inhibits the upregulation of adhesion molecules necessary for the movement of either T cells or monocytes, during the treatment of acute allograft rejection there is a disproportionate reduction in macrophage-monocyte infiltrate while quantitatively a normal number of T cells are recovered from the organ. Because MM therapy inhibits the macrophage response during and after therapy and only reduces T-cell infiltration during therapy, yet there is still target organ damage, then either only a few macrophages are required or T cells also can act as effector cells. With MM therapy, macrophage infiltrate is of a nonactivated phenotype expressing levels of CD4 that are 6 to 8 times less than those recovered from control animals (Fig. 3; for cross-references see ref. 6) and have a lower expression of CD45. Therefore, although macrophage–monocyte populations are thought to play a role in the development of organ-specific autoimmune disease, 11 their elimination or inactivation during MM therapy for EAU indicates that T cells may be equally effective as tissue-damaging effector cells. Although these data are representative of comparable experiments, the interpretation of exact numbers must be taken with some caution because of the low numbers tested, which precluded statistical analysis. However, the reproducibility supports the common trend of impaired macrophage infiltration before and after therapy and reduced lymphocyte infiltration during therapy. Because short-term MM therapy does not inhibit cytotoxicity or IL-2 production, the possible mechanisms of tissue destruction include activated T-cell cytotoxicity, cytokine (namely, tumor necrosis factor-α) cytotoxicity, or natural killer cell cytotoxicity. These data have supported other studies that highlight an effector role for T cells in not only the initiation of EAU but also in target organ destruction. 6

References


Abnormal Panretinal Response Pattern to Carbogen Inhalation in Experimental Retinopathy of Prematurity

Bruce A. Berkowitz 1 and John S. Penn 2

Purpose. Present technologies are not able to determine which retinas are at risk for the development of neovascularization in retinopathy of prematurity (ROP). In this study, the authors evaluated whether a novel magnetic resonance imaging (MRI) method could be used to identify differences between control retinas and those that will develop neovascularization in the newborn rat model of retinopathy of prematurity (ROP).

Methods. MRI and a 2-minute carbogen (95% O2/5% CO2) inhalation challenge (see ref. 11) were used to measure noninvasively the change in the posterior vitreous oxygen tension in specific locations across the full extent of the retina in day-12 rats raised in either room air (control, n = 7) or variable oxygen conditions (experimental ROP, n = 7). The experimental ROP animals were examined 2 days before the onset of neovascularization.

Results. In the ROP group, the response to carbogen was lower (P < 0.05) at every distance from the optic nerve than in the control group. Within the ROP group, the vascular midperipheral retinal reaction to carbogen, 1 to 2 mm from the optic nerve, was as low as that from the avascular periphery, 2 to 3 mm from the optic nerve. Although the vascular central retinal response to carbogen, 0 to 1 mm from the optic nerve, was greater than either the vascular midperipheral retina or the avascular periphery in the ROP group, theoretically this difference could be caused by oxygen diffusing from the hyaloidal circulation.

Conclusions. Carbogen-challenge MRI seems to be a useful tool for assessing the risk of retinal neovascularization in the newborn rat ROP model. This MRI method has potential clinical applicability, for example, because effective laser therapy with retinal sparing may be possible if focal photocoagulation, guided by an MRI map, is performed. (Invest Ophthalmol Vis Sci. 1998;39:840–845)
Blindness and vision loss in retinopathy of prematurity (ROP) is associated with an abnormal growth of retinal blood vessels. The pathogenesis of this retinal neovascularization remains unclear, although it is thought to be a result of retinal hypoxia. It is commonly assumed that in the neonate breathing room air, the vascular retina is normally perfused and, thus, better oxygenated than the avascular peripheral retina. The discovery of an hypoxia-inducible vascular endothelial growth factor (VEGF) with strong angiogenic properties and localized upregulation, in some studies, to the avascular retina in experimental models of ROP has provided additional support for a role of the presumed hypoxic avascular retina in proliferative retinopathies. These concepts form the basis of present clinical interventions that attempt to alleviate the assumed hypoxia by destroying the metabolically active avascular peripheral retina using cryotherapy or laser photocoagulation in infants with advanced ROP.

Unfortunately, such aggressive approaches have not been entirely successful. For example, limited clinical improvements in outcome (structural, 26% of eyes; and functional, 50% of eyes) were found at 42 months after cryotherapy. In addition, VEGF upregulation is not always associated with angiogenesis or exclusively avascular retina and can be stimulated in vitro by hypoxic physiological stimuli, such as reactive oxygen intermediates and vasoactive peptides (endothelin-1 and endothelin-3). These findings provide a compelling reason for additional studies of vascular and avascular retinal pathophysiology in an animal model of ROP under controlled laboratory conditions. Further, present technologies are unable to determine which retinas are at risk for the development of neovascularization in ROP.

Recently, we developed a magnetic resonance imaging (MRI) method that noninvasively measures a retinal physiological response to an inhalation challenge (namely, 100% oxygen or carbogen [95% O2/5% CO2]). This approach detects the resultant increase in the vitreous partial oxygen pressure over the room air value (∆P0₂) as an increase in the MRI signal intensity. Good agreement was found between the MRI-determined vitreous ∆P0₂ and that determined using an oxygen electrode under similar conditions.

The change in MRI signal intensity in the posterior vitreous (within 200 μm from the retina) is presumed to reflect the inner retinal change in oxygen tension during the inhalation challenge. If the inner retina is hypoxic because of an inadequate supply of oxygen (that is, if it is poorly perfused), the change in oxygen tension during the challenge is unlikely to be normal. Although an abnormal retinal response to an inhalation challenge could not be unambiguously interpreted as a measure of hypoxia at present, it may have practical utility for assessing the risk of retinal neovascularization. For example, instead of performing panretinal photocoagulation to treat neovascularization, effective laser therapy with retinal sparing may be achieved if a focal laser treatment, guided by an MRI oxygenation rate map, is performed.

In this study, carbogen (95% O₂/5% CO₂) breathing is used because it produces a larger MRI response relative to 100% oxygen inhalation in the newborn rat. Carbogen-enhanced MRI was applied to measure the panretinal response pattern before the growth of new preretinal blood vessels in the newborn rat model of ROP. For the present purposes, the important features of this model are that first it displays a large avascular region in the retinal periphery, and then retinal angiogenesis at the border of the vascular and avascular areas in 100% of eyes. The smallness of the newborn rat eye and the presence of a patent hyaloidal circulation limits the use of oxygen electrodes in this model.

METHODS

The animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The newborn rat model of ROP has been described in detail elsewhere. Briefly, Sprague-Dawley mothers and litters were housed in a modified pediatric incubator in which the oxygen levels were varied every 24 hours in a stepwise fashion between 50% and 10% oxygen for the first 12 days after birth. This protocol produces retinal angiogenesis during the next 6 days in room air in 100% of the eyes.

A second group of mothers and age-matched litters was maintained in room air. On the day of the experiment animals were removed from the incubators and were flown from Arkansas to Texas. MRI experiments began approximately 5 to 6 hours after the removal of the animals from the incubators and were performed as previously described. The last animal examined was studied approximately 14 hours after its removal from the incubators. This is approximately 1.5 days before histologic evidence of neovascularization. Although some associated retinal hemodynamic changes in advance of angiogenesis may have been produced during this time, the similar scatter for controls and ROP groups (see Fig. 3) suggests that this was not a confounding factor for these experiments.

Anesthesia was induced by a single intraperitoneal injection of urethane (1.5 g/kg, 36% solution, freshly made daily). Each rat was gently positioned on an MRI-compatible home-made holder with its nose placed in a plastic nose cone. Animals were allowed to breathe spontaneously during the experiment. To maintain the core temperature of the rat, a recirculating heated water blanket was used. Rectal temperature, pulse, and hemoglobin oxygen saturation (data not shown) were continuously monitored, as previously described, while the animal was inside the magnet.

MRI data were acquired on a 4.7 T system using a two-turn transmit/receive surface coil (1.5-cm diameter) placed over the eye and an adiabatic spin-echo imaging sequence (repetition...
time, 1 second; echo time, 22.7 msec; number of acquisitions, 1; matrix size, 128 × 256; slice thickness, 1 mm; field of view 28 × 28 mm; sweep width, 25,000 Hz, 2 minutes/image). A capillary tube (1.5 mm inner diameter) filled with distilled water was used as the external standard. Seven sequential 2-minute images were acquired as follows: six control images while the animal breathed room air; one image during carbogen breathing. Carbogen exposure was started at the end of the sixth image. Animals were killed at the end of the experiment by potassium chloride cardiac injection.

The MRI data were transferred to a computer (Macintosh IICi; Apple, Cupertino, CA). Image registration was performed using software written in-house for the program IMAGE (a freeware program available at http://rsb.info.nih.gov/nih-image). After registration, the six images of the animal breathing room air were averaged to improve the signal-to-noise ratio. All pixel signal intensities in the average room air image and the 2-minute carbogen image then were normalized to the external standard intensity. On a pixel-by-pixel basis, signal intensity changes during carbogen breathing were calculated, converted to ΔPO2 values, and displayed as a pseudocolor parameter map, as previously described. For anatomic reference, the retina/choroid and lens were blacked out (Fig. 1). Data along a 1-pixel thick line (200 μm) drawn in the preretinal vitreous space were extracted and displayed as a preretinal vitreous ΔPO2 band (Fig. 1). A direct comparison of retinal vascular architecture with the retinal response to carbogen was achieved by digitally overlaying this pseudocolor ΔPO2 band averaged over the animals in the ROP group (n = 7 from two different litters) or the control group (n = 7 from two different litters) onto a representative ADPase stained retinal flat mount (Fig. 1). A plot of the retinal response during 2 minutes of carbogen breathing versus the distance from the optic nerve then was constructed from these mean ΔPO2 bands by taking the data in 1.0-mm distance bins from the optic nerve to the ora serrata averaged over the inferior and superior retinas. Comparisons between treatment groups were performed using a two-tailed Student’s t-test at each distance from the optic nerve and using a one-way analysis of variance.

Separate non-MRI experiments were performed to measure blood gas values during room air and carbogen breathing (Table 1). For these experiments, control and 12-day-old ROP newborn rats were transported from Arkansas to Texas, as described above. Approximately 1 hour after urethane injection, during either room air (with and without a nose cone) or carbogen breathing (2 minutes), arterial blood was collected in a heparinized capillary tube from a cut in the exposed descending aorta. Blood gas analysis (AVL995 blood gas analyzer; AVL Scientific, Roswell, GA) of these samples is summarized in Table 1. In all cases, the core temperature of the rat was maintained.

### RESULTS

Figure 2 illustrates the average response band for each group (ROP and untreated control animals, n = 7 animals/group) overlaid on representative ADPase-stained retinal flat mounts. In the control group, a decreasing ΔPO2 gradient (high near the optic nerve, low toward the periphery) was apparent inferiorly and superiorly from the optic nerve. In the ROP group, the response to carbogen did not correlate with the vascular pattern because low values were apparent in the vascularized midperipheral retina (1-2 mm from the optic nerve or approximately 1 mm central to the peripheral extent of the vessels [2.03 ± 0.21 mm, mean ± SD, n = 139 measurements from four representative flat mounts]). In addition, the avascular peripheral retina (2-3 mm from the optic nerve) of the ROP group also demonstrated a low response compared with the vascularized area 2 to 3 mm from the optic nerve of the age-matched control animals.

Figure 3 is a plot of the retinal response to carbogen versus the distance from the optic nerve. Within the ROP group, there was no significant difference (P > 0.05) in ΔPO2 between the vascular midperipheral retina and the avascular peripheral retina. The ROP animals also demonstrated lower (P < 0.02) retinal response values than the control animals at each distance from the optic nerve. Overall, the panretinal response to carbogen breathing was lower for the ROP animals than for the untreated animals (one-way analysis of variance, P < 0.05). In the control group, there was no significant difference (P > 0.05) in ΔPO2 between the midperipheral and the peripheral retina. Both groups exhibited a greater (P < 0.05) ΔPO2 near the optic nerve than in the periphery.

### DISCUSSION

**Systemic Versus Local Response to Carbogen**

In this study, the panretinal response to carbogen breathing was lower for the ROP animals than for the controls. It is possible that this was a result of systemic physiological differences in the response to carbogen between the two groups of rats rather than a result of local ocular effects. To address this concern, various physiological parameters were measured and compared for each group. A significant (P < 0.05) difference in weight was found between groups (con-
FIGURE 1. Summary of the image analysis used to compare the panretinal response pattern during the carbogen challenge with the vasculature architecture. For anatomic reference, the retina/choroid complex and lens are blacked out in the parameter map. Data in a one-pixel thick region-of-interest, representing the preretinal vitreous space (purple line), were extracted to obtain a ΔPO₂ band. Comparison of the panretinal response with the vascular architecture was achieved by overlying the ΔPO₂ band on a representative ADPase-stained flat mount preparation.

Controls, 21.0 ± 0.4 g [mean ± SEM, n = 9]; ROP, 11.0 ± 0.3 g [n = 8]). However, the vitreous and ocular volumes also are smaller in the ROP group, and so the weight difference is not expected to account for the different retinal responses between groups. A significant (P < 0.05) difference in rectal temperatures was found between groups (controls, 37.2 ± 0.1°C [n = 9]; ROP, 36.8 ± 0.2°C [n = 8]). However, both values were within normal limits, and so this difference was not considered to play an important role in these findings. During the 2-minute carbogen breathing, Fao₂ and Paco₂ values for each treatment group were similar (Table 1). A significant relative acidosis was found in the ROP group. Acidosis could introduce some error into the interpretation of the MRI measurement. A change in blood pH per se is not expected to alter the visomotor reaction from retinal vessels or to affect the regulation of vascular

FIGURE 2. Visual summary of response results. The average ΔPO₂ bands from the control (left panel) and ROP groups (right panel) (n = 7 bands/group) were overlaid on representative flat mounts. The same color scale was used for both groups. Note that the panretinal response of the ROP animals was lower than that at each corresponding location of the control animals.
with these MRI data. Using a kitten ROP model, Ernest and Gold-biochemistry and structure of the newborn rat and guinea pig retinas. Such a comparison should be made cautiously given the different (APo₂, approximately 55 mm Hg). This difference may be caused by species variations or, perhaps, by a differential degree of photoreceptor development between the normally avascular guinea pig retina and the transiently avascular newborn rat retina. The avascular outer retina of the newborn may contain an immature photoreceptor layer of rats seems to be a local phenomenon.

Avascular Peripheral (2–3 mm from the Optic Nerve) Response to Carbogen

The avascular periphery of the ROP animals had a poor response to carbogen, relative to the vascularized retina region 2 to 3 mm from the optic nerve in the control group. To the best of our knowledge, there are no other reports that can be used for a direct comparison with these MRI data. Using a kitten ROP model, Ernest and Goldstick measured a lower oxygen level in the avascular retina (Po₂ = 1.4 mm Hg) than in the optic nerve (Po₂ = 15.6 mm Hg). In addition, they found an apparent abnormal retinal responsiveness after brief, repeated pure oxygen inhalation challenges. We also compared these MRI data in the newborn rat to the oxygen electrode data of Yu et al. obtained in the normally avascular guinea pig retina, although such a comparison should be made cautiously given the different biochemistry and structure of the newborn rat and guinea pig retinas. In the guinea pig, the inner retina had a Po₂ of 0.4 mm Hg during room air breathing and a ΔPo₂ ~ 30 mm Hg after 5 minutes of carbogen breathing. Our measurement was somewhat higher (ΔPo₂, approximately 55 mm Hg). This difference may be caused by chronic reduction in retinal perfusion, and thus in oxygen delivery. A third possibility is that the starting midperipheral preretinal Po₂ in the ROP animals was higher than that of the avascular retina and normal midperipheral retina, so that even if the same Po₂ were reached during carbogen breathing, the ΔPo₂ would be smaller. Although this last possibility cannot be entirely ruled out, it is difficult to imagine why the Po₂ overlying the vascular midperipheral retina in the ROP animals while they are breathing room air should be larger than control animals, and why, if it were larger, it would not oxygenate to a larger degree than other retinal regions. Experiments are planned to examine these three possibilities in detail.

Vascular Midperipheral Retina (1–2 mm from the Optic Nerve) Response to Carbogen

Somewhat surprisingly, the ΔPo₂ of the clinically patent vascular midperipheral retina of the ROP animals was as low as that of the avascular periphery; this response to carbogen was also lower than that for the normal vascular midperipheral retina. Identifying the mechanism(s) of the midperipheral retinal response anomaly is beyond the scope of this work. However, speculation on possible explanations for this phenomenon is warranted. First, it may be that the blood vessels in the midperiphery of the ROP animals respond better to oxygen and/or to carbon dioxide, compared with the midperipheral retinal region of control animals. So a smaller change in oxygen level would be produced during carbogen breathing. Indeed, the autoregulatory range of the immature retinal vessels of the newborn is narrower than in the adult, and it is easily damaged by magnesium and copper deficiencies, acute hypoxemia, and oxidative stresses. A second possibility, which is not mutually exclusive with the first, is that there is poor retinal vessel perfusion, so that little extra oxygen is delivered during carbogen breathing. It is known that, compared with aged-matched controls, the retinas of the experimental animals, at 2 weeks after variable oxygen exposure, have a lower retinal microvessel density and no deep capillary bed. These factors would combine to produce a chronic reduction in retinal perfusion, and thus in oxygen delivery. A third possibility is that the starting midperipheral preretinal Po₂ in the ROP animals was higher than that of the avascular retina and normal midperipheral retina, so that even if the same Po₂ were reached during carbogen breathing, the ΔPo₂ would be smaller. Although this last possibility cannot be entirely ruled out, it is difficult to imagine why the Po₂ overlying the vascular midperipheral retina in the ROP animals while they are breathing room air should be larger than control animals, and why, if it were larger, it would not oxygenate to a larger degree than other retinal regions. Experiments are planned to examine these three possibilities in detail.

Vascular Central Retina (0–1 mm from the Optic Nerve) Response to Carbogen

This study also found a lower response of the vascularized central (within 1 mm from the optic nerve) retina during carbogen breathing in the experimental animals relative to that region in control retinas. However, the interpretation of the results from this region is somewhat ambiguous because, even in the first 2 minutes of carbogen breathing, the central retina may be oxygenated not only from the retinal and choroidal circulations but also by oxygen diffusing through the vitreous from the hyaloidal circulation. At this age, the hyaloidal circulation in the newborn rat passes through the vitreous from the optic nerve to the lens; we did not find any remnants of this circulation on the retinal surface in this study. The hyaloidal influence can be appreciated by a simple calculation: oxygen will diffuse approximately 477 μm, or
The Retinal Response to Carbogen and Angiogenesis

In this study, low vascular midperipheral retinal and avascular periphery responses to carbogen breathing were found before the development of angiogenesis at the border of these two regions. We speculate that this apparent association is important. Support for this conjecture is provided by recent VEGF data obtained by Robbins et al. in the same newborn rat model of ROP. Before angiogenesis, they found an increased VEGF protein concentration in the vascular midperipheral retina (and in the avascular periphery) relative to those same locations in retinas of age-matched controls. That is, in this model of ROP, the VEGF pattern does not correlate with the vascular pattern but seems associated with the response pattern found in the present study. These observations are sufficiently intriguing to warrant further research into a relationship among the MRI ΔPO2, the VEGF upregulation, and the angiogenic outcome in the newborn rat model of ROP.

In summary, MRI seems to be a powerful noninvasive approach for assessing the panretinal response pattern to a carbogen challenge in the newborn rat. Compared with similar locations in the retina in control animals, this approach demonstrated a small avascular peripheral response and an abnormally low vascular midperipheral retinal reaction in a clinically relevant ROP model. These responses were observed before angiogenesis; experiments are planned to further delineate the relationship among the MRI ΔPO2 measurement, the VEGF upregulation, and angiogenesis in this model.

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