CYCLOSPORINE - OCULAR ABSORPTION, PHARMACOKINETICS & EFFECTS ON UVEITIS

By

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to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September,1986

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ABSTRACT

Inflammatory ocular disease is an important cause of blindness and uveitis accounts for 1.0% of blind patients in Canada.[1] This disease can be particularly troublesome to treat, because the nature of the causal factor or factors and mechanisms of progression are usually unknown.

Non-specific anti-inflammatory agents have been used orally and systemically with some success to treat uveitis,[2-8] but they may produce serious side effects both locally and elsewhere in the body.[9,12,14] With prolonged use tolerance to these drugs may develop, making them ineffective.

Recently a powerful immunosuppressive agent, Cyclosporine (Cy), used orally and systemically in the treatment of uveitis has shown promising results.[16-19,28] However, its routine use is limited because of a narrow therapeutic index and renal toxicity. [19,42,43]

Several studies have shown that subconjunctival injection of a number of antineoplastic agents enhanced ocular absorption [20-24] in a traditional pharmacological sanctuary,[13,14] and circumvented the associated systemic side effects. Therefore, if Cy were administered subconjunctivally it might be possible to avoid the side effects associated with the oral and systemic routes, and at the same time provide higher levels of Cy to the eye.

A protocol for the administration of Cy

subconjunctivally was developed in New Zealand white rabbits, to study toxicity, ocular pharmacokinetics following equidose administration subconjunctivally and systemically and the effects of Cy on an animal model of uveitis.

Subconjuntival administration of 5mg of Cy in 0.1cc (Sandimmune I.V.(R) 50 mg/ml) weekly was found to be the maximum tolerated dose by the rabbits' eye, and was superior to intravenous injection for ocular penetration while minimizing systemic exposure. The uveitis model showed that Cy was effective in reducing the inflammatory response and the earlier the application of Cy the milder the uveitis.

The results from our study support the contention that local administration of Cy would lead to higher levels of Cy absorption and circumvent the side effects of systemic administration. This may facilitate the routine use of Cy in ocular inflammatory disease.

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List of Abbreviations

BSA : bovine serum albumin

C : cornea

CE : conjunctival epithelium

CH : choroid

Cy : cyclosporine

CyC : dihydrocyclosporin C

CyD : cyclosporin D

EAU : experimental autoimmune uveitis

EAU-BSA : experimental autoimmune uveitis induced by bovine serum albumin

EAU-S-Ag : experimental autoimmune uveitis induced by retinal S-Antigen

FZ : focal zone of inflammation

I : iris

L : lens

LP : lamina propria

M - muscle

NA : necrotic area

ON : optic nerve

PMN - polymorphonuclear granulocyte

R - retina

RD - retinal detachment

SCL - sclera

SF - subretinal fluid

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INTRODUCTION

Ocular inflammatory disease constitutes one of the most important causes of blindness in people. Uveitis, in particular, accounts for 1.0% of the blind patients in Canada.[1] Uveitis is frequently of a non-specific nature and its exact etiology eludes clinical investigators. Even in instances where the specific cause of uveitis is known (eg. Herpes Simplex[49], Juvenile Rheumatoid Arthritis[50]), once the disease is established inflammation may persist in spite of treatment directed to causation.

Treatment of this disease is frequently on an empiric basis, with non-specific anti-inflammatory agents.[2-8] In a significant number of patients all traditional modalities may fail to control the inflammation and the eye may ultimately succumb to the secondary effects of chronic inflammatory disease. Often the end results in uveitis are cataracts, opacified media, atrophic retina, and cystoid macular edema with eventual loss of visual acuity.

The current therapy for ocular inflammatory disease in the human consists of local and systemic application of corticosteroids,[2,14] systemic administration of alternate anti-inflammatory agents such as indomethacin, phenylbutazone,[3,4] and systemic administration of immunosupprressive[5,6] agents such as azathioprine,[7] and cyclophosphamide.[8]

Although these agents have been used with some success in the treatment of ocular inflammatory disease, they have

not been without complications such as glaucoma induced by topical application of corticosteroids,[10,11,14] while systemic administration has resulted in Cushing's-like symptoms.[14,15] A greater risk is associated with systemic administration of immunosuppressives which can cause bone marrow suppression, secondary tumors and specific complications on other normally proliferating cell populations.[12,14,15]

Recently another immunosuppressive, Cyclosporine (Cy), has been tried as a possible candidate in the treatment of ocular inflammatory disease.[16,17,19,30] Currently Cy is employed to prevent the rejection of transplanted organs.[48] One of the intriguing characteristics of Cy is that it displays little myelotoxicity and it does not adversely affect the phagocytic cells of the mononuclear phagocyte system.[15,56]

Cy causes immunosuppression directed towards T-cell-mediated immunity.[56] Although the precise biochemical mechanism is not well understood, the effect may be achieved by inhibition of interleukin-2 synthesis or release, and inhibition of receptor acquisition for interleukin-2 by precusor Cytotoxic T-cells, as well as a diminished responsiveness of T-helper cells to interleukin-1.[56,58,60]

The effect of Cy on suppressing T-cell-mediated immune response is particularly interesting, because it has been shown retinal S-antigen can cause experimental autoimmune

uveitis (EAU) in animals that resembles the T-cell-mediated ocular inflammation seen in humans.[18,27,34]

In view of this, Cy has been given systemically(I.M.) for the treatment of EAU with success,[28,30,33] and it has also been employed orally in the treatment of patients with uveitis with encouraging results;[17-19] however, renal toxicity occured at a relatively low dose of 10 mg/kg per day.[17,42,43] If the side effects of Cy could be controlled or circumvented, it may provide wide clinical applications; as of now, the side effects limit its use in ocular inflammatory disease. Local application of Cy would be one way of achieving therapeutic levels of Cy within the eye while avoiding the systemic side effects.

Our laboratory has shown with a number of agents that ocular absorption could be significantly enhanced by subconjunctival injection of these agents when compared to equidose systemic administration, making it possible to circumvent the associated toxicities of systemic administration.[20-24]

Using the subconjunctival route for the administration of Cy, we evaluated vehicle and drug toxicity, ocular pharmacokinetics following equidose subconjunctival and intravenous administration, and the effects of subconjunctivally administered Cy on an animal model of uveitis to develop a protocol for the administration of Cy subconjunctivally.

We found that the commercially prepared Cy in the

vehicle Cremophor(R) EL produced less of an inflammatory reaction and the maximum tolerable dose by the eye of the commercial Cy was 5 mg in 0.1cc once per week. From the pharmacokinetic study we were able to show that significantly higher levels of Cy entered the eye by subconjunctival injection compared to an equidose intravenous injection. Equally important, the blood levels in the rabbits that received Cy subconjunctivally were within the recommended levels.

The uveitis (experimental autoimmune uveitis induced by bovine serum albumin) study showed that Cy was able to reduce the inflammatory response and the earlier the treatment with Cy the milder the uveitis.

With subconjunctival administration of Cy we were able to achieve higher ocular concentrations and at the same time greatly reduce the possibility of the associated systemic side effects of Cy, making Cy administered subconjunctivally a possible candidate for routine use in the treatment of ocular inflammatory disease.

CHAPTER 1

Ocular toxcity study of possible vehicles for cyclosporine

Introduction

Cyclosporine (Cy) , an immunosuppressive agent, has been used systemically in the treatment of chronic ocular inflammatory disease, which was resistant to conventional therapy.[17,18] However, the concentration of Cy needed to treat the ocular inflammation via this administration route also resulted in renal toxicity.[42,43]

Therefore,if Cy is administered locally, this side effect may be avoided. Ocular absorption has been shown to be significantly increased by local subconjunctival injection, when compared to the systemic route.[20-24] In order to exact a formulation for Cy to be injected subconjunctivally, an appropriate vehicle had to be found for Cy. The requirements for this vehicle were governed by the lipophilic chemistry of Cy[15,54] which limited the vehicle to an oil-based medium. Other requirements for the vehicle are that it produce little ocular reaction and that it be readily absorbed by the eye. The vehicles that were tested were castor, cotton seed, olive, peanut, and sesame seed oils as well as polyethylene glycol (PEG), which has both hydrophobic and hydrophilic properties.

Materials & Methods

Prior to starting the experiments, each rabbit in the study was examined by slit-lamp biomicroscopy to rule out the presence of pre-existing ocular disease. Six groups of five rabbits each (locally supplied New Zealand white females weighing 2.2 - 2.4 kg) were injected subconjunctivally, following topical anaesthesia (proparacaine HCl (0.5%), by placing a 25 gauge needle attached to a tuberculin syringe at a shallow angle parallel and posterior to the superior limbus of the right eye, and with a smooth motion the needle was inserted under the conjunctiva and 0.5 cc of a sterilized vehicle (by filtration throught a Millex-FG(R) 0.22 micron disposable filter unit) was slowly injected. The opposite eye served as a non-treated control.

The first group of 5 rabbits, each received 0.5 cc of castor oil, the second group cotton seed oil, the third group peanut oil, the fourth group olive oil, the fifth group sesame seed oil, and the sixth group PEG. The eyes were then examined by slit-lamp biomicroscopy each day for one week, and serially photographed. The degree of inflammation in each eye was graded according to the scheme presented in Table I, in which an overall average value of inflammation was obtained after evaluating each criterion for each rabbit in this experiment. The rabbits were also graded by 2 other examiners who had no knowledge of the grouping of the different rabbits. No significant

variability was found among the examiners (data not shown). The groups were sacrificed with anoverdose of pentobarbital after one week. The globes were enucleated, marked at the point of injection with a dye, fixed in 10% buffered formalin, embedded in paraffin, sectioned vertically and stained by Haematoxylin-Eosin for histopathological study.

Histologic assesment of density and area of inflammatory infiltrate was made and an overall average value of inflammation obtained. The histologic assessments were made by myself and a pathologist who had no knowledge of the grouping of the rabbits in the study. No significant variability was noted between the examiners (data not shown).

Table I

Conjunctiva		Ocular Discharge	Erosion of Epithelium	% Bleb present	Corneal Clarity	Iris Injection	Anterior Chamber	Overall Inflammation
Hyperemia	Chemosis							
none	none	none	none	0	clear	normal	normal	0
trace	trace	trace	trace	<10%	trace	trace	trace	1
mild	mild	mild	mild	10%-40%	mild	mild	mild	2
moderate	moderate	moderate	moderate	40%-70%	moderate	moderate	moderate	3
severe	severe	severe	severe	70%-100%	severe	severe	severe	4

Results

Clinical Ocular Toxicity (figures 1.1 and 1.2)

Subconjunctival injection of 0.5ml of the vehicles resulted in varying degrees of inflammation. In each case not much reaction was seen in the first 12 hours, except for mucus secretion, mild edema and hyperemia of the conjunctiva. Slit-lamp biomicroscopic examination in all cases showed no abnormalities within the anterior chamber or vitreous as to flare, cells and fibrin. The iris appeared normal, and the cornea was clear, and its surface did not stain with fluorescein.

With almost every vehicle the bleb took approximately seven days to be absorbed. The toxic effects of vehicles were confined to the conjunctiva, with hyperemia, chemosis and erosion of the conjunctival epithelium being present to varying degrees, depending on the vehicle.

In general cotton seed, olive, peanut and sesame seed oils showed the most aggressive clinical reaction with moderate to severe hyperemia and mild to moderate chemosis of the conjunctiva, as well as moderate to severe erosion of conjunctival epithelium in the rabbits. In these groups the inflammation peaked at about the third or fourth day, from which point the inflammation started to decrease.

Castor oil showed a less aggressive clinical reaction with mild to moderate hyperemia and chemosis of the conjunctiva with three of the rabbits in this group

showing mild to moderate erosion of the conjunctival epithelium. The inflammation in the castor oil group also peaked between days three and four, from which point the inflammation started to decrease. The plot of the overall inflammation over the one week period is presented in Figure 1.0 and representative photographs of each vehicle at one, four and seven days are shown in Figures 1.1 and 1.2.

The PEG group was the exception with the bleb having been absorbed within 24 hours of the injection and only showing trace hyperemia and chemosis of the conjunctiva over the first 48 hours, from there on the eye appeared normal.

Histopathologic Findings

The vehicles in our study produced varying degrees of inflammation, restricted to the conjunctiva and posterior soft tissues. The rest of the eye itself appeared uninvolved. An intermediate to severe reaction occurred with castor oil, which spread as far posteriorly as the optic nerve and caused an exuberant inflammatory infiltrate. An intermediate reaction was seen with cotton seed, olive, peanut and sesame seed oil; these agents typically reached the equator of the globe and induced a moderate inflammatory response. The least reaction resulted with PEG which appeared to be entirely absorbed with little or no inflammation.

Although the degree of inflammation varied, the pattern of the response was similar in all cases. Two hours following injection, a few polymorphonuclear granulocytes and lymphocytes could be seen streaming from dilated capillaries. By twelve hours, these had surrounded the oil droplets, which were gradually diffusing posteriorly. Twenty four hours after injection, these acute inflammatory cells were joined by macrophages, some of which appeared to be ingesting the oil. By one week, the inflammation exhibited a granulomatous pattern, with occasional foreign body giant cells, foamy macrophages, lymphocytes and plasma cells.

The histological findings of the various vehicles are shown in figures 1.3-1.8.

Figure 1.0

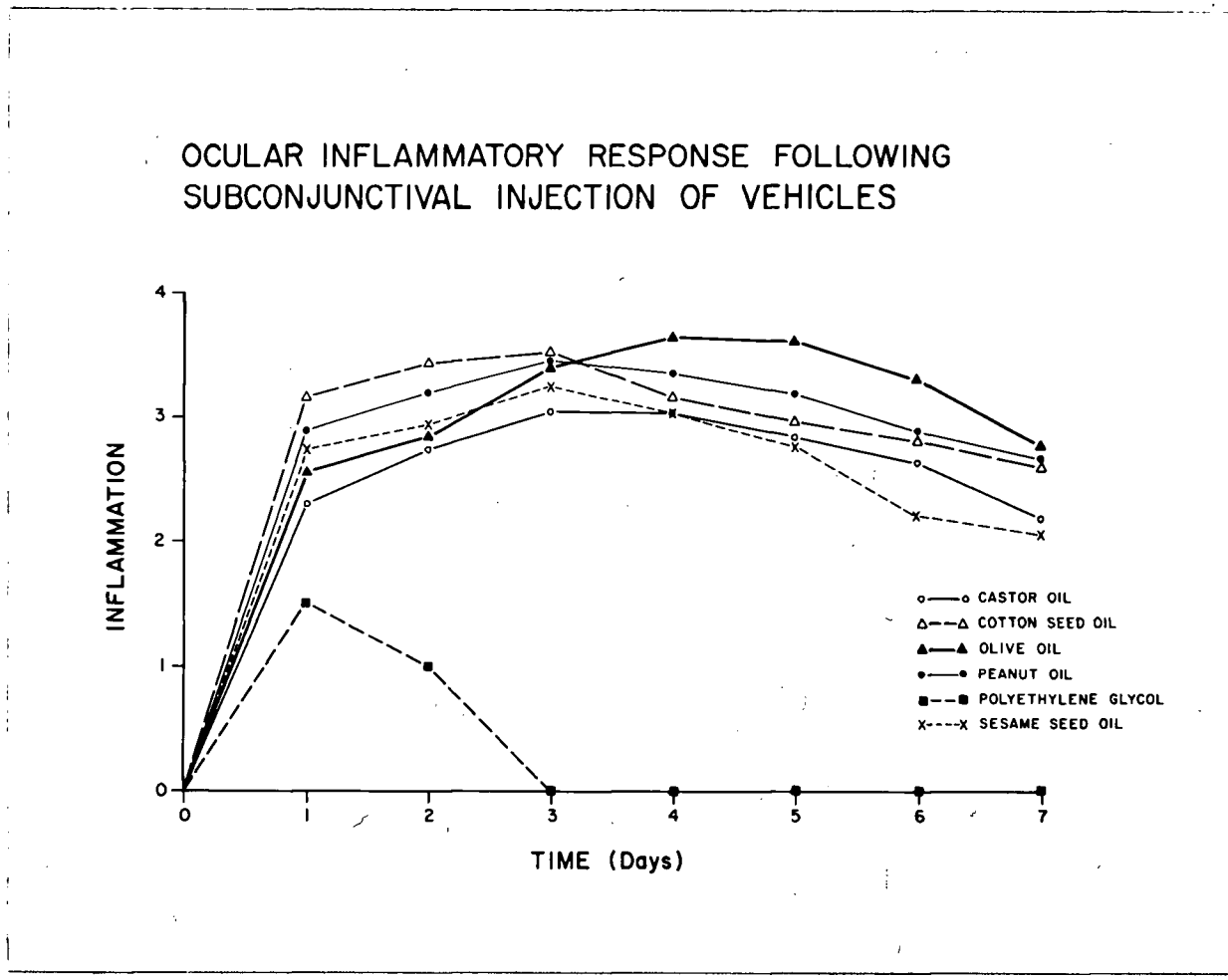
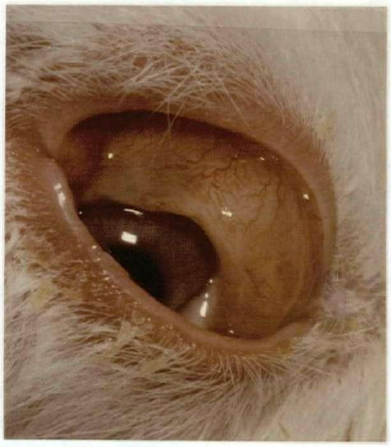
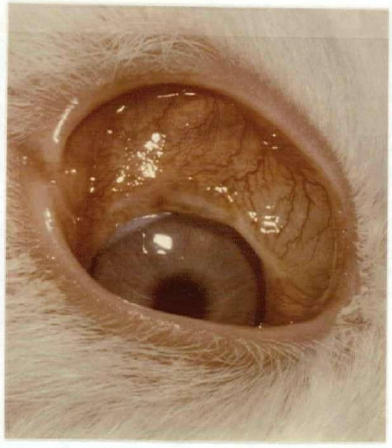

OCULAR INFLAMMATORY RESPONSE FOLLOWING
SUBCONJUNCTIVAL INJECTION OF VEHICLES
INFLAMMATION
0
1
2
3
4
0
1
2
3
4
5
6
7
TIME (Days)
CASTOR OIL
COTTON SEED OIL
OLIVE OIL
PEANUT OIL
POLYETHYLENE GLYCOL
SESAME SEED OIL

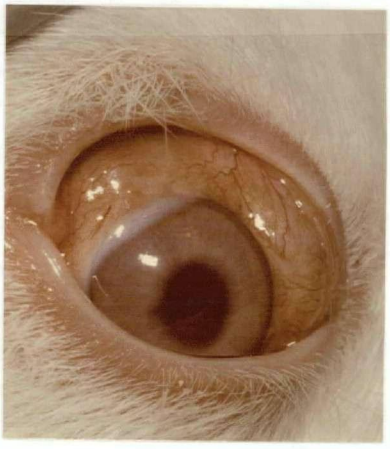
Figure 1.1
Clinical Ocular Inflammatory Response to the Vehicles Tested



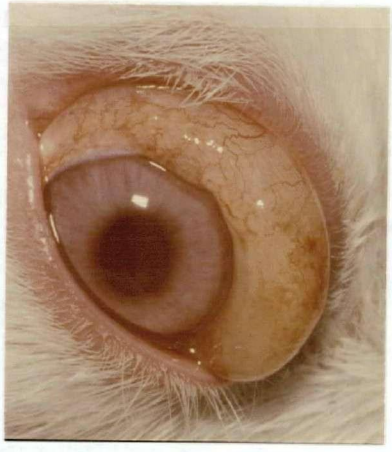
castor oil at day 1



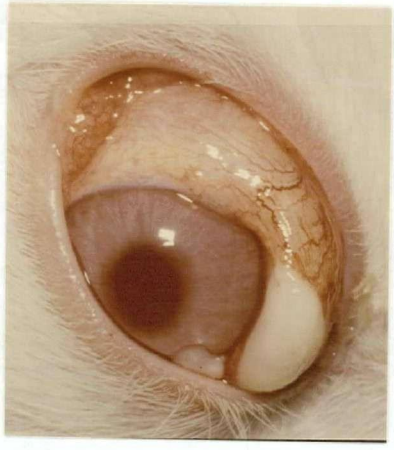
castor oil at day 4



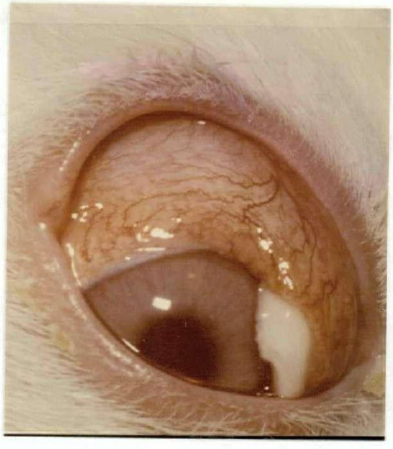
castor oil at day 7



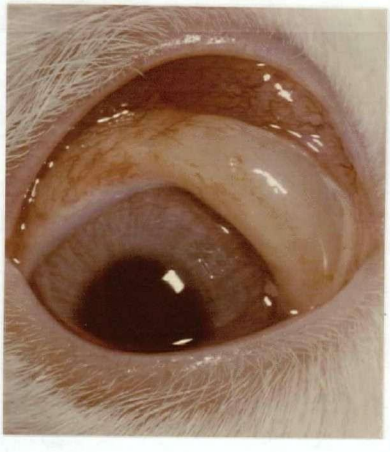
cotton seed oil
at day 1



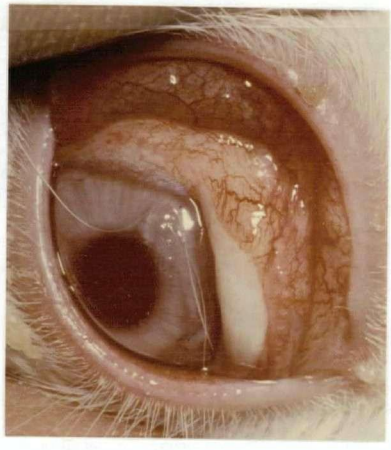
cotton seed oil
at day 4



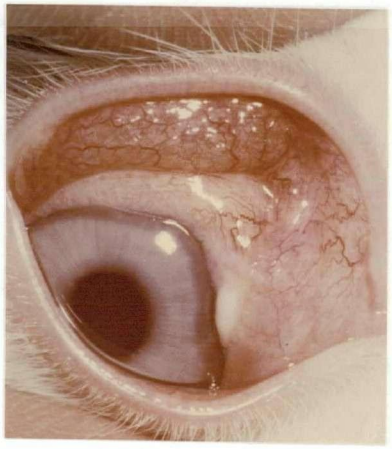
cotton seed oil
at day 7



olive oil at day 1

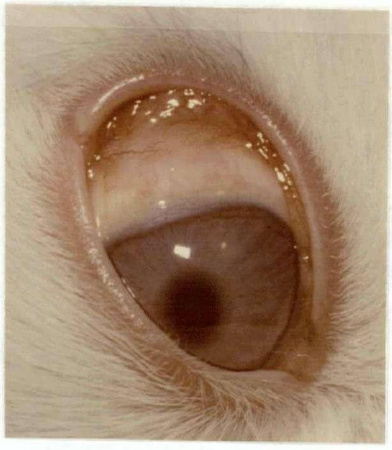


olive oil at day 4

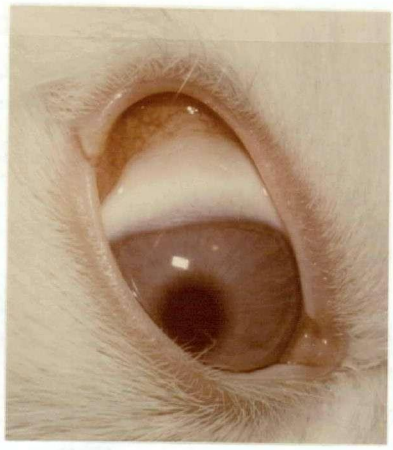


olive at day 7

Figure 1.2
Clinical Ocular Inflammatory Response to the Vehicles Tested Cont'd



PEG at day 1



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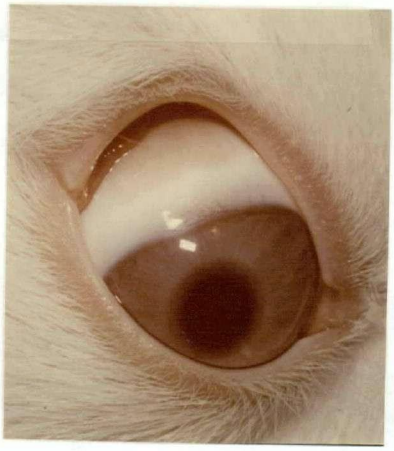
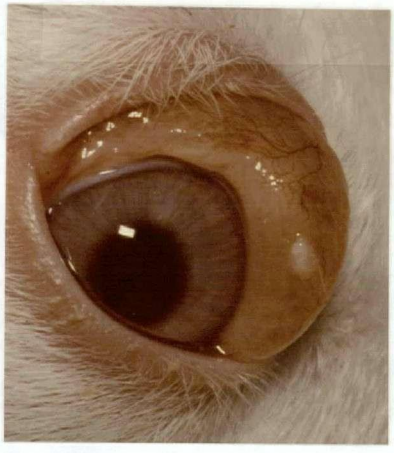
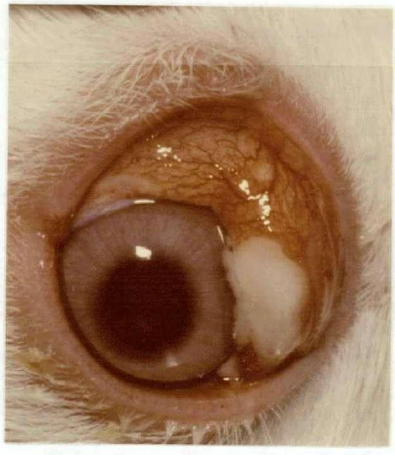


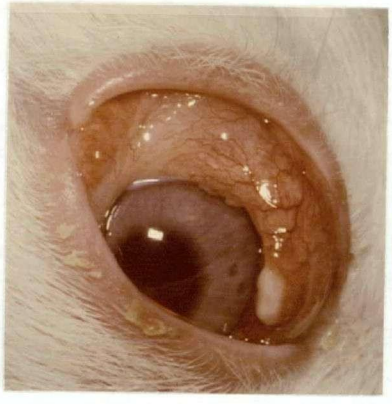
PEG at day 7



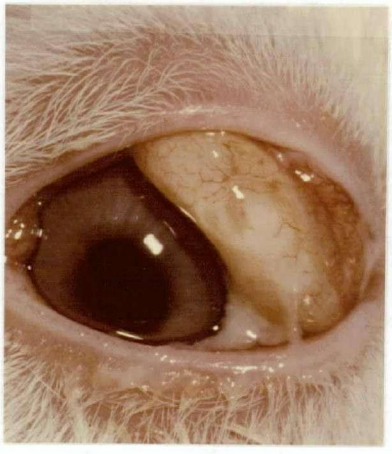
peanut oil at day 1



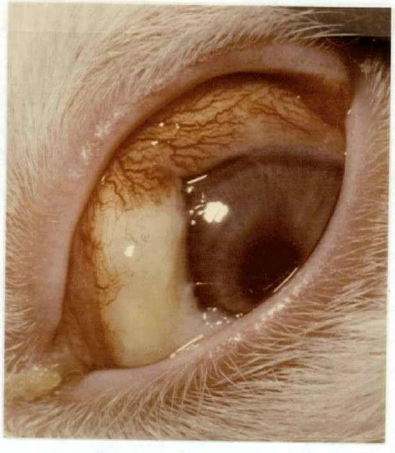
peanut oil at day 4



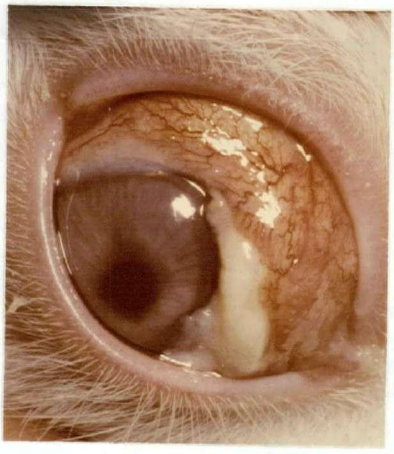
peanut oil at day 7



sesame seed oil
at day 1

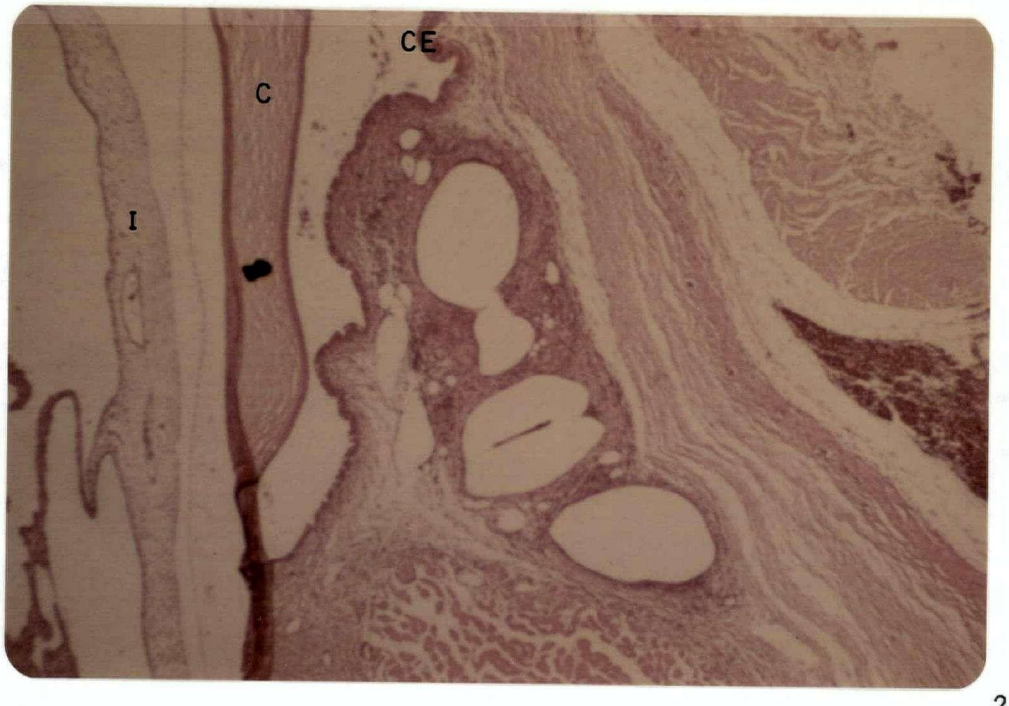


sesame seed oil
at day 4

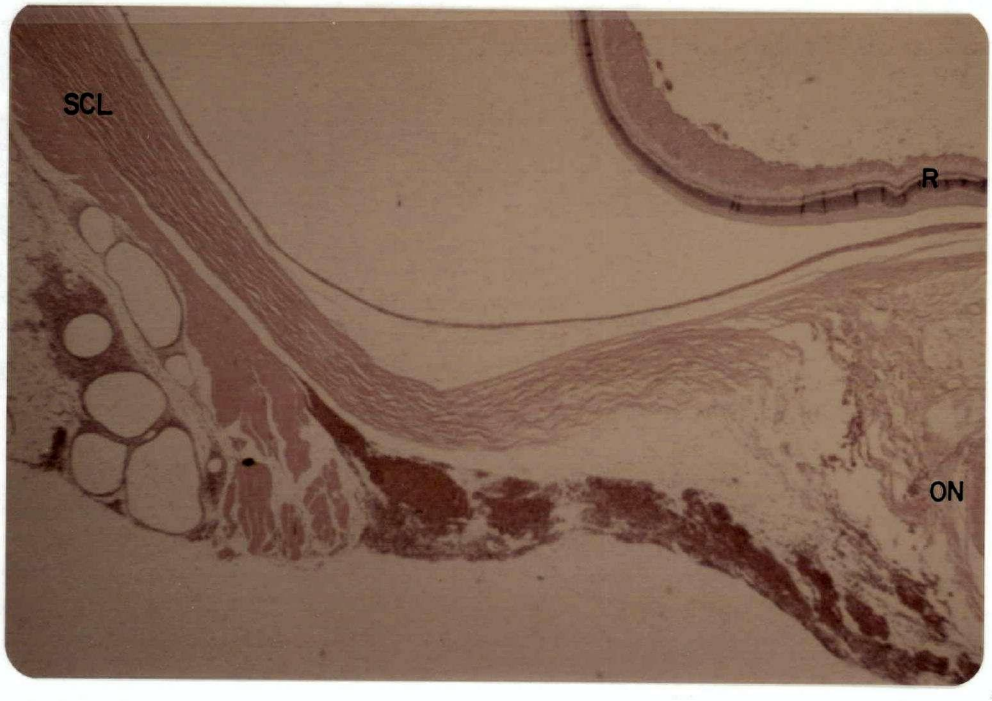


sesame seed oil
at day 7

Figure 1.3*
Histological effects of Castor Oil at one week



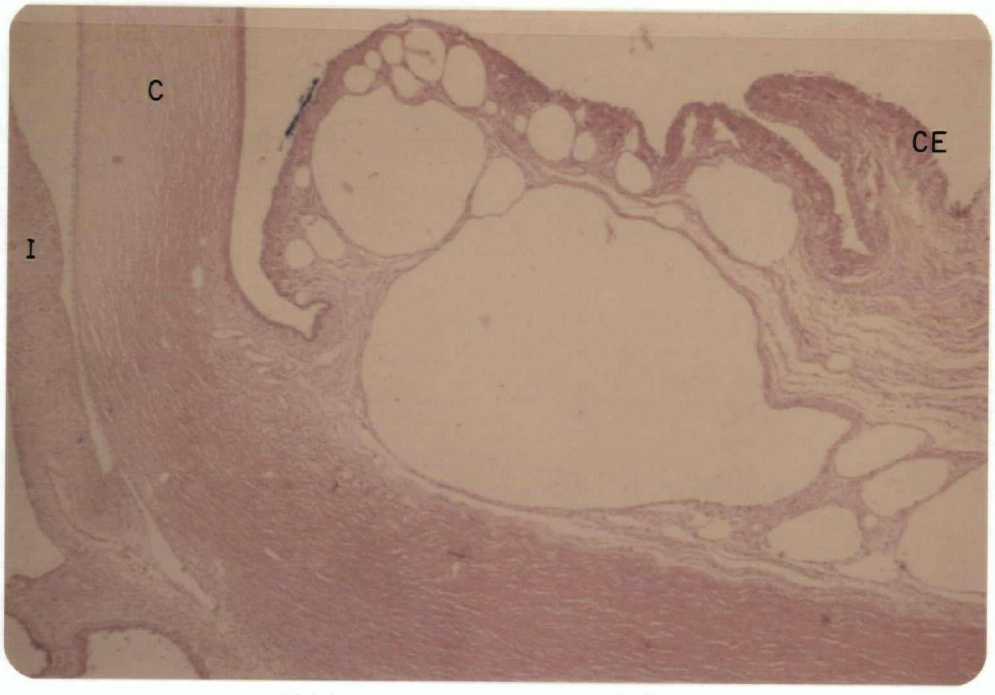
2.5x



2.5x

Castor oil spread posterior to the equator of the globe, almost reaching the optic nerve(ON). * see list of abbreviations for figures 1.3-1.8.

Figure 1.4
Histological effects of Cotton Seed Oil at one week



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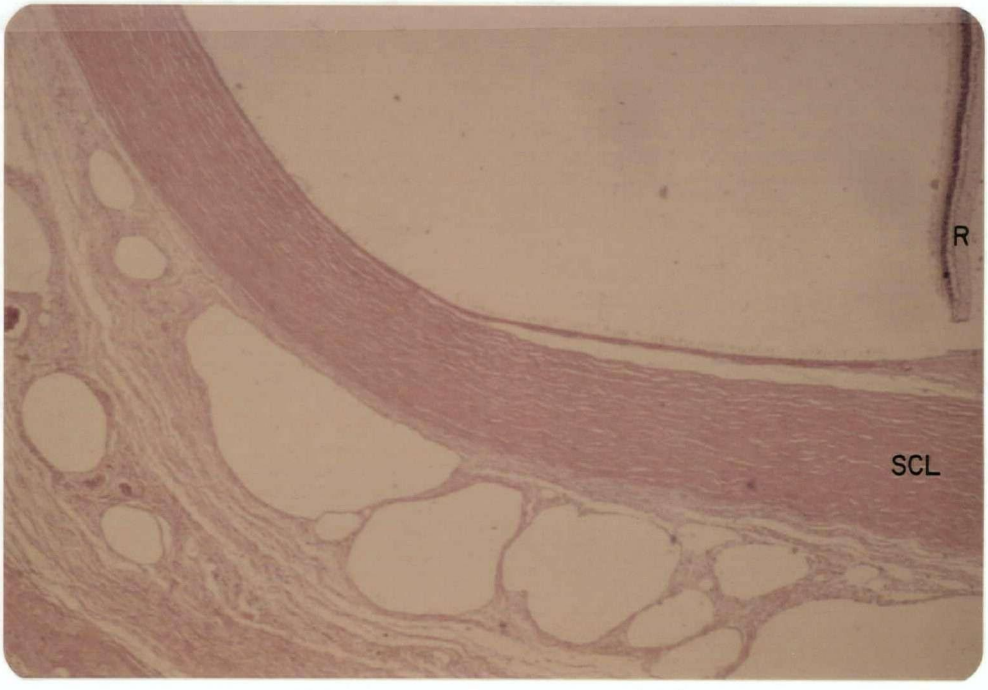
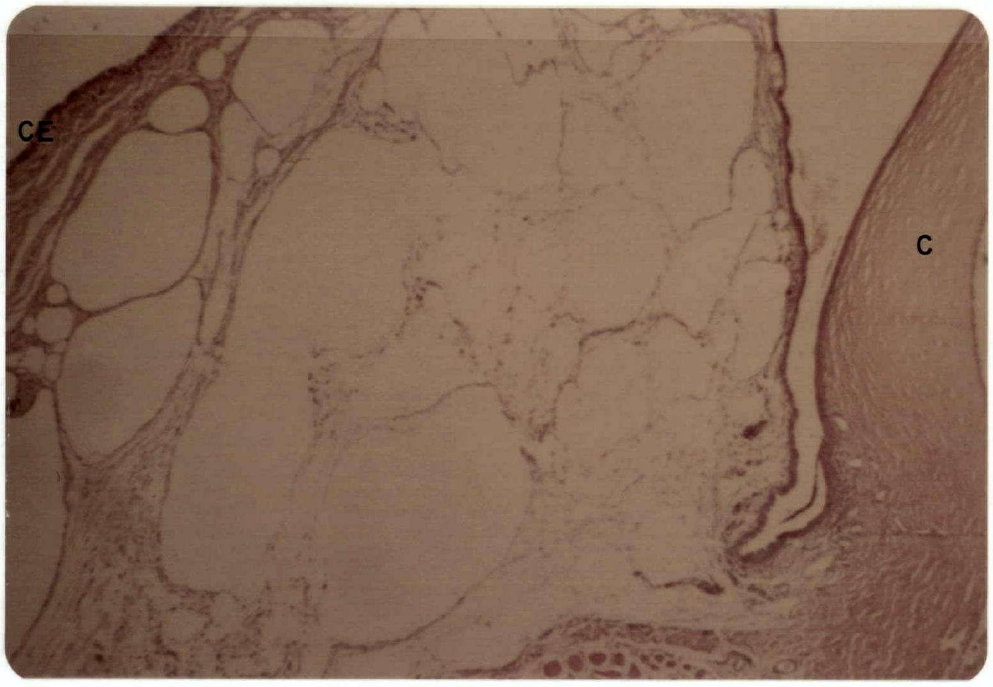


2.5x

The cotton seed oil can be seen reaching the equator of the globe.

Figure 1.5
Histological effects of Olive Oil at one week



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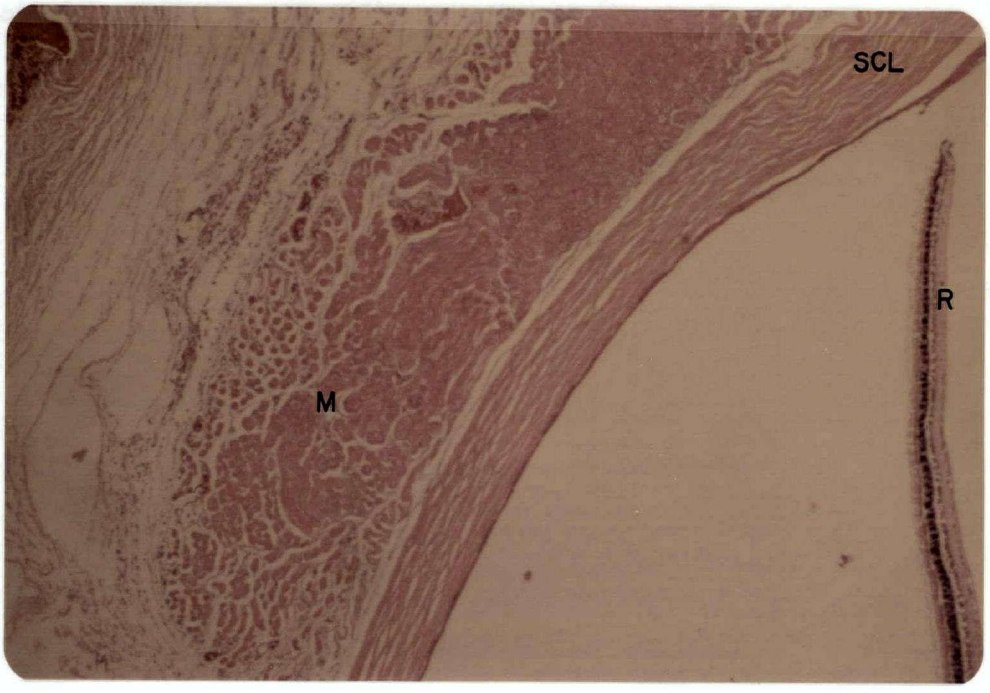
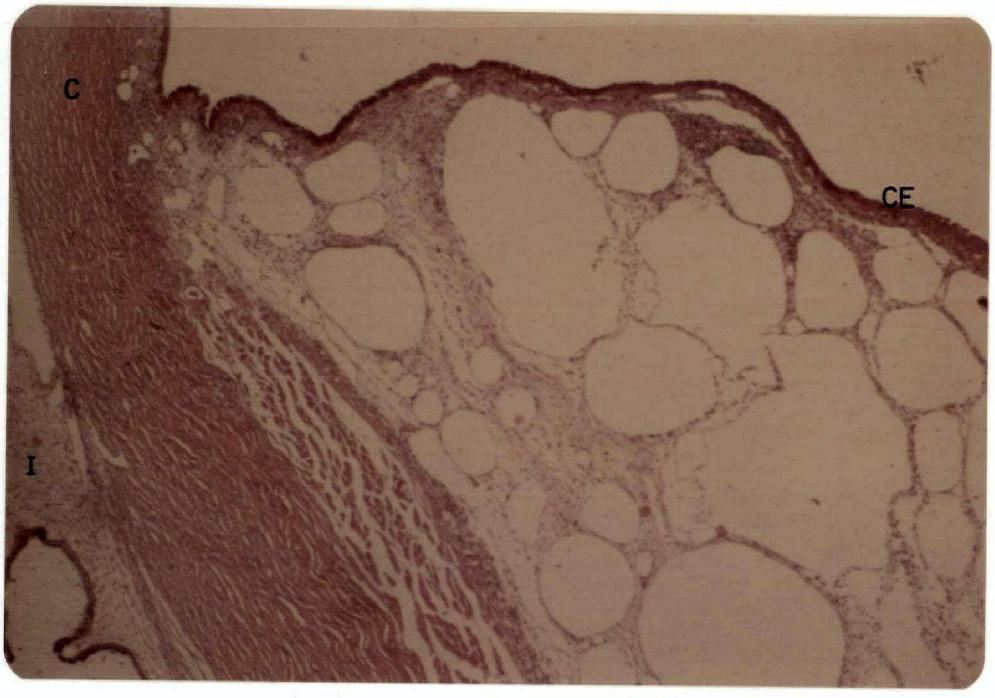


2.5x

Olive oil remained in the region of the superior rectus muscle(M).

Figure 1.6
Histological effects of Peanut Oil at one week



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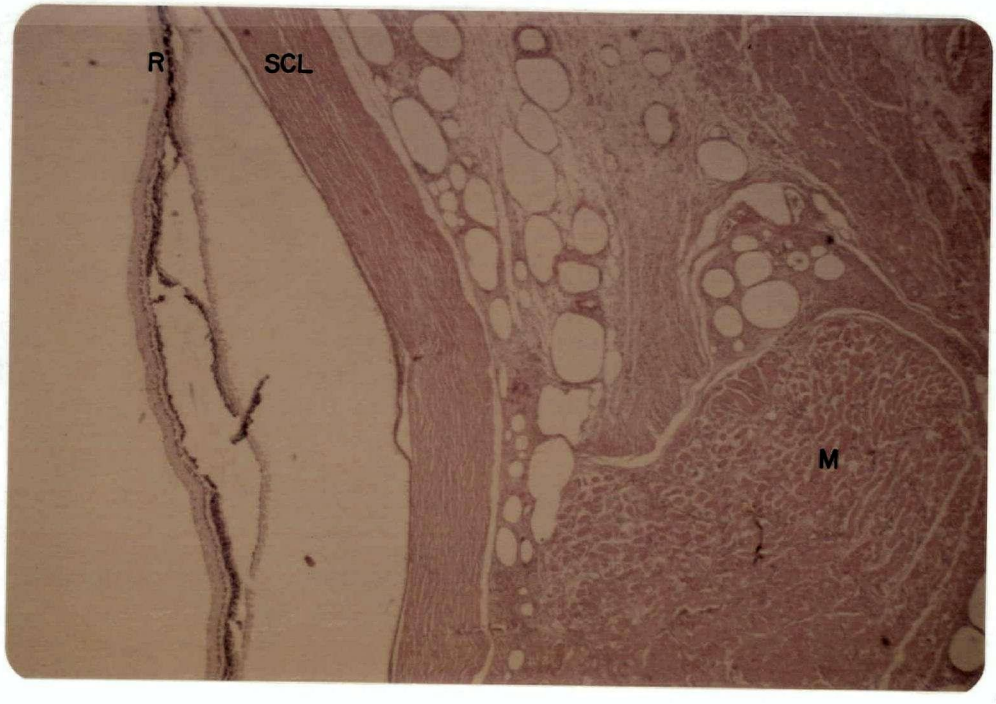
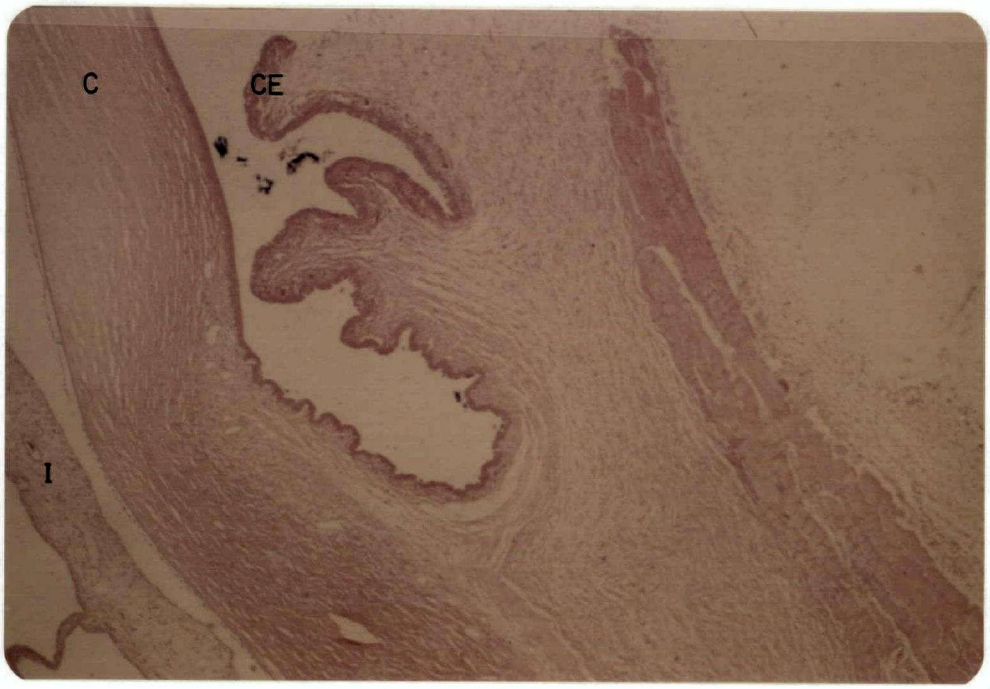


2.5x

Peanut oil spread to the equator of the globe, remaining mostly around the superior rectus muscle(M).

Figure 1.7
Histological effects of Polyethylene Glycol(PEG) at one week



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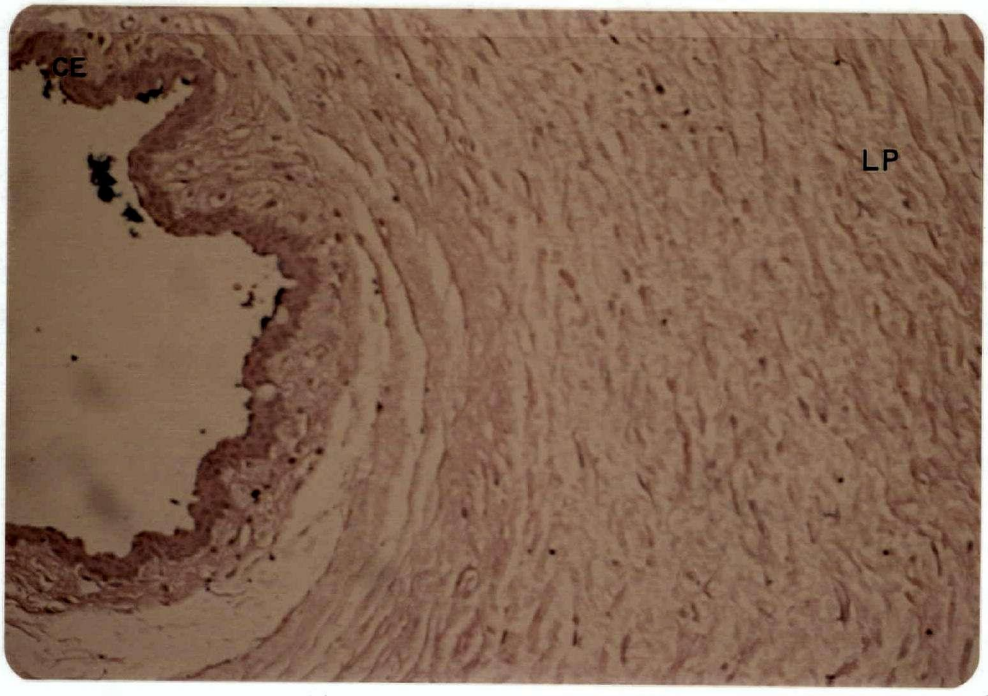
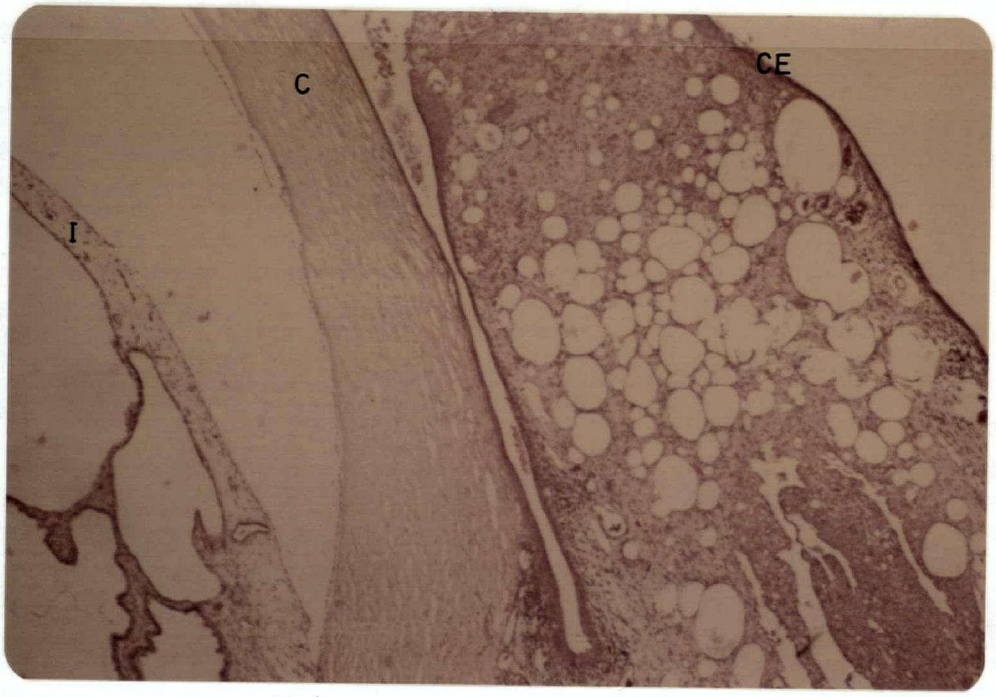


10x

Blue stain marks the site of injection. This area shows no evidence of inflammation at one week.

Figure 1.8
Histological effects of Sesame Seed Oil at one week



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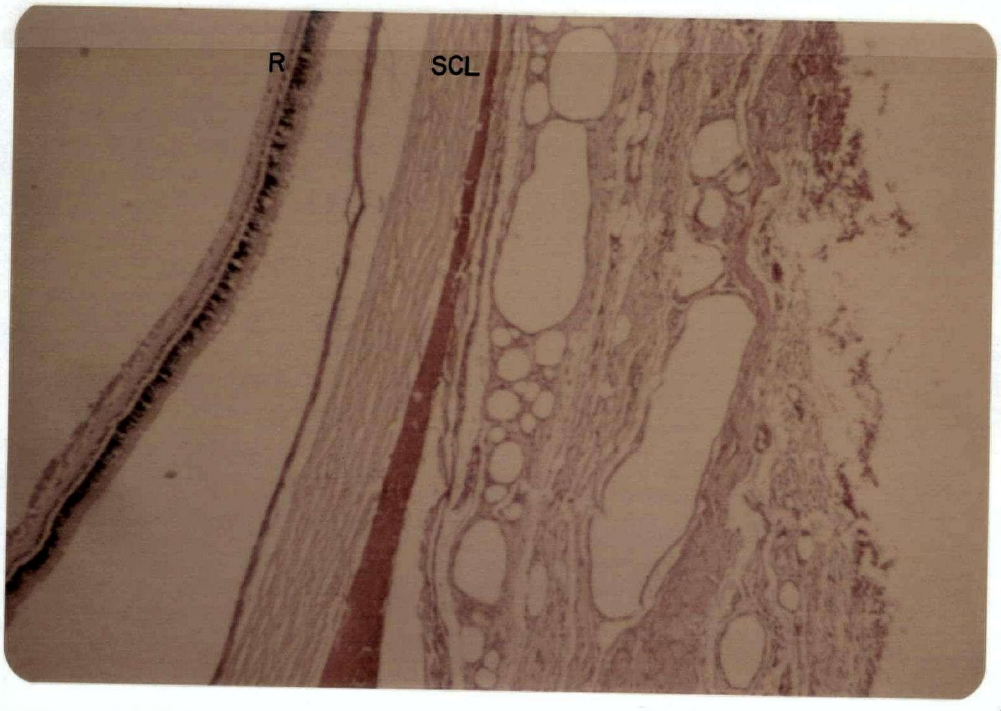


2.5x

Sesame seed oil can be seen reaching the equator of the globe.

DISCUSSION

In previous studies in this laboratory, the drugs that had been given subconjunctivally were soluble in saline.[21,23] Most of the ocular toxicity to the drug could be controlled by adjusting the amount of drug injected subconjunctivally.

Cyclosporine (Cy) presented us with a unique problem, because its chemistry demanded that the vehicle had to be lipophilic. For Cy to be injected subconjunctivally the ocular toxicity of two variables had to be determined, that of the vehicle and of Cy.

A number of vehicles were chosen as possible candidates for subconjunctival injection. These were selected from a group of vehicles that had previously been used as pharmacological solvents and in which Cy would be readily soluble.[46]

The vehicles selected for the study include: castor, cotton seed, peanut, olive and sesame seed oil as well as polyethylene glycol (PEG). These were sterilized and 0.5cc (this being the amount the rabbits' conjunctiva can tolerate) of each was injected subconjunctivally.

Daily observations were made by slit lamp biomicroscopy to ascertain the effects of the vehicles within and surrounding the eye. The major toxic effects were confined to the conjunctiva resulting in hypermia, chemosis and erosion of the conjunctival epithelium. Occasionally the vehicles would cause a corneal edema, but

this was infrequent.

The vehicles produced varying degrees of inflammation with PEG causing a trace inflammatory reaction histologically and clinically. This result is not entirely unexpected because PEG's are compounds of low toxicity and the PEG that was injected subconjunctivally was made up so that it had a tonicity equivalent to that found in the ocular chambers (0.9% NaCl) and was absorbed within 24 hours after injection. Unfortunately, the use of PEG was not possible because when I attempted to dissolve Cy in it, less that 0.5 milligrams dissolved in 10 cc. of PEG solution (data not shown).

The oils showed a greater affinity for Cy (on average 5 mg of Cy dissolved in 0.5 cc. of oil), but also produced a greater inflammatory response, partly because they were absorbed at a slower rate and the tonicity could not be adjusted to that of the eye. Cotton seed, olive, peanut and sesame seed oil produced an intermediate inflammatory reaction histologically, but the clinical findings showed a moderate to severe reaction. An intermediate to severe reaction was seen histologically with castor oil, but again these findings did not correlate well with the clinical findings which showed a mild to moderate inflammatory response.

These discrepancies may partly be explained by the spread of the oil posteriorly. In the case of castor oil, it spread as far back as the optic nerve, thereby causing

less of a reaction with the conjunctiva over the superior limbus, resulting in a mild to moderate response visible clinically. Because the oil had spread so far back, more oil was exposed to the ocular tissue; hence, a greater inflammatory reaction was seen histologically.

With cotton seed, olive, peanut and sesame seed oil, the vehicles spread up to the equator of the globe. In the case of these vehicles, most of the oil was restricted to the conjunctiva over the superior limbus, exposing this part of the conjunctiva for a longer period of time to the environment. Therefore, a moderate to severe clinical reaction was seen, and because less of the oil was exposed to the ocular tissues the ensuing inflammatory reaction that was seen histologically was less.

On average, the oils produced a similar response. However, since castor oil spread the furthest posteriorly we reasoned more Cy could be made available to the eye using this oil.

We felt using castor oil as a base for a vehicle for the delivery of Cy, the advantageous properties of castor oil can be exploited.

Cremophor(R) EL is such a commercially available vehicle, used in the I.V. form of Cy. The contents of Cremophor(R) EL consist of 33% alcohol, castor oil and polyoxyethylene glycol (POEG) 650mg. The POEG may act like a cushion to decrease the effects of the vehicle on the conjunctiva, and the alcohol provides two functions; it

allows more Cy to be dissolved in the castor oil so less vehicle has to be used, and at the same time it reduces the viscosity of the castor oil possibly making the vehicle less uncomfortable to the eye. The resultant of all these properties might be to further decrease inflammatory response to the vehicle, and to make possible the repeated injections of Cy subconjuntivally.

Conclusions

The oils on the average gave a similar response, but from a clinical perspective only castor oil appeared to be less severe. A commercially available vehicle which incorporated castor oil allowed for more delivery of the drug in less vehicle than could be obtained by using castor oil alone as a vehicle.

On this basis we reasoned theoretically the commercial preparation should produce less of a local response.

Unfortunately, at the time we were not able to obtain the Cremophor (R) EL without the Cy. Therefore, we could only speculate about the effects of the vehicle from our experience with the oils. One would expect much of the inflammatory reaction to be due to the vehicle and not Cy. We have since obtained the vehicle used in the Sandimmune I.V.(R) preparation and found that most of the inflammatory reaction was indeed due to the vehicle. In those rabbits that received Sandimmune I.V.(R) subconjunctivally, the inflammatory response was found to be less than with

the vehicle alone[81] (data and experiments not shown).

The original assumption, that most of the inflammatory reaction would be due to the vehicle used in Sandimmune I.V.(R) and not Cy was correct, and since the commercial preparation allows for more drug to be delivered in less vehicle than could be obtained using the oils alone, the inflammatory reaction could be greatly reduced.

CHAPTER 2

Dose determination study

Introduction

From the results obtained in Chapter 1, it was decided that the commercial preparation Sandimmune I.V.(R) containing the vehicle Cremophor (R) EL would be used. This was based on its availability as well as its ability to deliver more Cy (50 mg/ml). To complete the formulation of the subconjunctival dosage for Cy, the maximum tolerated dose had to be determined. This was done by testing a range of doses.

Materials and Methods

Three groups of five rabbits each (locally supplied New Zealand white females weighting 2.2 - 2.4 kg.) were examined by slit-lamp biomicroscopy prior to the experiment to rule out pre-existing ocular disease. These were injected subconjunctivally posterior to the superior limbus with 5, 10 or 25 mg of Cy {SANDIMMUNE I.V. (50 mg/ml of Cy in Cremphor)} using a 25 gauge needle attached to a tuberculin syringe {prior to injection the right eyes were anaesthetized with topical proparacaine HCl (0.5%)}. The opposite eye served as a non-treated control, as we were unable to obtain the Cremophor (R) EL without Cy. The eyes were examined by slit-lamp biomicroscopy each day for one week, and serially photographed. The degree of inflammation in each eye was graded according to the scheme

presented in Table II, in which an overall averaged value was obtained by assessing each criterion for the individual rabbits in this experiment. The groups were sacrificed with an overdose of pentobarbital after one week. The globes were enucleated, marked at the point of injection with a dye, fixed in 10% buffered formalin, embedded in paraffin, sectioned vertically and stained by Haematoxylin-Eosin for histopathological study.

Table II

Conjunctiva		Ocular Discharge	Erosion of Epithelium	% Bleb present	Corneal Clarity	Iris Injection	Anterior Chamber	Overall Inflammation
Hyperemia	Chemosis							
none	none	none	none	0	clear	normal	normal	0
trace	trace	trace	trace	<10%	trace	trace	trace	1
mild	mild	mild	mild	10%-40%	mild	mild	mild	2
moderate	moderate	moderate	moderate	40%-70%	moderate	moderate	moderate	3
severe	severe	severe	severe	70%-100%	severe	severe	severe	4

Results

Clinical Ocular Toxicity

The two highest doses of Cy were judged to be too toxic. The 10 mg subconjunctival dose of Cy produced within the first 24 hours moderate to severe hyperemia and chemosis of conjunctiva as well as moderate mucus secretion. The 25 mg subconjunctival dose of Cy produced erosion of the conjunctival epithelium and mild to moderate hair loss of the upper eyelid, secondary to inflammation and self-induced local trauma. Over the next six days the inflammation slowly resolved. The subconjunctival injection of a single 5 mg in 0.1cc dose of Cy was found to be the least toxic. It only produced trace to mild hyperemia and chemosis of the conjunctiva with trace mucus secretion. Most of these changes had resolved within 48 hours of injection of the Cy. From investigations performed with regard to the frequency of injections we noted that a single 5 mg in 0.1cc dose of Cy once per week would be clinically tolerable for our studies. We noted that injections of more than once per week led to intolerable local inflammation (data not shown). Observations on the effect of these doses is summarized in a daily plot of the overall inflammatory response for each day in figure 2.0.

The long-term study of the subconjunctival injection of 5 mg in 0.1cc of Cy once a week for two months showed no toxic effects that were not present with the

single subconjunctival injection of 5 mg in 0.1cc of Cy, except because of the repeated injections of Cy the conjunctiva reacted slightly more than was seen with just the single subconjunctival injection of 5 mg in 0.1cc of Cy, but the reaction largely resolved within 24 to 48 hours after injection.

Occasionally a transitory reaction was seen with all doses. This was moderate to severe edema of the upper eyelid of the rabbit, which usually resolved within 48 hours. In some instances, the edematous reaction was possibly due to the rabbit moving upon injection which inadvertently resulted in the needle penetrating the upper eyelid, causing the swelling of the eyelid. Representative photographs showing the effect of the various doses at one, four, and seven days are shown in figures 2.1-2.2.

Histopathological Findings

From our observations in Chapter 1 our data suggests some suppression of the inflammatory response when we used Sandimmune I.V.(R).

At two hours, the subconjunctival tissues of the experimental eye showed few polymorphonuclear granulocytes (PMN) by 12 hours, and by 24 hours, the experimental eye contained only a scattering of macrophages and lymphocytes. By one week the experimental eye showed only a few lymphocytes and macrophages.

Increasing the dosage of Cy to 10 and 25 mg resulted in additional toxic side effects. 10 mg injection of Cy caused epithelial irregularities and denaturation of collagen and muscle. 25 mg injections also caused localized scarring of the periocular tissues.

Subsequent experimental work done by our group with Sandimmune I.V.(R) preparation, suggested that indeed the inflammatory response with Sandimmune I.V.(R) was less than with the vehicle alone.[81]

These local side effects were less noticeable in the rabbits given 5 mg in 0.1 cc of Cy once weekly for 2 months, but they showed lymphocyte and plasmocyte infiltrates in the renal cortex interstitium and hepatic portal triads.

The histological findings of the various doses and the long-term effects of subconjunctivally administered Cy are shown in figures 2.3-2.6.

Statistical Analysis

An analysis of variance (nested) test showed that there was a significant difference between the various doses tested of the commercially available preparation. The level of significance between the doses was found to be $p=0.0001$.

Figure 2.0

OCULAR INFLAMMATORY RESPONSE TO DIFFERENT DOSES OF CYCLOSPORINE (SANDIMMUNE I.V.) SUBCONJUNCTIVALLY

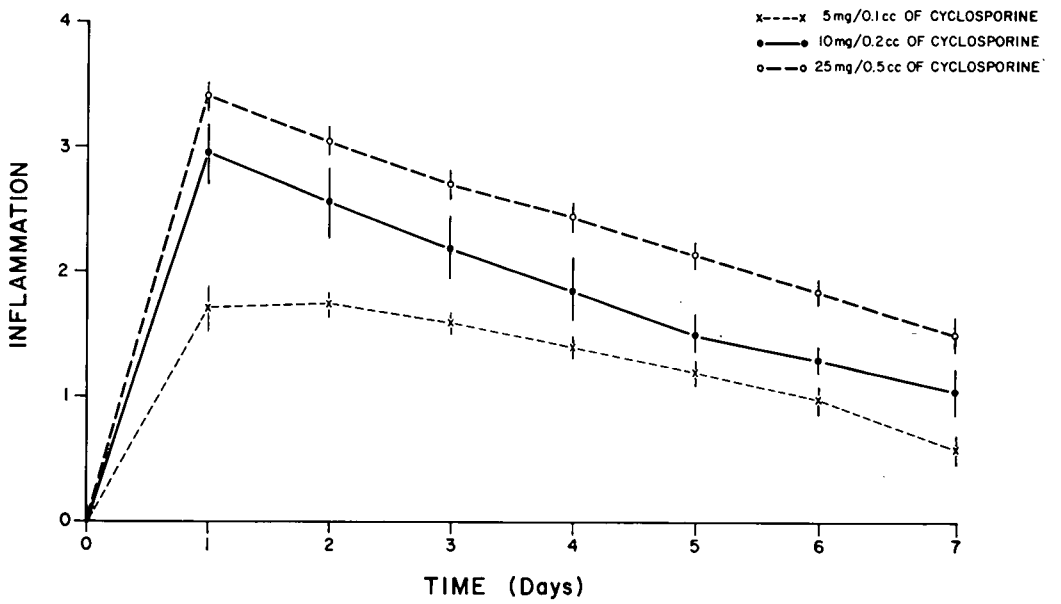
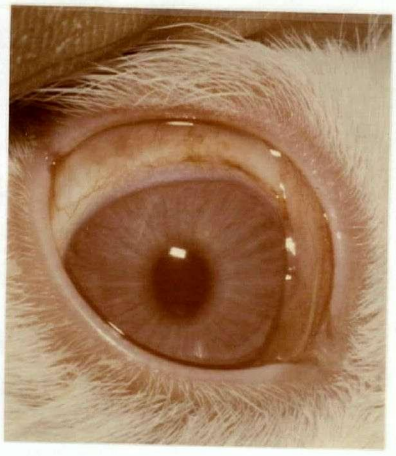
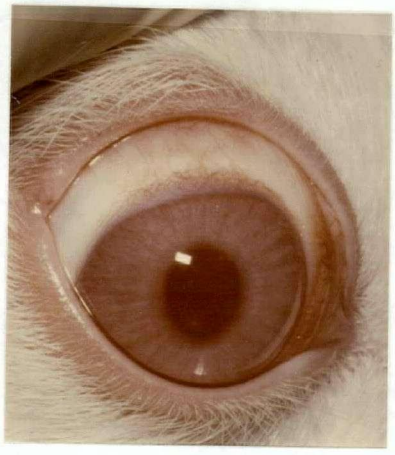


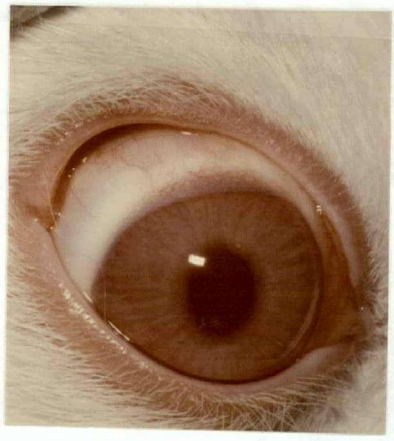
Figure 2.1
Effects of 5mg/0.1cc of Cyclosporine



at day 1

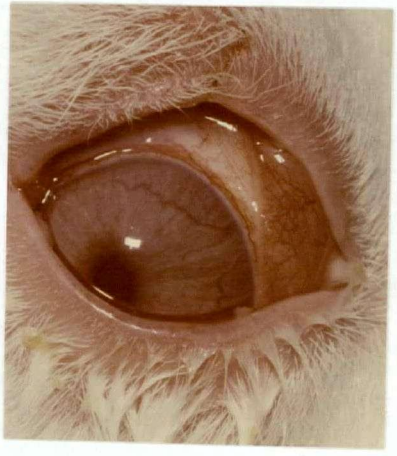


at day 4

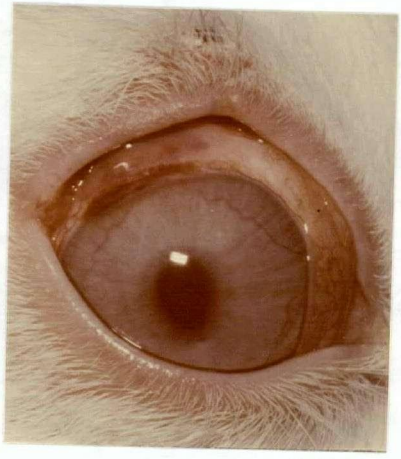


at day 7

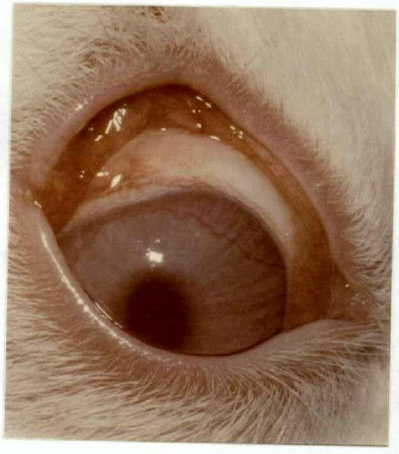
Effects of 10mg/0.2cc of Cyclosporine



at day 1



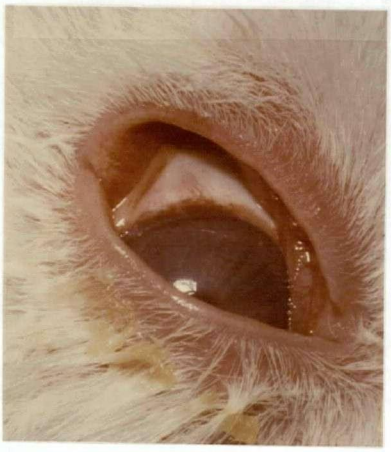
at day 4



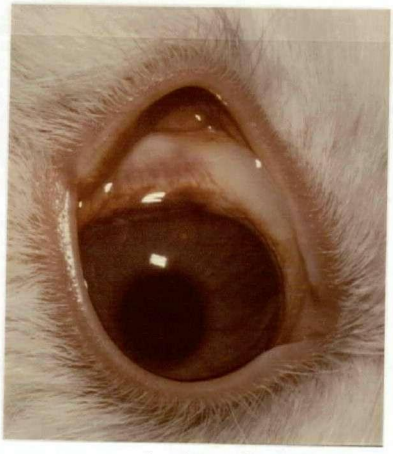
at day 7

Figure 2.2

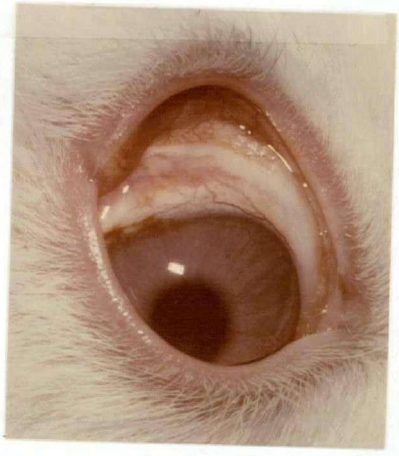
Effects of 25mg/0.5cc of Cyclosporine



at day 1

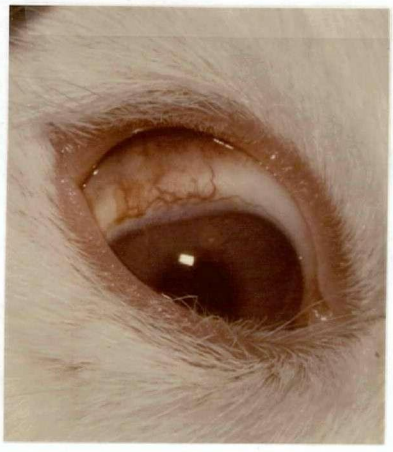


at day 4

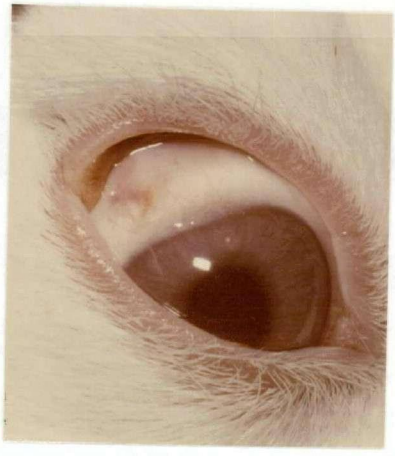


at day 7

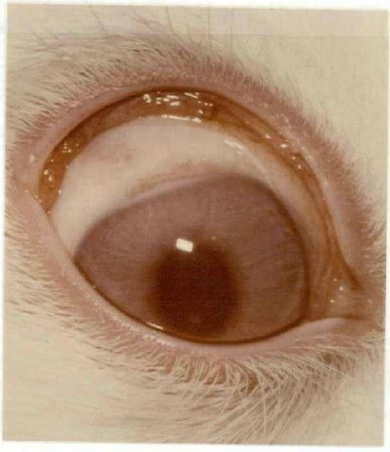
Effects of 5mg/0.1cc of Cyclosporine once per week for two months



at day 5

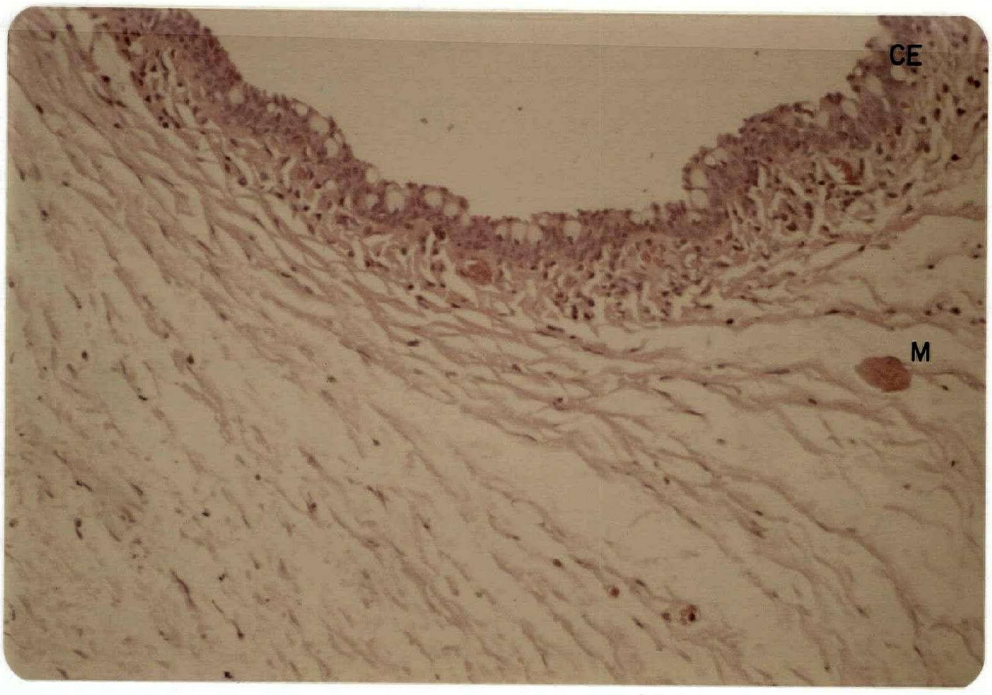


at day 30

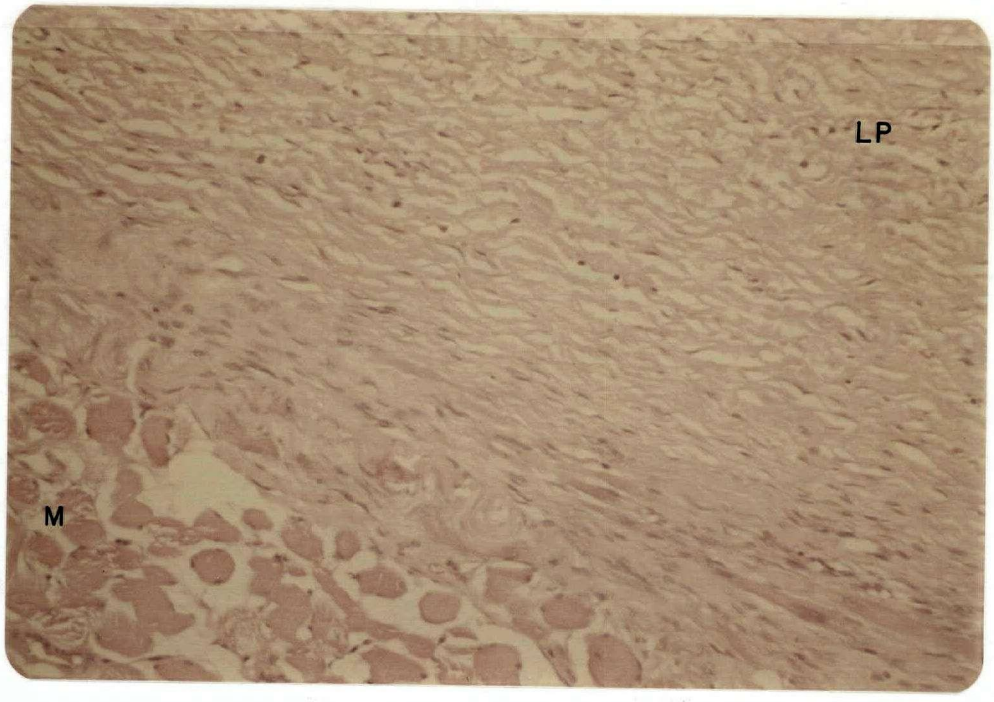


at day 60

Figure 2.3*
Histological effects of 5mg/0.1cc of Cyclosporine at one week



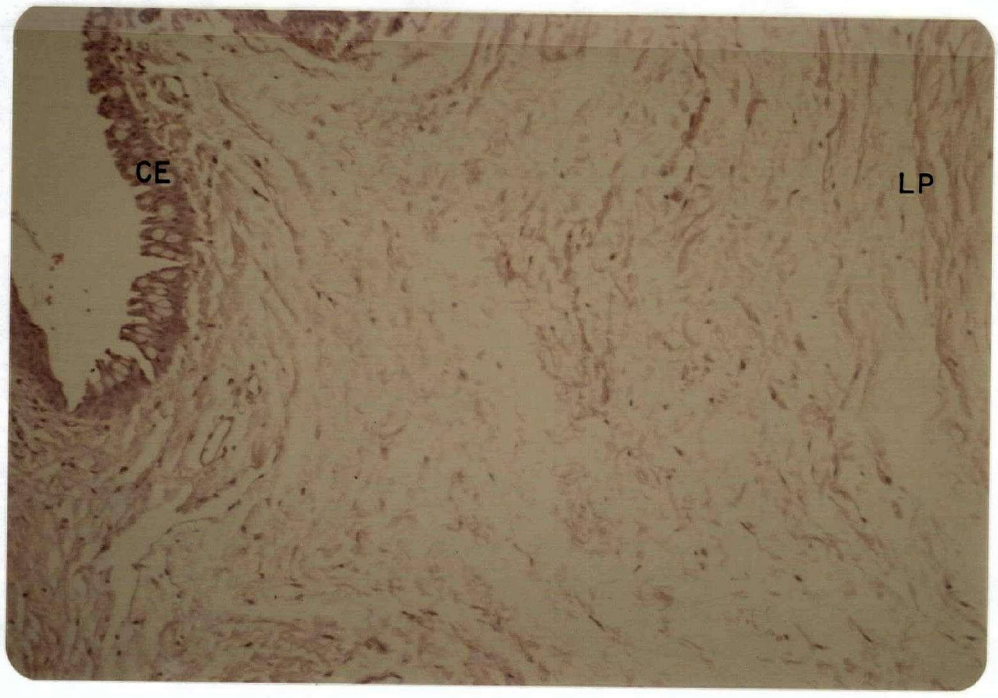
10x



10x

Only a few inflammatory cells are present at one week.* see list of abbreviations for figures 2.3-2.6.

Figure 2.4
Histological effects of 10mg/0.2cc of Cyclosporine at one week



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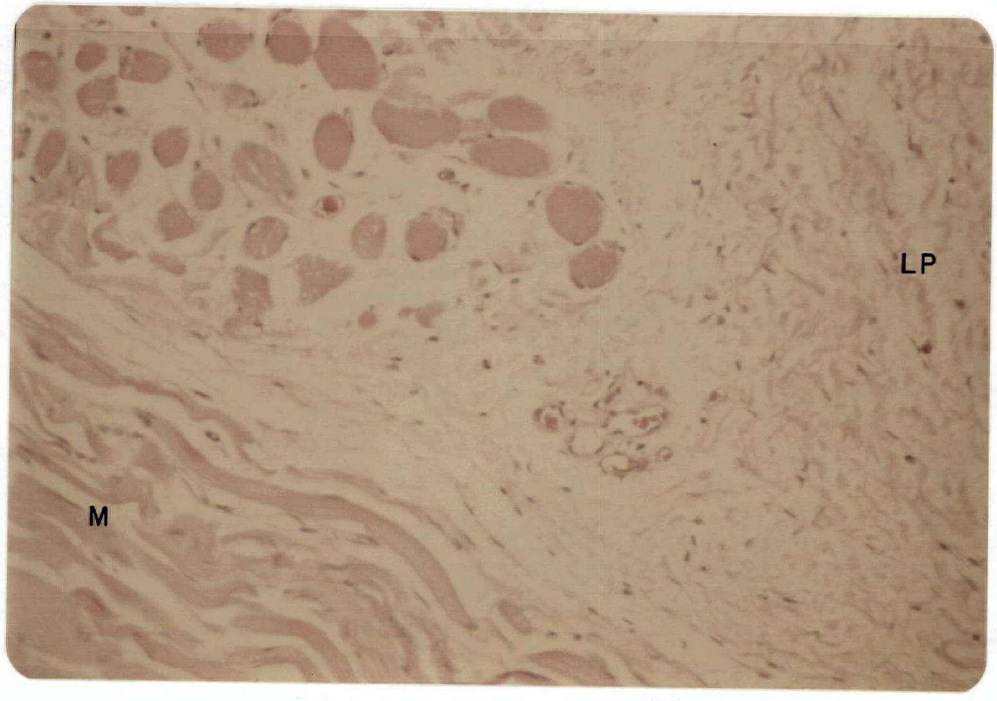
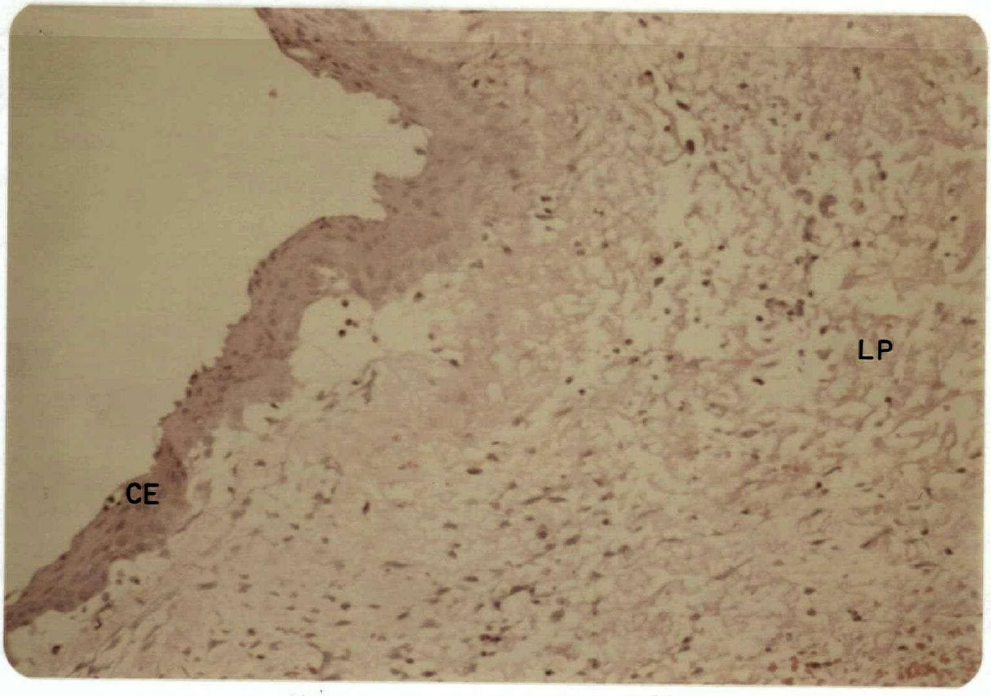


10x

A trace inflammatory response is present at one week as well as a loose organization of the collagen in the lamina propria.

Figure 2.5
Histological effects of 25mg/0.5cc of Cyclosporine at one week



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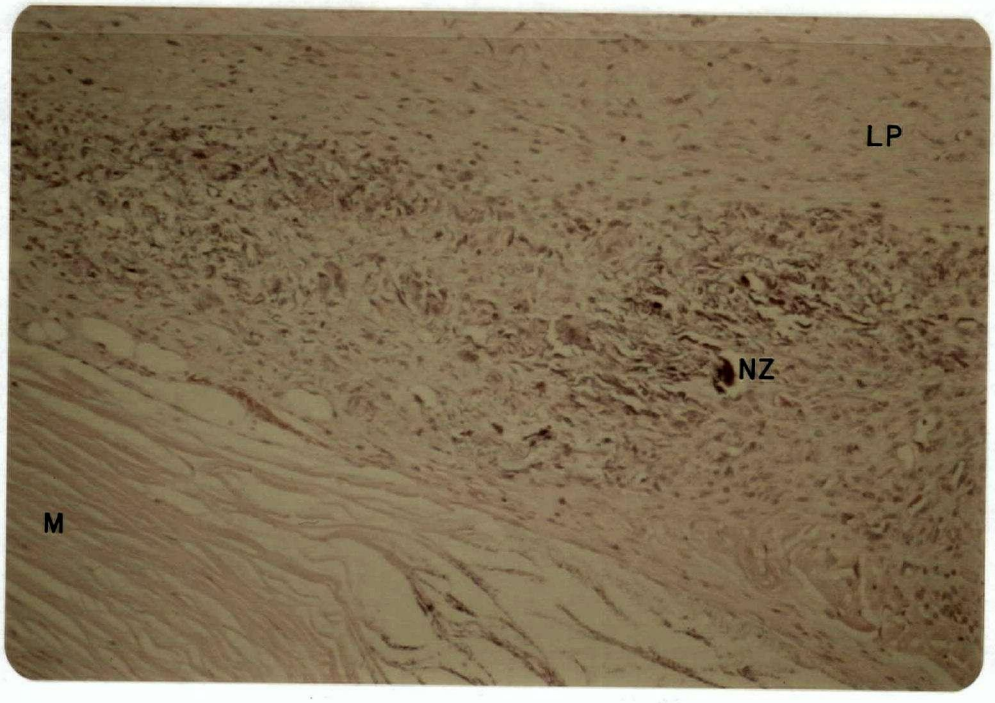
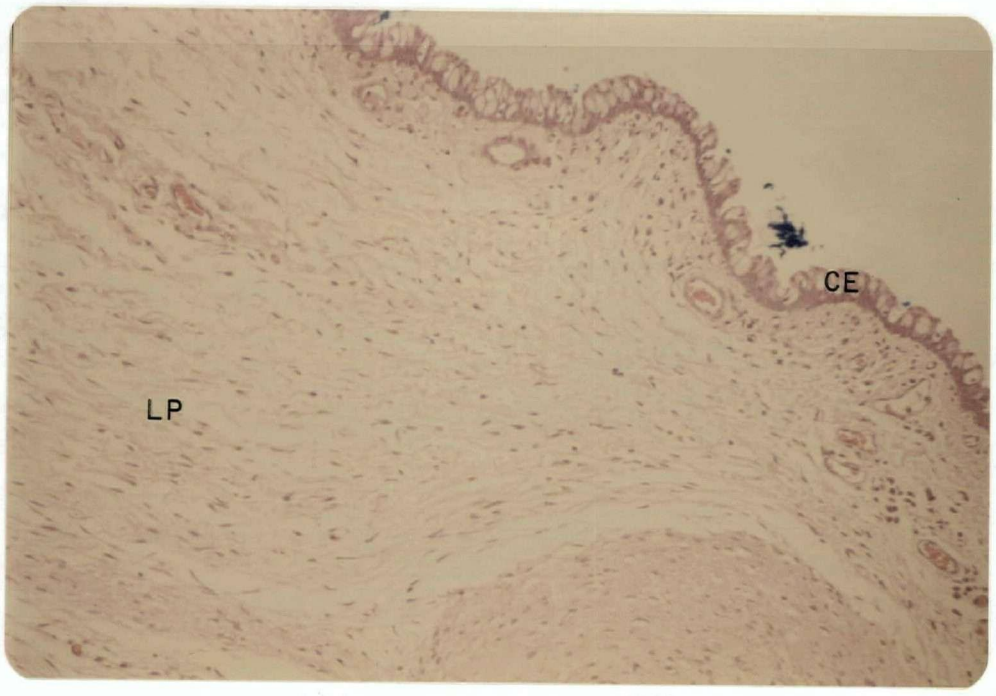


10x

A mild inflammatory response is present at one week. The collagen is disrupted and there is a necrotic zone(NZ) present in the lamina propria.

Figure 2.6

Histological effects of 5mg/0.1cc of Cyclosporine once per week in a rabbit that recieved this dose for two months



10x

Only a trace inflammatory response is present at the end of two months.

DISCUSSION

The dose determination study showed that 5 mg in 0.1cc of the commercially prepared Cy was the least toxic, causing only mild hyperemia which had largely resolved by 24 to 48 hours. With increasing doses (10 mg in 0.2cc and 25 mg in 0.5cc), the side effects became more apparent with moderate hyperemia and chemosis of the conjunctiva and exuberant mucus discharge, and at the highest dose hair loss of the upper eyelid due to local trauma and inflammation.

Although the inflammation with the higher doses had almost resolved by one week, the conjunctiva were still too sensitive and could not be reinjected at that time. With the low dose (5 mg in 0.1cc) administration the conjunctiva looked almost normal at one week, and from investigations performed on the frequency of injection (data not shown), this dose was found to be acceptable for injection once per week.

At higher doses there is some suggestion of suppression of the localized inflammatory response, but particularly with 25 mg in 0.5cc irregularity of the conjunctival epithelium, denaturation of the collagen and muscle as well as fibroblastic response were noted.

These effects can largely be attributed to the amount of vehicle injected and not to Cy. Since the Cy (Sandimmune I.V.$^{(R)}$) used was only available as 50 mg/ml, the amount of vehicle that was given was governed

by the concentration of Cy. As the dose was increased invariably the amount of vehicle was also increased, causing a greater inflammatory response of the eye.[81]

Even so, the inflammatory response seen with the higher doses was much less than with any of the vehicles tested, shown in an indirect way from the assumptions made in Chapter 1 regarding the commercial preparation.

For interest, we tested the effect of peanut oil and peanut oil containing 10 mg/ml of Cy, which was prepared locally. Five rabbits were injected subconjunctivally with 0.5 cc of peanut oil in the left eye (which served as the control eye) and 5 mg of Cy in 0.5cc of peanut oil to the right eye (which served as the experimental eye). There was evidence, from the differences between the histological observations of the control eye (received peanut oil only) and experimental eye (peanut oil and Cy), which supports the current suggested mechanism of Cy action.[56-60]

The following inferences could be made, from the difference in the cell populations between the control and experimental eye. Though further research is needed to conclusively show that the following does occur.

Cy seemed to cause inhibition of lymphocyte infiltration at the level of the T-cell. Current evidence to date indicates Cy is able to accomplish this by inhibition of interleukin-II production from T-helper cells, and blocking the receptor acquisition for interleukin-II by precursor cytotoxic T-cells, as well as

diminishing the responsiveness of T-helper cells to interleukin-I.[15,56-60]

Normally during acute inflammation polymorphonuclear granulocytes (PMN's) predominate in the first six to 24 hours being replaced by monocytes in 24 to 48 hours.[47] This infiltration was seen in the control eyes, but the experimental eyes showed fewer PMN's and macrophages which became more evident with time. This suggests that Cy could influence the migration of PMN's to the site of the injection, and since PMN's release chemotactic factors for macrophages, the resulting cascade seems to be inhibited by Cy. This is reflected by the cell population seen in the experimental eye. The mechanism by which Cy could interfere with the PMN's is unclear, but interference with C5a-induced chemotaxis has been suggested as unlikely.[61,62]

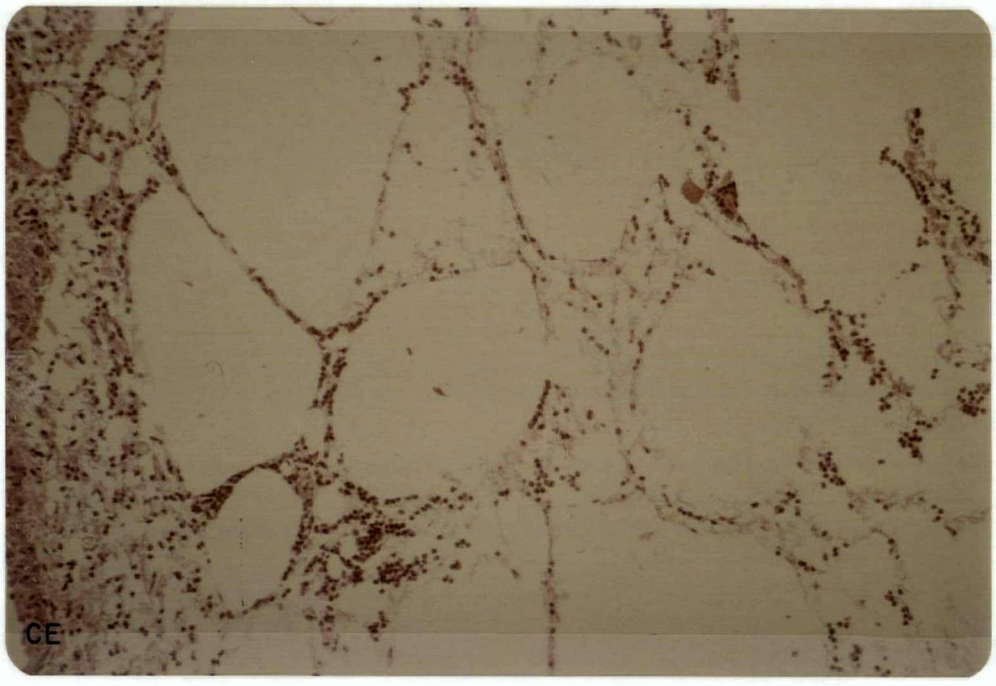
The currently held premise that the major actions of Cy are through the suppression of T-cells[58-60] is supported from the histologic observations of a decrease in the number of lymphocytes seen in the conjunctiva of the rabbits eye, with a possible minor effect on the suppression of PMN activity. These histologic findings are presented in Figures 2.7a through 2.7h.

The clinical and histological findings suggested a single dose (5 mg in 0.1cc) of the commercially prepared Cy once per week was tolerated better than more frequent administration. To ascertain whether repeated

Figure 2.7a

Histological effects of a subconjunctival injection of 0.5cc of peanut oil, control,(A) and peanut oil and Cy 5 mg/ml, experimental,(B) at 2 hours.
At this time there is a difference in the inflammatory infiltrate, though not dramatic, with more PMN's present in the control(A).

A.



B.

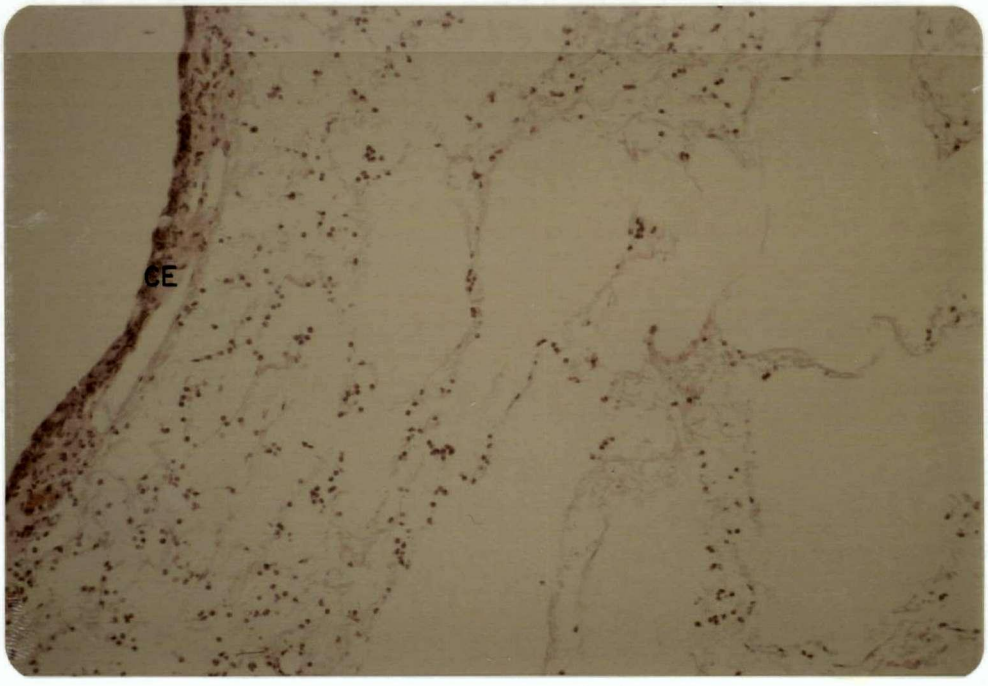


Figure 2.7b

Histological effects of a subconjunctival injection of 0.5cc of peanut oil, control,(A) and peanut oil and Cy 5 mg/ml, experimental,(B) at 4 hours.
The difference in the amount of inflammatory infiltrate is becoming more obvious with the control(A) showing more PMN's.

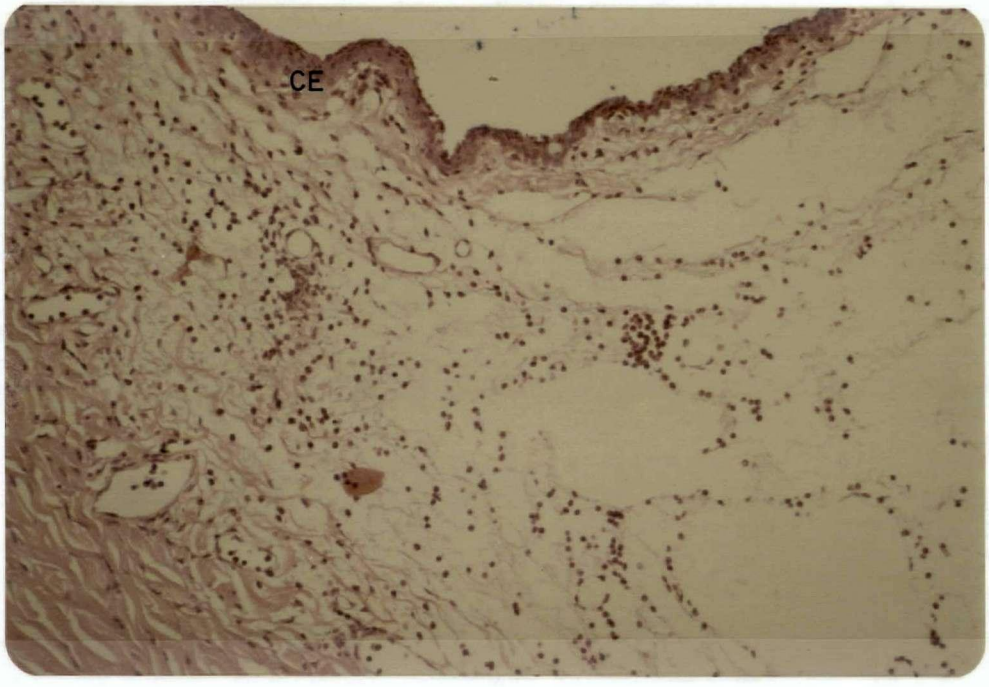
A.

B.

Figure 2.7c

Histological effects of a subconjunctival injection of 0.5cc of peanut oil, control,(A) and peanut oil and Cy 5 mg/ml, experimental,(B) at 12 hours.
There is a distinct difference in the number of inflammatorty cells present, mainly PMN's as well as compartmentalization of the oil in the control(A) and the experimental(B) appearing quiescent.

A.



B.

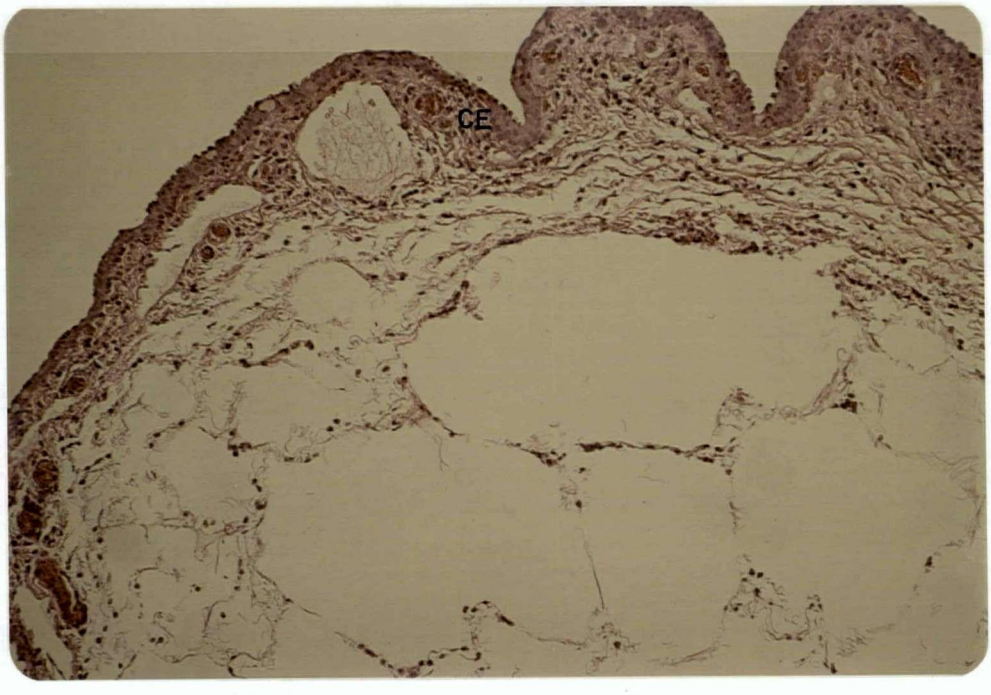
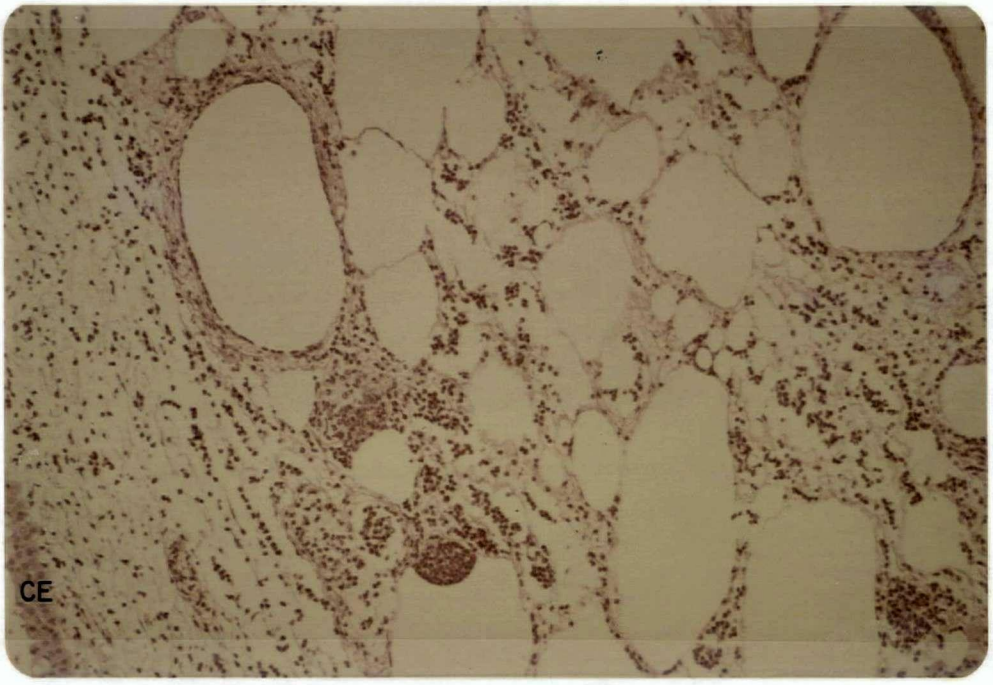


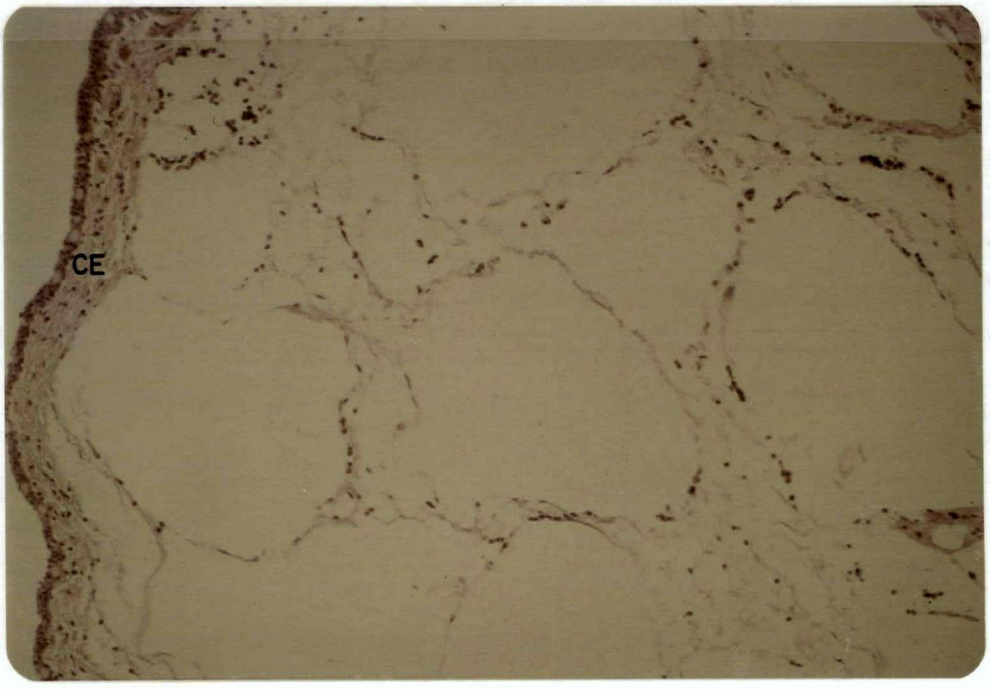
Figure 2.7d

Histological effects of a subconjunctival injection of 0.5cc of peanut oil, control,(A) and 5 mg of Cy in 0.5cc of peanut oil, experimental,(B) at 1 week.
The difference in the cell population between the control and the experimental eye is quite dramatic with the experimental one appearing almost acellular and the control showing quiet a heavy inflammatory infiltrate.

A.



B.



administration of this dose would have any adverse effects, a group of five rabbits received 5 mg of Cy in 0.1cc of Cy once per week for two months. The rabbits showed no side effects clinically that were not present with the single injection of 5 mg in 0.1cc of Cy. A post-mortem of the animals showed no gross changes in the organs. However, histopathology did show irregularity of the conjunctival epithelium, but to a lesser degree than was seen with the higher doses of Cy. In this case the epithelial irregularities can probably be attributed more to the repeated injections than to the vehicle.

In addition, lymphocytic and plasmacytic infiltrates were seen in the renal cortex interstitium and hepatic portal triads. Nephrotoxicity has been one of Cy's side effects when given orally or sytemically,[17,39,40,42,43] but it is unlikely that the alterations in the rabbits are due to Cy (see pharmacokinetic study in Chapter 3 for levels of Cy). Even if Cy is responsible for the effects, they would have been much greater if Cy had been given orally or systemically over the same period of time. These effects have been noted in patients receiving Cy orally for the treatment of uveitis, in which moderate to severe chronic renal histologic changes were observed, consisting of intersitial fibrosis and tubular atrophy.[42,43] The possible mechanism for renal injury can only be speculated on, but changes in glomerular capillaries,[37] tubular injury,[38] and a decrease in renal blood flow[41]

have been implicated as being caused by Cy, resulting in renal injury. It has also been postulated that Cy may be able to produce these effects because of the presence of basic cytosolic Cy binding proteins, discovered in lymphoid tissues. These proteins referred to as cyclophilins, have been found in high concentrations in the brain and kidney, organs that display toxic effects during treatment with Cy, possibly explaining the histological changes observed in the kidney.[55]

Though the aggregations of lymphocytes and plasmacytes in the renal interstitium and hepatic triads in the rabbits are of concern, they do not seem to be of a high degree, or lead to structural renal or hepatic damage. These mild effects as noted have been reversible on discontinuation of Cy therapy.[44]

Conclusions

A single injection of 5 mg in 0.1 cc of the commercially available Cy once per week, was found to be the maximum clinically tolerated dose. More frequent injections were noted to cause excessive local response. Our results using the commercially available Cy suggested that our initial hypothesis, that is it would be less toxic to the eye and allow for repeated administration, was correct.

CHAPTER 3

Pharmacokinetics of cyclosporine following subconjunctival versus intravenous administration.

Introduction

With systemic drug administration the eye and the central nervous system represent pharmacologic sanctuaries. Previous investigations have shown that subconjunctival administration is superior to intravenous for ocular penetration of antineoplastic agents.[20-24] To determine whether this was true for Cy, ocular, blood and urine levels of equidose Cy (the 5 mg in 0.1cc once per week of Cy (SANDIMMUNE I.V.), as determined from the dose determination study) following subconjunctival versus intravenous injection were compared in rabbits. We found that we were able to get higher amounts of Cy into the ocular chambers when Cy was given subconjunctivally. Compared to intravenous injection, subconjunctival injection resulted in four times the peak concentration of Cy in the vitreous and 0.1 times of that in the blood. Aqueous levels of Cy were undetectable in the rabbits given Cy intravenously.

Materials and Methods

Twelve groups of three rabbits each (locally supplied New Zealand white females weighing 2.0-2.2kg) were tranquilized by intramuscular injections of 0.2 ml/kg of

10:1 solution (100 mg/ml) of ketamine hydrochloride and acetylpromazine (Rogar S.T.B. and Ayerst Laboratories, Vancouver) every half hour for four hours. Normal saline was infused into the left marginal ear vein at a rate of 30-40 ml/hour to maintain urine flow. The rabbits were given 5 mg in 0.1cc of Cy as a bolus either subconjunctivally (posterior to the superior limbus in the right eye which had been previously anaesthetized with topical proparacaine HCl (0.5%)) or intravenously (in the left marginal ear vein). It should be noted that this is not the standard method for I.V. administration of Cy, rather it is by infusion over a period of time.

Then at 0.5, 1, 2, 4, 8 and 12 hours later, 1 ml blood samples were obtained by puncture of the medial artery of the right ear. The samples were placed into 1.5 ml microtest tubes and frozen. The total volume of urine collected during each interval was recorded, and samples of urine were placed into 1.5 ml microtest tubes and frozen.

At the appropriate time the animals were sacrificed with an overdose of pentobarbital and immediately after death the eyes were proptosed, and the corneas rinsed with saline and blotted dry. About 0.10 ml of aqueous humour was aspirated through the inferior limbus and placed in a microtest tube. The eyes were then enucleated, and the adherent episclera discarded. The globes were then rinsed with saline, blotted dry and snap frozen in liquid nitrogen. Later the eyes were allowed to partially thaw,

are were then incised with a scalpel; the frozen vitreous expressed and placed in a test tube and disrupted with a sonicator, the microtip being used for 30 seconds, and centrifuged at 2500 RPM for five minutes. The supernatant was then placed in a microtest tube and frozen.

Before each enucleation the surgical instruments were washed thoroughly with ethanol to remove any Cy that may be coating them, and the sonicator microtip was also washed with ethanol before sonication of each sample.

High-Pressure Liquid Chromatography (HPLC)

HPLC analysis of the samples was carried out with a Water Associates (Milford, Massachusetts) system consisting of two M6000 pumps, a model 710B WISP (Waters Intelligent Sample Processor) autosampler, a model 481 Lambada-Max variable wavelength detector, a M660 solvent programmer, a column heater and temperature control module, and a spectra physics 4290 integrator.

Pure Cy (a donation from Sandoz Ltd, Basle, Switzerland) and pure dihydrocyclosporin C (CyC - courtesy of Dr Freeman, University Hospital, University of Western Ontario), were used to prepare the stock solution of 50 ug/ml of Cy in 100% methanol and stock solution of the internal standard CyC was prepared as 10 ug/ml in 100% methanol. Standard concentrations of Cy were prepared ranging from 25 ng/ml to 10 ug/ml from the primary

stock solution by serial solution, from which appropriate standard curves were obtained (figure 3.4).

Extraction Procedure

Two different methods were required to determine the levels of Cy in the samples collected.

Method #1.

For the urine and ocular samples the method used for detemining the Cy levels was based on that developed by Lensmeyer and Fields[65] with a few modifications. The extraction is as follows with the modifications identified:

This method required in addition to the apparatus already mentioned the use of CN-Bond Elutes(R) (B.E), which are disposable solid phase columns (of 1.0 ml capacity and containing 100 mg of cyanopropyl phase packing) and the Vac Elut(R) extraction apparatus (both from Analytichem International, Harbor City, CA).

Reagents.

HPLC grade acetonitrile and methanol (from Caledon Laboratories Ltd., Geogretown, Ontario), deionized water prepared with the "Milli-Q" water purification system (Millipore Corp., Bedford, MA), and glacial acetic acid (from BDH Chemicals Canada Ltd., Vancouver) were used in preparing the reagents needed.

Solution A: The diluent consisting of water/acetonitrile (70/30 by vol) used to dilute samples.

Solution B: Water/acetonitrile (80/20 by vol) used for conditioning the CN - B.E.

Solution C: Acetic acid (0.5 M) /acetonitrile (80/20) used to remove interfering substances from the CN - B.E.

Solution D: Acetic acid (0.5 M) /acetonitrile (60/40) used to remove interfering substances from CN - B.E.

Mobile Phase - consisted of water /acetonitrile (60/40 by vol), which was filtered through a millipore filter system (using a 22 um durapore filter) and degassed prior to use.

Sample Treatment - To a 16 x 100mm disposable centrifuge test tube 250 ul of urine or ocular sample, 50 ul of the internal standard (10 ug/ml of CyC) and 2 ml of solution A were added. Then vortexed for 20 seconds and centrifuged for 5 minutes at 2500 RPM. {In Lensmeyer's method the internal standard was CyD and Solution A was 350 ug/l of CyD in water/acetonitrile (70/30 by vol).}

Bond Elute Conditioning - CN-B.E. was primed with two reservoir volumes (about 1.0 ml) of acetonitrile, and then

followed with two reservoir volumes of Solution B. Then the diluted sample was passed through the conditioned CN-B.E., making sure that the CN-B.E. did not dry between applications.

Interference Elution - After the meniscus of the sample passed through the top of the CN-B.E. packing, the CN-B.E. was washed with two reservoir volumes of solution C. After the last of Solution C had passed the top of the CN-B.E. packing, the CN-B.E. was then washed with 0.25 ml of Solution D and reduced pressure was maintained to remove any residual liquid from the CN-B.E.

Isolate Elution - 0.4 ml of acetonitrile was addedto the CN-B.E. and left for two minutes. The eluate was then collected into 16 x 100 mm disposable centrifuge test tubes, and under reduced pressure any residual liquid was be collected from the CN-B.E. The eluate was then evaporated under a stream of nitrogen at 40 degrees Celsius, and the dry extract was then reconstituted with 250 ul of mobile phase, vortexed for twenty seconds and centrifuged for 5 minutes at 2500 RPM. The reconstituted extract was then transferred to a limited-volume insert for chromatography. {In Lensmeyer's method the elutant was evaporated under a stream of air at room temperature, and the dry extract was reconstituted with 150 ul of mobile phase.}

Chromatography

50 microliters of the extracted sample was injected into the HPLC system which had a 25cm x 4.6mm Whatman CCS/C8 (5 micron particle size) analytical column. The mobile phase flow rate was 1.5 ml/minute, with detector range set at 0.01 AUFS at 210nm, chart speed was set at 0.25 cm/minute on the spectra physics integrator and the column heater was set at 72 degrees Celsius. (In Lensmeyer's procedure the analytical column used was a 25 x 4.6 mm Zorbax-cyanopropyl (5 micron particle size) and a 5cm x 4.6mm guard column packed with permaphase ETH (30 micron particle size), with detector range set at 0.02 AUFS at 214nm and recorder chart speed at 1 cm/min.)

Because the whole blood samples were frozen, substances formed upon thawing that plugged the CN-B.E., making extraction impossible using method#1 to determine the Cy levels in the blood samples.

The method used to determine the Cy level in the frozen whole blood samples was that developed by Carruthers, Freeman et al.[66] The method is as follows:

Method # 2:

Reagents

HPLC grade Diethyl ether (from J.T. Baker Chemical Co., Phillipsburg, N.J.), HPLC grade acetonitrile and methanol (from Caledon Laboratories Ltd., Geogretown, Ontario), and

deionized water prepared with the "Milli-Q" water purification system (Millipore Corp., Bedford, MA) were used to prepare the reagents needed.

Solution 1: 0.2 M HCL

Solution 2: 0.2 M NaOH

Solution 3: 1.2 M $(NH_4)_2SO_4$ in 40.0 ml of a solution consisting of 20% acetonitrile, 20% methanol, and 60% water.

Reconstitution Fluid - consisted of 3.0 ml of acetonitrile, 2.0 ml of methanol, 5.0 ml of water and 1.0 ml of Solution 3.

Mobile Phase: consisted of 46.95% acetonitrile, 20% methanol, 33.05% water and 0.5 ml of Solution 3 per 2.0 liters of mobile phase, which was filtered through a millipore filter system (using a 22 micron Durapore (Millipore)$^{(R)}$ filter) and degassed prior to use.

Sample Treatment - In a 16 x 100 mm disposable centrifuge test tube 250 microliters of whole blood, 50 microliters of the internal standard (10 ug/ml of CyC) and 2.0 ml of Solution 1 were added. Then vortexed for 20 seconds. To this mixture 6.0 ml of Diethyl ether was added

and shaken on a horizontal shaker at 100 RPM for 10 minutes. Then centrifuged at 2500 RPM for 5 minutes. The aqueous acid phase was then discarded with an ether-rinsed Pasteur pipette and 2.0 ml of Solution 2 was added to the Diethyl ether. This mixture was then vortexed for 30 seconds, and centrifuged for 5 minutes at 2500 RPM. The ether phase was then transferred with a Pasteur pipette to a 16 x 100mm disposable test tube. The ether was then evaporated under a stream of nitrogen at 40 degrees Celsius.

The dry extract was reconstituted with reconstitution fluid and vortexed for 20 seconds. The reconstituted extract was then transferred to a limited-volume insert for chromatography.

Chromatography

The analytical column used was a 25cm x 3.2mm I.D. containing Spherisorb-C8 packing (5 micron particle size), constructed by Dr. Freeman of University Hospital, University of Western Ontario. The flow rate was 1.0 ml/min., detector settings were 0.01 AUFS at 210nm, the column heater was set at 72 degrees Celsius and the chart speed setting on the spectra physics was 0.25 cm/min. 50.0 ul of the extracted sample was chromatographed and between each sample there was an acetonitrile wash to remove late elutors from the column. The acetonitrile wash was controlled by a M660 solvent programmer. The M660 gradient

controller was programmed as follows:

0-15 min 100% mobile phase

15-17 min 100% acetonitrile

17 min 100% mobile phase.

The running time was 17 minutes with an equilibration delay of eight minutes and a total run time of 25 minutes.

Results

Performance data on Method 1 and 2 are presented in Table III. The sensitivity limit (signals equal to twice the baseline noise) ranged from 15 to 25 ng/ml. The standard curves for each method (figure 3.4) were developed by plotting relative area ratio (Cy/CyC) vs concentration for Cy, and least squares regression was used assess linearity. The chromatograms for each type of sample extracted are presented in figures 3.0-3.3.

Table III

Performance Data on Methods 1 & 2

Method	n	Precision day to day concn, ng/ml	mean	S.D.	C.V. %	Analytical recovery Mean Recovery %	Linearity (linear up to at least 1400 ng/ml)	
1	20	100	106	6.8	9.9	90	y=(5.109E-4)x + 4.165E-3	r=0.9980
2	20	100	106	7.5	11.3	70	y=(2.075E-4)x + 2.789E-5	r=0.9989

x= Cy concentration in ng/ml

y= relative area ratio Cy/CyC

After Subconjunctival administration

The levels of Cy in aqueous humor of the right eye (that injected subconjunctivally) peaked at 718 ng/ml at one hour, then decreased to 119 ng/ml by 12 hours (figure 3.5). In the vitreous humor of the right eye the level of Cy peaked at 1078 ng/ml at two hours, then decreased to 253 ng/ml by 12 hours (figure 3.5). In the left eye (the controls) the levels of Cy were undetectable in the aqueous and vitreous humor.

The bioavailability of Cy in the ocular humors when administered subconjunctivally, calculated by an area-under-the-curve method,[45] is shown in Table IV.

The levels of Cy in whole blood peaked at 4 hours reaching only 104 ng/ml, then decreased to 54 ng/ml by 12 hours (figure 3.6). Cy levels in the urine were undetectable.

After Intravenous Administration

The Cy level in whole blood peaked at 1061 ng/ml at 0.5 hours, then decreased to 74 ng/ml by 12 hours (figure 3.6). The cummulative urine elimination of Cy over 12 hours in the rabbit was less than 1% of the injected dose (figure 3.7).

The data were pooled for the right and left eyes,since a paired t-test showed no significant difference ($p=0.05$) between them. The levels of Cy in the aqueous humor were

undetectable.

The levels of Cy in the vitreous humor peaked at 292 ng/ml at two hours, then decreased to 164 ng/ml by 12 hours (figure 3.5).

The bioavailability of Cy in the ocular humors when administered intravenously is shown in Table IV.

Table IV

Bioavailability of Cyclosporine in Ocular Humours
Humour, Bioavailability (ng/ml.hr)

Route of administration	Aqueous Humour Right	Vitreous Humour Right
subconjunctival	4.7	10.4
Intravenous	not detectable	5.3

Figure 3.0

A typical chromatogram of aqueous humor with the internal standard Dihydrocyclosporin C (A) eluting at 10 minutes and a 250ng/ml standard of Cyclosporine (B) eluting at 12 minutes.

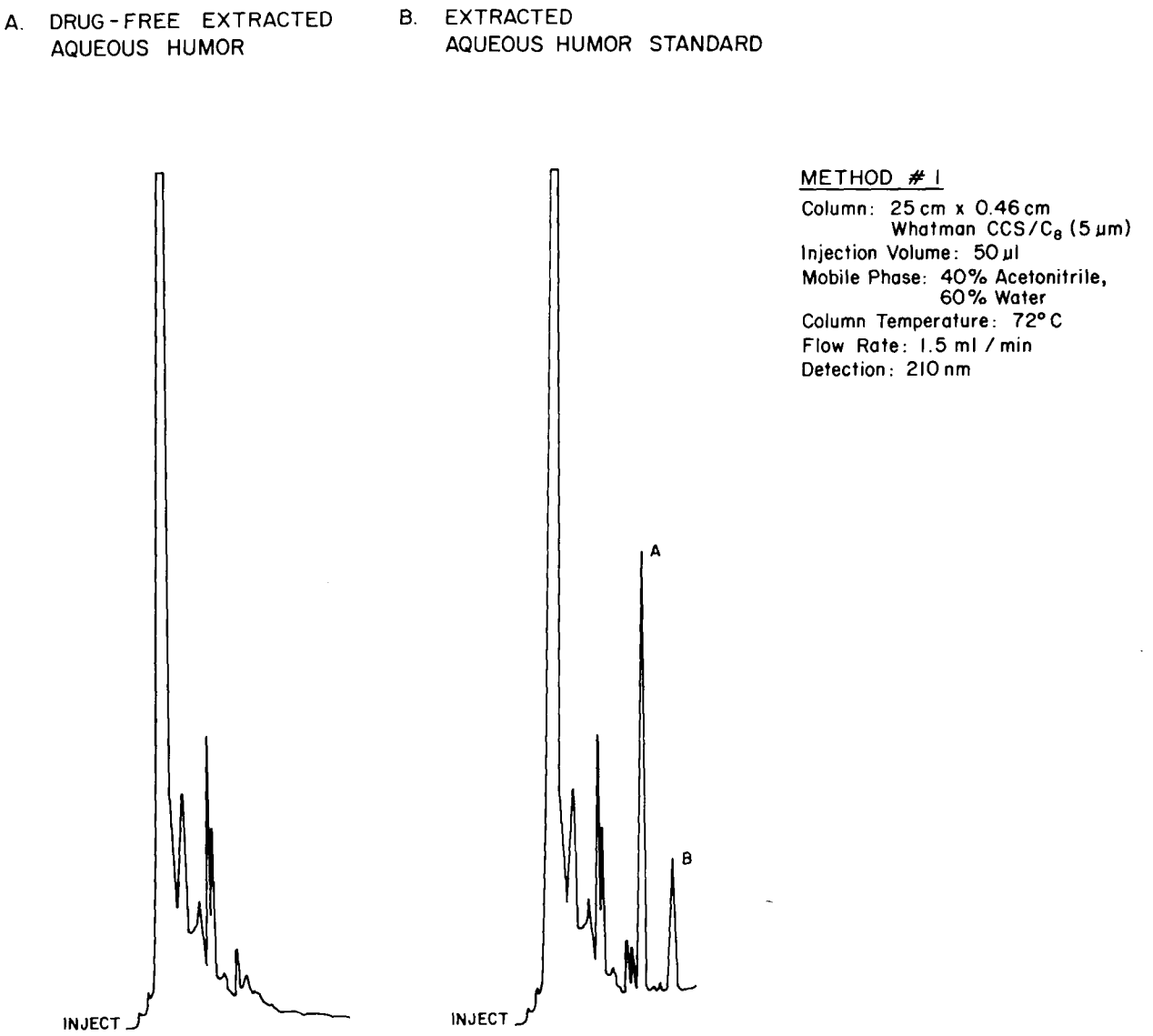


Figure 3.1

A typical chromatogram of vitreous humor with the internal standard Dihydrocyclosporin C (A) eluting at 10 minutes and a 250ng/ml standard of Cyclosporine (B) eluting at 12 minutes.

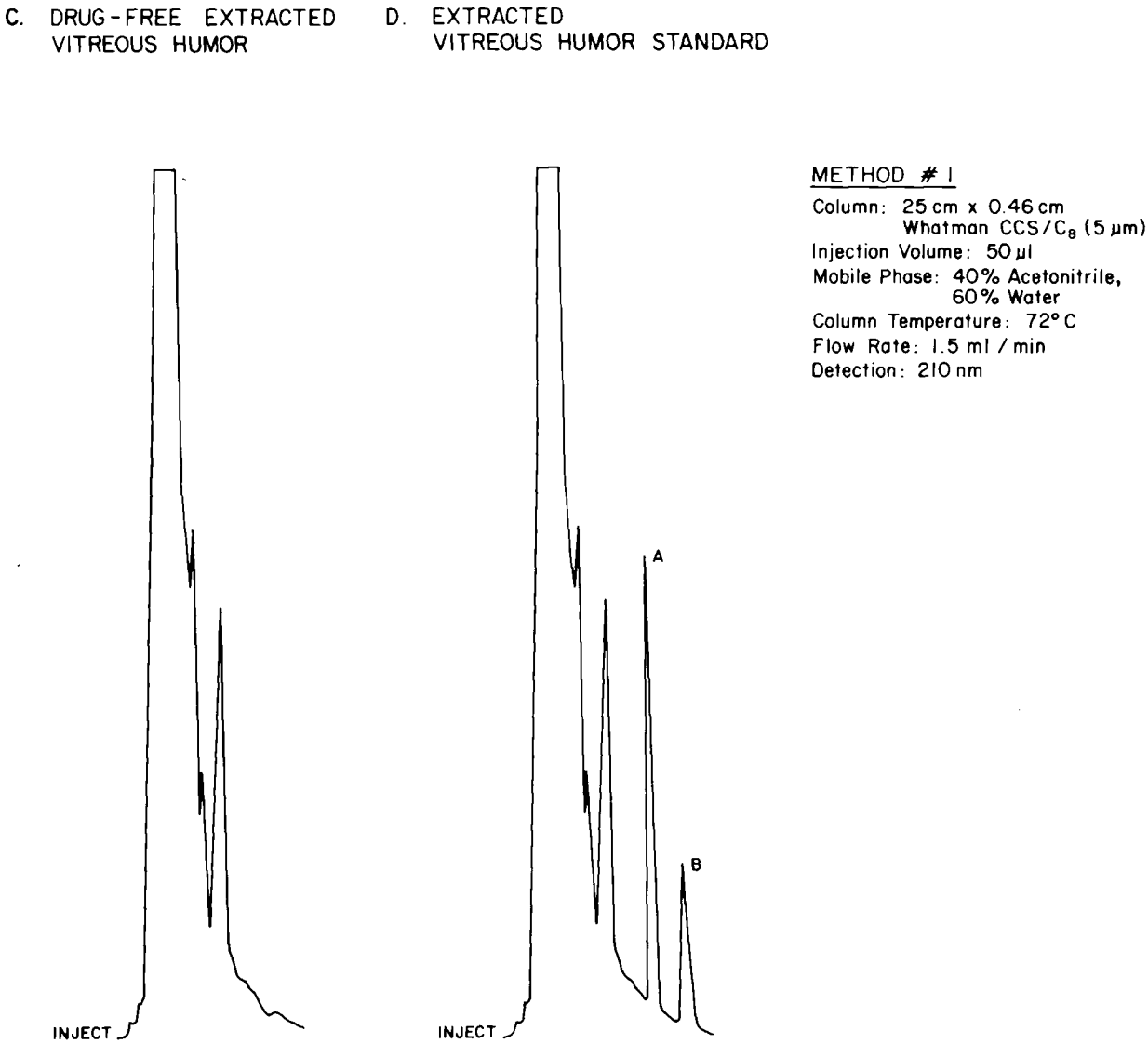


Figure 3.2

A typical chromatogram of urine with the internal standard Dihydrocyclosporin C (A) eluting at 10 minutes and a 250ng/ml standard of Cyclosporine (B) eluting at 12 minutes.

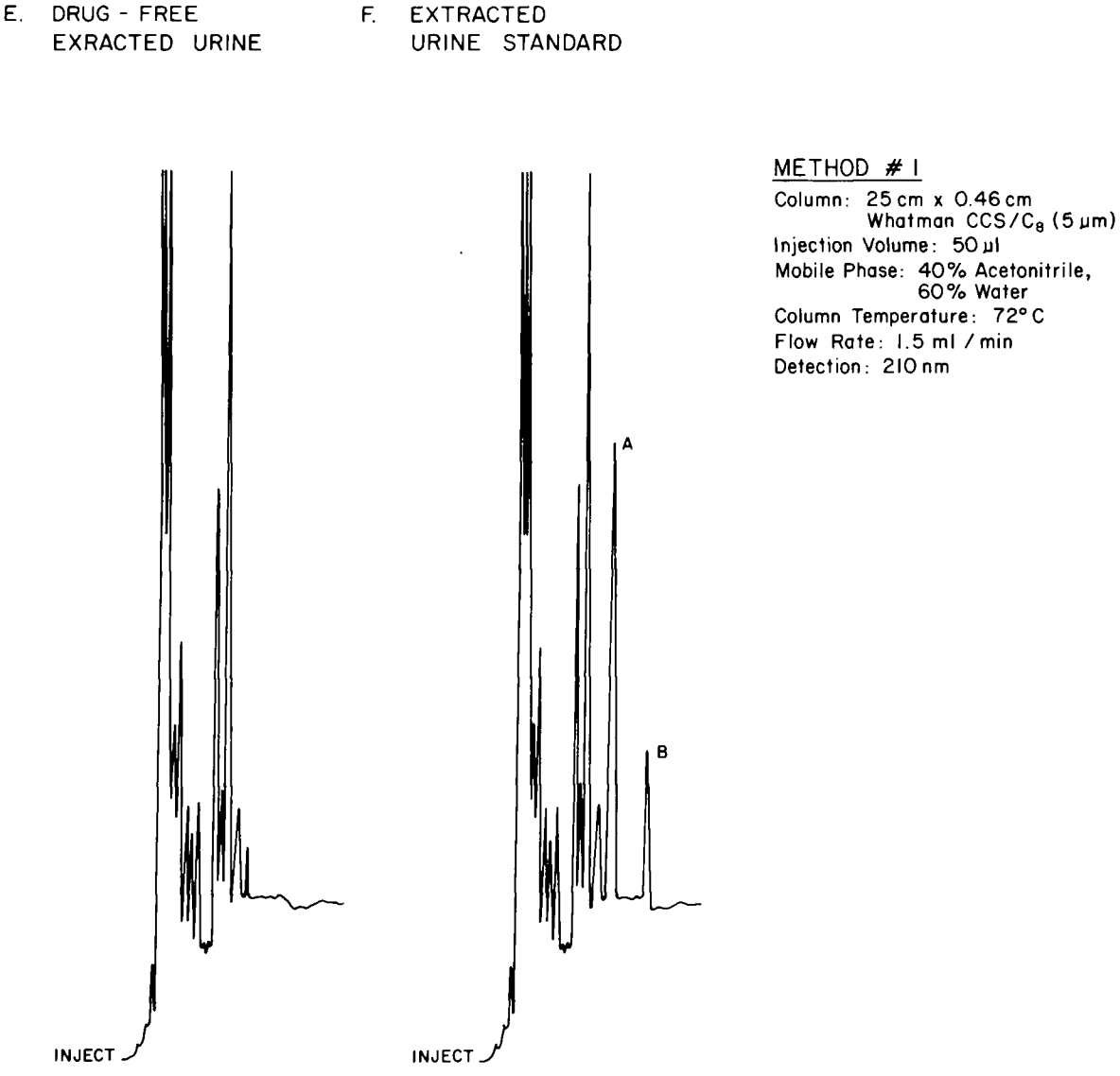
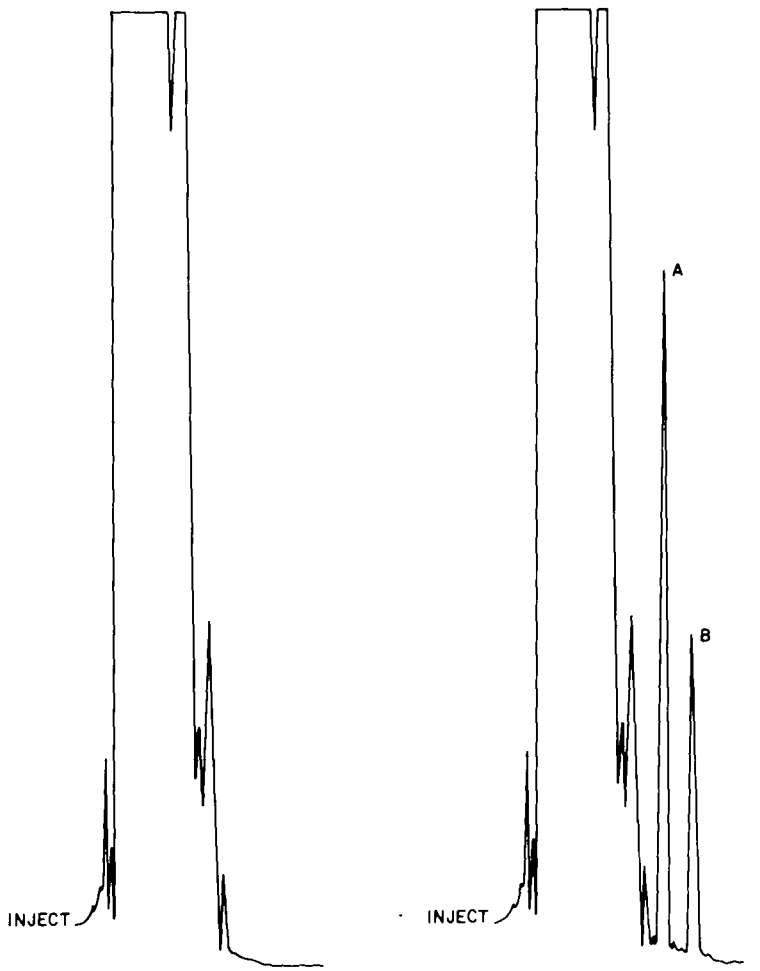


Figure 3.3

A typical chromatogram of whole blood with the internal standard Dihydrocyclosporin C (A) eluting at 10.30 minutes and a 500ng/ml standard of Cyclosporine (B) eluting at 13.00 minutes.

G. DRUG - FREE EXTRACTED WHOLE BLOOD

H. EXTRACTED WHOLE BLOOD STANDARD

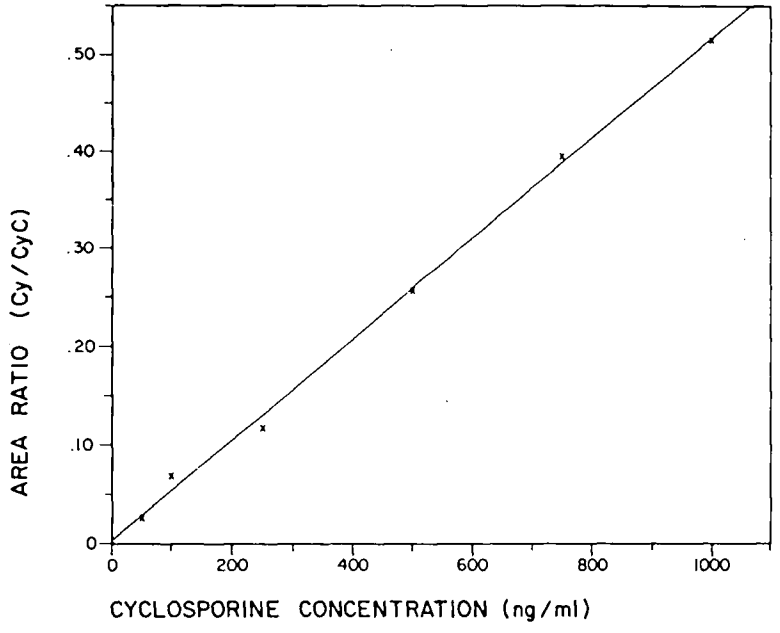


METHOD # 2

Column: 25 cm x 0.32 I.D.
(Spherisorb C_8, 5 µm)
Injection Volume: 50 µl
Mobile Phase: 46.95 % Acetonitrile,
20.00 % Methanol,
33.05 % Water
Column Temperature: 72°C
Flow Rate: 1.0 ml / min
Detection: 210 nm

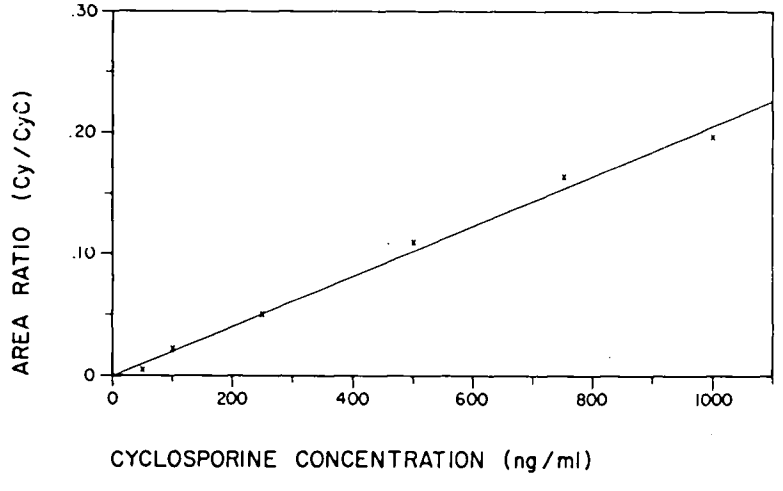
Figure 3.4

STANDARD CURVE FOR CYCLOSPORINE USING METHOD NO. 1



Y = (5.11E-4) + 4.17E-3

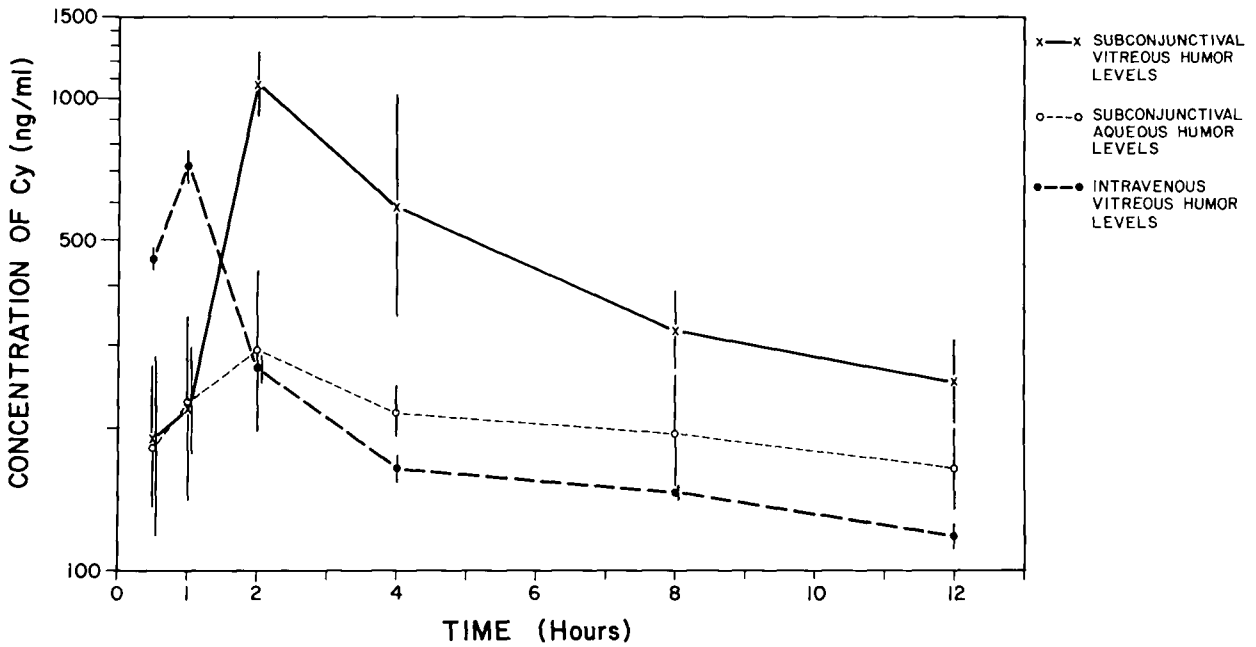
STANDARD CURVE FOR CYCLOSPORINE USING METHOD NO. 2



Y = (2.07E-4)X + 2.79E-5

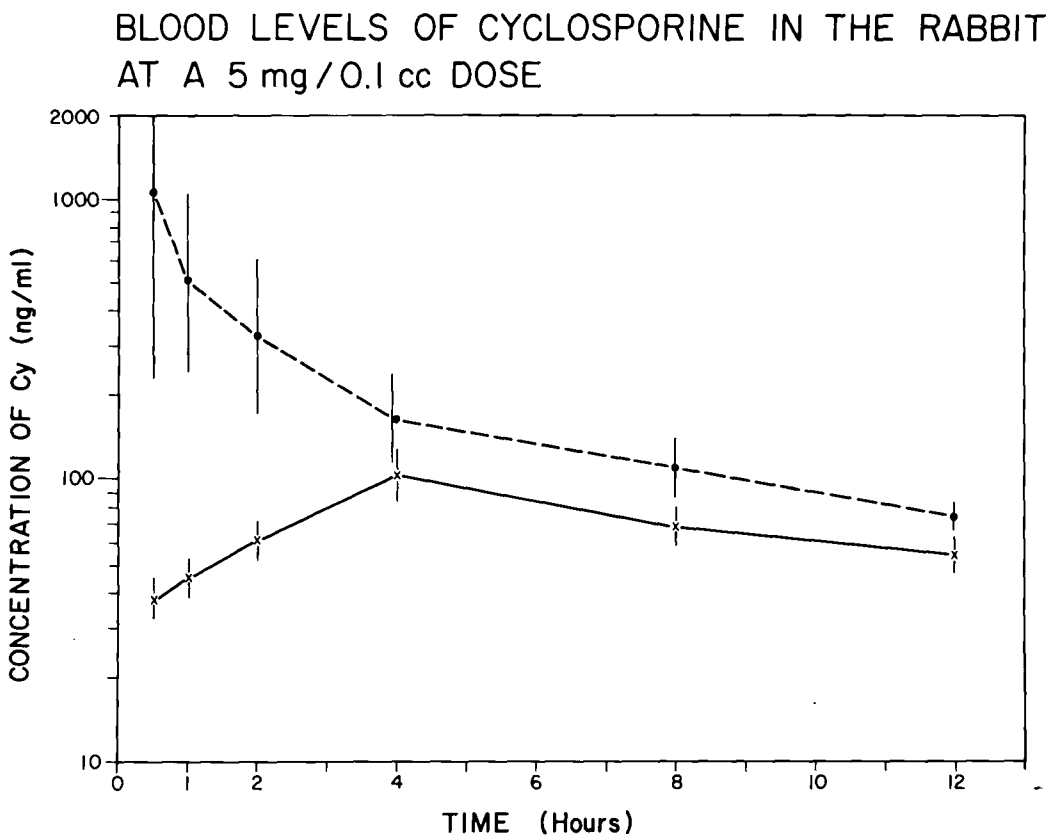
Figure 3.5

LEVELS OF CYCLOSPORINE
IN THE OCULAR CHAMBERS OF THE EYE



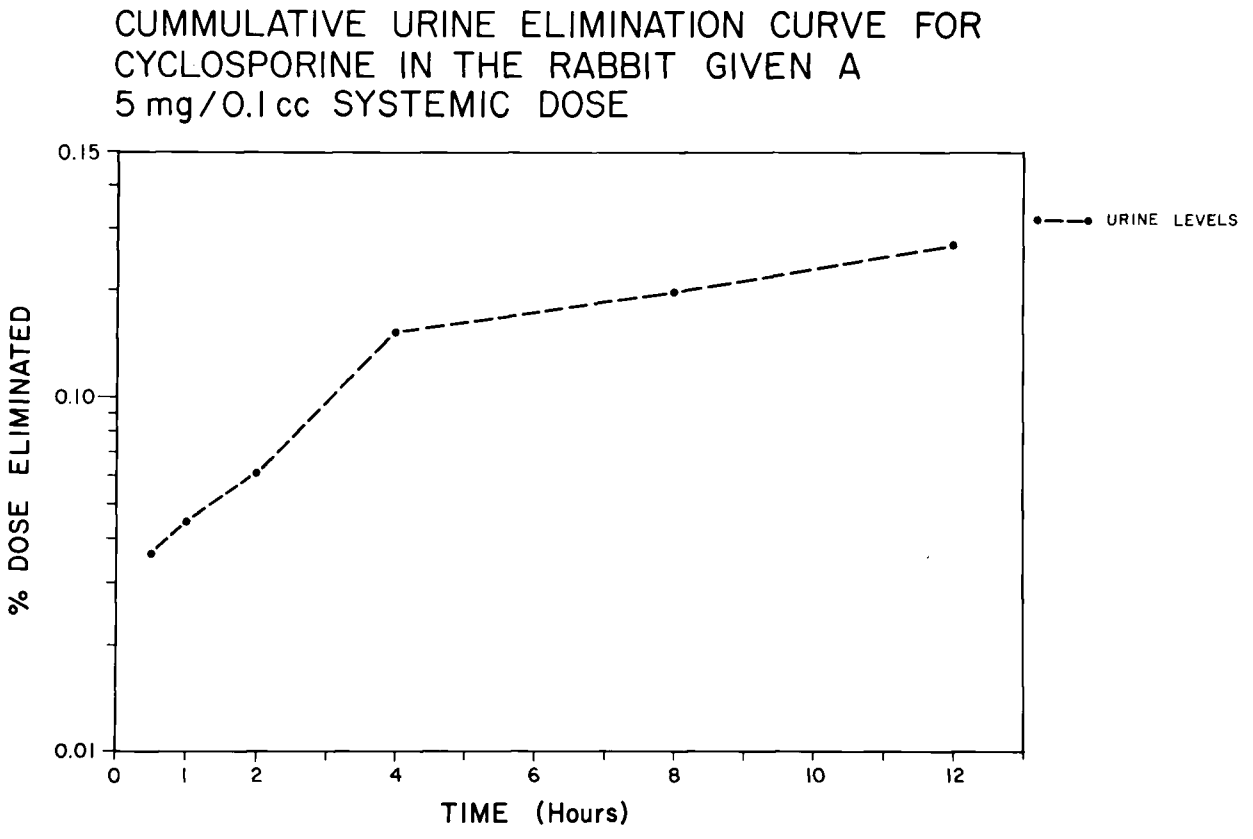
NOTE :- Aqueous humor levels were not detectable in rabbits given cyclosporine intravenously.

Figure 3.6



Levels of cyclosporine in the blood following subconjunctival (x) or intravenous (•) administration

Figure 3.7



NOTE :- Urine levels were not detectable in rabbits given cyclosporine subconjunctivally.

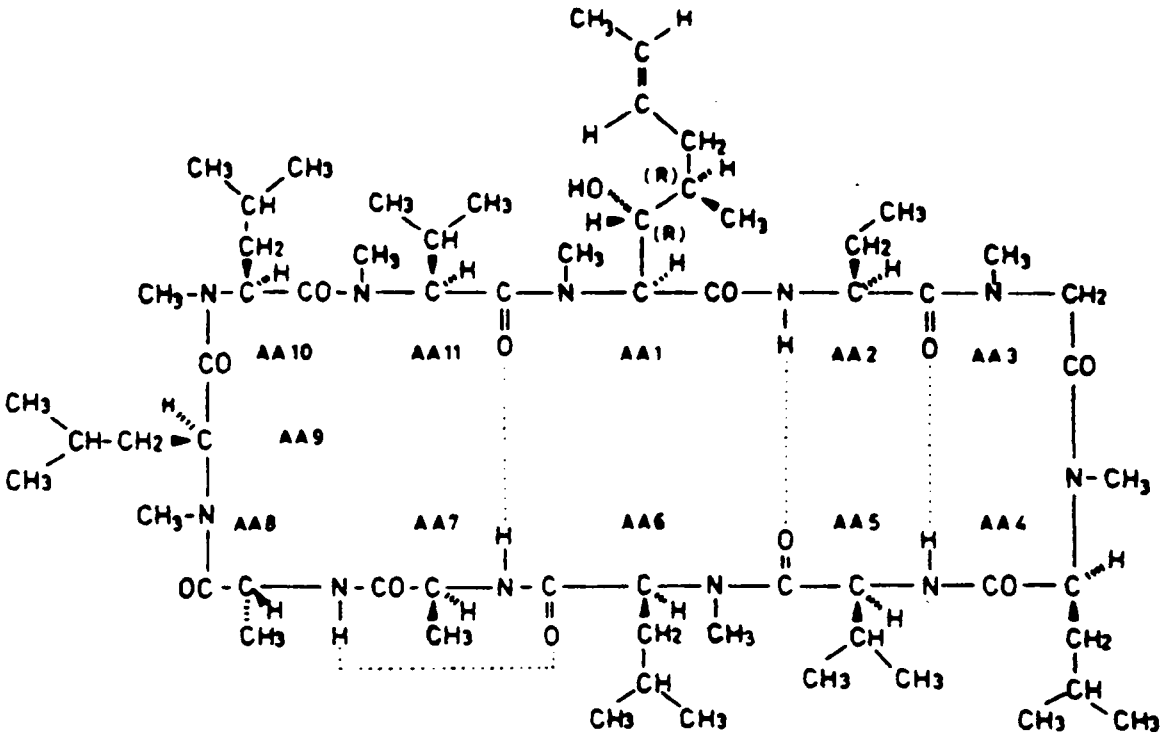
DISCUSSION

Cyclosporine (Cy) is a hydrophobic cyclic undecapeptide obtained from the fungus Tolypocladium inflatum. Although originally developed as an anti-fungal agent, it proved to be a potent immunosuppressive that could cause selective suppression of T-lymphocytes at an early stage of activation.[15,56] Currently, it has been found beneficial when given orally or intravenously for the prophylaxis and treatment of organ rejection in allogenic transplants,[48] and it shows promise in the treatment of autoimmune diseases.[15,51-53]

The structure of Cy reveals some unique features (figure 3.8); in position 1 it has a nine carbon amino acid, which was previously unknown and to which much of its activity is attributable. All of the amino acids that comprise Cy have the L configuration except for D-alanine in position 8 and sarcosine in position 3. Finally, seven of the amino acids are N-methylated, which may contribute to its lack of degradation when administered orally.[54]

Cy also exhibits large inter-individual variations in bioavailability and clearance rate. It displays a biphasic distribution pattern, the first representing the blood (in which 50% is found in erythrocytes, 40% in plasma, and 10% in leukcocytes[15]), and the second phase the peripheral tissues. In general the pharmacokinetics of Cy show that peak serum concentrations are achieved in 2 to 6 hours

Figure 3.8



Structure of Cyclosporine (Molecular Weight of 1202)

with a $T_{1/2}$ of 16 +/-8 hours, and an oral bioavailability of 34 +/-11%.[56,57] The metabolism of this drug is primarily by the liver, yielding numerous metabolites which are excreted into the bile, with less than 1% excreted intact into the urine.[15,56]

In addition, Cy has a narrow margin of safety with therapeutic concentrations between 100 and 400 ng/ml, and the major toxic effects of Cy such as nephrotoxicity and hepatotoxicity are manifested at concentrations greater than 400 ng/ml.[15,56,57]

Careful monitoring of Cy levels is essential to avoid the toxic side effects of Cy and to maintain immunosuppression. Two distinct procedures have evolved to measure Cy levels: radioimmunoassay (RIA)[63,64] and high performance liquid chromatography (HPLC).[65-67] Prior to 1982 Cy levels in plasma and blood were measured by RIA (which has a detection limit of 25-50 ng/ml), but the major problem with this procedure was that it could not distinguish between Cy and its metabolites. The result was a false impression that patients were either immunosuppressed or had toxic levels of Cy. The advantage of the RIA procedure was that it could be performed on a large number of samples easily, with a turn-around time of 4 to 6 hours for a batch of samples.

The second method of analysis by HPLC which allows for levels in whole blood to be determined was introduced by Sawchuk and Cartier,[67] and was able to distinguish Cy from

its metabolites and had a detection limit of 10 to 25 ng/ml depending on the sample.[65,67] The HPLC procedure was modified by others in order to increase the speed and sensitivity of the determination of Cy levels. In general, the methods of extraction of the samples and the type of analytical column (reverse phase C_{18} or C_8, cyanopropyl) have varied but all have required high temperature chromatography and detection has been by a U.V. detector at about 210nm.[65-70]

Important to monitoring Cy levels is not only the method, but the handling of the blood samples, because the concentration of Cy in plasma is widely variable and dependent upon the temperature of plasma separation.[71,72] Whole blood analysis is preferable,[65,73,74] since it is important to get a true picture of the levels of Cy, because of its narrow margin of safety.[15,56,57,75]

Though it is generally desirable to know the free levels of Cy as they reflect the actual amount of drug available for use by the body, there is controversy regarding method of measurement; that is, whether to use traditional plasma methods (free levels) or whole blood. In the clinical setting the method of measurement of whole blood Cy level is currently being favoured based on the rationale that this gives the clinician a better picture of the level of immunosuppresion being obtained. In order to avoid the variability associated with plasma level methods, and to follow the suggestions provided by MRC reviewers of

this project, we chose the whole blood method.

Therefore, the method chosen for the analysis of Cy levels was HPLC, because this method is able to distinguish between Cy and its metabolites and handle whole blood samples. The HPLC method developed by Lensmeyer and Fields[65] was used to measure Cy in the ocular and urine samples, but the extraction procedure used in this method was not able to handle the frozen whole blood samples. An alternate HPLC method developed by Carruthers and Freeman et al.,[66] was used to determine the level of Cy in the frozen whole blood samples. These methods were chosen because they were able to remove most of the interfering substances from the samples, and had good recovery (70-90%) and sensitivity (10 to 25 ng/ml) as well as a C.V. of 9-11% at 100 ng/ml.

The aim of this pharmacokinetic study of the subconjunctival vs the intravenous route was to substantiate whether local administration would enhance ocular absorption of Cy, and avoid the levels in the blood that have been associated with systemic administration that can result in nephrotoxicity.

The study showed that peak concentrations of Cy were 4 and 0.1 times in the vitreous and blood respectively when Cy was given subconjunctivally compared with Cy administered intravenously. In addition, aqueous levels were undetectable in rabbits that received Cy intravenously, and the bioavailability of subconjunctivally

administered Cy in the vitreous was two times greater than that achieved following intravenous administration. The ocular pharmacokinetics of the subconjunctival route also showed that the aqueous levels of Cy peaked at 1/2 hour and the vitreous levels at two hours. The delay in the peak concentrations between the ocular chambers can partly be explained by the histological findings from the vehicle and dose determination study, which showed castor oil spreading posteriorly. Since the bleb (containing the vehicle and Cy) for the first hour is near the limbic area, which is in close proximity to the aqueous chamber, the Cy levels in the aqueous are expected to peak before that in the vitreous, as was seen from the pharmacokinetic results. The large amount of Cy attained by the subconjunctival route may also have been enhanced by the local inflammation produced by subconjunctival injection. The local inflammatory response would alter both vascular and ocular permeability and may increase blood flow, thus enhancing absorption.

With regard to the trough levels obtained in the vitreous and aqueous following subconjunctival injection, they remained above the therapeutic range for 12 hours. In contrast, levels obtained by systemic administration at this particular dosage were only detectable in the vitreous and remained just above the therapeutic range for 12 hours but were below these obtained in the aqueous following administration of Cy subconjunctivally.

Equally important are the observed blood levels following this route of administration which were well below (4 times) the level (400 ng/ml) needed to avoid the toxic side effects of Cy.[15,56,57]

The rationale for pulsed local therapy in the case of ocular inflammatory disease is based on the concept that succesive treatment can gradually bring the disease under control since the affected lymphocyte population will not exchange quickly or easily with the general population in this sanctuary. In addition, numerous drugs have been shown to be absorbed better following local administration than following equidose systemic administration, and that systemic levels remain lower following subconjunctival versus equidose systemic administration in many drugs[20-24].

Conclusions

The results from this experiment provide a pharmacokinetic basis for the use of Cy subconjunctivally for ocular inflammatory disease. In general, the pharmacokinetics of Cy administered subconjunctival resembles that of other drugs administered in this manner.[20-24] The levels obtained vary with the drug being studied but in the case of Cy, it peaked later suggesting ocular absorption is better with hydrophilic than with hydrophobic drugs. Local administration may have a role to play in ocular inflammatory disease which has responded to oral or I.V. administered Cy, but which had to be

discontinued because of the nephrotoxicity associated with toxic systemic levels of Cy.

CHAPTER 4

Uveitis study

Introduction

Uveitis is an ocular inflammatory disease in which the nature of the causal factor is usually unknown. It is difficult to treat initially and the eye may ultimately succumb to the secondary effects of chronic inflammation during subsequent recurrences.

The basic mechanism thought to be involved in many types of uveitis is immunological. The treatment has included the use of anti-inflammatory agents[2-8] such as corticosteriods[2,14] and immunosuppressives.[5-8] These agents have been successful to varying degrees, but have also caused serious side effects like susceptibility to infection,[9] increased ocular pressures[10,11,14] and bone marrow suppression.[12,14,15]

Recently it has been demonstrated that experimental autoimmune uveitis (EAU) induced by retinal S-antigen resembles the immune-mediated ocular inflammatory response seen in humans, which is mainly T-cell-mediated.[18,27,34]

Cy has been used successfully systemically and orally in the treatment of T-cell-mediated uveitis in patients,[17-19] because it is able to block the early stages of T-cell activation. Unfortunately the high systemic and oral doses needed to get therapeutic levels of Cy into the eye, resulted in some serious complications such as nephrotoxicity.[17,42,43]

From the pharmacokinetic study of subconjunctival administration of Cy, we were able to show that higher amounts penetrated the ocular chambers, while avoiding the high blood levels of Cy.

To assess whether the subconjunctival route of administration would be effective in the possible treatment of uveitis, EAU was induced by injection of a single dose of bovine serum albumin (BSA) into the vitreous of rabbits. Acute uveitis developed within six to ten days, and persisted for at least a week, and then subsided. To assess the degree to which the inflammation might be suppressed, Cy subconjunctival therapy was initiated in one group of rabbits immediately after intravitreal injections. In another group Cy therapy was started one week after intravitreal injections.

Materials and Methods

Prior to the intravitreal injection all eyes were examined by slit-lamp biomicroscopy to rule out the presence of pre-existing ocular diseases. Fifteen locally supplied New Zealand white female rabbits (weighing 2.2 - 2.4kg) were tranquilized by intramuscular injection of 0.2 ml/kg of a 10:1 solution (100 mg/ml) of ketamine hydrochloride and acetylpromazine maleate (Roget S.T.B. and Ayerst Laboratories, Vancouver). After both eyes were anaesthetized with proparacaine HCl (0.5%), 0.10 ml ofa previously prepared solution of 50 mg/ml of BSA in Saline

(which had been sterilized by filtering the BSA solution through a Millex-GS(R) 0.22 micron disposable filter unit) was injected into the right vitreous, taking care to avoid the ciliary body and lens. Prior to the intravitreal injection of BSA an aqueous paracentesis was performed and 0.10 ml of aqueous humour was removed to prevent extravasation of the intravitreal inoculum. The opposite left vitreous received 0.10 ml of saline to serve as the control.

A 30 gauge needle attached to a tuberculin syringe was used to perform the aqueous paracentesis and the intravitreal injections.

The animals were divided into three groups of five rabbits each. Immediately after the intravitreal injections, Group 2 received subconjunctival injection of 5 mg of Cy in 0.1cc once per week and Group 3 received the subconjunctival injection of 5 mg of Cy in 0.1cc one week after inoculation with BSA. Group 1 served as the controls and received no treatment, except for 0.1cc of normal saline which was given subconjunctivally once per week. The 5 mg of Cy in 0.1cc and 0.1cc of normal saline were injected posterior to the superior limbus of the right eye by means of a 25 gauge needle attached to a tuberculin syringe once a week upon intitiation of therapy. The rabbits were examined daily by slit-lamp biomicroscopy, indirect ophthalmoscopy and serial photography. The rabbits were also studied by two other

examiners who had no knowledge of which rabbits received treatment and no significant variability was found among the examiners (data not shown).

The degree of inflammation in each eye was graded according to the scheme presented in Table V modified from Fox.[76] An overall value of inflammation was obtained after evaluating each criterion for each each rabbit in this experiment.

The experiment was terminated after three weeks and all of the rabbits were sacrificed with an overdose of pentobarbital. The globes were enucleated, fixed in 10% buffered formalin, embedded in paraffin, sectioned vertically and stained by Haematoxylin-Eosin for histopathological studies. Histologic assesment was made on the amount of cellular infiltrate, fibrin content and structural changes (e.g.posterior synechiae, retinal detachment). This was graded by myself and a pathologist who had no knowledge of the specific origin of the slides. No significant variability was found among the examiners.

Table V

Conjunctiva Hyperemia	Iris injection	Ciliary Flush	NV of Cornea	Posterior Synechia	Anterior Chamber			Posterior Chamber			Overall Inflammation
					Flares	Cells	Fibrin	Flares	Cells	Fibrin	
none	none	none	none	none	none	none	none	none	none	none	0
trace	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace	1
mild	mild	mild	mild	mild	mild	mild	mild	mild	mild	mild	2
mod	mod	mod	mod	mod	mod	mod	mod	mod	mod	mod	3
severe	severe	severe	severe	severe	severe	severe	severe	severe	severe	severe	4

NV = neovascuralization
mod = moderate

Results

Clinical Ocular Findings

Immediately after the aqueous paracentesis, intravitreal and subconjunctival injection (of Group 2) the anterior chamber became shallow and the iris appeared pale with a return to normal within 2 hours. There was a mild to moderate, non-specific, transient inflammatory reaction with intravitreal injections with both BSA and saline. The inflammatory reaction peaked at 24 hours and was characterized by mild pericorneal hyperemia, mild to moderate vasodilation of the iris vessels, mild to moderate flare and mild fibrin in the anterior chamber.

The inflammatory reaction slowly resolved over the next two days. Thereafter, the eyes remained stable until the fifth day at which point the animals developed varying degrees of unilateral uveitis. This transient inflammatory reaction is due to the trauma of injection and has been noted by others.[77] The results from the experimental rabbits in Groups 2 (that received subconjunctival Cy therapy immediately after intravitreal injection of BSA) and 3 (that received subconjunctival Cy therapy one week after intravitreal injection of BSA) were compared to the controls in (Group 1) for the onset and development of uveitis. Observations on the course of the uveitis are summarized in a plot of the daily composites of the overall inflammation for each day in figure 4.0.

Except for the transient inflammatory response to the

trauma of the needle all of the left eyes which had received an aqueous paracentesis and intravitreal injection of 0.10 ml of physiological saline, developed no uveitis.

The right eyes of the rabbits in Group 1 (controls) which were injected intravitreally with BSA and had no treatment developed visible trace to mild uveitis six days after the injections. This was characterized by mild iris and conjunctival hyperemia, mild perilimbal injection, cells, flare and fibrin in the anterior chamber. The uveitis progressed peaking by the 14th day when it had become quite severe. The anterior chamber at this point showed mild flare and moderate cells and fibrin. The posterior chamber had become quite involved with moderate to severe flare, cells and fibrin; as well, mild neovascularization of cornea was noted and in two of the rabbits posterior synechiae were observed.

After the 14th day the uveitis started to subside and a moderate inflammatory response mainly in the posterior chamber was present by day 21.

The groups that received treatment, (Groups 2 and3) showed a similar but significantly lesser inflammatory response than was observed in the controls (Group 1). In both the treated groups trace uveitis was noted seven days after intravitreal injection, peaking by the 14th day and subsiding thereafter. Group 2, which was treated immediately after the intravitreal injection, by the 14th

day showed a mild response which subsided to a trace inflammatory response by day 21. Group 3 which received treatment one week after the intravitreal injection of BSA showed a slightly greater response than Group 2. By the 14th day Group 3 showed a mild to moderate inflammatory response which subsided to a trace to mild inflammmatory response by day 21. As with Group 1, between days seven and 14, both Groups 2 and 3 showed that the uveitis was mainly restricted to the posterior chamber with slight involvement of the anterior chamber.

In summary, subconjunctivally administered Cy was found to exhibit an immunosupressive activity and the earlier the treatment with Cy the milder the uveitis. This was the case with Group 2 that was treated at the time of the intraviteral injection. Representative photographs showing the progress of uveitis in the three groups at seven, 14 and 21 days are shown in figure 4.1.

Histological Findings

The 3 groups were graded for inflammation depending on the amount of cellular infiltrate, fibrin content and structural changes.

The common inflammatory cells included polymorphonuclear granulocytes, macrophages, lymphocytes and plasmocytes, while the structural changes included retinal detachment and posterior synechiae.

Group 1 showed trace inflammation of the anterior chamber, and moderate inflammation of the perilenticular region, iris and angle. Two of the rabbits also developed posterior synechiae. The choroid showed moderate to severe inflammation, while the retina was moderately inflamed with partial or complete detachment. The vitreous contained minimal inflammation but moderate to severe fibrin deposits.

In group 2 the anterior chamber was devoid of inflammation, while the perilenticular area, iris and angle, showed only trace to mild inflammation. One rabbit had developed a posterior synechia. The choroid displayed mild to moderate inflammation and the retina showed trace to mild inflammation with focal serous detachments. The vitreous contained few inflammatory cells and mild to moderate fibrin deposits.

In group 3 there was a slightly greater inflammation than in group 2, but much less than in group 1. There was trace involvement of the anterior chamber, and mild to moderate inflammation of the perilenticular region, iris angle. One of the rabbits in this group also developed a posterior synechia. The choroid showed a moderate inflammatory response and the retina displayed mild inflammation with areas of focal serous detachments. The vitreous also showed minimal inflammation with moderate fibrin deposits present.

Representative histological photographs comparing the

varying degrees of inflammation between the controls and experimental rabbits are shown in figures 4.2-4.5.

Statistical Analysis

An repeated measures analysis of variance(nested) test showed that there was a significant difference between the treated and control rabbits. The level of significance between the groups was found to be p=0.0001. There was also found to be a significant difference between the two treated groups of rabbits, with the level of significance between the groups being p=0.047.

Figure 4.0

THE EFFECT OF SUBCONJUNCTIVAL CYCLOSPORINE ON UVEITIS INDUCED BY BOVINE SERUM ALBUMIN

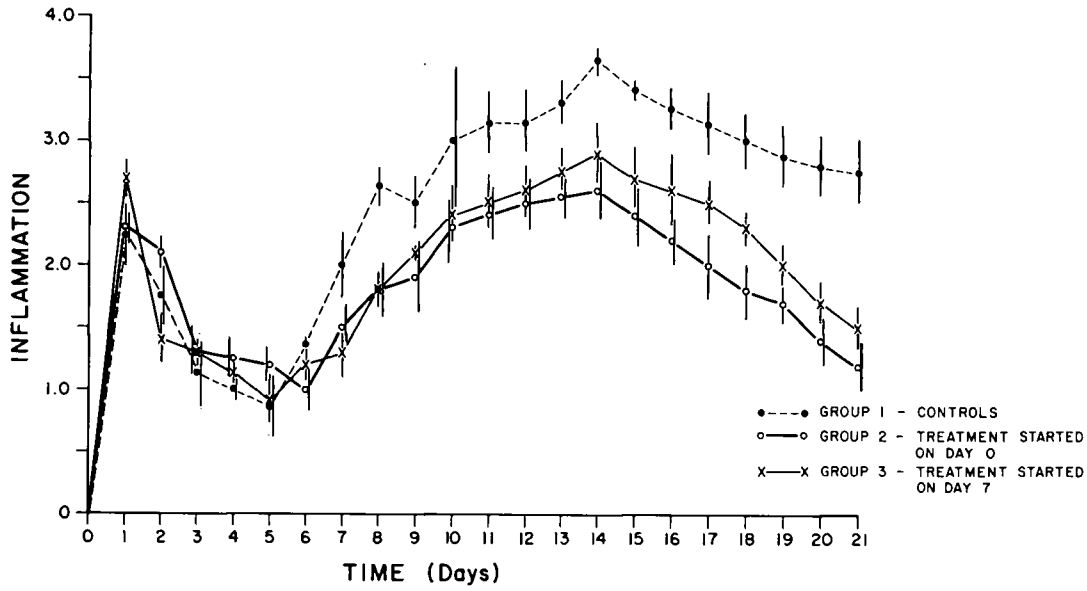
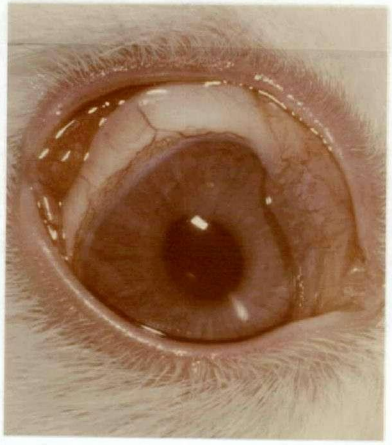
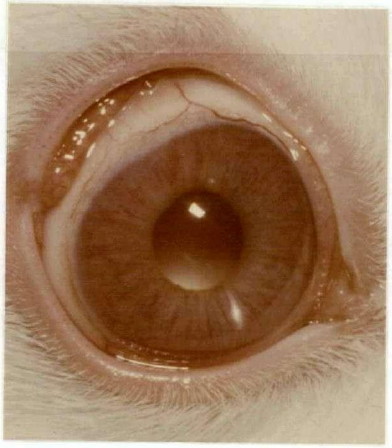


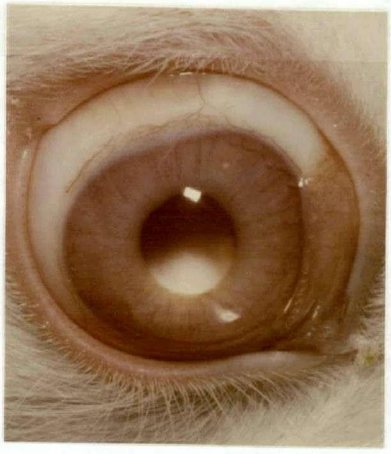
Figure 4.1
Effects of Bovine Serum Albumin induced uveitis and 5mg/0.1cc of Cyclosporine administered subconjunctivally



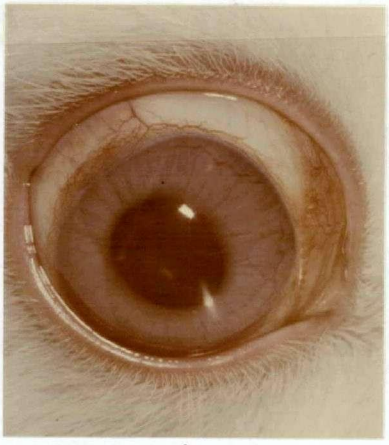
Group 1 (received BSA only) day 7



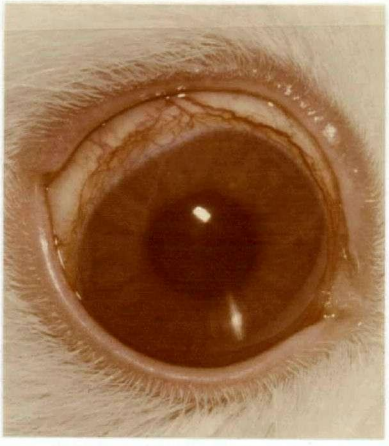
Group 1 day 14



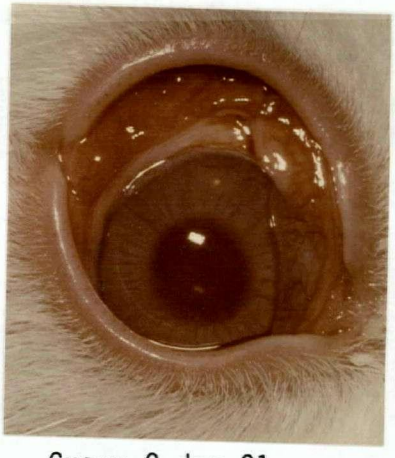
Group 1 day 21



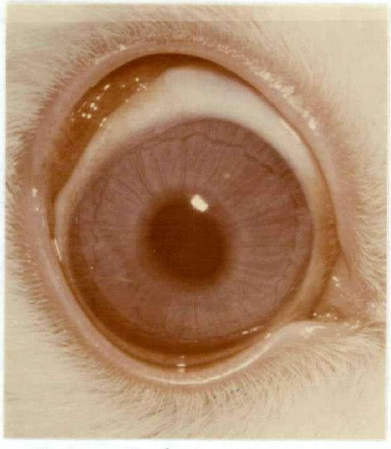
Group 2 (treatment started at day 0) day 7



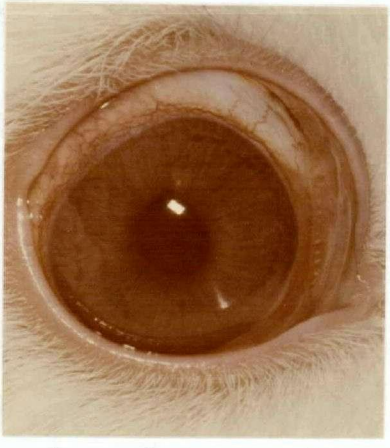
Group 2 day 14



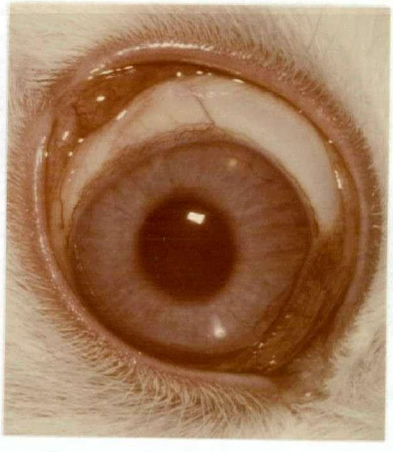
Group 2 day 21



Group 3 (treatment started at day 7) day 7

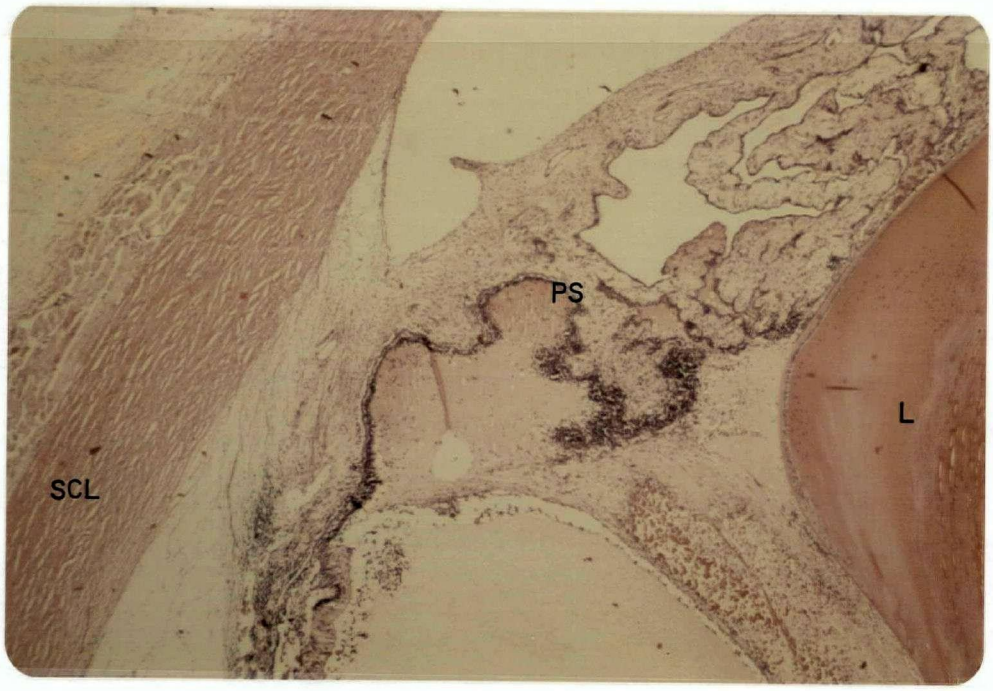


Group 3 day 14

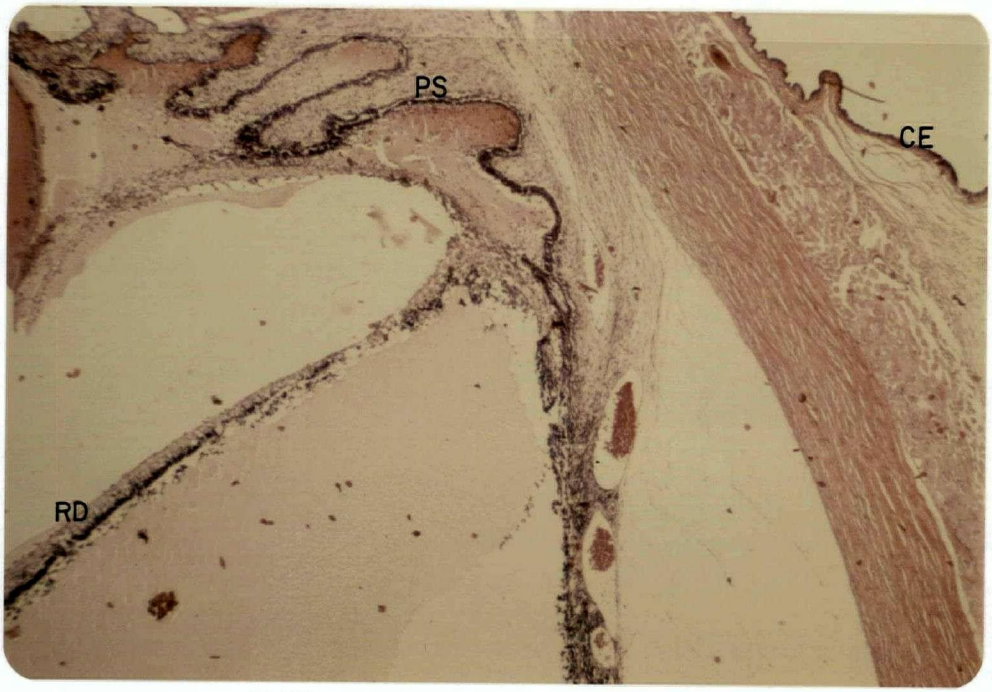


Group 3 day 21

Figure 4.2*
Histological effects of Bovine Serum Albumin(BSA) in Group 1 (received no treatment)



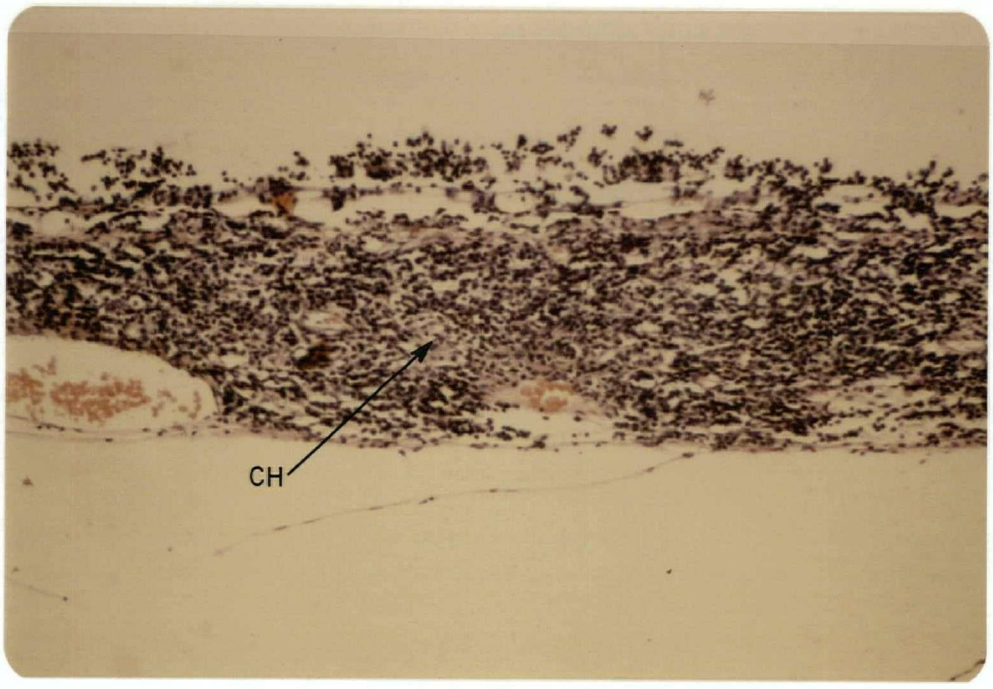
2.5x



2.5x

In some of the non-treated rabbits posterior synechia(PS) were present with a moderate to severe inflammatory response and retinal detachment(RD).* see list of abbreviations for figures 4.2-4.5.

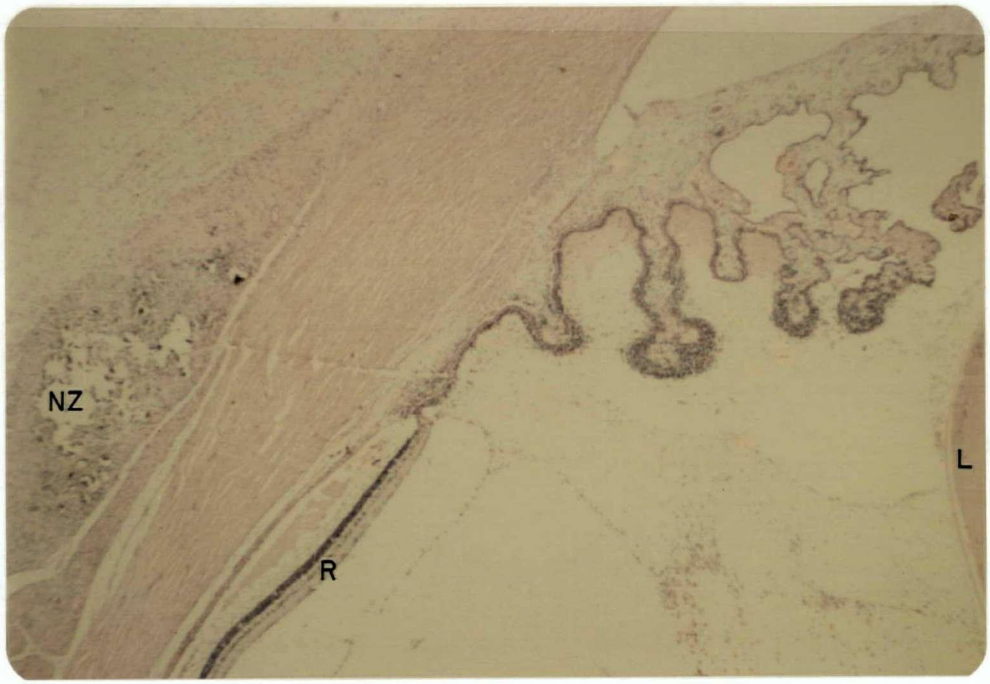
Figure 4:3
Histological effects of BSA in Group 1 on the posterior segment of the eye



25x

The choroid(CH) in the non-treated rabbits showed a severe chronic non-granulomatous uveitis.

Figure 4.4
Histological effects of Cyclosporine on uveitis induced by BSA in Group 2 (treatment strated on day 0)



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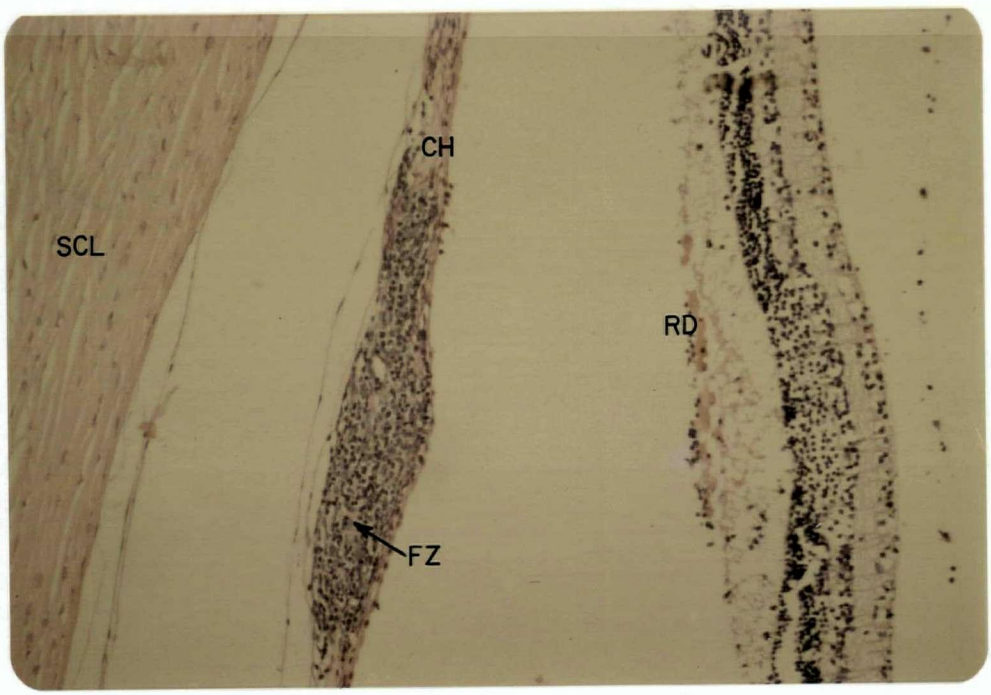
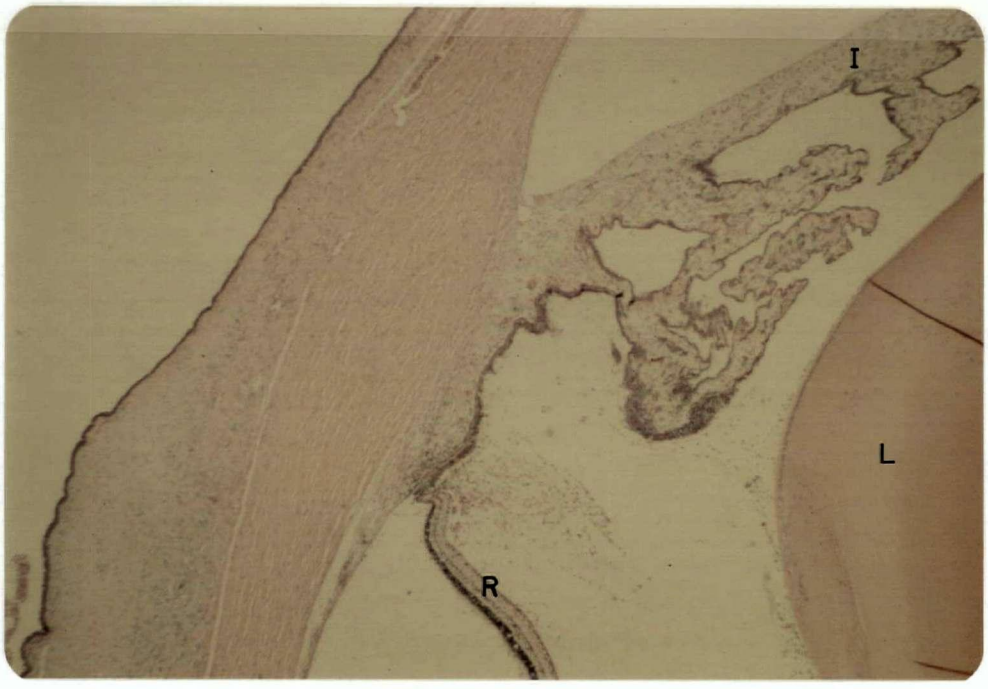


10x

A mild inflammatory response is present in the perilenticular area with focal zones(FZ) of inflammation in the choriod(CH) as well as focal serous retinal detachments(RD).

Figure 4.5
Histological effects of Cyclosporine on uveitis induced by BSA in Group 3 (treatment started on day 7)



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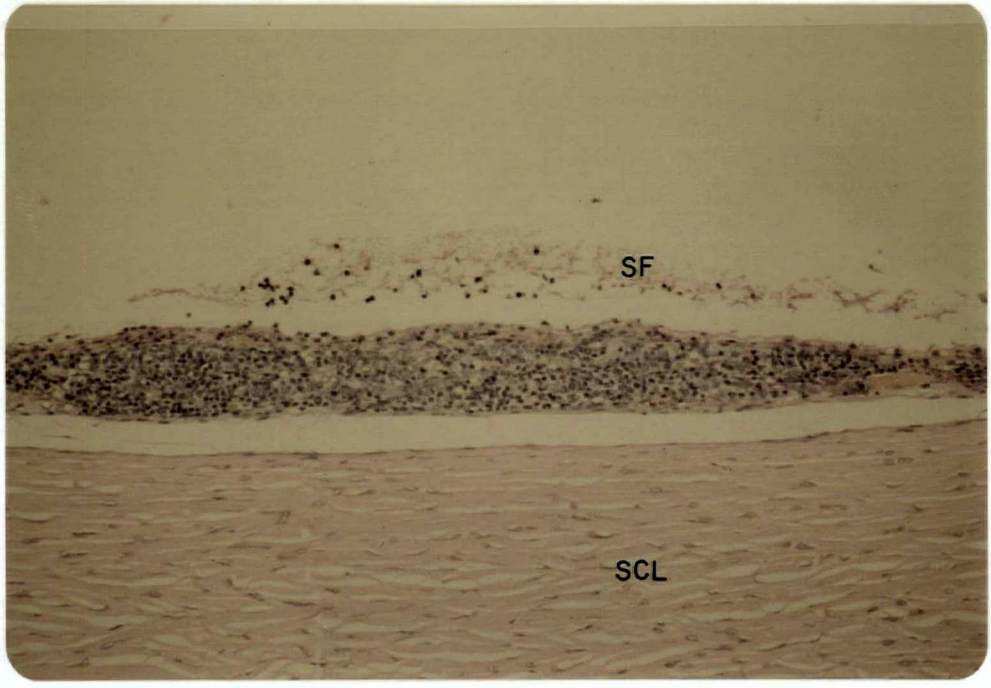

10x

A slightly greater inflammatory response with focal zones(FZ) of inflammation in the choroid(CH) and focal serous retinal detachments(RD) are present in these rabbits compared to Group 2(treatment was started on day 0).

DISCUSSION

Uveitis consists of a group of ocular inflammatory diseases of which the pathogenesis remains uncertain. There have been numerous attempts to find suitable potent anti-inflammatory drugs which will be effective in the treatment of uveitis.[2,3,7,8,14] Currently, the accepted therapy for uveitis has consisted of local and systemic application of corticosteriods,[2,14] a lternate anti-inflammatory drugs,[3,4] and systemic administration of immunosuppressives.[5-8]

The eye represents a pharmacological sanctuary.[13,14] In order to obtain the necessary therapeutic levels within the eye, high levels of these drugs orally and systemically are required to elicit an effect. These doses have resulted in observed side effects such as Cushing's-like symptoms due to systemic administration of corticosteroids,[15] myelotoxicity due to systemic administration of immunosuppressives[12,15] and local application of corticosteroids have also resulted in glaucoma.[10,11,14] The problem is compounded because some ocular inflammatory diseases have been found to be resistant to current therapy and with prolonged use of these drugs, tolerance may develop making them ineffective.

In order to improve treatment, an understanding of the processes resulting in uveitis is required. The underlying mechanism involved has been postulated as immunologic (autoimmune) in origin. Circulating and intraocular

immune complexes that have been found in patients with uveitis lends support to this contention.[35,36]

Along these lines animal models have been developed to study uveitis, and have resulted in the isolation of retinal S-antigen (S-ag).[25] S-ag has been identified as the 48,000 molecular weight protein found in the outer segments of the photoreceptor.[26] In experimental systems, autoimmune uveitis can be induced by S-ag (EAU-S-ag). Both humoral and cellular immunity respond to S-ag causing a delayed-type hypersensitivity.[31] Experimental induction of uveitis using S-ag(EAU-S-ag) has not always been succesful and is particularly difficult to induce in a rabbit model.

Studies have shown that T-lymphocytes play the major role in the development of EAU. This position has been supported by the transfer of EAU from uveitogenic T-cell lines to rats. This occurred without the involvement of humoral immunity suggesting that it is not necessary for the development of EAU.[31,32] The type of EAU-S-ag, resembles the ocular inflammatory response seen in some types of human uveitis, particularly uveitis involving the posterior segment of the eye.[18,19,27,34] In some of these patients, the T-lymphocytes were also shown to be reactive to S-ag in vitro.[19,34]

This finding has led to the use of Cyclosporine (Cy) in the treatment of uveitis, which can cause specific suppression of T-cells,[58-60] without causing myelotoxicity associated with other immunosuppressives

such as azathioprine[7,15] and cyclophosphamide.[8,15]

Cy has been found effective in the treatment of ocular inflammatory disease, and in the case of EAU-S-ag, findings suggest that Cy can break the cycle of S-ag specific T-cells needed for the induction and continuation of the disease.[28,29] The mechanism by which this may be possible is inhibition of the stimulation of T-helper cells without affecting the T-suppressor cell population. The relative increase in the suppressor population may be due to the break in the cycle of T-cell recruitment which may also be specific for S-ag. This suggests that the disease may remain under control even after Cy has been discontinued.[18]

Unfortunately, in the clinical situation the disease often recurs, partly because the uveitis seen in the clinic may not be predominately due to a cellular immune response,[31] and because the high doses of Cy that are required to be given orally and systemically to reach therapeutic levels within the eye result in renal toxicity, this limiting its use.[17,42,43]

Previously, the local immunosuppressive value of a group of antineoplastic drugs that were injected subconjunctivally were assessed for their effectiveness against EAU induced by bovine serum albumin (EAU-BSA). The results showed that these drugs were able to delay the onset and reduce the severity of the EAU. However, these drugs produced local toxicity, which although reversible

with discontinuance of the drugs would not have been acceptable in the clinical circumstance. Yet, subconjunctival administration of the drugs circumvented the side effects produced when given systemically (appendix I).

This approach was used to develop a protocol for the subconjunctival injection of Cy, in the hope that the side effects associated with systemic administration may be avoided, making possible the routine use of Cy in ocular inflammatory disease.

The maximum tolerable subconjunctivally administered dose by the rabbits' eye (5 mg in 0.1cc once per week) of Cy, was used to test its effectiveness in EAU-BSA. Induction of EAU was first tried by using S-Ag and the procedure outlined by Wacker[25] and others,[29,30,33] in order to have a model that resembled human uveitis on which to test Cy administered subconjunctivally. Unfortunately, the disease did not develop in any of the rabbits, and we therefore chose to induce uveitis by BSA,[77,78] which causes a much more acute uveitis and the resulting inflammatory response which is more likely due to the activation of both humoral and cellular immunity,[79] in contrast to a predominatly cellular immune response as seen in EAU-S-ag.[28,32,34] Since Cy caused immunosuppression primarily by inhibiting stimulation of T-cells, and only has a minimal effect on T-cell-independent B-cell function,[56,57] the effect o f Cy would be reduced in

EAU-BSA. Cy therapy was administered to a group of rabbits at the time of inoculation with BSA, another group of rabbits received treatment seven days after inoculation, and a control group which were inoculated with BSA received no treatment.

The results showed that in EAU-BSA subconjunctival injection of Cy was able to delay the onset and reduce the severity of EAU without causing any apparent side effects. The data suggests that the rabbits that received Cy therapy at the time of inoculation had an inflammatory response that was less severe than that of the group that received Cy therapy seven days after inoculation with BSA. This was to be expected, as Cy caused the suppression of T-cells during the early stages of activation and had little effect on the suppression of primed T-cells.[15,56,58,59] Therefore, the earlier the application of Cy the more effective it is likely to be in the treatment of the disease.

Although there was only a slight difference between the two treated groups, there is a significant difference between the control and experimental (treated) groups. In the treated groups there was also a significant difference and the similarity in the inflammatory response can be partly explained by the fact that EAU-BSA is not predominately mediated by cellular immunity.

An opportunity arose to test Cy injected subconjunctivally in a veterinary clinic. Four cats that

had chronic corticosteroid-resistant anterior uveitis (appendix II)[80] were treated for two months by subconjunctivalinjection of Cy according to the protocol developed in the rabbits, the only exception being that the cats received 0.1% topical dexamethasone twice daily (BID) to lessen the effects of the subconjunctival injection. The uveitis had disappeared in all the cats, but two months after discontinuing Cy therapy, while being maintained on 0.1% topically dexamethasone (BID) the uveitis flared up again in three of the four cats. Possibly, the cause of uveitis was systemic in origin and a population of uveitogenic T-cells were still present systemically, resulting in the recurrence.

This showed the feasibility of routine application of Cy when administered subconjunctivally. In addition, the results support previous studies that Cy and dexamethasone act synergistically.[33] Cy acts predominatly by inhibiting the activation of T-helper cells, while dexamethasone inhibits inflammation by inhibiting prostaglandin production and responsiveness of T-cells, though at a later stage after the T-cells have been activated.

Conclusions

Subconjuntival injections of Cy were effective in reducing the inflammatory response in EAU-BSA and the results suggest that the sooner the treatment with Cy the more effective it is likely to be. Subconjunctival injections of Cy were also found to be effective in the treatment of chronic corticosteroid-resistant anterior uveitis in cats without causing systemic side effects.

SUMMARY

The research tried to provide an alternative route of administration for Cy, that would give the benefits of Cy while avoiding the toxic effects of it in the treatment of ocular inflammation. The results from the pharmacokinetic and uveitis study demonstrated that the subconjunctival route was able to meet both requirements, with the only limiting factor being that Cy could only be administered once per week by this route. New combinations of vehicles or formulations that are less lipophilic would be beneficial in this respect.

Although the levels of subconjunctivally administered Cy may not remain at the therapeutic levels within the eye over the one week period between injections, pulse therapy is known to be quiet effective in the case of the eye. This has been shown with other agents injected subconjunctivally. This may be because from an immunological point of view the eye is unique, in so far as it is not able to replace the uveitogenic T-cells as fast as other organ sites; therefore, each successive treatment further reduces the uveitogenic T-cell population within the eye eventually resulting in the elimination of the disease from the eye.

The subconjunctival route of administration of Cy may hopefully pave the way for use of Cy in the treatment of ocular inflammmatory disease in the clinic either primarily or as an adjunct. One, however, must be aware that the

disease may not be a local phenomenon, but rather systemic in origin as was possibly the case in three of the clinically affected cats we treated in which the disease recurred one month after discontinuing therapy with subconjunctivally administered Cy.

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APPENDIX I

THE EFFECTS OF MULTIPLE DOSE SUBCONJUNCTIVAL ANTINEOPLASTICS ON ACUTE IMMUNE UVEITIS

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M.N. Bussanich

G. Gudauskas

C. Kumi

INTRODUCTION

Hypersensitivity has been recognized as a factor in the cause of some human ocular inflammatory diseases especially uveitis, although the amount and nature of antigen involved are usually unknown.[1] Corticosteroids have been traditionally used to treat ocular inflammations with success but complications such as susceptibility to infection[3] and increased intraocular pressures[4,5] may occur. Alternative non-toxic anti-inflammatory therapy, either as an adjunct or used independently would be an advantage.

Pharmacokinetic experiments suggest that penetration of subconjunctivally administered drugs into the ocular compartments can be up to 100 times higher than the same dose given systemically[6,7,8,9]. Subconjunctival injections may provide therapeutic drug levels to the eye while avoiding the higher doses when given systemically.

It has been demonstrated that an experimental allergic uveitis can be induced by injection of a single dose of bovine serum albumin into the vitreous of rabbits[2] and acute uveitis develops within 6 to 10 days, and persists for at least a week and then subsides. The present experiments were undertaken to determine whether subconjunctival antineoplastic therapy initiated immediately after the intravitreal injections could suppress the degree of inflammation.

MATERIALS AND METHODS

Prior to the intravitreal injection all eyes were examined with a slit lamp to rule out the presence of pre-existing ocular diseases. Twenty locally supplied New Zealand albino rabbits (2.2 - 2.4 kg) were tranquilized by i.m. injection of Ketamine/Acepromazine maleate (100 mg/ml) (10:1). After

topical anaesthesia with proparacain HCl (0.5%), a solution of crystalized bovine serum albumin (50 mg/ml in 0.9% NaCl solution) was filtered through a Millex^{R} - GS 0.22um disposable filter unit and 0.15 ml of the filtrate injected into the right vitreous. The opposite left vitreous received 0.15 ml physiological saline to serve as controls. A 30 gauge needle attached to a tuberculin syringe was inserted through the ocular coats taking care to avoid the posterior lens capsule. Only a small amount of injected fluid exuded from the injected sites after removal of the needles.

Animals were divided into 5 groups of 4 rabbits each. Immediately after the intravitreal injections, therapy with subconjunctival injections of saline, methotrexate, 25 mg/ml; cystosine arabinoside, 150 mg/ml; 5-Fluorouracil, 25 mg/ml and 6-Mercaptopurine, 40 mg/ml was initiated. All subconjunctival injections were made twice a week for 3 weeks. A volume of 0.5 ml of each drug solution was injected posterior to the superior limbus of the right eye by means of a 30 gauge needle attached to a tuberculin syringe. Daily clinical observations included careful slit lamp observation of cornea for evidence of perilimbal injection and quantitation of anterior chamber cells (expressed as 0 - 4+), flare (expressed as 0 - 4+) and fibrin. Conjunctival hyperemia, edema, mucus secretion and injection was noted. The iris was monitored for swelling, injection and reactivity and the lens and vitreous were studied daily. Serial photography was taken to document the clinical course. Peripheral blood counts were done on each animal once a week to monitor the extent of hematopoietic toxicity. Additionally, a generalized severity of inflammatory response was graded on a scale of 0 - 4+ after a modification of the method of

Wirotsko and Halbert[2] as follows:

0 = normal.

1+ = trace iris hyperemia, trace flare and occasional cells.

2+ = mild iris hyperemia, ciliary flush, mild flare, few cells in the anterior chambers, peripheral injection.

3+ = moderate amount of flare, cells in the gross, precipitate over lens and cornea, fibrin in the anterior chamber, perilimbal injection, posterior synechia, vitreous opacity.

4+ = extreme iris hyperemia, miotic pupils, intense photophobia, fibrin in the anterior chambers, dense cellular precipitate, posterior synechia, perilimbal injection.

The composite of the gradings of a) general clinical observations, b) flare and cells in the anterior chamber for all animals in each group were plotted against time. (Fig. 1). The experiment was terminated after 22 days. All rabbits were sacrificed with an overdose of pentobarbital sodium. Representative eyes were enucleated, fixed in 10% buffered formalin, sectioned and stained for histological studies.

RESULTS

Immediately after the intravitreal injections the anterior chambers became shallow and the iris appeared pale with a return to normal within 2 hours. There was a mild, non-specific, transient inflammatory reaction with intravitreal injections with both bovine serum albumin and saline. This lasted for a day and was characterized by a mild pericorneal hyperemia, slight vasodilatation of the iris vessels and flare in the anterior chambers. This reaction

is due to the trauma of injection and has been noted by others.[2] Thereafter all treated eyes remained quiet for at least 5 days at which point the animals developed varying degrees of unilateral uveitis.

The experimental results compared the onset and development of uveitis in rabbits treated with subconjunctival saline versus those treated with antineoplastic agents. Observations on the course of uveitis are summarized in a plot of the daily composites of the gradings 0 - 4+. (Fig. 1-left). Except for the transient inflammatory response to the trauma of the needle all left eyes which received .15 ml normal saline developed no uveitis.

The right eyes of animals injected intravitreally with bovine serum albumin and treated with subconjunctival saline developed grossly visible uveitis 6 days after the injections. This was characterized by iris and conjunctival hyperemia, perilimbal injection, and cells and flare in the anterior chambers. The inflammation peaked between the ninth and eleventh days after the intravitreal injections. After the thirteenth day the inflammation began to subside.

With cytosine arabinoside, uveitis was observed 8 days after the intravitreal injections. The uveitis peaked between the thirteenth day and the sixteenth day and subsided thereafter. Animals injected with methotrexate developed an initially mild uveitis between the seventh and eleventh days with a moderate rise in severity until the seventeenth day when it began to subside. With 5-Fluorouracil a mild uveitis developed on the tenth day and lasted for 6 days. Animals injected with 6-Mercaptopurine had a milder uveitis throughout the period of the experiment. Inflammation was minimal

until the sixteenth day when a clinically significant uveitis developed lasting 4 days.

In summary, all 4 drugs were found to exhibit anti-inflammatory action in varying degrees with later onset and milder uveitis than controls. 6-Mercaptopurine appeared to be the most effective in suppressing the degree and onset of the inflammation, followed by 5-Fluorouracil. The eyes treated with cytosine arabinoside and methotrexate were more inflamed than those treated with 6-Mercaptopurine and 5-Fluorouracil. It was noted that the degree of inflammation in all eyes, including those treated with saline, was about the same during the latter quarter of the experiment.

TOXICITY STUDIES

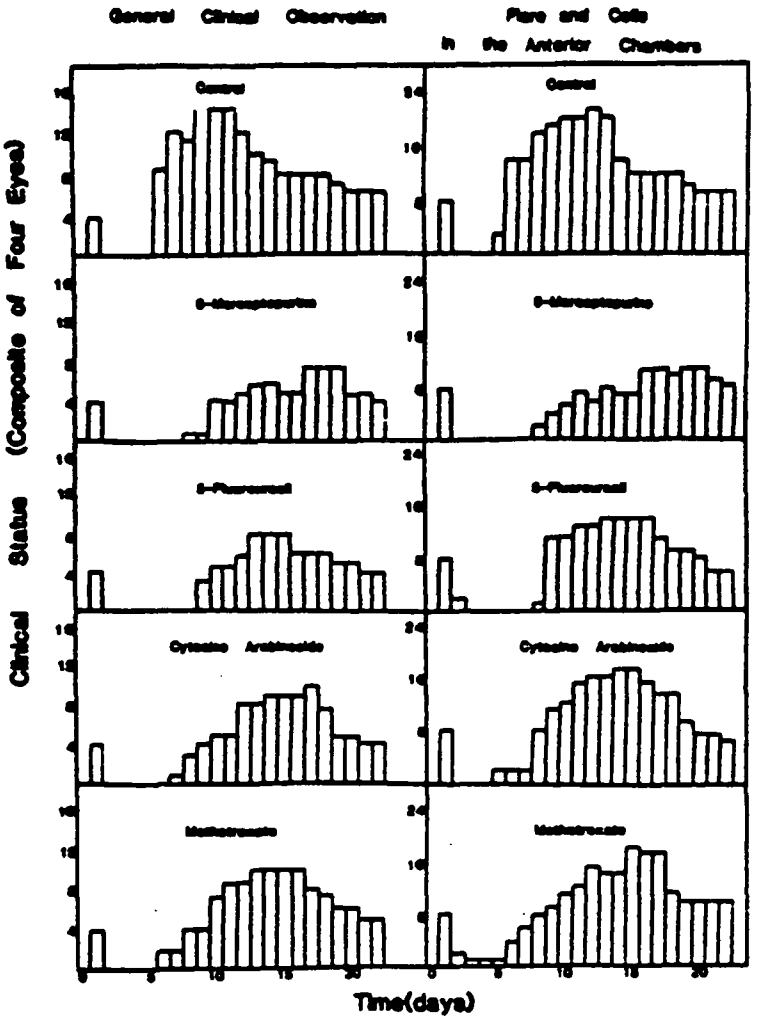
Systemic Toxicity.

There was no significant sign of hemtopoietic toxicity at the levels of drugs injected as monitored once a week by peripheral blood counts for the duration of the experiment.

Ocular Toxicity.

The multiple dose injection of methotrexate resulted in no significant signs of toxicity. With cytosine arabinoside there was a mild conjunctival hyperemia and chemosis which resolved after the therapy was withdrawn. Injections with 6-Mercaptopurine caused occasional hemorrhages in the conjunctiva and in a number of animals there was focal necrosis of the conjunctiva. 5-Fluorouracil was clearly toxic, leading to significant corneal edema and subsequent vascularization of peripheral cornea. In addition, conjunctival chemosis and periocular hair loss was noted.

OCULAR INFLAMMATORY RESPONSE
FOLLOWING SUBCONJUNCTIVAL ANTINEOPLASTICS



CONCLUSIONS

There have been numerous attempts to find suitable potent anti-inflammatory drugs which will be effective for the treatment of non-specific uveitis[12] without exhibiting the side effects of corticosteroids.[3,4,5] Some of these side effects may be avoided by the use of non-steroidal anti-inflammatory drugs.[13] Some cytotoxic agents, including cytosine arabinoside, methotrexate and 6-Mercaptopurine are known to have both anti-inflammatory and immunosuppressive properties[14] and may be useful in the treatment of uveitis. 6-Mercaptopurine has been used systemically to suppress immune uveitis[2,15,16,17].

We have attempted to assess the local immunosuppressive value of this group of antineoplastic drugs when injected subconjunctivally. Our experiments have demonstrated that 6-Mercaptopurine and to a lesser degree 5-Fluorouracil were able to delay the onset and reduce the severity of allergic uveitis induced by intravitreal injection of bovine serum albumin in rabbits. Cytosine arabinoside and methotrexate also appeared to reduce the severity and delay the onset of inflammatory response. In the case of both 5-Fluorouracil and 6-Mercaptopurine we felt that there was significant local toxicity at dose levels employed and although these toxicities were reversible with discontinuance of the drugs they would not be acceptable in the clinical circumstance. Further investigation of subconjunctivally injected 6-Mercaptopurine in particular might be worthwhile, perhaps in modified dose levels or as an adjuvant to other forms of therapy.

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APPENDIX II

Combined local chemotherapy for spontaneously occurring anterior uveitis in cats

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Abstract

Spontaneously occurring corticosteriod resistant anterior uveitis in four domestic cats was treated with subconjunctival injection of cyclosporin A and 0.1% dexamethasone topically for a period of eight weeks. Thereafter, the cats were maintained on 0.1% dexamethasone topically. The inflammation in all the cats had been stable for up to 2 months following treatment, but had relapsed while being maintained on topical dexamethasone twice daily.

Introduction

We have previously demonstrated the superiority of ocular penetration following local injection of several commonly used antineoplastic agents[24,25]. Not only do these drugs penetrate in higher concentrations than are achieved with systemic administration of equal doses,[20-23] but the paraocular route avoids the metabolic breakdown of the active components of the drugs characteristically seen with systemic administration. Herein we report the treatment of chronic anterior uveitis by combined local therapy with Cyclosporin A and 0.1% dexamethasone.

Method

The four cats chosen for the study had previously been treated topically and systemically with corticosteriods for anterior uveitis with an unsatisfactory response. They received Cyclosporin A subconjunctivally once a week and 0.1% dexamethasone twice daily, for 8 weeks. The eye was anesthetized with proparacain HCL(0.5%), and injected with 0.1cc of 5mg/0.1cc of Cyclosporin A[a] was given subconjunctivally above the superior limbus using a 25 gauge needle attached to a tuberculin syringe.

The cats were followed for a 8 weeks by slit-lamp biomicroscopy, indirect ophthalmoscopy and serial photography. The degree of inflammation was graded according to the following scheme modified from Fox[12]:

Ciliary injection	corneal clarity	corneal erosion	iris injection	Ant. Chb. haze	Vitreous & Retina	Hypopyon	Overall inflammation
none	clear	none	none	clear	chorioretinal detail sharp	none	0
trace	trace	trace	trace	trace	chorioretinal detail blurred	trace	1
mild	mild	mild	mild	mild	visible but no clear detail	mild	2
moderate	moderate	moderate	moderate	moderate + cells	no detail + cells	moderate	3
severe	severe	severe	severe	severe cells & fibrin	obscured	severe	4

[a] SANDIMMUNE I.V. 50mg/ml; SANDOZ Ltd, BASLE, SWITZERLAND.

Cat 1

A domestic short haired 13 year old neutered male, was first seen in October 1984 with bilateral uveitis and hypopyon. It had a normal CBC and blood chemistry and the serum was negative for feline leukemia. The cat had been treated from October 1984 to September 13, 1985 with 0.1% topical dexamethasone alternating with oral prednisone at a dose of 5 mg/day for one month at a time, however, the disease was not subsiding and we had noted an increase in keratic percipitates (KP's) with inflammation of the iris (fig. 1a).

In view of the increase in inflammation the cat was placed on Cyclosporin A subconjunctivally once per week and 0.1% dexamethasone topical twice daily (BID), for 8 weeks. At the end of the treatment the CBC and blood chemistry were unchanged, the inflammation had decreased with only small KP's present and the cat was maintained on 0.1% topical dexamethasone (fig. 1b). Follow-up showed that the cat remained stable for 2 months but relapsed there after while on topical dexamethasone. The overall progress of the cat is documented in figure 1c.

Cat 2

A domestic short haired 10 year old neutered male, was first seen in September, 1985 with bilateral uveitis, endothelial KP's, nodules on the iris and hypopyon in the right eye. Blood chemistry and CBC was normal and the serum was negative for Feline leukemia and toxoplasmosis.

The initial treatment was with 0.1% topical dexamethasone BID for three weeks and at the end of the this period, examination showed only a slight decrease in the inflammation (fig. 2a). Since the disease was not responding to the steriod treatment alone, the cat was placed on Cyclosporin A subconjunctivally once per week and 0.1% topical dexamethasone (BID), for 8 weeks; thereafter, maintained on 0.1% topical dexamethasone BID.

At the end of the Cyclosporin A treatment, CBC and blood chemistry was normal except for the decrease in the white blood count from 7500/mm^3 to 5000/mm^3. The progress of the cat on the Cyclosporin A and subsequent follow-ups can be seen in figure 2c, demonstrating improvement of the uveitis while on the treatment (fig. 2b) and subsequent recurrence eight weeks after discontinuing therapy.

Cat 3

A domestic long haired 9 year old spayed female, was first seen on Februray 11, 1985 with bilateral uveitis, iris nodules and a fibrin clot attached to the right lens. The cat was placed on 0.1% topical dexamethasone and 5 mg of prednisone orally. The CBC and blood chemistry was normal and the serum negative for feline leukemia. On Februray 23, 1985 the eyes had become worse and by March 7 the anterior chamber had improved, but the cat had developed a superficial corneal erosion which slowly healed over one week.

The eyes remained stable until early October 11, 1985 when the cat had a relapse in both eyes with marked inflammation and a fibrin clot on the surface of the lens (fig. 3a).

Because of the severe bilateral flare-up the cat was placed on Cyclosporin A subconjunctivally once per week and 0.1% topical dexamethasone BID, for 8 weeks. By the end of the 8 week treatment there had been considerable improvement in both eyes with only a few small KP's (fig. 3b). The effects of Cyclosporin A can be seen in figure 3c.

Blood chemistry and CBC done at end of the Cyclosporin A treatment was normal. The cat was maintained on 0.1 %topical dexamethasone BID and remains stable for one month.

Cat 4:

A domestic short haired 8 year old neutered male was first seen on Nov 9, 1985 with right unilateral uveitis. The iris was congested, had nodules on it, and fibrin was noted on the lens capsule with a moderate amount of flare and cells in the anterior chamber vitreous (fig. 4a).

This cat was placed on Cyclosporin A subconjunctivally once per week and 0.1% topical dexamethasone BID, for 8 weeks. By December 7, 1985 it had developed a corneal erosion. The fifth injection of Cyclosporin A and topical dexamethasone was not given until the following week.

The cat had improved considerably by the end of the treatment period (fig. 4b). After discontinuing of the treatment the cat was placed on topical dexamethasone BID.

There was a minor relapse on the last week of therapy with Cyclosporin A and dexamethasone. The hypopyon had returned and the eye was becoming inflammed again, but on subsequent follow-ups the inflammation had regressed and one month following treatment with Cyclosporin A and dexamethasone there was only a trace of inflammation.

The eye still remains stable 16 weeks afte treatment with Cyclosporin A and dexamethasone, the longest period of the four cats in this study. The progress of the cat while on the treatment and subsequent follow-ups can be seen in figure 4c.

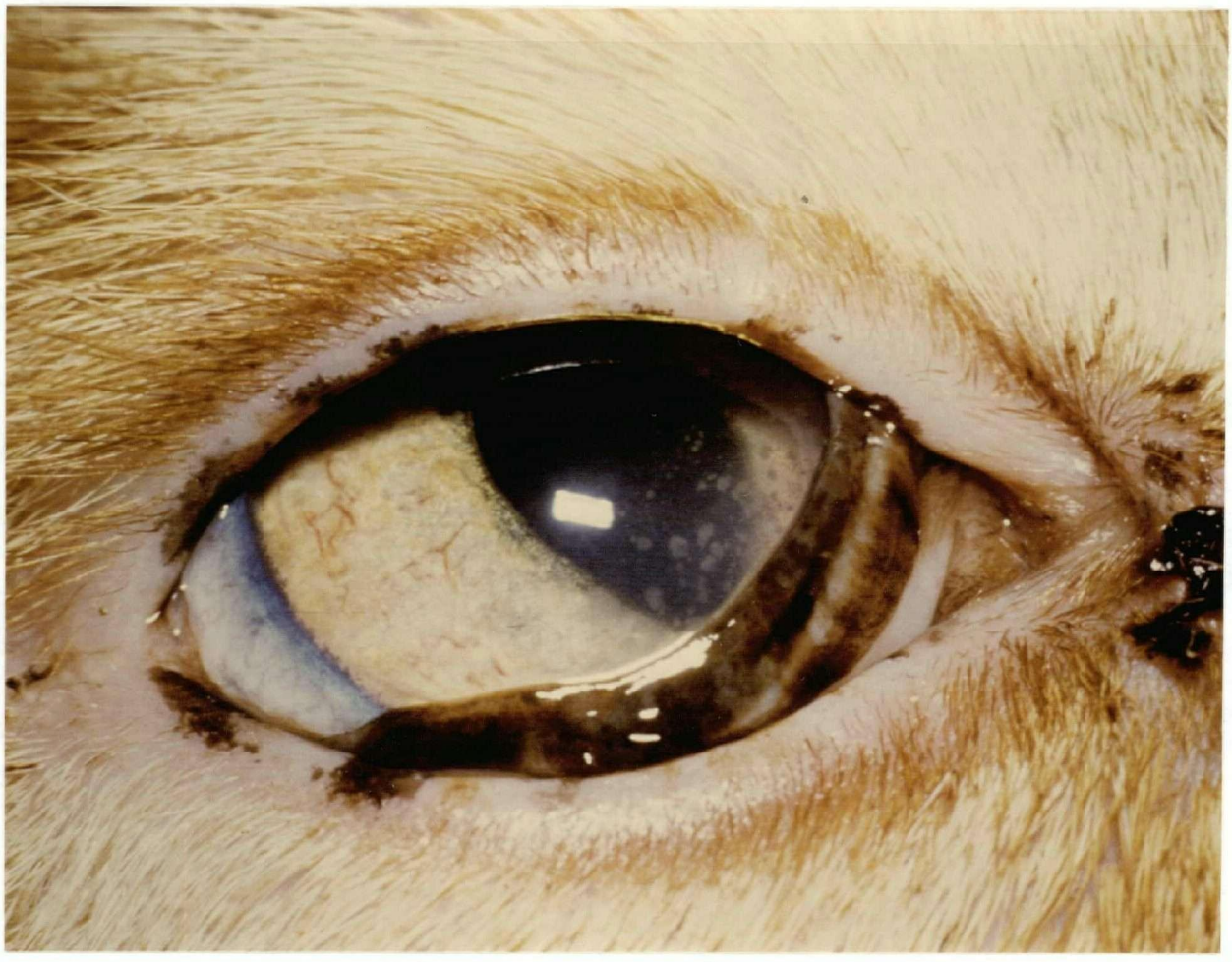


Figure 1a

Right eye of Cat 1 showing keratic percipitates (arrow) before initiation of treatment.

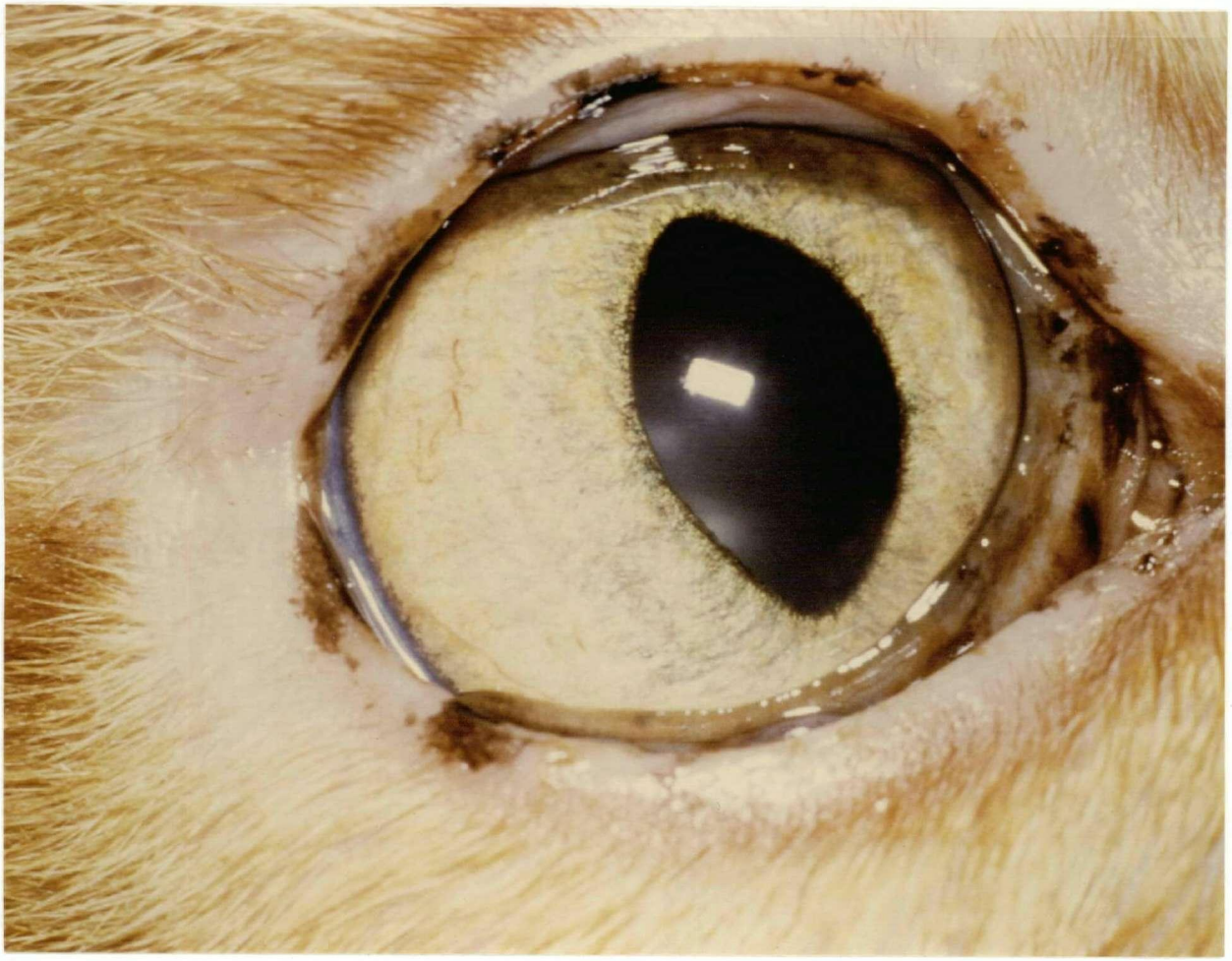
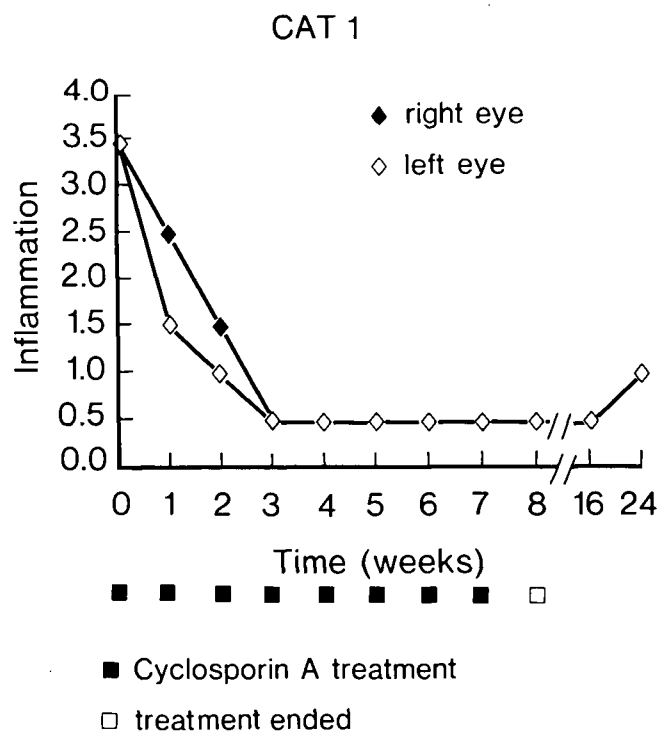


Figure 1b

Right eye of Cat 1 showing lack of inflammation at completion of treatment with subconjunctival Cyclosporin A and topical 0.1% dexamethasone (eighth week).

Figure 1c


CAT 1
Inflammation
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
right eye
left eye
0 1 2 3 4 5 6 7 8 16 24
Time (weeks)
Cyclosporin A treatment
treatment ended

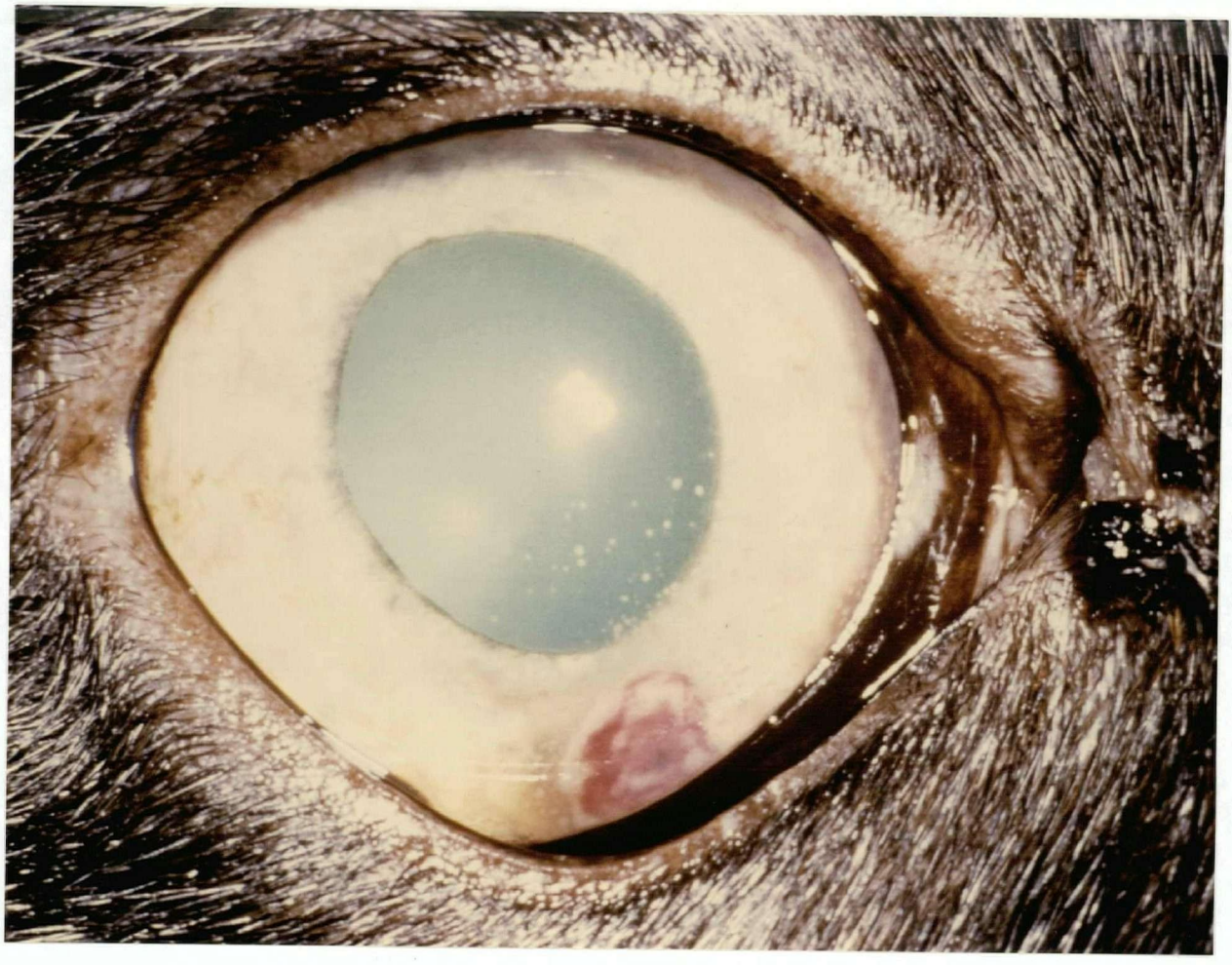


Figure 2a

Right eye of Cat 2 showing Hypopyon and small keratic percipitates before initiation of treatment.

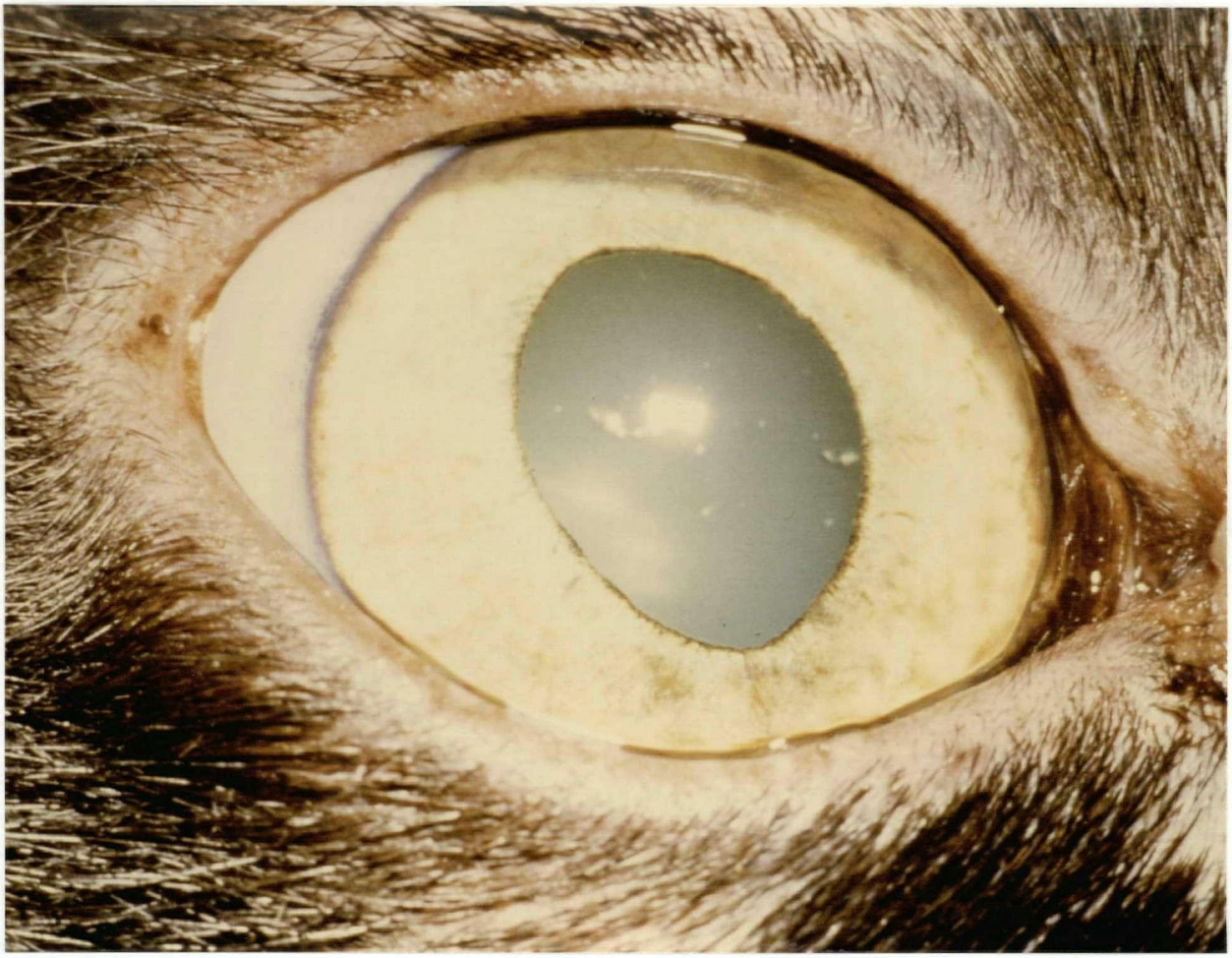
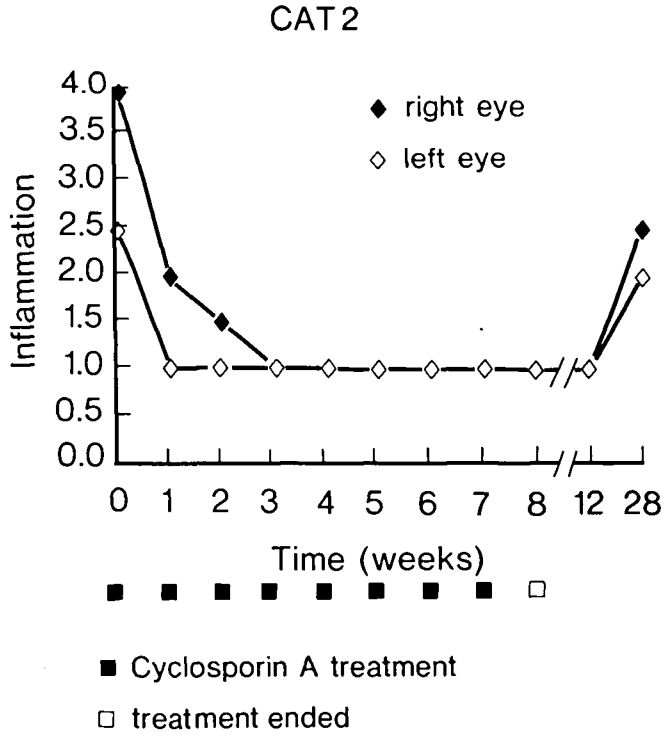


Figure 2b

Right of Cat 2 showing resorption of the hypopyon and decrease in the number of keratic percipitates, at completion of treatment with subconjunctival Cyclosporin A and topical 0.1% dexamethsone (eighth week).

Figure 2c


CAT 2
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Inflammation
◆ right eye
◇ left eye
0 1 2 3 4 5 6 7 8 12 28
Time (weeks)
■ Cyclosporin A treatment
□ treatment ended

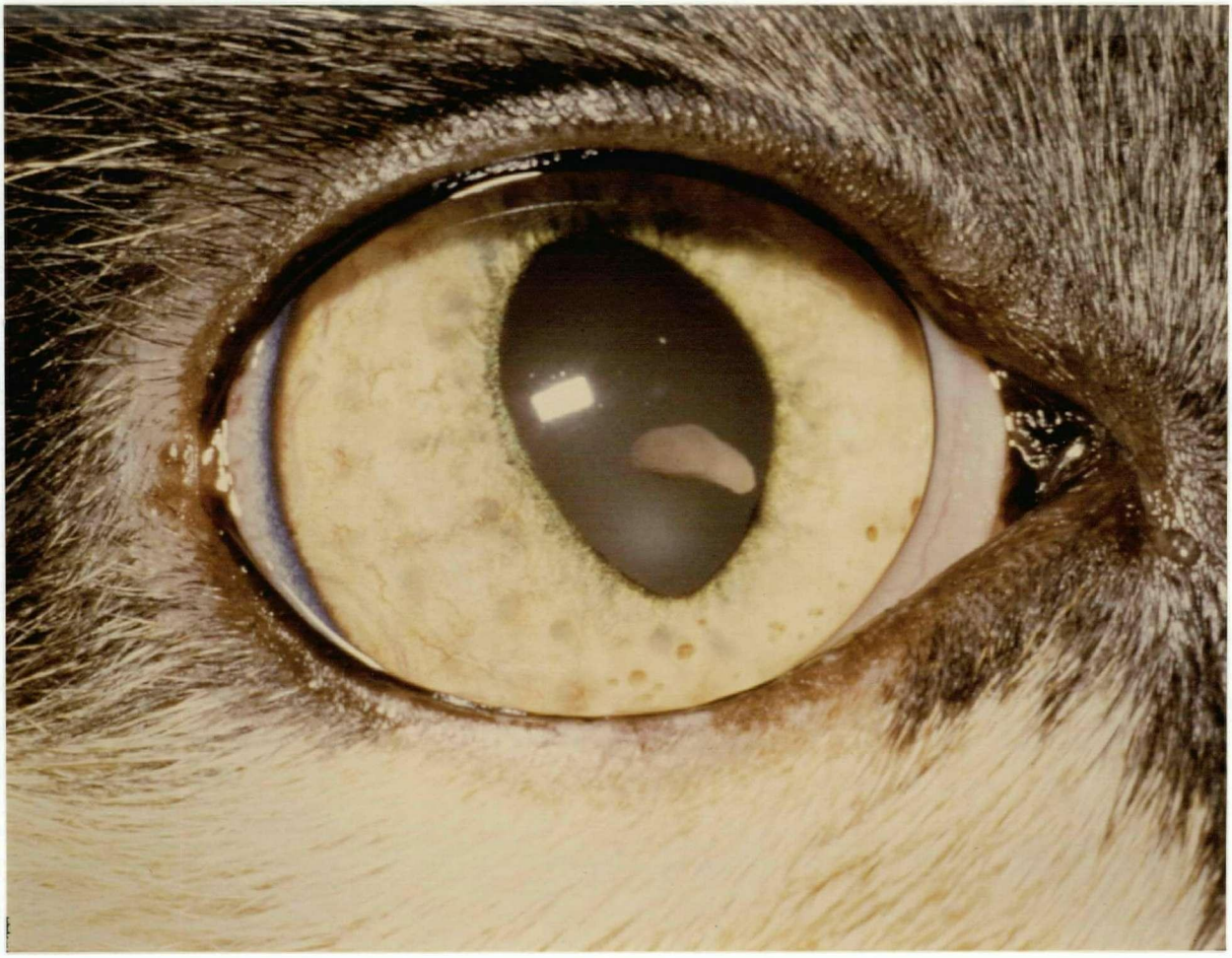


Figure 3a

Right eye of Cat 3 showing the presence of a fibrin clot attached to the lens, small keratic percipitates and nodules on the surface of the iris (arrow), before initiation of treatment.

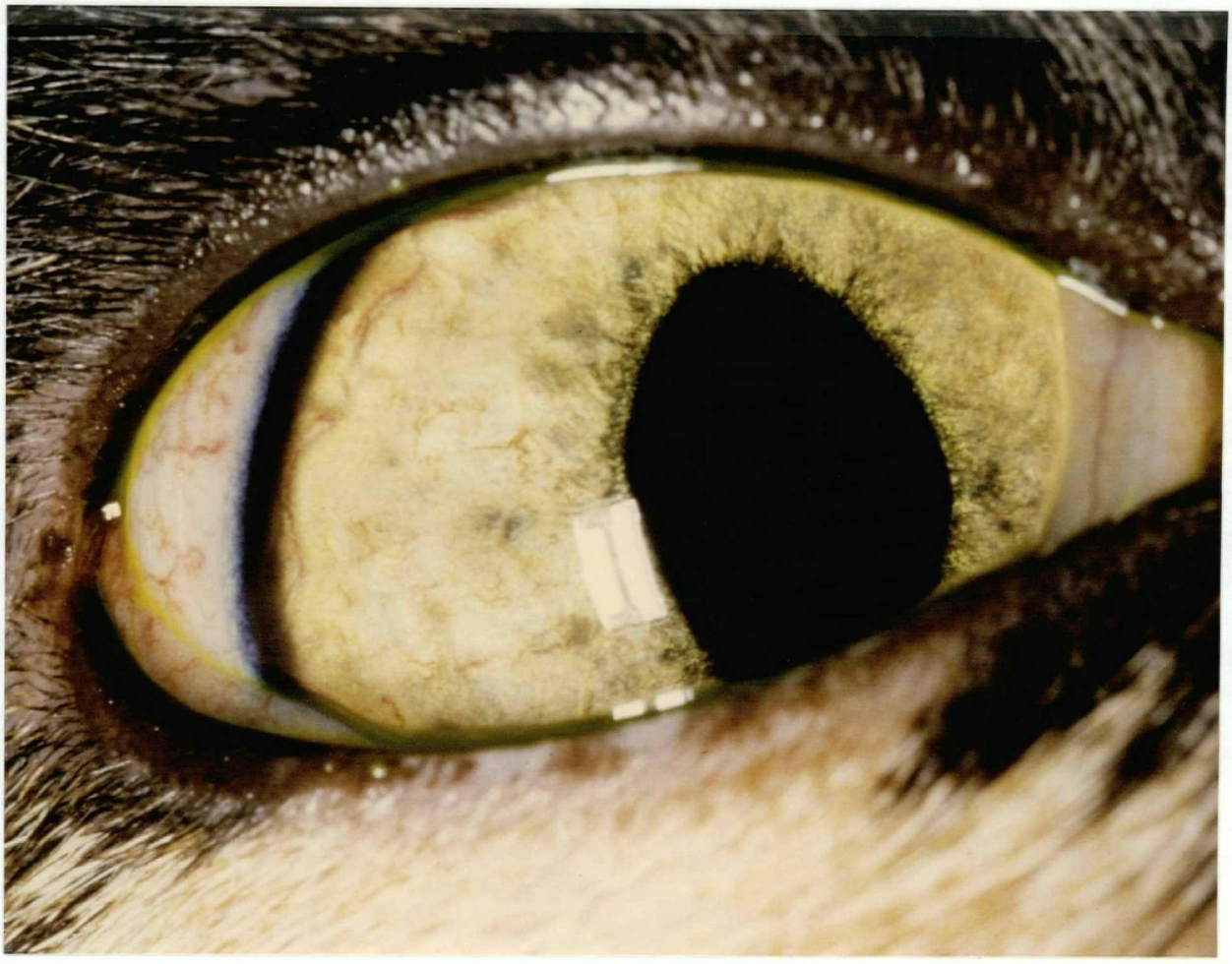
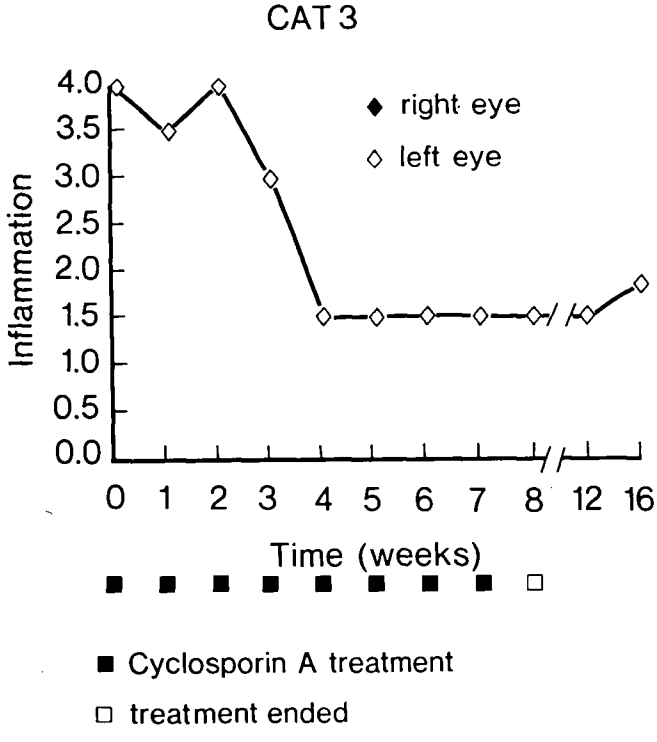


Figure 3b

Right eye of Cat 3 showing decrease of inflammation at the end of treatment with subconjunctival Cyclosporin A and topical 0.1% dexamethsone (eighth week).

Figure 3c


CAT 3
Inflammation
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
◆ right eye
◇ left eye
0 1 2 3 4 5 6 7 8 12 16
Time (weeks)
■ Cyclosporin A treatment
□ treatment ended

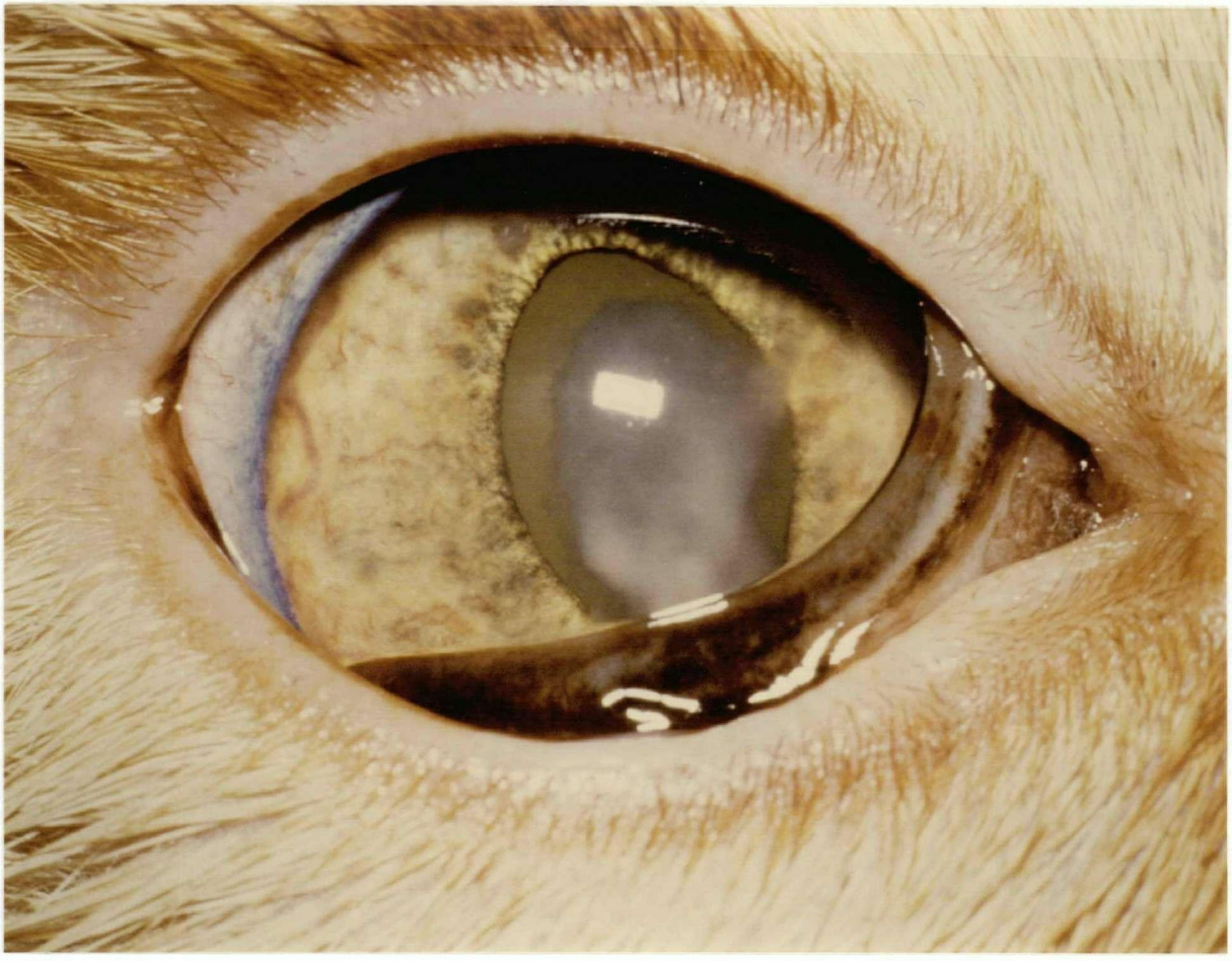


Figure 4a

Right eye of Cat 4 showing the presence of a fibrin clot on the surface of the lens, with nodules on the iris surface (arrow) before initiation of treatment.

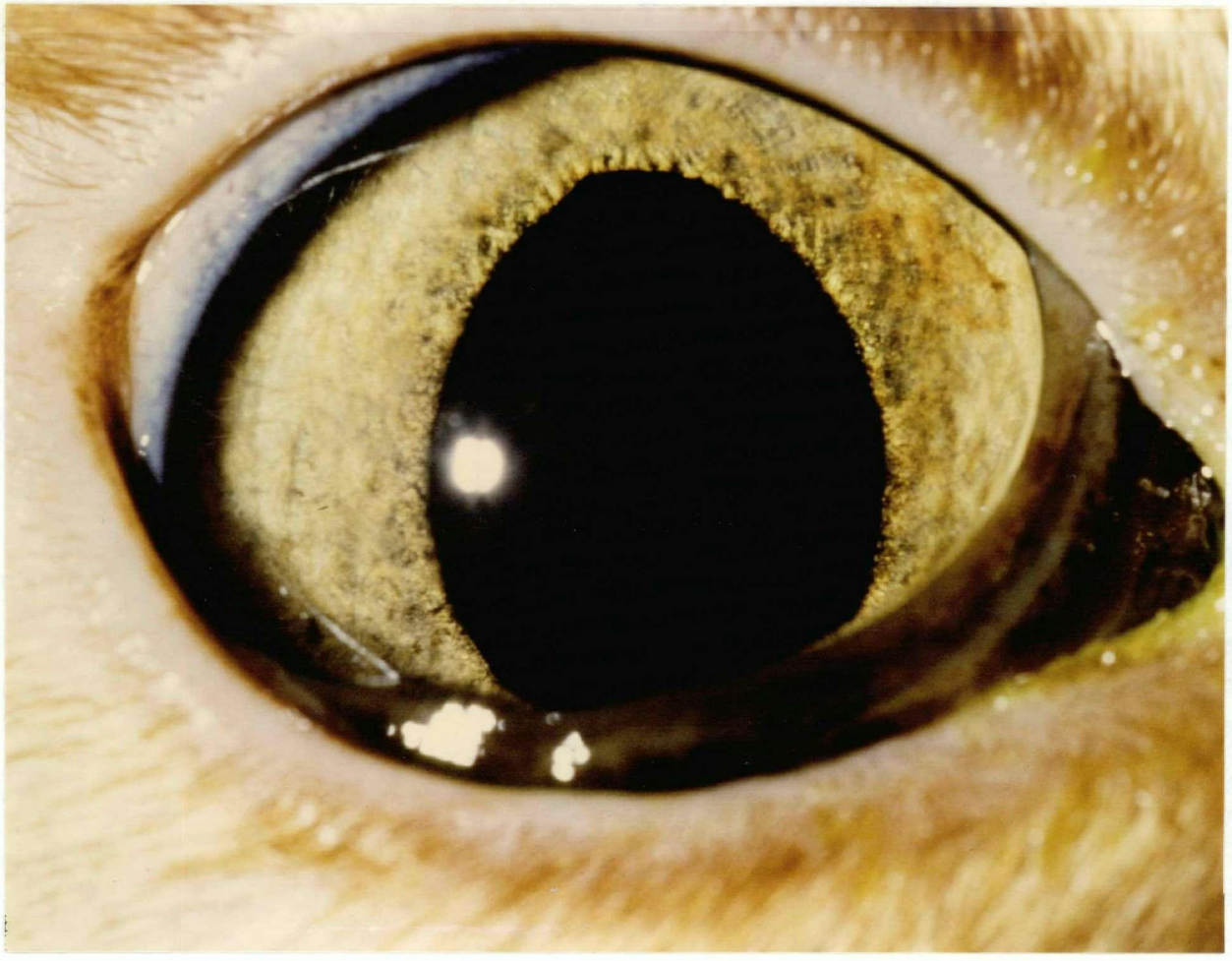
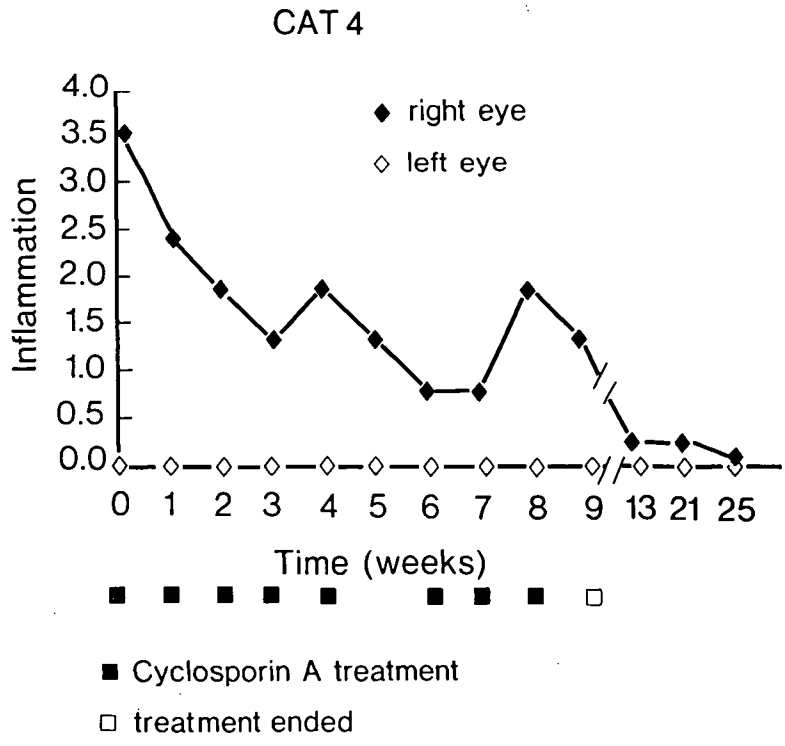


Figure 4b

Right eye of Cat 4 showing decrease of inflammation at the end of treatment with subconjunctival Cyclosporin A and topical 0.1% dexamethasone.

Figure 4c


CAT 4
right eye
left eye
Inflammation
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9 13 21 25
Time (weeks)
Cyclosporin A treatment
treatment ended

Discussion :

Because the etiology of uveitis is not well defined, it can be difficult to treat and recurrent. It is therefore not suprising that uveitis accounts for 1.0% of blind human patients in Canada.[1]

The mainstay of treatment has been steroids, in some case supplemented by immunosuppressive agents.[2] Cyclosporin A has been employed systemically (i.m.) and orally in the treatment of uveitis.[3-6] It has been effective in treating T-cell mediated inflammation.[7-9] The mode of action of Cyclosporin A is felt to be inhibition of the release of interleukin-2 from T-helper cells thereby suppressing the inflammatory reaction.[10,11]

Since retinal S-antigen is thought to be a major factor in the development of T - cell mediated uveitis,[13,14] Cyclosporin A should be effective in some types of uveitis,[3,6,15] especially in the acute phase. The problems associated with treating uveitis with Cyclosporin A have been it's side-effects. Particularly nephro and hepatotoxicity which occurrs at a relatively low dose of 10 mg/kg.[16-19]

We have shown with a number of agents that ocular absorption can be significantly enhanced by sunconjunctival injection of the drug when compared to equidose systemic administration.[20-25] In this way it may be possible to circumvent the associated toxicities of systemic administration.

Subconjunctival administration of Cyclosporin A was developed by us using a rabbit model. As a result of toxicities studies of numerous vehicles the dose and makeup of Cyclosporin A that we used was 0.1 cc of 5 mg/ 0.1cc of Cyclosporin A, administered subconjunctivally once per week.

We were able to successsfully control chronic steroid resistant spontaneous anterior uveitis by using Cyclosporin A once per week and 0.1% topical dexamethasone BID, for eight weeks and than maintaining the cats on 0.1% topical dexamethasone twice daily. The eyes on average remained stable for about two months (except for cat 4 which had right unilateral uveitis and remains stable to date). The 0.1% topical dexamethasone was given with the Cyclosporin A, because Cyclosporin A alone would cause mild swelling of the upper eye lid, which would last about 24 to 48 hours, but with 0.1% topical dexamethasone the swelling lasted for less than 24 hours.

It would seem from the way the uveitis responded to the Cyclosporin A that it would be more effective in the acute phase of the disease.

From this study the combined therapy of Cyclosporin A and 0.1% topical dexamethasone acted synergistically and was able to control the uveitis successfully for a period of up to 2 months after discontinuing treatment.

Further studies on the subconjunctival administration of Cyclosporin A are under current investigation.

Acknowledgements:

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