Treatment of Laser Induced Retinal Injuries

Midterm Report
(September 30, 1987 through March 31, 1989)

N. Naveh, M.D.
Michael Belkin, M.D.

June 21, 1989

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Treatment of Laser Induced Retinal Injuries

Previously we have shown that the ocular response to laser-induced ocular injury is characterized by an enhancement in arachidonic acid metabolism and that steroid treatment curtailed this response. In the present study we investigated the involvement of leukotrienes B4 (LTB4) and the following parameters were studied: LTB4 production by the retina/choroid, its accumulation in the vitreous, and changes in vitreal protein levels. The effect of an antileukotriene drug on the severity of the ocular inflammatory response was also studied.

To overcome the problem that the amounts of LTB4 produced by the retina in vitro were too small to be detected by the biochemical assay used by us, a model for the study of LTB4 production by the retina-choroid was established, using Ca^{2+} ionophore A 13187. The choice of the antileukotriene drug to be used in our future work was done following an in vitro study in which the efficacy of two antileukotriene drugs were compared (B1755C versus norguiaretic acid (NDGA)). NDGA has been proven to be the more effective antileukotriene.
The changes in LTB₄ production and its vitreal accumulation, as well as vitreal protein levels, were studied in eyes exposed to Nd:YAG laser irradiation at various time intervals during a two-week period. Our results confirm our hypothesis, and indicate that the ocular response to laser-induced retinal injury involved an enhancement in chorioretinal LTB₄ production, and its subsequent accumulation in the vitreous to above pre-laser values. It is postulated that LT involvement in the ocular response to trauma might be responsible for the immediate incapacitation and the late vision reduction. Our finding that the ocular response to laser-induced retinal injury involves an enhanced leukotriene B₄ response, might be responsible for edema formation and accumulation of various toxic substances in the vitreous, with resultant immediate incapacitation of the affected individuals. Treatment to reduce leukotriene production might attenuate vision reduction.
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In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals": prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life and Sciences, National Research Council (NIH Publication, No. 85-23, revised 1985).
Summary

This midterm report covers the work performed from September 30, 1987 through March 31, 1989, and includes data obtained during:

1) A period of non-funded, extension (Feb. 87-September 1987).
2) Information reported in our recent annual report dated May 1989, covering work done for the period of 30 Sept. 87 to 1 Oct. 88).
3) Data obtained up to the period of March 31, 1989).

During the period covered by this midterm report we:

1) Established a reproducible model for the study of leukotrine B$_4$ production by retina-choroid.

2) Compared the efficacy of two antileukotriene drugs (NDGA and BW755) and chose NDGA as the drug of choice for the treatment of laser injuries.

3) Studied changes in leukotriene B$_4$ levels in the vitreous of laser injured eyes.

4) Studied the effect of repeated laser exposure on the leukotriene response.

5) Initiated the study on the effect of NDGA on leukotriene B$_4$ response.
Introduction

Our study of "Steroid treatment of a laser induced retinal injury" summarized in an annual report (1), demonstrated that Neodymium (Nd):YAG retinal burn was associated with an inflammatory reaction at the laser lesion site; An extensive infiltration by polymorphonuclear cells of both the retina and choroid was associated with excessive production of prostaglandin E₂ (PGE₂) by the retina-choroid, which were immediately released into the vitreous causing an increase in PGE₂ vitreal levels to above pre-laser values. The vitreal response involved also an enhanced protein concentration indicative of a "break" in the blood retinal barrier. At later phases after exposure, the injury site underwent scarring.

Steroid treatment of laser exposed animals which was started immediately after laser exposure was only partially effective in reducing the chorioretinal response to trauma, but it significantly curtailed the vitreal reaction; Steroids inhibited altogether the vitreal PGE₂ response but had only a partial inhibitory effect on protein leakage into the vitreous. However, steroid treatment had no effect on the retinal polymorphonuclear cellular infiltration and it suppressed only transiently the excessive PGE₂ production by the damaged choroid-retina.

The fact that steroid treatment was unable to abolish completely the choroid-retina response in eyes subjected to laser irradiation established the need for a search for a better and more effective therapeutic regimen. An effective therapeutic regimen should minimize the ocular response to laser irradiation so that the immediate incapacitation and the chronic scarring process affecting visual rehabilitation will be diminished.
This requirement for a new medication led us to extend our study and look into the behavior of leukotrienes $B_4$ (LTB$_4$) during the ocular response to laser injury.

We hypothesized that laser induced retinal injury might be associated with excessive leukotriene (LT) production. Our hypothesis is based on the fact that the retinal injury was related to an enhanced PGs production which is indicative of excessive availability of free arachidonic acid released from its binding to cellular membrane, which serves as a substrate for LT production (2).

Increased levels of LTB$_4$ in the aqueous humor of rabbits was demonstrated following ocular trauma (3,4) in uveitis in humans (5). Though no direct correlation was demonstrated between elevated LT levels and the ocular inflammatory response (6).

The significance of LTs in the tissue inflammatory reaction is exemplified in studies in which LT antagonists or LT synthesis inhibitors provided some protection in situations such as endotoxemic shock or various forms of trauma (7,8).

LTs generated in the brain affect cerebral circulation to promote vasopasm and edema (9,10). The retina, which is also a nervous tissue might be affected in a similar manner, so that its exposure to excessive LT levels might have a detrimental effect on vision. Therefore, it must be kept in mind that the laser induced retinal lesion is a pathological process in which both PGs and LTs play a major role.

In order to assess the mediatory role of LT in retinal injury, we suggested to study the effect of two antileukotriene drugs, Norgualaretic acid (NDGA) and BW755C, on the laser induced retinal injury. NDGA inhibits LT production through inhibition of the lipooxygenase pathway and has no
effect on PGs formation (11). BW755C affects simultaneously the formation of both LTs and PGs (12).

However, in any pathophysiological process where LTs and PGs have been argued to participate, many other biologically active substances including thromboxanes, oxygen radicals, interleukenes, etc. are also produced. Therefore, it is expected that successful therapeutic strategies would include drugs aimed against multiple mediators, in addition to modulation of the LT actions (13).
Materials and Methods

A total of 192 pigmented rabbits of either sex (2 to 2.5 kg.) were used in this year's study. Rabbits were divided into five study groups: (48, 54, 41, 30 and 21 rabbits in each group, respectively).

1) Study on retinal in vitro LTB₄ production.
2) Study on the effect of two antileukotriene drugs on LTB₄ in vitro retinal production.
3) Study on changes in retinal and vitreal LTB₄ levels of laser exposed eyes.
4) Study on repeated laser exposure.
5) Study on the effect of NDGA on vitreal LTB₄ of laser exposed eyes.

Animal care and treatment in this investigation were in compliance with the ARVO Resolution on the Use of Animals in Research.

Laser procedure

Neodymium:YAG laser exposure - A Q-switched Neodymium (Nd):YAG laser (Lasag:Thun, Switzerland) was used to perform 15 retinal laser applications through a Russel-Fankhouser three mirror lens. These applications were aimed at the posterior pole of the right eye of each rabbit nasally to the optic nerve head, at areas devoid of blood vessels, as far apart as possible. The other eye was left untreated. The endpoint in establishing a Nd:YAG laser burn was a visible whitening of the retina at the burn site with slight reddening of the underlying choroid. Excluded were eyes with retinal or vitreal hemorrhage. Each exposure consisted of a single pulse of single burst in the multimode at energy level of 0.1-0.4 millijoules.

Nd:YAG laser exposed group - Rabbits which underwent Nd:YAG laser irradiation were divided into 4 subgroups, studied at 1, 3, 7 and 14 days after exposure.
Thirty minutes before the laser procedure, the animals were anesthetized by 35 mg/kg Ketamine and 5 mg/kg Xylazine injected intramuscularly following pupil dilatation with Tropicamide 0.5%. Local anaesthesia with Benoxinate 0.1% preceded the laser treatment. Following animals sacrifice, with intravenously injected overdose of phenobarbiturate, the right eye of each rabbit was enucleated, and samples of the vitreous body and the retina-choroid were obtained for LTB₄ and protein measurements as described.

**Unexposed control group** - Control rabbits were not exposed to any laser irradiation and their vitreous, and retina-choroid of the right eye of each rabbit were obtained for LTB₄ determination as described for the Nd:YAG laser group.

**Retina-choroid preparation** - Following enucleation, the corneas were cut all around the limbus, and the iris and ciliary body were removed by pulling gently at the iris base. The lens was removed and the vitreous expelled as described (1) and placed separately in a vial. The retina attached to the choroid (a retina-choroid preparation) was separated from the sclera and each preparation was placed in another vial containing the buffer for further studies on LT production, described below.

**Leukotriene B₄ determination** - The retina-choroid preparation was incubated in 0.6 ml Krebs Ringer Bicarbonate Hepes Buffer, pH 7.4 in a period, the tissue was removed and samples were withdrawn for LTB₄ determination. The vitreous of each eye was similarly incubated following the addition of 0.3 ml of the same buffer. At the end of the incubation period, a sample was withdrawn for LTB₄ determination. LTB₄ was determined using a Radioimmunoassay kit (New England Nuclear) with a specific antibody.
Protein determination - Protein was measured in the vitreous body using the modified Lowry method (13).

Experimental design

Experimental design for the first two groups was similar:

Group 1 - A model for in vitro study of leukotrienes by retina-choroid.

Group 2 - Comparison of the in vitro inhibitory effect of two antileukotriene drugs.

Studying these two subjects involved investigation of the in vitro changes in LTB4 production by retina-choroid.

In vitro studies - Each retina-choroid preparation was incubated separately for 30 minutes at 37°C in a shaking bath in 0.6 ml of a Krebs Hepes Buffer, Ph 7.4 with or without the addition of Ca²⁺ ionophore A23187 at various concentrations (0.1 to 5.0 micromoles). At the end of the incubation period a sample was withdrawn for LTB4 determination.

The antileukotriene effect of NDGA and BW755C on chorioretinal LTB4 production was studied using a retina-choroid model previously described, so that in this set of experiments we used a retina-choroid preparation incubated in Ca²⁺ ionophore containing media. The appropriate drug was also added to the incubation medium using various doses (see results section). At the end of a 30 minute incubation period, samples were withdrawn for LTB4 determination. The amounts which accumulated in the incubation medium containing the retina-choroid was considered as representing the amounts produced by the tissue, and this was dubbed "LTB4 in vitro production".

Group 3. Changes in retinal and vitreal LTB4 response in laser injured eyes

This was studied in vivo, in two groups of rabbits; an unexposed control group and a laser exposed group. In the laser exposed group, the retina-choroid was obtained at 1, 3, 7, and 14 days after exposure and then
incubated, as described. Samples from the incubation media containing the retina-choroid were withdrawn for determination of LTB$_4$ in vitro production while samples from the vitreous were used for determination of vitreal protein and LTB$_4$ levels.

Group 4. Effect of repeated laser exposure on LTB$_4$ vitreal response - Animals were lasered in a manner similar to that used in groups 3 and 5 and two weeks later were lasered again, and this time the laser applications were aimed at areas free of visible laser lesions. The animals were sacrificed at 1, 3, 7 and 14 days after the second laser exposure.

Group 5. The in vivo effect of an antileukotriene drug (NDGA) on the vitreal LTB$_4$ response in laser exposed eyes - Laser procedure followed that used in groups 3-5, but within 1 hour after exposure NDGA was administered intramuscularly, and this treatment was repeated daily.
Results

Group 1 - Study of the in vitro chorio-retinal LTBA4 production.

LTBA4 in vitro production by the choriod-retina yields amounts which are too small to be detected by the biochemical assay used by us. Therefore, measure to enhance retinal LTBA4 in vitro production were required.

Production was enhanced by adding Ca+ ionophore A13187, a drug widely used as an activator of LTBA4 production. The effect of varied concentrations of Ca+ ionophore was studied (from 0.1 to 5.0 micromoles) in 48 rabbits divided into 6 groups (Table 1).

The addition of Ca+ ionophore (0.1 to 1.0 micromoles) caused a dose dependent increase in retinal LTBA4 production (Table 1), and maximal in vitro production levels were achieved at 1 micromole. With higher levels of Ca+2 ionophore production was enhanced to a lesser degree and at 5 micromolar no excitatory effect was noted. On the addition of 1 micromole Ca ionophore LTBA4 in vitro production levels reached levels of 389±132 pg/gm wet weight (Table 1) which were easily detected by our biochemical assay. We find this method of using Ca ionophore for measurement of in vitro chor oretinal LTBA4 production to be a satisfactory model.

Group 2 - Comparison of the in vitro efficacy of two antileukotriene drugs (BW755C and NDGA).

The study of the inhibitory effect of the two antileukotriene drugs - BW 755C and NDGA on LTBA4 in vitro production by the choroid-retina was carried out to decide their relative efficacy. The drug possessing a greater inhibitory effect, is likely to be more active as an antinflammatory agent.
Comparison of the two antileukotriene drugs involved 54 rabbits and was performed in a dose dependent manner. Each drug was studied at 3 different concentrations (0.1, 1.0, and 10 micrograms/ml). The appropriate drug was added separately to a vial containing a single retina choroid incubated in a media with Ca^{+2} ionophore (1.0 micromole). Following a 30 minute incubation period in 37°C in a shaking bath, a sample from the media was withdrawn for LTB_{4} determination.

In evaluating our results, we considered as baseline the amounts of LTB_{4} produced by choroid-retina treated with Ca^{+2} ionophore only, but with no other drug added (352±128 pg/gr. wet wt.). Both BW755C and NDGA had a significant inhibitory effect on LTB_{4} production which was evident at each of the concentrations studied (Table 2).

NDGA at low concentration (0.1 microgm/ml) inhibited baseline LTB_{4} production by 90% and was more effective than the corresponding BW755C group (27±13 and 107±30 pg/gm wet wt., respectively). At higher dose (1.0 microgm/ml) both drugs exhibited a similar effect: demonstrating an inhibitory effect which reduced levels to less than 10% baseline values. The standard deviation in the two latter groups were equal or greater than the mean and this was caused by the fact that production ranged from 0 to 40. Based on our data, we decided to use NDGA as our drug of choice for inhibition of LTB_{4} production in our animal studies.

Group 3 - Changes in vitreal and retinal LTB_{4} in laser injured eyes.

The determination of LTB_{4} in the choroid-retina and vitreous of eyes subjected to Nd:YAG laser irradiation encountered two main problems: The problem of the naturally low leveled retinal production was overcome by the addition of Ca^{+} ionophore A13187 to the media of the incubated retina, following the method described in "Results", section A.
The second problem of low vitreal LTB₄ levels was solved by increasing the number of laser application to 15 using the same power setting (method section, laser procedure).

Baseline (pre-laser) vitreal content of LTB₄ was very low and ranged from 0 to 33 pg/gm weight (wt) with an average of 19±9 pg/gm wt. Following laser exposure, vitreal LTB₄ levels were elevated to above pre-laser values and peaked on day 3, at which time they were 280% higher than pre-laser values (Figure 1).

Nd:YAG laser induced retinal damage was associated with an enhanced chorioretinal LTB₄ production throughout the first week following exposure. An augmented production (200% higher than baseline) was evident already at the first day, and remained elevated also on day 3. Maximal production values were achieved on day 7 and reached levels 2.7 times higher than pre-laser values. Control levels were resumed on day 14.

Group 4 - Repeated laser exposure.

This study on exposure to repeated laser irradiation might be of military significance: Laser exposure to low energy levels, might go unnoticed during the initial phase (if the lesion site does not include the macular area). Therefore, the unaware soldier will be sent back to combat where he might be exposed to additional laser irradiation. We suggested that a repeated laser exposure might result in an accumulative damage to the blood retinal barrier, similar to that described by Bito (1974 with a progressive accumulation of toxic substances in the vitreous body and a subsequent reduction in visual acuity.

In the present series of experiments, the animals were lased once and two weeks later were exposed to a second similar session of laser irradiation. During the second laser exposure, the laser was aimed at areas
free of visible laser lesions (as described under experimental design). Following the second laser exposure, the animals were sacrificed.

Leukotriene B\(_4\) was determined in the media containing the chorioretina or the vitreous of each eye, while protein was measured only in the vitreous as described.

Our results (Fig. 2) demonstrate that repeated laser exposure is related to an increase in LTB\(_4\) vitreal concentrations to above pre-laser values. Peak levels, 7 and 4.5 folds higher than baseline were noted during the second week after exposure (days 7 and 14 respectively), while earlier levels did not exceed pre-laser. The pattern of changes of vitreal LTB\(_4\) content in eyes subjected to repeated exposure was different from that observed in eyes exposed to the laser only once; In the latter group, vitreal LTB\(_4\) values peaked earlier after exposure and maximal levels were lower. Later during the second week baseline levels were resumed (Fig. 2).

This pilot study serves as a partial confirmation to our suggestion that repeated laser exposure to low energy levels might be associated with a more pronounced vitreal response that that observed in eyes exposed to laser only once. This enhanced LTB\(_4\) response occurring late after exposure, might indicate that repeated laser exposure could be unexpectedly harmful to the eye.

Group 5 - Effect of NDGA on laser injury.

In this group we tried the effect of an antileukotriene drug - NDGA on the ocular inflammatory response following laser exposure. The parameters studied were: the changes in LTB\(_4\) in vitro production by the choroid-retina and its accumulation in the vitreous.

The decision as to which antileukotriene drug should be used as an effective antinflammatory agent, was based on our previous work (see group
2). Our data on the comparison between two antileukotriene drugs; the BW755 and NDGA showed the supremacy of the NDGA. Therefore, in our study on the efficacy of an antileukotriene drug in reducing the ocular response to laser injury we used NDGA. The experimental design followed that already was described and involved exposure to the Nd:YAG laser and NDGA was given intramuscularly within 1 hour following injury and daily during the two week observation period. The exposed animals were sacrificed at 1, 3, and 7 days after exposure, the eyes were enucleated and samples of retina-choroid, and vitreous body were obtained.

In assessing our results, the pre-laser values were considered as baseline (100%) and levels in the treated and untreated groups were given as percentage of baseline. Our results on the effect of NDGA treatment on the vitreal LTB₄ response in laser exposed eyes (Fig. 3) indicate that NDGA reduced the accumulation of LTB₄ in the vitreous during the first week after exposure.

The inhibitory effect of NDGA persisted for a whole week, thus protecting the retina from the adverse effect of excessive levels of LTB₄.
Conclusive remarks

The findings obtained during this period study which have a bearing on future studies are:

1) The newly developed method for determination of retinal LTB₄ production, which otherwise was undetectable, is going to serve us in the coming two years. This method is reproducible and might serve for analysis of the antiinflammatory properties of various drugs.

2) Our finding that NDGA, an natileukotriene drug caused a 90% inhibition of LT synthesis by the retina, made us choose it as a treatment to reduce to laser induced response in our future in vivo studies.

Military Clinical Significance

Our finding that the ocular response to laser induced retinal injury involved an enhancement in chorioretinal LTB₄ production and its subsequent accumulation in the vitreous has not been reported so far, and has direct clinical implications.

LTs involvement in the ocular response to trauma might be responsible for edema formation and accumulation of various toxic substances in the eye affecting the retina, with resultant immediate incapacitation of the affected individuals. Measures to inhibit LT production might reduce attenuate the scarring process so that vision reduction will be minimized.

In addition our finding that repeated laser exposure is related to a more pronounced vitreal inflammatory response is of significance in cases in whom the first laser exposure went unnoticed. Is a soldier will be prone to another, more harmful exposure which might have a long lasting effect on his vision.
Our finding that NDGA, an antileukotriene drug suppresses, the vitreal
LTB₄ response is promising and might indicate its potency as an
antiinflammatory agent with resultant protective effect in laser injury.
Further studies are required to substantiate this.
Table 1
The effect of Ca\(^{++}\) ionophore A23187 on the in vitro retinal production of Leukotriene B\(_4\).

<table>
<thead>
<tr>
<th>Ca(^{++}) ionophore Concentration (micromole)</th>
<th>0.1</th>
<th>0.3</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinal Leukotrienes levels (pg/gr wet weight)</td>
<td>123±50</td>
<td>316±60</td>
<td>285±134</td>
<td>389±132</td>
<td>93±76</td>
<td>0</td>
</tr>
<tr>
<td>(mean±SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number of eyes studied</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

*Control Leukotriene Levels 12±7 pg/gr wet wt.

*Retinal leukotrienes production by retina-choroid untreated by Ca\(^{+}\) ionophore.
Table 2

A comparison of the inhibitory effect of two antileukotriene drugs (BW 755C and NDGA) on leukotriene $B_4$ production by the retina-choroid.

*Leukotriene $B_4$ production by the retina-choroid (pg/gram wet wt) Mean ± SD

<table>
<thead>
<tr>
<th>Concentration (microgram/ml)</th>
<th>BW 75 Mean ± SD</th>
<th>NDGA Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 microgram/ml</td>
<td>107±30</td>
<td>27±13</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td>1.0 microgram/ml</td>
<td>35±64</td>
<td>23±20</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td>10 microgram/ml</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
</tr>
<tr>
<td>Control</td>
<td>352±128</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=6</td>
<td></td>
</tr>
</tbody>
</table>
Table 3

Changes in vitreal and Retinal leukotrienes B_{4} following Neodymium:YAG laser induced retinal injury.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Vitreal LTB_{4} pg/gr wt. (mean±SD)</th>
<th>Retinal LTB_{4} production pg/gr wet wt. (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28±14 n=5</td>
<td>390±104 n=5</td>
</tr>
<tr>
<td>3</td>
<td>79±31 n=6</td>
<td>307±99 n=6</td>
</tr>
<tr>
<td>7</td>
<td>24±15 n=4</td>
<td>575±228 n=6</td>
</tr>
<tr>
<td>14</td>
<td>35±13 n=4</td>
<td>174±29 n=5</td>
</tr>
</tbody>
</table>
Leukotriene B₄ in the Vitreous and its in vitro production by the Retina–Choroid

![Graph showing data on Leukotriene B₄ concentration over time.]

*Statistically significant difference from baseline.*
Figure 2.

Vitreal Leukotriene B4 concentrations following repeated Laser exposure

Vitreal Leukotriene B4 (percentage of baseline)

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Single Exposure</th>
<th>Repeated Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>3</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>7</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>14</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>
Fig. 3

Vitreal Leukotriene B\textsubscript{4} concentration
effect of NDGA

![Graph showing the concentration of vitreal leukotriene B\textsubscript{4} over time for NDGA treated and untreated samples.]

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