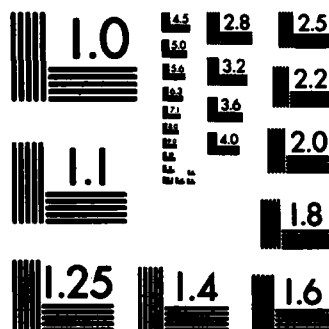
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) ✓ The overall goal of this project is to determine quantitatively and qualitatively the influence of organophosphate (OP) compounds on ocular tissues, especially the retina. Our initial efforts have been to develop tissue and organ culture systems of retina and lens cells. These systems have been based on our studies with a human retinal tumor cell line (retinoblastoma-Y79) developed in our laboratories. We have successfully		

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20. ABSTRACT (continued)

isolated a growth factor from the tumor cell line which is required for the growth of Y79 cells. In serum-free studies we have determined the growth requirements for the retinoblastoma cells and have shown that the growth factor, which is secreted by the cells, must bind to the cell surface in order to activate growth. The same growth factor stimulates growth of lens cells in culture. Preliminary studies have shown that it is a requirement for maintenance of retinal cells in culture in the absence of serum. In addition we have carried out studies using a transformed hamster retina. These cells were transformed with SV40 and now grow permanently in culture. They also produce a growth factor which we are starting to characterize and compare with the human retinoblastoma growth factor. Using our data from the human retinoblastoma and transformed hamster retina we are developing culture systems for the growth of normal retinal cells in culture for use in the studies with organophosphates.

Preliminary experiments have been performed on the transport of H^3 -diisopropylfluorophosphate (DFP) through the rabbit cornea in order to determine its rate of penetration through the cornea to the aqueous humor. Our results show that it takes approximately 20 minutes for the DFP to appear in the aqueous humor and this process seems to be independent of the concentration of the OP. Thus we feel this is probably a bulk transport phenomenon.

To examine the effects of OPs on retinal physiology, we have recorded electroretinograms (ERGs) from isolated, superfused retinae of the marine toad, Bufo marinus. The data indicate that superfusion with DFP differentially alters the a and b-wave components of the ERG. Superfusion with DFP irreversibly decreases a-wave amplitude. Amplitudes of b-wave responses first increase 2-3 fold and subsequently decrease. Superfusion with normal Ringer's does not return normal response amplitudes. The kinetics of the decline in a-wave amplitude and the rise in b-wave amplitude are different. In the future, we will attempt to define the specific cell types affected by DFP by recording intracellularly from different retinal cells and by employing H^3 -DFP to autoradiographically localize the sites at which DFP exerts its effects.

Fluoride ion is generated by the hydrolysis of DFP. We have examined the effects of various concentrations of F^- on the cyclic nucleotide system of rod photoreceptors. Fluoride has differential effects on the activation of photoreceptor cyclic GMP phosphodiesterase (PDE). In the dark, F^- activates PDE by exerting its effect on a GTP binding protein. In the light, F^- blocks the ability of light and guanine nucleotide to activate PDE through this same protein. Both these phenomenon are extremely sensitive to F^- concentration. We are attempting to better understand F^- effects on the structural properties of the proteins of the cyclic nucleotide cascade.

AFOSR Contract Number F49620-82-C-0050
 First Year Technical Report
 July 1983

**STUDIES OF ORGANOPHOSPHATE EFFECTS
 ON RETINAL PHYSIOLOGY, CELL BIOLOGY AND BIOCHEMISTRY**

**YALE UNIVERSITY SCHOOL OF MEDICINE
 NEW HAVEN, CT 06510**

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Research Objectives

- A. Development of a retinal organ culture system for the testing of organophosphates (OP) (Short term).
- B. Development of retinal tissue culture system for the study of various OP's (Long term).
- C. Determination of corneal penetration of OP's.
- D. Study the molecular mechanisms of OP's effects (Long term).
- E. Determination of the ability of OPs to move from the retinal blood supply to different ocular tissues.
- F. Determination of the cellular localization of OP in intact retina in culture.

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Status of Research

A. Retinal organ culture

1. Development of media which maintains retina in a viable state.

- a. Insulin studies. Using human retinoblastoma cells (Y79) in culture we carried out extensive studies to determine the concentrations of insulin which stimulates these cells to grow. Insulin was found to be half maximal in the stimulation of growth at a concentration of 30ng/ml. This is a concentration of about 5×10^{-9} M. This concentration is now being used with normal retinal cells in culture.
- b. Transferrin studies. We are studying the protein transferrin to see its effects on the stimulation of growth of Y79 cells especially from the standpoint of its ability to transport metal ions into the cells. Transferrin was found to be half maximal in stimulation at about 200ng/ml which is 2.5×10^{-9} M. Transferrin was tested with several metal ions and it was found that not only is transferrin important for the transport of iron into the cell but also selenium. We arrived at a selenium concentration that was optimal at about 3×10^{-9} M.
- c. Small metabolites. Other compounds tested which might have a role in the growth of retinal cells were Taurine and ornithine. Taurine was found to stimulate in a range of 5-500 μ M. Taurine was of interest since it is the amino acid found in the highest concentration in the retina. Other compounds tested were ornithine, progesterone, G_2 -gangliosides, G_3 -gangliosides and vasopressin. Only taurine showed any affect on the growth of the retinoblastoma cells in serum free solution.

- d. Retinoblastoma derived growth factor (RDGF). We have previously shown that by growing human retinoblastoma cells in a serum-free environment we can induce them to produce their own growth factors. This growth factor (RDGF) was tested on normal retinal cells in culture and found to sustain their growth in a serum-free environment for over three weeks. Thus, it was of interest to carry out further purification in order to study the mechanism of action, in hopes of obtaining more information about the control of retinal cell growth. We determined that RDGF exists in low ionic strength solution (0.1 M NaCl) as a molecular weight aggregate while in a high ionic strength solution (0.5 M NaCl) the aggregates break apart into a monomer. We were able to show that in normal tissue culture media the growth factor probably exists as a dimer. Since the growth factor (RDGF) is a high molecular weight aggregate in 0.1 M NaCl we can pass it through a Sephadex column in low salt and thus remove all the low molecular weight proteins. The growth factor is then stepped to high salt solution and passed through the same column so that it moves as a monomer. At this point all high molecular weight protein is removed. In this way we can obtain a quick purification of the growth factor.
2. Growth of cells from explant culture. We have shown that we can take normal rabbit retina (3 months old) and grow them in culture. The culture conditions are RPMI 1640, Fetal Calf Serum 10%, Na_2SeO_3 3nM, plus antibiotics. This system plus a system incorporating the retinoblastoma derived growth factor is used to maintain growth of the explanted cultures in media for over 3 weeks.

3. Further work. At the moment only preliminary results have been seen using the synthetic media with retinas in culture. In the future we will also be attempting to use human retinas with the synthetic media to see if we can maintain individual retinal cells, of the different cell types, in culture. These cells will be used for metabolic and electrophysiological studies.

B. Retinal Tissue Culture - Rabbit retina. We removed the retinas from young rabbits (approx. 3 months age). These retinas have been dissociated with trypsin and grown in tissue culture. Tissue culture experiments fall into two main types: (a) Plastic beads: In order to transfer the retinas we found that trypsinization will progressively kill off the retina in passage, however, by using small plastic beads we get the retinal cells to grow up onto the beads. These beads can be shaken loose from the flask and then placed into a new flask. The retina cells will grow off the beads onto the new flask. In this way the retina can be propagated continuously for many months. (b) Synthetic media: We are presently testing synthetic media using the retinoblastoma derived growth factor (RDGF) and using insulin, transferrin, and selenium concentrations determined with the human retinoblastoma cells (Y79) growing in culture. Preliminary results indicate that retina growing under these conditions is as viable as retina growing in the presence of fetal calf serum.

1. Transformed hamster retina. We have obtained retinas from hamsters that were transformed with Herpes Type II. These retinal cells grow rapidly in culture (doubling time approx. 15 h). We are carrying out growth studies on these cells and find that they are also producing a growth factor. This growth factor is different from the growth factor produced by the retinoblastoma cells and looks

promising from the standpoint of possibly being able to grow other retinal cell types. In addition we are attempting to clone the hamster retina cells since they consist of several different cell types. These cells will then be studied by electrophysiological as well as biochemical techniques to determine the effects of organophosphates on these cells. In addition the growth factors are being used to conduct studies with normal retinal cells to determine their ability to grow in the presence of these different growth factors for OP studies.

2. Retinoblastoma. We have recently obtained two other retinoblastoma cell lines. At present these cells are being studied for their ability to produce growth factors which may be of use in growing normal retinal cells in culture.

C. Determination of corneal penetration of OPs.

1. We have designed a chamber which will hold a rabbit cornea so that we can add tritium labelled diisopropylfluorophosphate (DIFP) to one side of the cornea and then measure its appearance on the other side of the cornea while at the same time maintaining the cornea in an oxygenated normal environment.
2. OP penetration. We find that both in the absence and presence of cold diisopropylfluorophosphate (DIFP), labeled DIFP will pass through a rabbit cornea at the same rate. This indicates that the DIFP probably moves through the cornea by a bulk transfer process and not a facilitated one. We find that the labeled DIFP appears on the anterior chamber side of the cornea within approximately 20 min. This result appears to be very consistent from cornea to cornea. We plan to repeat these experiments with human corneas when they become available.

D. Studies on the molecular mechanisms of OP effects.

Electroretinogram (ERG). Initial experiments testing the effect of DIFP on the ERG in the isolated toad retina have been performed. In these experiments DIFP (1-4mM) was superfused onto the isolated toad retina. Response amplitude and intensity-response curves were measured before, during and after treatment for both the a and b-wave components of the ERG. A-wave amplitudes exhibit a monotonic decline with a 1/2 time of approximately 80 minutes. There is no shift in the intensity response curve indicative of an alteration in sensitivity.

The b-wave exhibits a biphasic change in response amplitude after DIFP treatment. Forty to 60 minutes following DIFP treatment there is a striking increase (more than doubling) of response amplitude. After 65 minutes of DIFP exposure there is a rapid drop in b-wave response amplitude. Since the b-wave response requires input from the receptors, the apparent increase in b-wave amplitude is an underestimate of the real amplitude increase because the a-wave has diminished 30-40% by 60 minutes.

Phosphodiesterase (PDE). Since F^- is a well known effector in the cyclic nucleotide system, and this ion is generated when DIFP is hydrolysed, we felt it important to study F^- effects on the cGMP PDE of the rod outer segment. We examined F^- effects on PDE activation in both light and dark rod outer segment membranes. In the dark, maximal activation (40% of light level) of PDE was achieved at 2.5mM F^- . If guanine nucleotides [GTP or Gpp(NH)p] were present, greater activation (50-60%) was achieved. In the light, F^- inhibited activation by GTP or Gpp(NH)p approximately 80%.

GTPase. The mechanism of PDE activation involves the release of an inhibitory protein by means of the 39000 dalton subunit of the GTPase. In the presence of guanine nucleotide and light the GTPase can be solubilized from the rod outer segments (ROS) membrane. Since F^- is known to activate adenylate cyclase through a guanine nucleotide binding protein we suspected that in the dark F^- might release the GTPase from the ROS membrane. When F^- was added to dark ROS and the membranes centrifuged and the supernatant solution analysed by SDS gel electrophoresis we found that F^- solubilized the GTPase from the ROS. We have not yet demonstrated that PDE activity in the ROS increases after F^- treatment or that the inhibitory protein is present in the F^- supernate. These experiments are in progress.

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Purification and characterization of retinoblastoma derived
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Growth of retinoblastoma cells in a serum free environment

Reid, T.W., Antonetti, D.L. and Lee, M.S.

Evidence that the growth factor produced by retinoblastoma cells
growing in culture must bind to the external surface of the cell

Reid, T.W. and Antonetti, D.L.

Presentations

Purification and characterization of retinoblastoma derived growth factor based upon its self-association

Pahuja, S.L., Albert, J., Mullins, B.T. and Reid, T.W.
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