

# Prevention of Experimental Choroidal Neovascularization With Intravitreal Anti-Vascular Endothelial Growth Factor Antibody Fragment

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**Objective:** To evaluate the safety and efficacy of intravitreal injections of an antigen-binding fragment of a recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor (rhuFab VEGF) in a monkey model of choroidal neovascularization (CNV).

**Methods:** In phase 1 of the study, each animal received intravitreal injections, 500 µg per eye, of rhuFab VEGF in one eye (prevention eye), while the contralateral eye received rhuFab VEGF vehicle (control eye) at 2-week intervals. On day 21, laser photocoagulation was performed to induce CNV. In phase 2, the vehicle-treated eye was crossed over and both eyes received 500 µg of rhuFab VEGF beginning 21 days following laser-induced injury at days 42 and 56. The eyes were monitored by ophthalmic examinations, color photographs, and fluorescein angiography.

**Results:** rhuFab VEGF did not cause any ocular hemorrhages. All eyes treated with rhuFab VEGF developed

acute anterior chamber inflammation within 24 hours of the first injection that resolved within 1 week, and this inflammation was less severe with subsequent injections. The incidence of CNV, defined angiographically, was significantly lower in the prevention eyes than the control eyes ( $P < .001$ ). Subsequent treatments were associated with less leakage in eyes with established CNV that were crossed over from the control eyes to the treatment eyes ( $P = .001$ ).

**Conclusions:** Intravitreal rhuFab VEGF injections prevented formation of clinically significant CNV in cynomolgus monkeys and decreased leakage of already formed CNV with no significant toxic effects.

**Clinical Relevance:** This study provides the nonclinical proof of principle for ongoing clinical studies of intravitreally injected rhuFab VEGF in patients with neovascular age-related macular degeneration.

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**C**HOROIDAL neovascularization (CNV) is the major cause of severe visual loss in patients with age-related macular degeneration (AMD). New blood vessels grow from the choroid and penetrate through the Bruch membrane into the subretinal pigment epithelial and subretinal space. These vessels can leak and bleed, leading to exudative retinal detachment and hemorrhage. Eventually, this process progresses to a fibrovascular scar with destruction of photoreceptors and vision loss.

Until recently, the only beneficial treatment demonstrated in clinical trials was laser photocoagulation to obliterate the newly formed blood vessels.<sup>1</sup> However, this treatment causes full-thickness retinal damage and, in the case of subfoveal lesions, leads to immediate loss of central vision. Most CNV lesions manifest in a subfoveal location and are lesions that are frequently large,

ill defined, or occult and do not qualify for laser photocoagulation.

In an attempt to provide more selective and effective treatment for CNV in neovascular AMD, several innovative therapies have been studied, including photodynamic therapy (PDT) and agents that target angiogenesis. The goal of these treatments is to effectively treat CNV without damaging adjacent retinal tissue or by inhibiting growth factor-mediated neovascularization. In PDT, a photosensitizing drug is injected intravenously and activated by a nonthermal laser light in affected vessels in the retina. Using the appropriate drug dose, light dose, and timing of irradiation, relatively selective occlusion of CNV vessels can be achieved with minimal effect on the retina. Several photosensitizing drugs have been studied, including verteporfin,<sup>2,3</sup> lutetium texaphyrin,<sup>4</sup> and tin ethyl etiopurpurin (SnET2/Purlytin).<sup>5</sup> Verteporfin PDT (QLT

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## MATERIALS AND METHODS

### ANIMALS

Ten cynomolgus monkeys (*Macaca fascicularis*), obtained from Covance Biomedical Products Inc, Alice, Tex, were used in accordance with the guidelines of Association for Research in Vision and Ophthalmology on the use of animals in research and according to the guidelines of the Animal Care Committee at the Massachusetts Eye and Ear Infirmary.

Monkeys were anesthetized for all procedures with intramuscular injections of ketamine hydrochloride, 20 mg/kg; acepromazine maleate, 0.125 mg/kg; and atropine sulfate, 0.125 mg/kg. Supplemental anesthesia of 5 to 6 mg/kg of ketamine hydrochloride was administered as needed. In addition, 0.5% proparacaine hydrochloride was used for topical anesthesia. Supplemental anesthesia, with intravenous pentobarbital sodium solution (5 mg/kg), was administered before enucleation. Animals were euthanized with an intravenous pentobarbital sodium veterinary euthanasia solution (J.A. Webster Inc, Sterling, Mass) administered intravenously.

### ANTIANGIOGENIC DRUG INJECTIONS

rhuFab VEGF was provided by Genentech Inc, South San Francisco, Calif. rhuFab VEGF was preserved in a lyophilized powder form in a sterile vial and stored at 2°C to 8°C. The composition of the reconstituted rhuFab VEGF was 25 mg/mL of rhuFab VEGF in 10mM histidine, 2.5% trehalose, and 0.01% polysorbate 20 (pH 5.5). The lyophilized powder was reconstituted in the vial before each use with sterile water for injection and physiologic buffer to yield a concentration of 10 µg/µL, which was confirmed by UV absorption. The control eye was injected with a vehicle consisting of all components except the rhuFab VEGF protein.

### METHOD OF ADMINISTRATION

Intravitreal injections of 50 µL per eye with either rhuFab VEGF or vehicle were performed on both eyes through the pars plana using a 30-gauge needle and tuberculin syringe after instilling topical anesthesia and 5% povidone iodine solution. Before each dose administration, the vial stopper was wiped with 70% alcohol and allowed to air dry. The drug was withdrawn through a 5-µm filter, and a new (sharp) 30-gauge needle was used for intraocular injection. After the injection, bacitracin ophthalmic ointment was instilled in the fornices. The injection sites were varied to avoid trauma to the sclera. A 2-week interval was chosen based on previous toxicology studies.<sup>15</sup>

### FREQUENCY AND DOSING

In phase 1, the right or left eye of each animal was randomly assigned to receive intravitreal injections of rhuFab VEGF at a dose of 500 µg (50 µL per eye), and this eye was termed the prevention eye. The dose was based on previous toxicology studies.<sup>15</sup> The fellow eye was assigned to intravitreal injections of rhuFab vehicle and was termed the control eye. All eyes received 2 intravitreal injections before laser treatment with either rhuFab VEGF or vehicle alone on days 0 and 14. On day 21, all eyes underwent argon green laser photocoagulation to induce CNV lesions. On day 28, 1 week after laser, the prevention eye received another injection of drug and the control eye received vehicle. Phase 2 of the study began on day 42 or 3 weeks after laser induction, when CNV would be expected to have developed. Following fluorescein angiography on day 42, both eyes of each animal received intravitreal injections of rhuFab VEGF at a dose of 500 µg (50 µL per eye), and this was repeated on day 56 (**Table 1**).

### INDUCTION OF EXPERIMENTAL CNV

The CNV membranes were induced in the macula, an area between the temporal vascular arcades, of cynomolgus monkeys with argon green laser burns (Coherent Argon Dye Laser 920; Coherent Medical Laser, Palo Alto, Calif) using a slitlamp and plano fundus contact lens. Nine lesions were symmetrically placed in the macula of each eye by a masked surgeon (M.G.K. and M.A.A.). The laser variables included a 50- to 100-µm spot size, 0.1-second duration, and power ranging from 350 to 700 mW. The power used was assessed by the ability to produce a blister and a small hemorrhage. If no hemorrhage was noted, an additional laser spot was placed adjacent to the first spot following the same laser procedure. Color photographs and fluorescein angiography were used to detect and measure the extent and leakiness of the CNV.

### OCULAR EXAMINATIONS

The eyes of the animals were checked for relative pupillary afferent defect and then dilated with 2.5% phenylephrine hydrochloride and 0.8% tropicamide. Both eyes were examined using slitlamp biomicroscopy and indirect ophthalmoscopy on days 0, 14, 28, 42, and 56 (before drug injection); days 1, 15, 29, 43, and 57 (24 hours after injection); day 21 (before laser); days 35 and 49 (intermediate days); and day 63 (enucleation and death).

Continued on next page

Phototherapeutics, Inc, Vancouver, British Columbia), the only approved drug for PDT to date, has been shown to reduce the risk of moderate vision loss in patients with subfoveal CNV, particularly those with predominantly classic CNV<sup>6,7</sup>; however, the recurrence rate and the number of required treatments are high and not all subfoveal lesions benefit from treatment.

A different approach to the treatment of ocular neovascularization is antiangiogenic therapy. Angiogenesis refers to a process of new blood vessel formation that involves a complex interaction of different factors that can

be either stimulatory or inhibitory and includes growth factors, extracellular matrix elements, and intracellular or cellular adhesion molecules. Some of these factors have been shown to be associated with CNV. Antiangiogenic agents inhibit neovascularization either by promoting the action of endogenous inhibitors of angiogenesis or by blocking the effect of angiogenic stimulators.

One of the potential targets for antiangiogenic therapy is vascular endothelial growth factor (VEGF), which is a secreted polypeptide with mitogenic effects on vascular endothelial cells.<sup>8</sup> It has been shown to be present in surgi-

## COLOR PHOTOGRAPHY AND FLUORESCIN ANGIOGRAPHY

Fundus photography was performed on all animals on the same days as the ocular examination except for days 28 and 56. Photographs were obtained with a fundus camera (Canon Fundus CF-60Z; Canon USA Inc, Lake Success, NY) and 35-mm film.

The Imagenet Digital Angiography System (Topcon 501 A and Imagenet system; Topcon America Corp, Paramus, NJ) was used for fluorescein angiography. Red-free photographs of both eyes were obtained followed by fluorescein angiography using 0.1 mL/kg of body weight of 10% sodium fluorescein (Akorn Inc, Abita Springs, La) at a rate of 1 mL/s. Following the fluorescein injection, a rapid series of images were obtained in the first minute of the posterior pole of first the right eye and then the left eye. Additional pairs of images were obtained at approximately 1 to 2 and 5 minutes. Between 2 and 5 minutes, 2 images of the midperipheral fields (temporal and nasal) were taken of each eye. Fluorescein angiography was performed at baseline (day 0) and days 7, 14, 29, 42, 49, 57, and 63.

## ANALYSIS OF OPHTHALMIC DATA

Photographs and angiograms were evaluated for evidence of angiographic leakage, hemorrhages, or any other abnormalities. The fundus hemorrhages were graded based on a grading system with retinal hemorrhages that involved less than 3 disc areas defined as grade 1, hemorrhages between 3 and 6 disc areas defined as grade 2, and hemorrhages of more than 6 disc areas defined as grade 3. The association of hemorrhages with CNV membranes or laser induction site was also assessed. Clinically significant bleeding was defined as any fundus hemorrhage greater than or equal to a 6-disc area.

Ocular inflammation was assessed by slitlamp biomicroscopy. Anterior chamber and vitreal cells were counted with a 2-mm slitlight at a high magnification and graded using the schema of the American Academy of Ophthalmology (**Table 2**).

The CNV lesions were graded by reviewing fluorescein angiograms performed on days 35, 42, 49, 56, and 63 by 2 masked and experienced examiners (E.S.G. and J.W.M.) who graded by consensus opinion. The CNV lesions were graded according to the following scheme, using standardized angiographs for comparison. Grade 1 lesions had no hyperfluorescence. Grade 2 lesions exhibited hyperfluorescence without leakage. Grade 3 lesions showed hyperfluorescence in the early or midtransit images and late leakage. Grade 4 lesions showed bright hyperfluorescence in the transit and late leakage beyond the treated areas. Grade 4 lesions were defined as clinically significant.

Statistical analysis was performed using the Population-Aggregated Panel Data with Generalized Estimating Equations and the incidence rate ratio (IRR). The incidence rate was defined as the number of grade 4 lesions that occurred during a given interval divided by the total number of lesions induced. In phase 1, the IRR referred to the ratio of incidence rate of grade 4 lesions in the prevention eyes to the incidence rate in control eyes. An IRR of 1 would signify no difference between incidence rates. A number much smaller than 1 would indicate a reduction in the incidence of grade 4 lesions in the prevention group vs control group. In phase 2, we compared the incidence of grade 4 lesions in the control eyes vs the treatment eyes. This means that the incidence of grade 4 lesions was compared over time in the set of eyes that were first assigned to the control group but on days 42 and 56 were treated with rhuFab VEGF and became treatment eyes.

## SERUM PHARMACOKINETICS AND ANTIBODY ANALYSIS

Blood (approximately 2 mL) was collected from a lower-extremity vein before rhuFab VEGF injection and approximately 24 hours and 7 days after the injections. All samples were maintained at room temperature and allowed to clot, then chilled until centrifuged within 1 hour of blood collection. Serum was transferred to 1.5-mL conical tubes and stored at  $-60^{\circ}\text{C}$  to  $-80^{\circ}\text{C}$ .

Pharmacokinetics analysis of rhuFab VEGF was performed using the rhuFab VEGF antigen enzyme-linked immunosorbent assay method. Antibody analysis was performed using the anti-rhuFab VEGF antibody enzyme-linked immunosorbent assay method.

## HISTOPATHOLOGIC ANALYSIS

The globes were carefully removed from each animal, dissected clean of orbital tissue, rinsed in isotonic sodium chloride solution, and placed in modified Karnovsky fixative consisting of 2% glutaraldehyde and 2.5% formaldehyde in 0.1M cacodylate buffer 7.4 on ice. Within 10 minutes, the globes were opened and the anterior segment removed and the posterior pole placed in fixative overnight and then changed to buffer (0.1M cacodylate) until processed for light microscopy.

Each eye was prepared for light microscopy by sectioning into blocks, which contained lesions of interest. Tissues were postfixed in 2% osmium tetroxide in 0.1M cacodylate buffer for 2 hours at room temperature then dehydrated in a series of ethanols, infiltrated with propylene oxide and Epon, and embedded in Epon. Blocks were cut for 1- $\mu\text{m}$  sections and stained with 0.5% toluidine blue in borate buffer.

cally excised human CNV<sup>9,10</sup> and in the aqueous and vitreous humor of patients with retinal neovascular disorders, such as diabetic retinopathy and retinal vein occlusion.<sup>11</sup> Antibodies to VEGF have been shown to inhibit neovascularization in an experimental model of retinal ischemia and iris neovascularization in cynomolgus monkeys.<sup>12</sup>

We wished to study the effects of intravitreal injections of an antigen-binding fragment of a recombinant humanized monoclonal antibody directed toward VEGF (rhuFab VEGF) in a monkey model of laser-induced CNV.

rhuFab VEGF is the Fab portion (the antigen-binding portion) of anti-VEGF monoclonal antibody. It is a recombinant antibody that consists of 2 parts: a nonbinding human sequence, which makes it less antigenic in primates, and a high-affinity binding epitope derived from the mouse, which serves to bind the antigen.<sup>13</sup> Its molecular weight of 48 000 makes it a much smaller molecule than the full-length monoclonal antibody with a molecular weight of 148 000.<sup>13</sup> Unlike the full-length antibody, rhuFab VEGF has been shown to penetrate the internal limiting mem-

**Table 1. Experimental Design\***

Day	Prevention Eye	Control/Treatment Eye
0	rhuFab VEGF (500 µg per eye)	Vehicle (50 µL per eye)
7	rhuFab VEGF	Vehicle
21	Laser	Laser
28	rhuFab VEGF	Vehicle
42	rhuFab VEGF	rhuFab VEGF (500 µg per eye)
56	rhuFab VEGF	rhuFab VEGF
63	Enucleation	Enucleation

\*rhuFab VEGF indicates recombinant humanized monoclonal antibody fragment directed toward vascular endothelial growth factor.

**Table 2. Anterior Chamber Inflammation Grading System\***

Grade	Definition
0	No inflammatory cells
1+	Up to 10 inflammatory cells
2+	10-20 cells
3+	20-30 cells
4+	Too numerous to count

\*American Academy of Ophthalmology. *Basic Clinical Science Course*. American Academy of Ophthalmology; 1995;9:86.

brane and access the subretinal space in animal models when injected intravitreally.<sup>13,14</sup> Therefore, rhuFab VEGF potentially offers better retinal and choroidal distribution and a better therapy than its full-length antibody counterpart.

The purpose of this study was to assess the safety and efficacy of intravitreal injections of rhuFab VEGF in the laser-injury CNV model. This model uses argon green laser to induce CNV in the monkey macula and has been used in the past to study PDT. We have previously shown that there is a good correlation between fellow eyes in the number of CNV lesions with significant angiographic leakage (M.G.K., unpublished data, December 1999) and therefore designed a study using the contralateral eyes as controls. Safety and efficacy were evaluated in 2 phases of the study. Phase 1, the prevention phase, called for the initiation of rhuFab VEGF treatment before laser induction of the CNV and 1 week after laser to inhibit the formation of CNV, which typically appears by 2 to 3 weeks after laser injury. Phase 2, the treatment phase, began on day 42 or 3 weeks after laser when CNV lesions would be expected in the control eyes from phase 1. Therefore, in phase 2 the effect of rhuFab VEGF treatment on attenuating the extent and leakiness of existing CNV lesions was assessed.

## RESULTS

### SAFETY OF INTRAVITREAL rhuFab VEGF INJECTIONS

Clinical examination and review of fundus photographs did not show any hemorrhages before the laser photocoagulation, with the exception of one eye that developed a mild vitreous hemorrhage after the first intravitreal injection through the pars plana. This eye was hypotonous before intravitreal injection due to a paracentesis for aqueous hu-

**Table 3. Total Number of Fundus Hemorrhages for All Animals According to Grade**

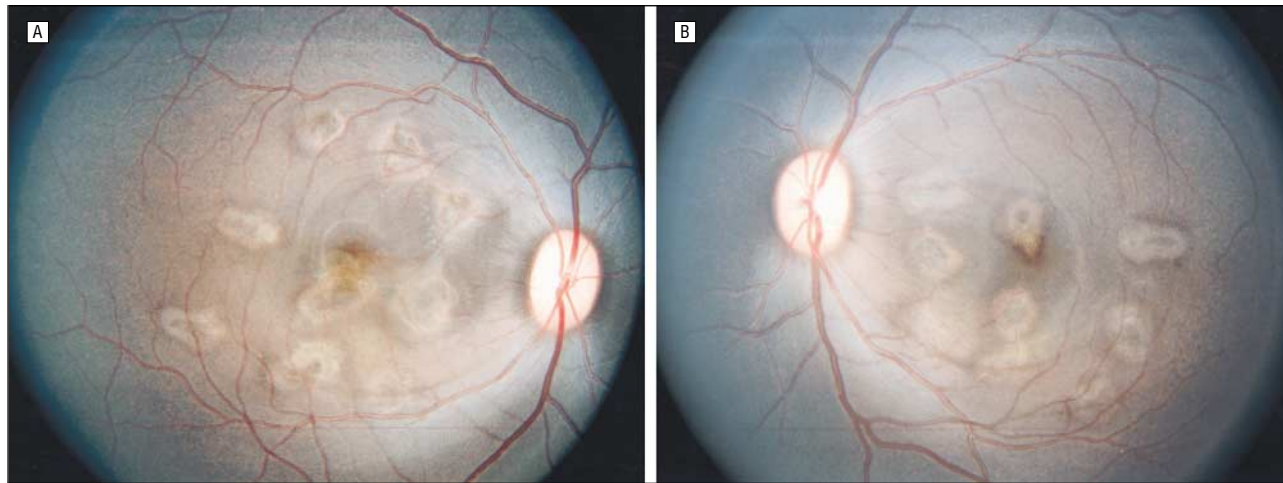
	Grade 1 (<3 Disc Areas)	Grade 2 (3-6 Disc Areas)	Grade 3 (>6 Disc Areas)
Prelaser days	0	0	1
Day 21 (laser)	5	1	0
Day 28	6	1	0
Day 35	7	0	0
Day 42	2	0	0
Days 56 and 63	0	0	0

**Table 4. Anterior Chamber (AC) Cellular Inflammatory Response After Recombinant Humanized Monoclonal Antibody Directed Toward Vascular Endothelial Growth Factor (rhuFab VEGF) and Vehicle Intravitreal Injections**

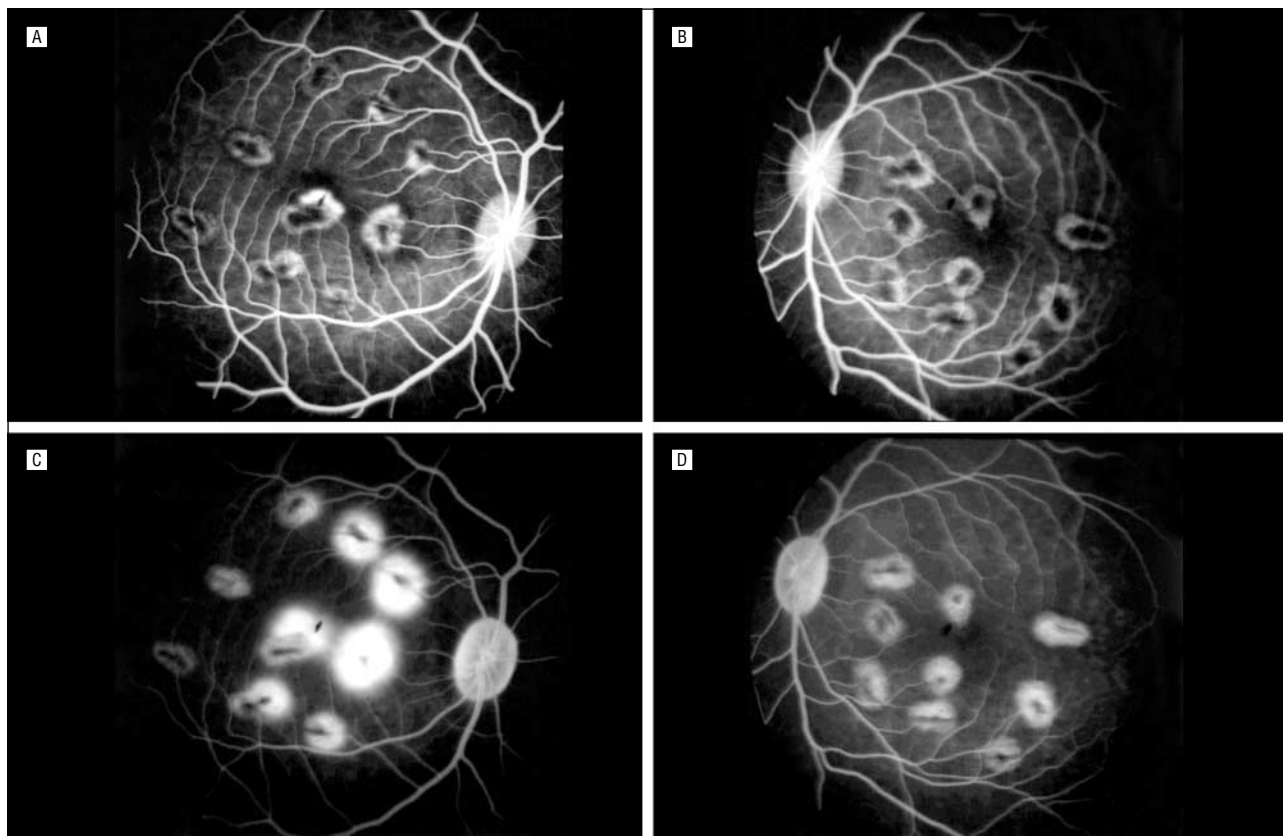
Day	Prevention Eye		Control/Treatment Eye	
	Injection	AC Cells	Injection	AC Cells
0	rhuFab VEGF		Vehicle	
1		1 to 4+		0 to 1+
7		0 to trace		0
14	rhuFab VEGF		Vehicle	
15		1 to 2+		0
21		0 to trace		0
28	rhuFab VEGF		Vehicle	
29		0 to 3+		0 to trace
42	rhuFab VEGF		rhuFab VEGF	
43		0 to 2+		3+ to 4+
49		0		0
56	rhuFab VEGF		rhuFab VEGF	
57		0 to 3+		0 to 3+
63		0		0

mor collection. The hemorrhage resolved within the next 2 weeks and did not recur with the future injections. Within 1 week of the laser, retinal hemorrhages were seen associated with laser injury sites as expected both in the rhuFab VEGF- and vehicle-injected eyes (**Table 3**). On day 28 (1 week after laser), there were 6 grade 1 hemorrhages observed in all animals. There was only 1 grade 2 hemorrhage noted at this time, and by day 35 (1 week later), this hemorrhage was less than 3 disc areas and became grade 1. All of these hemorrhages resolved within the next 4 weeks. No retinal or choroidal hemorrhages were noted associated with intravitreal rhuFab VEGF or vehicle injections in either prevention or treatment eyes.

All eyes treated with rhuFab VEGF developed acute anterior chamber cells within 24 hours of the first intravitreal injection. As given in **Table 4**, prevention eyes developed 1 to 4+ cells on day 1 after the drug injection. Inflammation resolved within 1 week (day 7). Subsequent injections produced less inflammation when eyes were examined 24 hours later (days 15, 29, 43, and 57). Eyes injected with vehicle showed minimal or no inflammation. However, on day 42 (in phase 2 of the study), control eyes were crossed over to receive rhuFab VEGF, and these eyes developed 3+ to 4+ anterior chamber cells within 24 hours. Following the second administration of rhuFab VEGF to these eyes (at day 56), inflammation



**Figure 1.** Posterior fundus color photographs demonstrating choroidal neovascular lesions 2 weeks after laser induction on day 35. A, Control eye (recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF] vehicle); B, prevention eye (rhuFab VEGF).



**Figure 2.** Fluorescein angiogram of the same animal as in Figure 1 on day 35 demonstrating laser-induced lesions. A, Early frame of the control eye (recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF] vehicle). B, Early frame of the prevention eye (rhuFab VEGF). C, Late frame of the control eye (rhuFab VEGF vehicle). D, Late frame of the prevention eye (rhuFab VEGF). Late frames of the fluorescein angiogram demonstrate presence of grade 4 lesions in the control eye but not in the prevention eye.

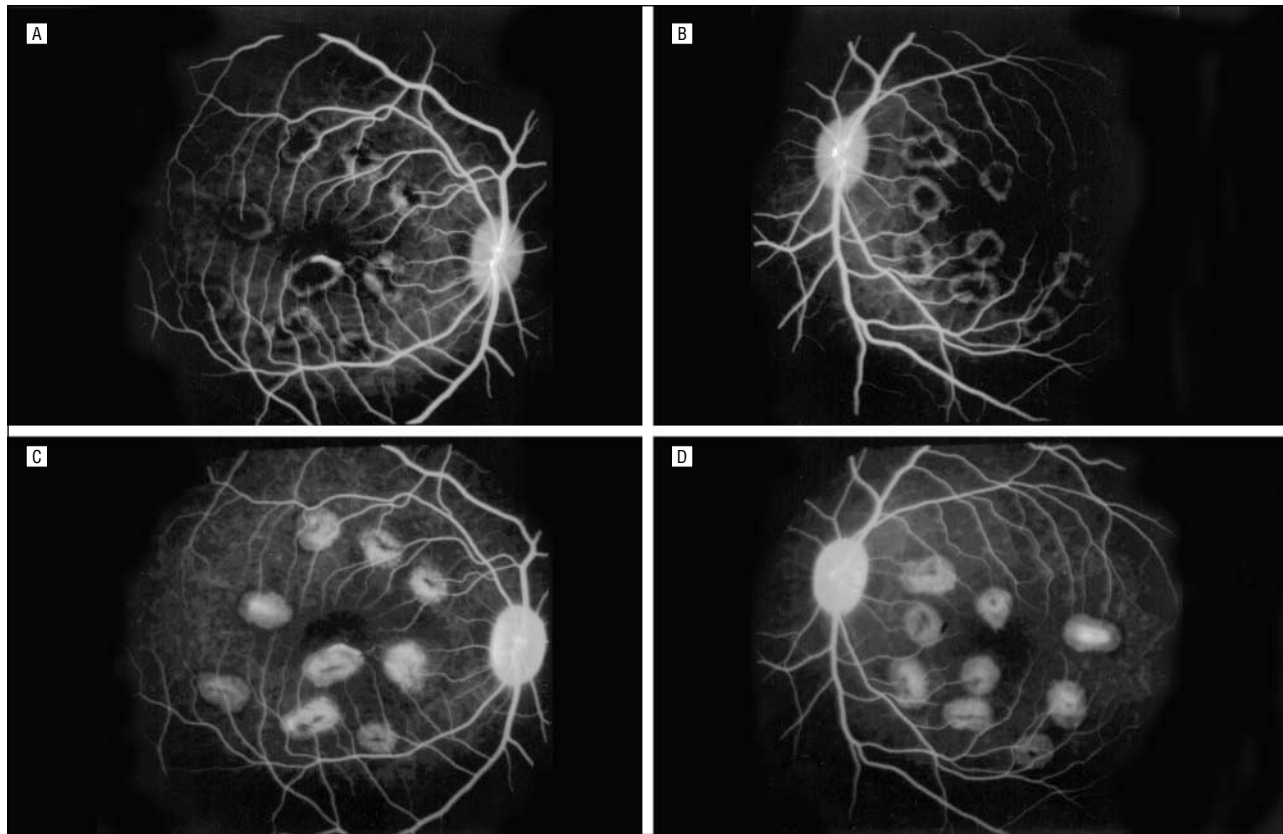
was less pronounced. No other clinical or angiographic abnormalities were observed.

#### EFFICACY OF INTRAVITREAL rhuFab VEGF INJECTIONS

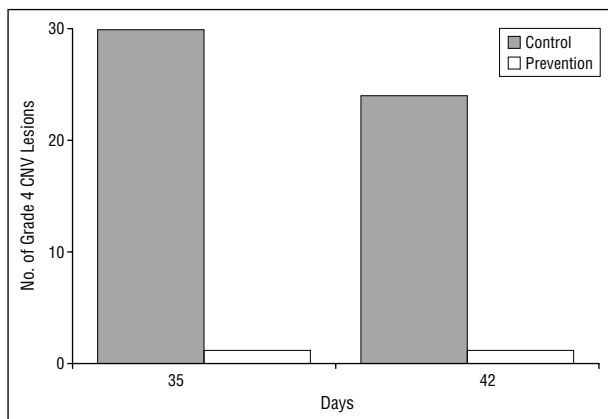
Fluorescein angiograms of both eyes in each animal were evaluated according to the grading system described in the “Materials and Methods” section for phase 1 and phase

2. Examples of the photographic and angiographic appearance of paired eyes in phase 1 and 2 are shown in **Figures 1, 2, and 3**.

In phase 1, analysis of the CNV lesions at days 35 and 42 (2 and 3 weeks after laser induction) showed a reduction in the likelihood of reaching grade 4 leakage ( $P < .001$ ) in the rhuFab VEGF prevention group compared with the vehicle control group (**Figure 4**). The IRR for the prevention group and the control group was



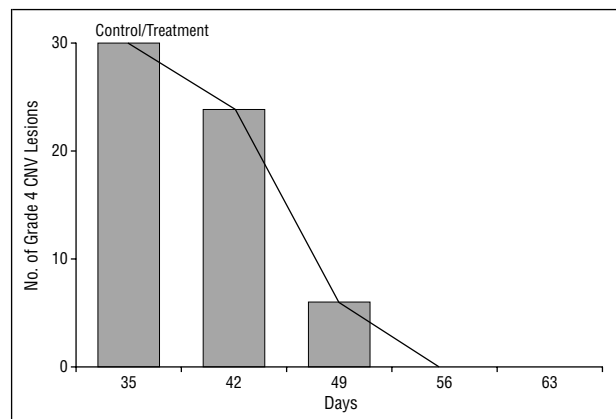
**Figure 3.** Fluorescein angiogram of the same animal as in Figure 7 at the time of enucleation (day 63). A, Early frame of the control eye (recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF] vehicle crossed over to rhuFab VEGF treatment). B, Early frame of the prevention eye (rhuFab VEGF). C, Late frame of the control eye (rhuFab VEGF vehicle crossed over to rhuFab VEGF treatment). D, Late frame of the prevention eye (rhuFab VEGF). Late frames of the fluorescein angiogram demonstrate decreased leakage in both eyes.



**Figure 4.** Phase 1. Total number of grade 4 choroidal neovascular (CNV) lesions in the prevention group (recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF]) (empty bar) vs control (rhuFab VEGF vehicle) (shaded bar) 2 weeks after laser induction (day 35) and 3 weeks after the laser induction (day 42).

0.041 (95% confidence interval, 0.009-0.176), indicating that intravitreal rhuFab VEGF injections prevented formation of clinically significant CNV. There was no laterality effect ( $P = .48$ ) between the eyes.

In phase 2, all eyes in the control group were injected with 500  $\mu\text{g}$  of rhuFab VEGF on days 42 and 56, and these eyes became the crossover or treatment group. The number of grade 4 lesions in the control/treatment group is presented in **Figure 5**. Using the

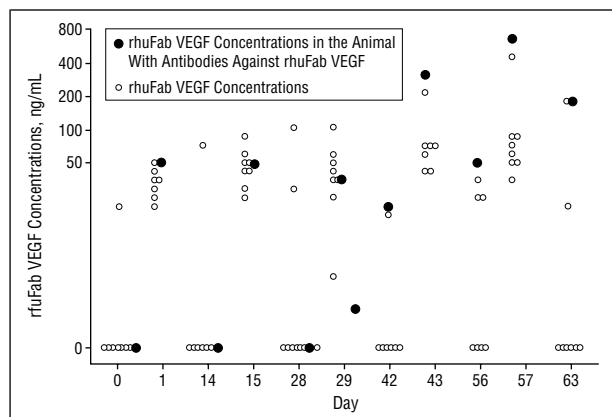


**Figure 5.** Phase 2. Total number of grade 4 lesions in the control (recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF] vehicle)/treatment group (rhuFab VEGF) over time. The control eye was injected with rhuFab VEGF vehicle until day 42. After fluorescein angiogram was performed on day 42, the eyes in this group were injected with rhuFab VEGF. The bar graph demonstrates the number of grade 4 lesions over time before and after rhuFab VEGF injection.

population-aggregated panel data model, the chance of being classified as grade 4 on days 49, 56, and 63 (treatment eyes) compared with days 35 and 42 (control eyes) was assessed. The rate of grade 4 lesion occurrence was reduced in the treatment group and was statistically significant ( $P = .001$ ; IRR=0.074; 95% confidence interval, 0.032-0.174), indicating decreased

**Table 5. Summary of Histopathologic Data on Selected Choroidal Neovascularization Laser-Induced Lesions**

Lesion No. (Animal No.)	Eye Group	Fluorescein Angiogram Grade		Width, µm	Height, µm	No. of Capillaries	No. of Acini	No. of Macrophages	Retinal Pigment Epithelial Coverage	Subretinal Fluid
		Day 35	Day 63							
1 (4511)	Prevention	2	2	300	0	0	0	12	Unable to assess	No
2 (4511)	Control/treated	2	2	500	0	0	0	22	Unable to assess	No
3 (4511)	Control/treated	4	2	560	80	4	0	27	Yes	No



**Figure 6.** Recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF] serum levels detected over time. The box plot illustrates that the rhuFab VEGF levels increase 1 day after injection but, generally, decrease within a week. With the subsequent injections, the serum drug levels tend to increase further.

leakage of already formed CNV membranes with rhuFab VEGF treatment. There was no laterality effect ( $P = .15$ ) between eyes.

#### PHARMACOKINETICS AND ANTIBODY ANALYSIS

Anti-rhuFab VEGF assay performed on blood serum showed that 1 of 10 animals developed antibodies to rhuFab VEGF. The antibodies were first detected on day 42 and persisted until the day of death. The rhuFab VEGF antigen assay showed that the average detectable drug level in the vitreous was 32 ng/mL after the first injection and increased with subsequent treatments as shown in **Figure 6**. These levels continued to increase when both eyes were injected with rhuFab VEGF on days 42 and 56. Levels decreased within 7 days of treatment but increased further with subsequent injections, indicating accumulation.

#### HISTOPATHOLOGIC ANALYSIS

Histopathologic evaluation was performed on representative lesions, and the data are summarized in **Table 5**. The first lesion presented in Table 5 is from an eye in the prevention group and was graded as grade 2 on the fluorescein angiogram 2 weeks after the laser induction (day 35) and at the time of death on day 63. It consisted of a depression in the retina with fibroblasts and lacked the normal outer nuclear layer. There were some macrophages present in the choroid but no capillaries (**Figure 7A**). This lesion looked similar to lesion 2 (Table 5), which

was also assessed as grade 2 on the angiogram, but came from an eye in the control/treatment group. Lesion 3 is from a control eye in the prevention phase that developed a grade 4 lesion with profound angiographic leakage 3 weeks after laser induction but then decreased leakage to grade 2 after treatment with rhuFab VEGF. At the end of the study, this treated lesion was smaller than the typical untreated CNV lesions studied previously in the same model (M.G.K., unpublished data, December 1999). Within the lesion there were few capillaries and pigment-laden macrophages, with the occasional fibroblast, but the lesion was small and covered by retinal pigment epithelium (**Figure 7B**).

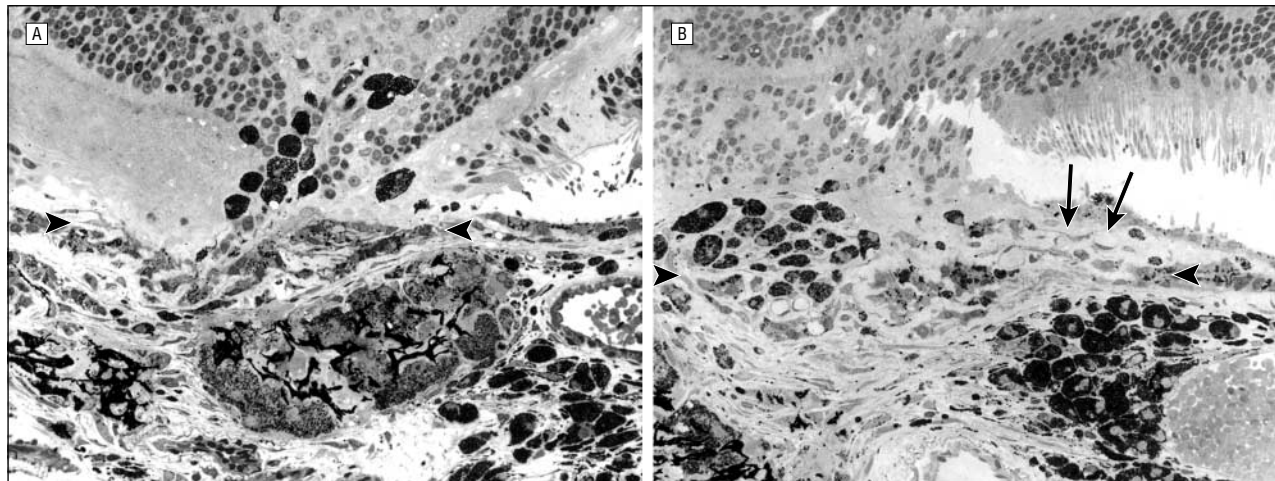
#### COMMENT

Intravitreal injections of 500 µg of rhuFab VEGF administered every 2 weeks in a laser-induced CNV model in 10 cynomolgus monkeys showed no significant toxic effects and prevented formation of clinically significant CNV. The results also suggested that rhuFab VEGF may have a beneficial effect in treating established CNV as seen in neovascular AMD.

In our study, transient anterior chamber inflammation that resolved without any sequelae and retinal hemorrhages associated with laser induction were observed as expected and reabsorbed over several weeks. A previous study of rhuFab VEGF injection in normal cynomolgus monkey eyes showed a similar safety profile regarding ocular inflammation.<sup>15</sup> In that study, intravitreal administration of rhuFab VEGF every 2 weeks into eyes of otherwise untreated monkeys showed transient inflammation at doses up to 2000 µg per eye, doses that were higher than the levels used in our study. Perivascular lesions that had been reported at the high doses in some animals were not observed in this study. This could also be related to the longer dosing interval of 13 weeks as opposed to 9 weeks in our experiment. Additionally, O'Neill et al<sup>15</sup> showed that intravitreal administration of rhuFab VEGF had no effect on electroretinographic variables, including visual evoked potential.

The phase 1 part of the study indicated that rhuFab VEGF prevented the formation of angiographically leaking CNV. Three of 10 animals did not develop grade 4 lesions in either eye. However, all the other 7 animals showed significantly fewer grade 4 lesions in the eyes receiving rhuFab VEGF than the eyes receiving vehicle.

The phase 2 part of the study suggests a treatment benefit for established CNV lesions. Three weeks after laser induction, eyes previously in the control group were



**Figure 7.** Photomicrographs of a 1- $\mu$ m section of a lesion from the eye in Figure 3. A, A lesion assessed as grade 2 on the fluorescein angiogram (prevention, rhuFab VEGF treatment). There are a few macrophages and proliferating retinal pigment epithelial cells forming a barrier at the level of the Bruch membrane (arrowhead) to which remnants of the outer nuclear layer are opposed. There are no capillaries. B, A lesion assessed as grade 4 at 2 weeks after laser induction (control recombinant humanized monoclonal antibody directed toward vascular endothelial growth factor [rhuFab VEGF] vehicle) that was then treated with rhuFab VEGF and evaluated as grade 2 at the time of death (treatment). There are capillaries (arrow) and macrophages covered by proliferating retinal pigment epithelial cells. The Bruch membrane is indicated by arrowhead. The subretinal space is an artifact of processing (toluidine blue stain, original magnification  $\times 20$ ).

crossed over to receive rhuFab VEGF, with both eyes receiving active rhuFab VEGF treatment. The number of grade 4 lesions that were counted after the rhuFab VEGF injection on day 42 was significantly lower than the number of grade 4 lesions before rhuFab VEGF injection. This suggests a significant treatment effect with rhuFab VEGF. However, previous studies have shown that the natural history of these lesions is to angiographic regression with loss of leakage as early as 2 or 3 weeks after the laser induction, with a mean regression period of 13 weeks.<sup>16</sup> Although our data suggest that rhuFab VEGF successfully attenuated the appearance of leaking CNV lesions, spontaneous regression of these lesions may have played a role.

Levels of rhuFab VEGF in the blood are highest on the first day following rhuFab VEGF injection, but then they decrease rapidly by the seventh postdose day. At day 43, the increase in serum rhuFab VEGF may be attributed to switching the previously control eye to active treatment. Only 1 of the 10 animals developed antibodies to the drug in the serum. In a previous toxicology study, 15 of 28 animals developed antibodies toward rhuFab VEGF; however, these animals were treated for a longer period (13 weeks) and were treated at doses up to and including 2000  $\mu$ g per eye at 2-week intervals.<sup>15</sup> It is not unexpected to elicit an antibody response following the administration of a heterologous protein. Additionally, since antibodies bind the rhuFab VEGF, it is not surprising that the animal with rhuFab VEGF antibodies exhibited elevated serum levels of the rhuFab VEGF more than other monkeys due to reduced clearance of the complex. It should be noted that these antibodies were nonneutralizing and as expected were generated toward the humanized backbone of the rhuFab VEGF and not the binding epitope.

In this study, all of the representative histopathologically examined lesions were relatively smaller than lesions studied previously in this CNV model (M.G.K., unpublished data, December 1999). This finding sug-

gests that rhuFab VEGF inhibited growth of CNV lesions and also led to regression of established CNV lesions. However, this finding is confounded by the relatively long period of follow-up (6 weeks after laser induction) coupled with natural history of laser-induced CNV lesion regression in this model.

This study showed that intravitreal injections of rhuFab VEGF in the cynomolgus monkeys were safe and prevented formation of clinically significant CNV. Also, rhuFab VEGF may have a beneficial effect in treating established CNV. In view of these positive results in the animal model, rhuFab VEGF may be a promising agent in treating human CNV lesions associated with neovascular AMD. Clinical trials of intravitreal injections with rhuFab VEGF in patients with neovascular AMD are currently ongoing.

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### Ophthalmological Numismatics

Allvar Gullstrand, 1862-1930, was professor of ophthalmology at Uppsala, Sweden, and inventor of the slitlamp microscope. For his contributions to the physiology and function of the human eye, he received the Nobel Prize for Medicine in 1911. This silver medal was struck by Erik Lindberg in 1935 for the Royal Swedish Academy of Science. The obverse (**Figure 1**) depicts Gullstrand's bust facing right. It is surrounded by an inscription. The reverse (**Figure 2**) is a cross in the sky surrounded by clouds. *Arcana Oculorum Videntibus Oculis Perspexit* is the inscription on the reverse. This translates to, "He was the secrets of the eye with his vision."

Courtesy of: Jay M. Galst, MD, 30 E 60th St, New York, NY 10022.



Figure 1.



Figure 2.