Thomas Ogden and Israel Goldberg,
Research Proposals: A Guide for Success
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The Abstract and Specific Aims

The Abstract and the Specific Aims are combined in this chapter because they are very similar. The Specific Aims should be written first to fit within one page, and then trimmed as necessary to fit within the Abstract box and augmented with brief statements of significance and experimental methods. Do not squeeze it into the box by using a smaller type font. The instructions on form page 2 of PHS 398 specify,

> State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. *DO NOT EXCEED THE SPACE PROVIDED.*

The Abstract and Specific Aims are the two most important pages in the proposal. One or the other of these pages may be the only part of the proposal that some of the reviewers will read. Consider again the scenario of the Study Section meeting. The members are seated around a large table. Two of them are the assigned reviewers who have studied the proposal in detail and who will read their written reviews to the group. These presentations take 5–10 minutes each, and during this time the other 15 or so members will browse through the proposal. If any part of the proposal is actually read it is probably the Abstract or the Specific Aims, and it is more likely to be the Specific Aims that is read because of its larger format. It is also common for members generally familiar with the area

of the proposal, but not actually assigned to review it, to read its Specific Aims before the Study Section meeting, in order to be better prepared to discuss it.

Study Section members have their own styles of review, but most probably start by quickly scanning the Specific Aims. Thus, this section also has a major impact on the primary reviewers and ultimately on the priority scores. If the Specific Aims are tightly written and beautifully logical and informative, they give a very good first impression of the proposal.

THE SPECIFIC AIMS

Preparation of a research proposal should start with the Specific Aims; the rest of the proposal merely amplifies what is presented there. After reading a well-written Specific Aims, an experienced reviewer will understand the problem addressed, the hypothesis being tested, and the feasibility and power of the experimental approach, and will have a feeling about their importance. If the Specific Aims fails to communicate these ideas, the reviewer is left frustrated and depressed by the realization that this essential information will have to be forcefully extracted from the depths of the proposal.

Failure of the Specific Aims has a devastating and cascading effect on the review. After struggling with it, the reviewer goes on to the Background and Significance section. The review of the literature and discussion here may be pertinent but lost on a reviewer who does not understand what the proposal is all about. As reviewers, at this point we usually abandon any attempt to follow a line of logic and turn to the Experimental Design and Methods section to see if we can at least figure out what will be done. Sometimes it is necessary for a reviewer to list proposed experiments, and assign them to a Specific Aim in order to understand the thinking of the investigator. Then the Background and Significance section is reread in search of that elusive thread of logic one hopes is there. All in all, it is very difficult for mere science to overcome such a psychological handicap imposed on the reviewer. The proposal with a poorly written Specific Aims will surely not receive the priority score it might merit on the basis of its science. The PHS 398 guidelines say, "List the broad, long-term objectives and what the specific research proposed in this application is intended to accomplish, e.g., to test a stated hypothesis, create a novel design, solve a specific problem, or develop new technology. *One page is recommended.*"

We prefer to write a Specific Aims page in four pieces, which are then perfected, trimmed, and merged. These are roughly: (1) general goal/significance, (2) a theoretical framework or model, (3) hypotheses, and (4) tests of the hypotheses; this last is the actual Specific Aims.

General Goal and Significance

The problem is stated and is shown to be important. This must be done in one or two sentences. It is not necessary to belabor the obvious. For instance, the mere mention of AIDS is sufficient to establish significance; save the gruesome statistics for the Background and Significance section. An indication of the direction of the study is expressed in the goal statement. This should be broad enough to give the impression that this study is part of a larger research plan that will continue beyond the bounds defined in the Specific Aims. A long-term goal might be identified generally as simply the alleviation of the problem.

Example

> Macular degeneration is the most common cause of lost reading vision in the elderly. The pathogenesis of this disease is poorly understood but involves the development of subretinal neovascularization and changes in the choroidal circulation. Our long-term goal is to develop methods for the prevention and treatment of macular degeneration based on understanding of molecular mechanisms that are the basis of the pathology in the retina.

This example identifies the problem, its significance, the field of study, and the long-term goal in only 67 words. It also provides a basis for assignment of the proposal to the National Eye Institute or possibly to the National Institute on Aging, both of which have better funding rates for R01 grants than do some other NIH Institutes. The problem is important by definition, since the NEI has identified it as an area where research is needed.

In making this opening statement it is essential to avoid abbreviations. When we first saw this paragraph it read, "ARMD is the most common cause of decreased VA in the elderly. The pathogenesis of this condition is poorly understood but involves the development of SRN and changes

in the choroidal circulation. . . ." Although the primary reviewers undoubtedly would know the meaning of these abbreviations, many of the other Study Section members might not. The effect of this is to dissuade the latter from reading further in the Specific Aims. Instead they will use the time to prospect for red flags.

It is equally useless to simply list a series of experiments, or even worse, a series of methods, as the Specific Aims without providing enough background for the reviewer to understand the problem being studied. A well-written Specific Aims section informs as it goes along, so that no questions essential to its understanding are left unanswered.

A Theoretical Model

Having identified the problem, present a broad theoretical construct or model to which the problem can be related. In disease-related research, the model usually pertains to pathogenesis and will probably logically connect several different hypotheses. It may be difficult to generate such a model in some types of research that are still in a descriptive phase of development, but theory can add great depth to the proposal. Its absence is a blatant red flag, and alerts the reviewer to the possibility that the proposal will lack focus and depth.

A possible model for the above example of subretinal neovascularization (SRN) is,

> Factors released by degenerating retinal pigment epithelial cells (RPE) lyse the underlying basement membrane, exposing the choriocapillaris, and attracting macrophages. These release angiogenic factors to cause endothelial proliferation and migration and so stimulate the choroidal vessels to invade the subretinal space. The SRN amplifies the RPE degeneration.

This theory is actually a set of causally related hypotheses, one or more of which can be the subject of the proposal.

Diagrams are rarely seen on Specific Aims pages. This is unfortunate, because a diagram is sure to catch the eye of the nonassigned reviewers. Better yet, a Specific Aims that is sufficiently brief to accommodate a diagram is very well written indeed! A possible diagram of this model is shown in Figure 5.1.

The theory must be plausible, and it is useful if it has been around long

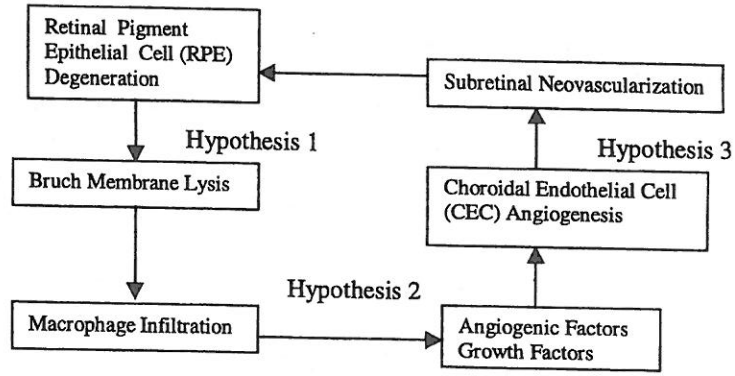


Figure 5.1. Diagram of a SRN theoretic model.

enough to generally be accepted. But many theoretical models are much too broad to be a suitable subject for research. However, any of the several hypotheses in such models could well be the focus of a study. To investigate all parts of a broad model would result in an excessively broad project that might well be downgraded in review because of lack of focus.

One of the most common complaints about weak proposals is that they are too "diffuse." This usually means that the Specific Aims are not sufficiently closely interdependent. A diffuse proposal is usually superficial, since several different investigations cannot be pursued efficiently and in depth within the usual allotted time. Diffuse proposals often lack a theoretic framework that would serve to keep the work focused. Having expressed a basic theory, the temptation to study all parts of it at the same time should be avoided. It is, after all, the path along which you will continue your research after the successful conclusion of the present study.

Interdisciplinary research is also in great demand by the NIH Institutes because it is a good way to foster the application of new technologies to old problems. It is difficult to write an interdisciplinary proposal that is not diffuse. A neuroscientist interested in the biology of transmitters may enlist the aid of a molecular biologist to generate some oligonucleotide probes for specific transmitter receptor genes. What transmitters of the 20–30 possibilities should be studied? As few as possible! Also it would be catastrophic for the proposal to suggest that the work be done in nervous tissue of Alzheimer patients, as this would shatter the focus of the study. Problems of particular interest to the molecular biologist must not be allowed to surface in the proposal, as this will decrease its focus.

Obviously a great deal of diplomacy is required to set up a strong, focused multidisciplinary study.

Another catchphrase of the NIH Institutes is "clinical relevance." There is a great temptation to combine clinical and basic science studies in the same proposal, probably due to a general perception that clinical relevancy increases the prospects for funding. Such a study is by definition diffuse, and great care must be taken to convince the reviewer that each part of the study goes into as much depth as possible with the material at hand. It is certainly true that the Institute Advisory Council, based on high program relevance, may elect to improve the priority score assigned to a proposal by the Study Section. But this is not done often and usually happens only when research is proposed in a relatively unfrequented area. High program relevance does not automatically lead to increased scores if the area is already being studied adequately. It is much more common for a proposal to suffer the stigma of being diffuse because an attempt at clinical relevancy was forced.

Hypotheses

Having established the problem and a logical structure within which it can be considered, one or more specific hypotheses should be stated. This is the most important part of the Specific Aims section, and is often missing or stated in such general terms as to be useless. Unless a specific hypothesis can be stated and tested, research is nothing more than a fishing expedition. Admittedly, descriptive research begins the study of a new field and is essential as a base for in-depth studies, but there are very few such new fields of study. A trap that awaits us all is the "interesting observation" that beckons to us like the Sirens of Ulysses. Research that is designed to investigate something just because it is there may be very interesting to the PI, but rarely to enough of the Study Section to generate a fundable priority score. Phenomenological proposals are weak and tend to end up on the rocks. It is worth repeating that a proposal is strengthened if a hypothesis is clearly identified, if it relates logically to a broad theoretic model, and if the proposed experiments will actually test it.

Some hypotheses are hardly worthy of the name. "Colorectal cancers are detected more often with the flexible sigmoidoscope than with the rigid sigmoidoscope" is a hypothesis of sorts, but is trivial and hardly worth a research effort to test. It was proposed as the basis for a retrospective study of data from over 800 sigmoidoscopies in a large county

hospital. The flexible instrument reaches higher into the colon than the rigid sigmoidoscope. The difference in detectability could be related to variation of the incidence of cancer with position along the colon, a possible problem of epidemiology. This proposal was greatly strengthened when it was rewritten as an epidemiological study to test the hypothesis that the incidence of high colonic cancer in men is reduced by long-term use of a high-fiber diet. The same data were used for the study, but the approach was changed.

We live in an age of powerful experimental tools. The availability of a technology has the tendency to stimulate research that uses it. Proposals that are based on technological advances rather than on important hypotheses cannot help but be weak. A good example was the introduction of a powerful procedure used by molecular biologists called the polymerase chain reaction, or PCR. This procedure amplifies minute amounts of DNA in a tissue section, thereby permitting the recognition of virus particles. With the emphasis on AIDS research, there was a rush to seek evidence of HIV in a variety of different tissues. "The hypothesis to be tested is that HIV is present in the [you name it] of patients with AIDS Related Complex" was a formula for far too many studies, some of which were actually funded since the NIH was compelled to use the funds given to it by Congress for AIDS research. A hypothesis is not strong unless it is related to a significant theoretical model of the disease.

A hypothesis worthy of consideration can be tested directly or gives rise to corollaries or predictions that can be tested. Untestable hypotheses are worse than useless; they are destructive in that they may consume time and effort without a concomitant advance of knowledge.

Excessive listing of hypotheses signals lack of focus. A single important hypothesis is best; most proposals list two or perhaps three (four is one too many).

Specific Aims (Tests of the Hypotheses)

Specific Aims are then stated. These are the tests of the hypotheses presented in terms of experiments or groups of experiments. These should be listed numerically and should be reiterated verbatim and in order in the Experimental Design and Methods section of the proposal. The Specific Aims should be just that, specific. They must be brief and indicate the general nature of the technology used (but should not include discussion of the actual methods). This section usually fills about a third of the

page. The reason for each of the aims should be obvious from consideration of the hypotheses and their corollaries. There is never enough room on this page to really explain the rationale of each aim. But this is done in exhaustive detail later in the proposal. It is only necessary that each aim fit within the structure of the theory. Avoid editorializing: "These studies may lead to the development of novel strategies for the treatment of whatever." Do not cite references. If the reviewer has to look up a reference in order to get through the Specific Aims, it is a failure. Three Specific Aims are usually enough!

Example

The following Specific Aims was submitted to the NIH. It is instructive for several reasons. You might wish to evaluate and rate it before reading the critique of it.

> Alzheimer's disease (AD) is a dementing disorder of unknown etiology. The diagnosis of "presumed" or "probable" AD is made through clinical diagnosis, in recognition that AD can only be definitively diagnosed histopathologically. Characteristically, memory is initially impaired, followed by visuo-spatial deficits, and, finally, involvement of all cognitive functions (Hutton, 1987).
>
> We hope to address a number of Specific Aims by the completion of this project:
>
> 1. Is there selective involvement of a particular component or class of cells in the visual system of AD patients? If so, can this be related to the pathophysiology of AD in the rest of the brain? If there is a predilection for loss of a class of ganglion cells in AD, this may yield insight to the reasons for predominant degeneration of large neurons in other areas of the brain (Terry et al, 1981).
> 2. Can visual testing be used, in conjunction with present neurological and psychometric evaluations, as a screening procedure to identify AD?
> 3. Can visual testing or histopathological assessments of the visual system be used to identify subtypes of AD? If so, this might provide insights leading to possible management and treatment strategies for AD.
> 4. We will gain insights into both anatomical and functional AD subgroups through correlative histopathological and clinical assessments of the visual system in the age-matched controls (normals) used in this study.
> 5. Significant new data relevant to the effect of age on the visual system will be gathered.

Critique

This is a weak Specific Aims. The first line is excellent, but the rest of the opening paragraph is fluff without a clear relationship to the proposal.

The brief description of the defects of AD speaks down to the reviewers, who are certainly well informed about it. The reference is superfluous.

There is no hypothesis or theory offered.

To write, "We hope to do this or that," is weak. It may be honest, but it is bad grantsmanship. It leaves room for doubt as to whether what follows will be achieved. A major concern of the reviewer is the question of what the PI will have left if part of the proposal does not work. The Specific Aims should never contain anything that is controversial, equivocal, or negative.

Aim 1 could have been stated as a hypothesis and test combined. "We will test the hypothesis that large neurons are selectively destroyed in AD by measuring the sizes of ganglion cells in the retinas of AD patients and of age-matched controls." The rest of Aim 1 is editorializing. The suggestion that this retinal study might be correlated with the results of other research on brain tissue is speculation. Such correlations are notoriously difficult, and to throw one in here seems to be window dressing. Speculations in the Specific Aims are very destructive since they interfere with its purpose, which is to provide an executive summary of the project. Speculation is by its very nature weak and argumentative. The less of it in a proposal the better.

Specific Aim 2 is clearly a non sequitur. What does a screening procedure have to do with large cell loss? Actually there is an association, but it is speculative. There is a suggestion that visuomotor skills are dependent on large ganglion cell input from the retina to the brain. The investigators hope to find visuomotor deficits in AD patients, and if these can be seen early in the disease, the tests could be used for screening. Unfortunately, the opening paragraph states that memory loss is the initial event in AD, and loss of visuomotor function comes later. Clearly a test of memory loss would be a better screening procedure. This is not suggested since there is no apparent correlation between memory loss and large cell loss.

Specific Aim 2 could have been stated thus: "A corollary of this hypothesis suggests that large ganglion cell dependent visuomotor function of AD patients should be defective. We will test this with eye track recordings in patients and age-matched controls."

Specific Aim 3 is combined speculation and window dressing. What is meant by "subtypes of AD"? At present, as stated in the opening paragraph, we cannot even diagnose AD without histopathology, so how can we talk about clinical subtypes? Of course diagnosis by histopathology

cannot help "management and treatment strategies of AD." This aim is best eliminated.

Specific Aim 4 assumes that Aim 3 was successful, and is otherwise editorializing, as is Aim 5. Both should be eliminated.

A restructuring of this Specific Aims could be built around the following, excerpted from above and expanded:

> Alzheimer's disease (AD) is a dementing disorder of unknown etiology. Recent studies have shown that AD is associated with loss of larger brain cells and with optic nerve degeneration. Since the retina is actually part of the brain and has been studied in far greater functional and anatomic detail, it may provide an ideal model in which to investigate the relationship of a cell's size to its susceptibility to damage in AD.
>
> Specific Aims:
>
> 1. We will test the hypothesis that large neurons are selectively destroyed in AD by measuring the sizes of ganglion cells in the retinas of AD patients and in age-matched controls.
> 2. A corollary of this hypothesis suggests that large ganglion cell dependent visuomotor function of AD patients should be defective. We will test this with eye track recordings in patients and in age-matched controls.

This is considerably less than a full page. It should be expanded to emphasize the desirability of a collaborative study involving a basic scientist and a clinician, and the availability of a large patient base. Ideally, the theoretical model would contain hypotheses about the functional relations between AD etiology and neuron size, or about the mechanisms that drive these relations. These should lead to the prediction that there should be a predilection of AD to affect large rather than small neurons.

A successful Specific Aims section can be read in about 3 minutes. It leads the reader to understand the goals of the project and its importance, the theory behind the study, the hypotheses to be tested, and the tests to be used.

The following is a relatively well-written Specific Aims:

> A number of clinical diseases have been associated with disorders of retinal pigment epithelium (RPE) transport and barrier function. The long-term goal of this research is to fully characterize these properties of human RPE to facilitate treatment and perhaps prevention of these diseases.
>
> During the last period we also developed and standardized a new method by which fluid fluxes can be measured directly rather than calculated from isotope fluxes, which are subject to cumulative experimental errors. We plan to incorporate this method into our proposed studies, to test the following **hypotheses:**
>
> a) Cultured fetal human RPE, under normal conditions, transports fluid from its apical side to its basal side utilizing a $Na+$, $K+$, $Cl-$ cotransport system as well as a $Na+$, HCO_3- cotransport system.
>
> b) The activities of these transport systems are modulated by intracellular cAMP concentrations.
>
> c) Cultured fetal human RPE mediated transepithelial fluid movement is modulated by beta adrenergic agonists, histamine, prostaglandin E1, and vasoactive intestinal peptide (VIP) that alter intracellular cAMP concentration. In addition, agents that alter intracellular cAMP metabolism, such as the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), also alter human RPE mediated transepithelial fluid movement.
>
> To test these hypotheses, we propose studies with the following specific aims:
>
> 1. To characterize cultured fetal human RPE transepithelial transport by extending Ussing chamber studies using pharmacologic probes, ion manipulation, and isotope flux studies.
> 2. To determine how cultured fetal human RPE transepithelial transport is modulated by intracellular cAMP.
> 3. To determine the degree to which cultured fetal human RPE transepithelial transport may be regulated by extracellular receptors (such as those to beta adrenergic agents) and to determine the degree to which cultured fetal human RPE transepithelial transport is affected by agents (such as IBMX) that alter intracellular cAMP metabolism.

In the original, this Specific Aims section just filled one page. The hypotheses could be improved by deleting the phrases about methods and emphasizing the hypothesized movement of fluid in real life. The Specific Aims themselves could be improved by eliminating the editorializing, since these comments are repeated in the Methods section. This was for a competing renewal proposal, so reference to past productivity and continuity of work is good.

THE ABSTRACT

The Abstract (called "Description" in PHS 398) should contain (1) the essence of the Specific Aims; (2) a few short sentences concerning the health relatedness of the research; and (3) its scientific significance in terms of its long-term goals. Such statements are often added to the Specific Aims as well. This is useful, provided that the essential parts of the section are not shortened to make room for this addition.

The following is an acceptable abstract in that it expresses a hypothesis and states the experimental approach to its testing. The significance

of the proposed work is also presented. But it fails the appearance test. There are no spaces. Hypothesis, Method, and Significance are not highlighted. It is jammed into the box.

Magnesium (Mg) deficiency may play an important role in the pathogenesis of enhanced vascular reactivity in hypertension. The overall hypothesis to be evaluated is that Mg deficiency caused by glucose intolerance, insulin resistance, or other factors in hypertensives leads to increased vasomotor tone via altered release of vasoactive cycloxygenase and lipoxygenase products of arachidonic acid and enhanced angiotensin II (AII) action. To evaluate the effects of Mg deficiency in normal subjects we will induce the condition by administration of a low Mg diet. Vascular and adrenal sensitivity to AII, platelet aggregation, and eicosanoid levels will be studied prior to and after Mg deficiency is established. Since evidence suggests that Mg deficiency can modulate insulin action, the effect of this deficiency on glucose tolerance will also be studied. In another project the effect of insulin on intracellular Mg levels will be studied using a new fura 2 Mg dye technique. These studies will be performed in groups of subjects with varied blood pressure and insulin levels. Also the effects of acute intravenous and chronic oral Mg loading on the above parameters will be studied in similar subject groups. We will directly study the effect of Mg on AII, insulin, and insulin-like growth factor action in isolated and cultured adrenal glomerulosa cells. Concentration of Mg will be varied and signal transduction and steroidogenic effects will be evaluated. These studies will provide insight into mechanisms important to the pathogenesis of altered vascular reactivity of subjects with hypertension or hyperinsulinemia.

An Abstract that completely fills the box, without line spaces or indentations, affords a repulsive aspect for a tired reviewer, who may well decide to skip it. An Abstract of three or four paragraphs separated by line spaces and containing the words "Hypothesis" and "Specific Aims" in bold, on the other hand, catches the eye, promises interesting informative reading, and has a positive impact on the reviewer (see below).

Magnesium (Mg) deficiency may play an important role in the pathogenesis of enhanced vascular reactivity in hypertension. The overall **HYPOTHESIS** to be evaluated is that Mg deficiency caused by glucose intolerance, insulin resistance, or other factors in hypertensives leads to increased vasomotor tone via altered release of vasoactive cycloxygenase and lipoxygenase products of arachidonic acid and enhanced angiotensin II (AII) action.

Specific Aims: (1) Determine the effects of low Mg on vascular and adrenal sensitivity to AII (platelet aggregation and eicosanoid levels, and glucose tolerance). (2) Determine the effect of insulin on intracellular Mg levels (fura 2 Mg dye technique). These studies will be performed in subjects with varied blood pressure and insulin levels. (3) Determine the effects of acute intravenous and chronic oral Mg loading on the above parameters. (4) Determine the signal transduction and steroidogenic effects of Mg on AII, insulin, and insulin-like growth factor action in isolated and cultured adrenal glomerulosa cells.

Significance. These studies will provide insight into mechanisms important to the pathogenesis of altered vascular reactivity of subjects with hypertension or hyperinsulinemia.

Appendix D-1
Design Example

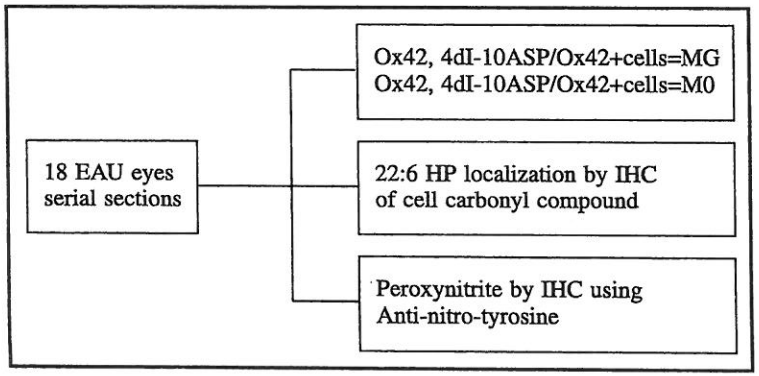
Specific Aim 2: Evaluate generation of cytotoxic agents (*cytokines and oxidants*) by the migrated microglia in the early phase of EAU.

Hypothesis: iNOS expression and peroxynitrite generation by migrated microglia occur early in the development of experimental EAU.

Experiment 3. Determine expression of iNOS by the migrated microglia and generation of peroxynitrite by these cells in the early phase of EAU.

Rationale: In the preliminary studies, we have demonstrated that at the early phase of EAU, the retinal microglia migrate to the outer retina/photoreceptor cell layer. We have also shown that microglia isolated from normal adult rats produce O_2^-, •NO, and peroxynitrite on stimulation in vitro, and in vivo microglia isolated from axotomized/EAU rats generate peroxynitrite. In this study, we further define these experiments to confirm the oxidant species peroxynitrite by determining iNOS expression, peroxynitrite being the combination product of O_2^- and •NO. Localization of iNOS and peroxynitrite in the migrated microglia will be also attempted.

Design:



1. Induce EAU in 18 chimeric rats with optic nerve axotomy.
2. Sacrifice 6 animals each on days 8, 9, and 10.
3. Detect intracellularly generated peroxynitrite by nonfluorescent dihydrorhodamine, which in the presence of peroxynitrite forms a fluorescent rhodamine (Preliminary Studies, Experiment IX).
4. Counterstain with Ox42 and rhodamine to further confirm the identity of microglia. Similarly a different section of the retina will be processed to localize iNOS.

Data analysis/Significance: Examination of the sections with appropriate excitation/emission filters should localize the intracellular presence of peroxynitrite. The presence of fluorescent rhodamine in Ox42-positive cells in the outer retina will suggest the generation of peroxynitrite by either microglia or macrophages. However, using the adjacent section, the co-localization of 4Di-10ASP axotomy dye and rhodamine will indicate the 4Di-10ASP-positive cells to be microglia and the dye-negative cells to be macrophages. The PCR study will reveal the presence or absence of any circulation-derived cells (macrophages) in the EAU retina.

Appendix D-2
Design Example

Experiment 9 (Specific Aim 2). Effects of Ang1 and Ang2 over-expression in RPE and CEC co-culture.

Hypothesis: When Ang2>Ang1, VEGF-induced 3D networks continue to grow and are sensitive to growth factor withdrawal. When Ang2<Ang1, VEGF-induced 3D networks show slower growth and are less sensitive to growth factor withdrawal.

Rationale. RPE and CEC can each be transduced using retroviruses to over-express genes of interest and can be selected to provide pure over-expressing cultures. Cells over-expressing Ang1 or Ang2 will be used in 3D co-cultures to show effects on CEC tubular networks. Over-expression of Ang1, relative to Ang2, in CEC and possibly RPE, should destabilize the RPE and, in the presence of VEGF, promote angiogenesis. The vessels should remain immature and will be sensitive to growth factor withdrawal. Over-expression of Ang1, relative to Ang2, in RPE and possibly CEC, should mature vascular networks and provide resistance against the effects of growth factor withdrawal.

Design.

1. Culture bovine CEC and human RPE; use early passages.
2. Develop retroviral vectors for Ang1 and Ang2.
3. Transduce cells with vectors, select with G418, collect high titer viral supernatants.
4. Co-culture CEC and RPE.
5. Treat co-cultures with varying concentrations of Ang1 and Ang2.
6. Measure volume of tubular formations.

Analysis and potential problems. Vector production and concentration are routine in this lab (see Preliminary Data). Cells will be examined for toxicity, although none is expected. Control experiments will employ "empty vector" retrovirus and B-gal retrovirus. Our system is sufficiently sensitive to detect a 25% difference in vascular tube volume.

Appendix D-3
Design Example

Table 1 *Experimental Design for Specific Aims 1 and 2*

Gp	Rabbits[1]	*P. acnes* Inoculum[2]	Serum[3]	Aqueous[4]	Vitreous[5]	IOL[6]	PLC[7]
Natural History Studies (Experiment 1)							
A	32 pseudo-phakic	Live	224	64	32	16 BC 16 FITC	16 BC 16 FITC
B	32 aphakic	Live	224	64	32		16 BC 16 FITC
C	16 pseudo-phakic	Saline (control)	112	32	16	8 BC 8 FITC	8 BC 8 FITC
D	16 aphakic	Saline (control)	112	32	16		8 BC 8 FITC
Nonviable *P. acnes* Studies (Experiment 2)							
E	16 pseudo-phakic	Heat killed	112	32	16	8 BC 8 FITC	8 BC 8 FITC
F	16 pseudo-phakic	Lysed	112	32	16	8 BC 8 FITC	8 BC 8 FITC

[1]Number of rabbits receiving unilateral ECCE surgery with (pseudophakic) or without (aphakic) IOL implantation, and followed clinically for 6 months (Specific Aim 1).

[2]Inoculum preparation injected into anterior chambers post-ECCE surgery (Specific Aim 1).

[3]Number of serum samples (7/rabbit) collected during 6-month follow-up period for the ELISA determination of antibody user to *P. acnes* and/or lens protein (Specific Aim 2).

[4]Number of aqueous specimens, collected at time of surgery and enucleation, for bacterial culture (Specific Aim 1) and ELISA determination of antibody liters to *P. acnes* and/or lens protein (Specific Aim 2).

[5]Number of vitreous specimens, collected at time of enucleation, for bacterial culture (Specific Aim 1) and ELISA determination of antibody titers to *P. acnes* and/or lens protein (Specific Aim 2).

[6]Number of IOLs collected at the time of enucleation for bacterial culture (BC) and immunofluorescent (FITC) identification of *P. acnes* (Specific Aim 1).

[7]Number of posterior lens capsules (PLC) collected at the time of enucleation for bacterial culture (BC) and immunofluorescent (FITC) identification of *P. acnes* (Specific Aim 1). The remainder of the enucleated eye will be submitted for histopathologic studies.

Timetable for Specific Aim 1: Experiment 1 will be performed over the first 17 months of the proposal period. We will complete the Group A and B animals in a series of four trials using 16 rabbits at a time (8 each, pseudophakic and aphakic). The Group C and D animals will be completed similarly in two trials. Experiment 2 will be performed over months 13 through 21 of the proposal period. We will complete Group E and F animals in two consecutive trials beginning at 2-month intervals as described for Groups A–D. There will be some overlap of time with the viable *P. acnes* long-term natural history studies described above. We will begin a trial every 2 months to provide for the appropriate use of space in the animal housing facilities and to optimize the scheduling and completion of the research week.

Appendix D-4
Design Example

AIM 1: Establish the cellular and molecular mechanisms by which HGF stimulates the formation of multilayered groups of RPE from the monolayer.

Overall Aim 1 Rationale: We hypothesize that HGF, produced by activated RPE cells, is a major mediator of phenotypic change in adjacent RPE; a relatively immotile monolayer of adherent RPE sitting on Bruch's membrane is changed into a motile population of relatively discohesive cells within a provisional ECM. Consistent with this hypothesis is the expectation that cells of the monolayer will respond differently to HGF than cells in culture. It is likely that effects on junctional proteins will be prominent in monolayer explants, while effects on ECM production, integrins and proteases may be more prominent in cultured cells.

Experiment 1: Define and compare temporal patterns of gene expression in cultured human RPE and human RPE monolayer explants after HGF treatment.

Hypothesis: RPE cultures and monolayer explants will each show distinct patterns of modulated gene expression after HGF treatment.

Rationale: Data mining and knowledge generation from expression array profiles will characterize differential HGF responses of the RPE monolayer from those of RPE cultures. Thus expression arrays will independently test the basic hypotheses of this grant and will be useful guides for subsequent experiments. Critical analysis of this data, followed by biologic validation, will reveal novel targets of HGF action.

Design:

1. Passage 2–4 human fetal RPE cultures and human RPE explant cultures
2. Expose to recombinant human HGF (20 ng/ml) for 0, 3, 6, 24 or 48 hours. Control cultures; vehicle alone.
3. Explant cultures will be dissected from fresh donor eyes (pg. 47).
4. Isolate total RNA using the Microarray core facility for generation and labeling of cDNA, and hybridization on Affymetrix U95A oligonucleotide arrays (representing over 12,000 human genes). All tests will be repeated three times from different donors.

Analysis:

1. Compare patterns of gene expression between samples at any one time point and then over time (3, 6, 24, and 48 hours) for the treated and untreated samples using weighted-linear regression t-tests.
2. "Treatment group" will be a predictor variable, coded as 1 if treated and 0 otherwise. We will stratify on replicates (1, 2 or 3) to account for the matching.
3. Measures in the untreated samples will be compared across all time points using repeated measures ANOVA.
4. Test whether average expression is constant over time, or whether a change is detected using the F-test. To compare treatment effects over time, we will perform a

general heterogeneity test followed by a test for differences in trends (slopes). A significant effect for the Treatment variable but not for the slopes will show an initial treatment effect that is maintained (constant) over all time points. A significant difference in slopes will show a treatment effect that changes over time.

5. The data from the RPE cultures will also be compared to the data from the RPE explant monolayers at each time point and over time using a linear regression model. Building on the model described for cell cultures, we can test for differences between RPE culture with RPE monolayer by introducing an indicator variable for the monolayer.

Expectations and Alternatives:

1. Both cultured RPE and monolayer explants will show significant alterations of gene expression after HGF treatment although the cultured cells and the explants will show different patterns of altered gene expression.

2. Differences may change over time as the RPE in the monolayer become dissociated and start to separate from the monolayer.

3. Variability in gene expression based on the retinal site is reduced by varying the site for each time point between experiments. We do not anticipate this to be a major problem since we have not previously seen significant differences between superior and inferior retina.

4. Through use of stringent statistical criteria only a small proportion of the genes on the chip will be identified as being of interest. The subset of genes to be studied in detail with biologic validation will be decided after temporal analysis of multiple time points with focus placed on genes that can be directly related to PVR pathogenesis.

Appendix D-5
Design Example

Experiment 7: Document photoreceptor damage by electron microscopy (E.M.) at the site of microglial infiltration.

Rationale: Damage to photoreceptors by the oxidants includes condensation and disruption of the outer segment discs of photoreceptors. This is the earliest morphologic change that can be documented by E.M. In recently reported methods, the axotomy dye 4Di-10ASP was photoconverted aerobically, and dye positive microglia were then readily identifiable in the E.M. by the inclusion of photoconverted dense granular 4Di-10ASP. Any morphologic damage of outer segments at the sites adjacent to microglia can then also be recognized.

Design: Eighteen chimeric/axotomized/EAU (days 8, 9 and 10) eyes (procedures on p. 37) will be processed for ultrastructural identification of 4Di-10ASP-positive microglia, using the oxygen-enriched photoconversion method. One half of the retina from the enucleated eye will be photoconverted following appropriate fixation for the E.M. visualization. The remaining half of the unfixed retina will be processed for DNA extraction and PCR amplification to detect the Y-chromosome, as described in our preliminary experiments (p. 25).

Data analysis/Significance: Based on our preliminary experiments (p. 27), we do not anticipate the infiltration of blood-derived macrophages in this early phase of EAU. This will be confirmed by PCR for Y-negativity, and only Y-negative retinas will be used for the study. Therefore, the photoreceptor damage adjacent to the microglia, which contain dense 4Di-10ASP deposits detectable by E.M., will indicate a pathogenic role for these cells.