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Oxygen-induced retinopathy in the rat: hemorrhages and dysplasias may lead to retinal detachment

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ABSTRACT

Rearing neonatal rats in hyperoxia induces the development of retinal hemorrhages and retinal dysplasia. Albino rats were placed in 80% oxygen immediately after birth and were exposed for either 5, 10, or 14 days, followed by sacrifice or exposure to normoxia for an additional 2, 4, 5, 7, 8, 10, 38, 45 or 56 days. Control rats were simultaneously raised in room air and sacrificed at the same times. All animals were enucleated and their eyes processed for light and electron microscopy. Eyecups were trimmed to facilitate cross-sectioning of the retina in the vertical meridian. No control rats showed signs of retinal hemorrhages or of dysplastic folds or rosettes. Nor did the retinas of rats killed immediately after oxygen exposure contain hemorrhages, but the incidence of retinal folds or rosettes in this group was 54%. For rats exposed to combinations of hyperoxia and brief normoxia (10 days or less), 40% suffered hemorrhages and 50% developed retinal folds or rosettes. Although hemorrhages were more prominent in rats subjected to longer periods of oxygen (73% of all rats exposed for 14 days followed by brief normoxia vs. 6% of those exposed for 5 days followed by brief normoxia), the incidence decreased with time post-exposure in room air. Hemorrhages occurred in 100% of the rats raised in oxygen for 14 days followed by 2 days in room air, and decreased to 50% by 7 days in room air and to 0% by 38 days, indicating a spontaneous resolution with time. In each case, the blood appeared to leak from the newly-forming vessels of the deep capillary net, with most of the red blood cells migrating to the subretinal space. Retinal fold or rosette formation, indicative of developmental dysplasia, occurred in a fraction of virtually all groups of exposed rats, and persisted at the longest post-exposure periods. These two manifestations of oxygen-induced retinopathy are emphasized because they lead to an abnormal separation of the retina from the epithelial layer, which may increase the likelihood of the most serious consequence of ROP — retinal detachment. In fact, all rats that endured post-exposure periods of 38 days or longer before sacrifice exhibited retinal detachment.

INTRODUCTION

A major drawback of animal models of retinopathy of prematurity (ROP) is their failure to progress to retinal detachment. In the rat, this may be due in part to the relatively large, spherical lens, which occupies nearly half of the volume of the eye (1). For the greater part, though, it is assumed that the failure of newborn animals raised in oxygen to develop retinal detachments is due to their relative inability to produce extensive pre-retinal neovascularization. However, aside from the end-stage retinal detachment that can occur in infants, animal models have shown other retinal manifestations of oxygen exposure that closely mimic ROP. Areas of vascular non-perfusion, retinal hemorrhages, and mild neovascularization have, at one time or another, been described in newborn kittens (2,3), puppies (4), mice (5), and rats (6, 7) when exposed to various periods of oxygen and room air.

We have succeeded in inducing retinal detachment in oxygen-reared newborn rats following an extended post-exposure period in room air. It is our conclusion that these detachments were not the result of traction subsequent to pre-retinal neovascularization. Rather, they were the end result of exudative detachments caused by retinal hemorrhages that occurred after rats were returned to room air, in combination with oxygen-induced dysplastic growth of the outer retinal layers.

We sought to study these two phenomena and their association with retinal detachments by characterizing their temporal sequence during and after oxygen exposure. In our initial attempts to determine the presence of retinal hemorrhages, we employed ink-

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perfusion of the retinal vessels, followed by dissection and flat-mounting of the retina. This method of vascular assessment has been the standard over decades of ROP research, and it is a primary means by which oxygen was first shown to play a causal role in inducing retinopathy. Three of the above referenced studies (2, 4, 6) have pointed to the leaking of ink from retinal vessels during perfusion as likely evidence for the prior presence of hemorrhages. However, this technique is highly susceptible to pressure-induced artifact. It is possible to reproduce the appearance of these retinal "hemorrhages" in room air-raised rats simply by increasing the perfusion pressure.

Since we have hypothesized that oxygen-induced hemorrhages may play a causal role in subsequent retinal detachment in the new born rat, it was of great importance to document the incidence of the hemorrhages, as well as a variety of additional characteristics that could not be addressed with ink-perfusion. Included in our assessment were attempts to determine the following: 1) whether or not oxygen-induced retinal hemorrhages arise at the level of the deep capillary bed, the superficial capillary bed or both; 2) where the exudate goes after the hemorrhage occurs, and how long the leaked blood remains in the retina; 3) the means by which the blood is removed from or leaves the retina; and 4) the specific cause of the leaks on a cellular level. All of these issues remain to be resolved for animal models of retinopathy of prematurity. Yet, understanding these simple facets of retinal bleeding is not only critical to establishing a model, but very few of these issues have been addressed in infants, so new information about the human disease may be forthcoming as well.

To determine the definite presence of oxygen-induced retinal hemorrhages in rats and to understand their characteristics, we have resorted to cross-sectional retinal histology. We report here the presence of retinal hemorrhages in rats raised in elevated oxygen for three separate exposure durations, each followed by various periods in room air. The hemorrhages appear to arise from vessels within the deep capillary net which lines the inner nuclear layer on its proximal and distal surfaces, and from which red blood cells migrate rapidly to the subretinal space.

In addition, we report the occurrence of retinal folds or "rosettes" which appear in a large number of our oxygen-treated rats. The appearance of such anomalies is conventionally interpreted as evidence of developmental dysplasia and it is associated with several other spontaneously occurring and experimentally-induced disorders of the developing eye in both humans and animals. Among the *induced* dysplasias are those caused by viruses (8, 9) or by *in utero* exposure to irradiation (10, 11) or lysergic acid diethylamide (12).

Each of the two aforementioned phenomena (retinal hemorrhages and dysplastic folds) leads to an unnatural separation of the neuro-retina from the underlying epithelial tissue. The kind of separation caused by sub-retinal hemorrhage is called an *exudative retinal detachment*, and should not be confused with that which is thought to be the primary detachment in infants with cicatricial ROP – *tractional retinal detachment*. Still, even the former type of detachment may weaken the normal bond that exists between the retina and the epithelium, predisposing the retina to the latter type of detachment in the presence of tractional force. Indeed, we have found non-rhegmatogenous retinal detachment in all rats (n = 12) allowed to remain for 38 or more days in room air after oxygen exposure. There is no sign of pre-retinal neovascularization, retrolental membranes or any other tractional elements in these eyes. Based on the present experimental findings, the possible relevance of subretinal hemorrhages and retinal folds to retinal detachment in infants with ROP warrants consideration.

MATERIALS AND METHODS

Thirteen litters consisting of 122 Sprague Dawley albino rats were used in these experiments. All experiments conformed to the Guiding Principles in the Care and Use of Animals (DHEW Publications No. (NIH) 80-23). Immediately after birth a litter of newborn rats was either placed with its mother in an oxygen incubator (modified Isolette®), or was maintained with its mother in

Table 1. Sample sizes (numbers of rats) of treatment groups that underwent histological assessment.

OXYGEN EXPOSURE DURATION (Days)	POST-EXPOSURE ROOM AIR DURATION (Days)							
	0	2	4 or 5	7 or 8	10	38	45	56
5	4	4	3	4	5	-	-	-
10	3	3	6	6	-	-	-	-
14	4	5	4	6	-	4	4	4
TOTALS	11	46				12		

room air. A total of 69 newborn rats was subjected to various periods of 80% oxygen, of which 58 received *subsequent room air* maintenance. Oxygen exposure periods lasted for 5, 10 or 14 days, and were followed by immediate sacrifice or by exposure to brief normoxia for an additional 2, 4, 5, 7, 8, or 10 days, or to an extended period of 38, 45, or 56 days (Table 1). Fifty-three control rats were simultaneously raised in room air and sacrificed at the same times. A select few animals (six) were assessed by ink-perfusion using a method published in detail elsewhere (13). The remaining rats were enucleated and their eyes processed for light and electron microscopy. Eyecups were trimmed to facilitate cross-sectioning of the retina in the vertical meridian.

Preparation of tissue for sectioning involved fixation of the whole eyecup in 2.5% glutaraldehyde overnight at 4°C followed by post-fixation in OsO_4 for 90 minutes at room temperature. Tissues were dehydrated in a graded ethanol series and infiltrated with EPON/propylene oxide. After allowing this mixture to evaporate overnight with the tissue submerged, the eyecups were embedded in 100% EPON araldyte and heated to 60° overnight. Tissues from control and oxygen treated rats of a given age were processed simultaneously. Sections were taken for light (0.5 µm thick) and electron (900 Å thick) microscopy. Thick sections were stained with 1% Toluidine blue.

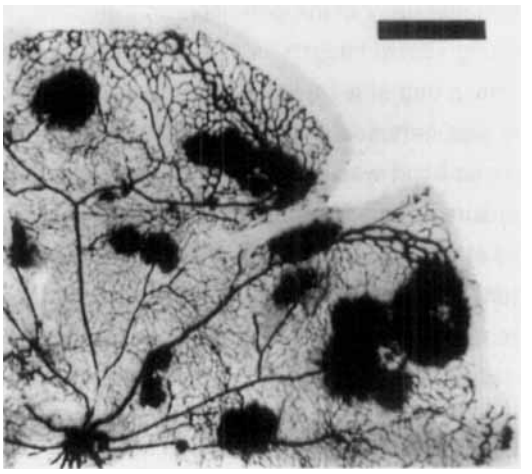


Figure 1. Ink leaks can be produced in control retinas by perfusing at pressures above 120 mm Hg. This retina is from a 14-day old rat raised in room air. Scale bar is 1 mm.

For the sake of consistency and to maintain a manageable body of data, all sections illustrated or discussed in this report were taken from the retinal vertical meridian. *The authors realize this sampling is limited in scope and urge the reader to use caution when assessing the estimations of incidence in the*

Results section. In each thick section the entire meridian, from ora serrata to ora serrata, was included in the assessment. Sections were collected every 10 μm for approximately 100 μm of the retinal distance through which the optic nerve enters the globe. Between five and seven sections were scrutinized from each eye. Both eyes from each rat were examined and a hemorrhage or fold in one eye was considered sufficient to note a positive result for that rat. In no case were the two eyes of an animal considered separate data points. Sections were examined by three individuals who were masked with respect to treatment. Thin sections were then taken from points of interest along the vertical meridian and were stained with lead citrate and uranyl acetate.

RESULTS

The inadequacy of ink-perfusion as a means of determining vessel integrity is illustrated in Figure 1. The retina is that of a 14-day old rat raised in room air. Its body was perfused while it was deeply anesthetized, but while its heart was still beating strongly. In spite of this pressure buffer, the animal's retinal vessels were ruptured at a pressure of 150 mm Hg. However, consistent full perfusion of retinal vessels generally requires 120 mm Hg, so there is little margin for error.

By resorting to histological sectioning it could be determined that no control, room air-raised rats contained retinal hemorrhages or dysplastic folds in the vertical meridian (0 of 53). Nor did the retinas of rats killed immediately after various periods of oxygen exposure contain hemorrhages (0 of 11), but the overall incidence of retinal folds or rosettes in these combined groups was 54% (6 of 11). For all rats exposed to some combination of hyperoxia followed by brief normoxia (10 days or less), 40% suffered hemorrhages (18 of 46) in the vertical meridian and 50% developed retinal folds or rosettes (23 of 46).

The incidence of retinal hemorrhages in rats subjected to the various treatments is described in Figure 2A. Hemorrhages were more prominent in rats subjected to longer periods of oxygen. Seventy-three

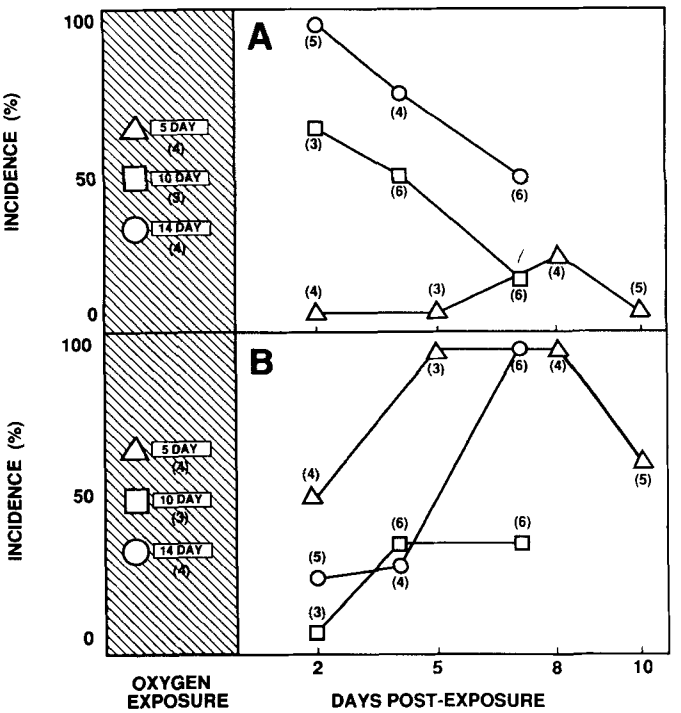


Figure 2. This figure illustrates the incidence of retinal hemorrhages (A) and retinal folds (B) in rats submitted to various periods of 80% oxygen and room air. The cross hatched area designates 5, 10 or 14 day exposures to the hyperoxic treatment. Room air post-exposure periods are indicated on the abscissa. Sample size for each treatment is parenthetically noted.

percent of all rats exposed for 14 days, followed by brief post-exposure normoxia, sustained hemorrhages (11 of 15) compared to only 6% of those groups exposed for 5 days followed by brief normoxia (1 of 16). The number of hemorrhages in the vertical meridians of those retinas containing them varied from as many as five per retina (2 rats from the 14-day oxygen, 2-day room-air group and 2 from the 14-day oxygen, 4-day room-air group) to as few as one (several treatment groups). The incidence of hemorrhages decreased with time post-exposure in normoxia. Hemorrhages occurred in 100% of the rats raised in 80% oxygen for 14 days followed by 2 days in room air (5 of 5), decreased to 50% by 7 days post-exposure (3 of 6) and was 0% by 38 days post-exposure (0 of 4), indicating a spontaneous resolution with time. The retinal location of hemorrhages was most often the

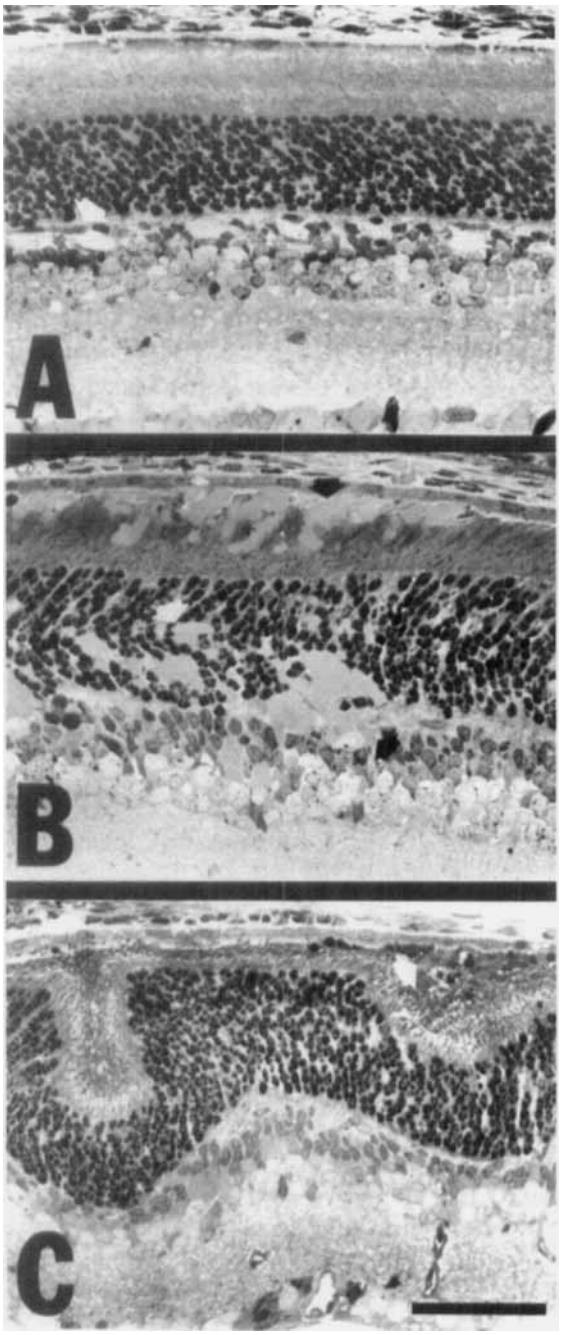


Figure 3. Retinal cross sections from 16-day old rats raised either in room air only (A) or in 80% oxygen for 14 days followed by room air for 2 days (B and C). In panel B an example of retinal hemorrhaging is illustrated. Red blood cells are evident at the level of the deep capillary net, throughout the outer nuclear layer, and in the subretinal space (arrows). In panel C retinal folds have formed during development. The fold on the right contains blood in the subretinal space (arrow). The white arrow in A indicates the location of the deep capillary net. Scale bar is 50 μm.

peripheral-most third, with less frequent occurrence in the mid-peripheral third and no evidence of hemorrhages in the central third of the retina.

The incidence of retinal dysplasia in rats subjected to the various treatments is illustrated in Figure 2B. There was no clear pattern in the incidence of folds or rosettes with time in oxygen or with post-exposure period. However, since no room air-raised rats formed retinal folds or rosettes, while more than half of all rats treated with oxygen did form them, an oxygen-induction hypothesis is reasonable.

Light micrographs of a portion of the vertical meridian of retinas from three 16-day-old rats reveal the appearance of the oxygen-induced retinal hemorrhages and folds (Figure 3). Room air-raised control rats (A) were free of any retinal abnormality. Rats raised in 80% oxygen followed by a brief period in room air (B) often exhibited large retinal hemorrhages extending from the level of the deep capillary net (white arrow in A, lower black arrow in B), throughout the outer nuclear layer (white arrow in B), to the subretinal space (upper black arrow in B) where red blood cells and infrequent macrophages were located. In the periphery of these retinas, there were often folds, indicative of developmental retinal dysplasia (C). There were frequent examples of a combination of hemorrhaging and dysplastic development (white arrow). There were also many examples of true "retinal rosettes" (Figure 4). The location of these rosettes was most often the far retinal periphery, near the ora serrata. Infrequent mid-peripheral folds were observed, but none were found in the central third of the retina.

The cellular make-up of an oxygen-induced retinal hemorrhage can be studied at higher magnification in Figure 5. This hemorrhage, which was located in the subretinal space, contained both red blood cells and macrophage or macrophage-like cells (arrows). The newly formed photoreceptor outer segments appeared to be degenerating as evidenced by a general lack of morphological integrity. Serial thin sections were collected and were scrutinized in the vicinity of sub-retinal hemorrhages in order to determine the integrity of

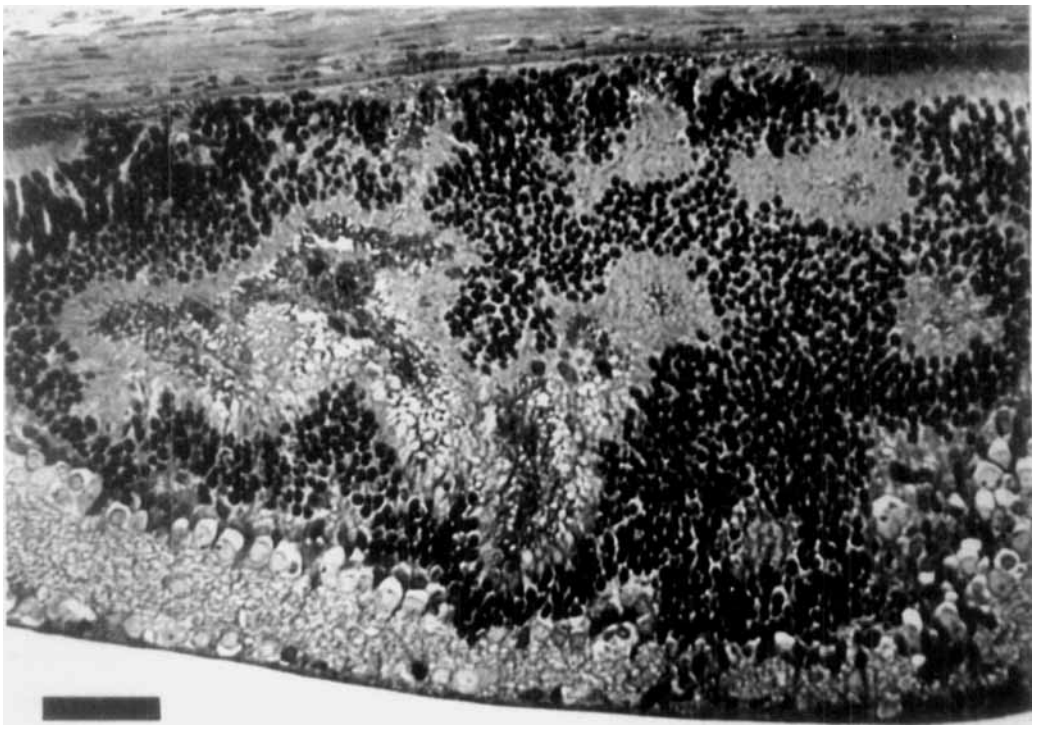


Figure 4. A retinal cross-section from a 16-day old rat raised in 80% oxygen for 14 days followed by room air for 2 days. Rosettes, primarily involving rings of the outer nuclear layer, inundate this area of retina in the far periphery. Some of the rosettes contain photoreceptor outer segment material within them. Scale bar is 50 μm.

Bruch's membrane and the choriocapillaris. In no section was there any evidence of choroidal hemorrhage, nor was there any breach of Bruch's membrane.

Evidence for the source of the hemorrhages was sought in cross-sections of retinal capillaries. Electron micrographs of two capillary cross-sections can be found in Figure 6. The top panel illustrates a capillary from the retina of a 16-day-old room air-raised rat. At the time of retinal fixation its lumen was open and unobstructed and it contained a red blood cell. On the cellular level, its endothelial cells exhibited healthy mitochondria and endoplasmic reticulum, and adjacent cells exhibited intact tight junctions (arrows). The bottom panel exhibits a retinal capillary from a rat raised in 80% oxygen for 14 days, followed by room air for 2 days. At the time this retina was fixed there were hints of impending endothelial necrosis. Perhaps most notable is that adjacent endothelial cells did not exhibit tight junctions (arrow and inset). Mitochondria were swollen or misshapen and large cytoplasmic vacuoles were present in surrounding tissues (arrow heads). The vessel lumen had collapsed, distorting the red blood cell contained therein — a condition not observed in sections of control tissue.

In three retinas hemorrhages were seen to pool at the inner limiting membrane (Figure 7). No erythrocytes were observed in the deep retina implying that the source of the three hemorrhages was the superficial vessel bed. The inner limiting membrane appeared intact in every section taken from these retinas. The three retinas were from two rats that received 14 days of oxygen, followed by 2 days of room air and another that received 10 and 4 days, respectively.

Non-rhegmatogenous retinal detachments (Figure 8), occurred in rats maintained in room air for extended post-exposure periods before sacrifice. It is likely these detachments occurred some time before enucleation

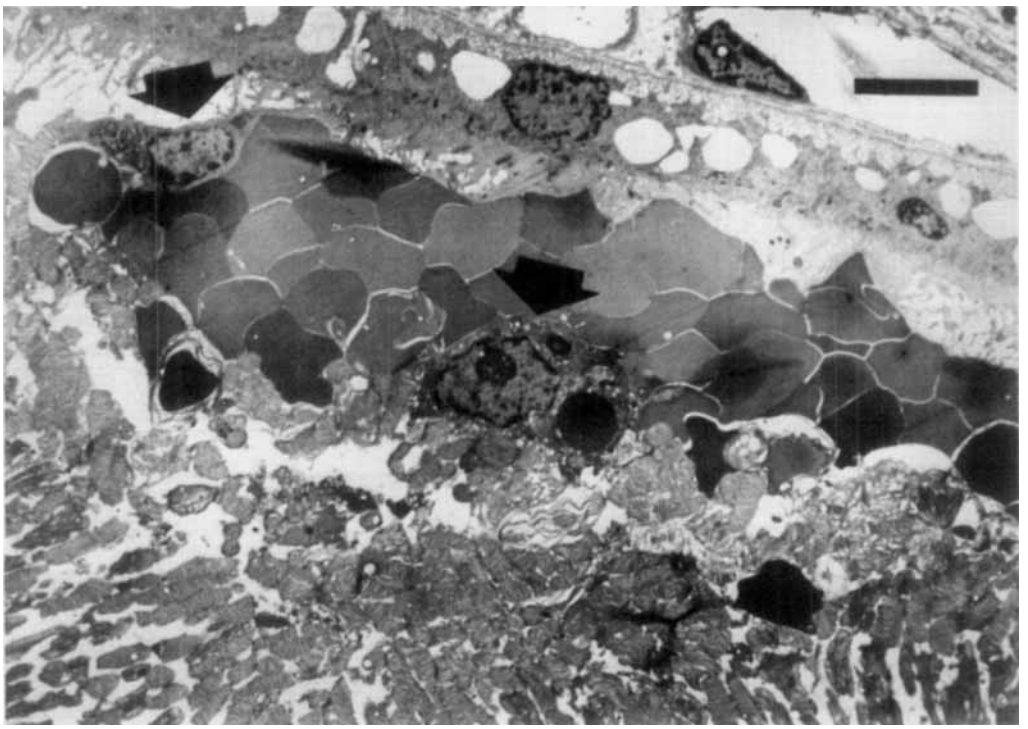


Figure 5. An electron micrograph of a hemorrhage in the retina of a rat raised in 80% oxygen for 14 days followed by room air for two days. The hemorrhage contains both red blood cells and polymorphonucleic cells (arrows). There is no indication of a compromise in the integrity of the tissue between the choroidal vessels and the retina, and therefore no reason to credit the choroid as the source of the bleeding. Scale bar is 5 μm.

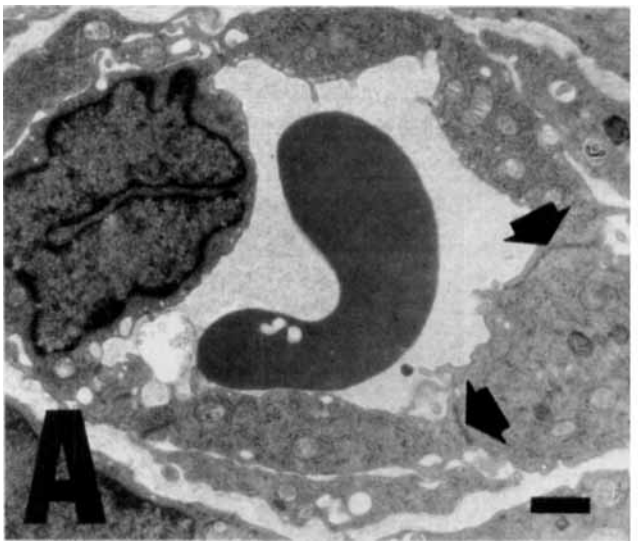
and fixation, since the photoreceptors in the vicinity display clear signs of autolysis while those in adjoining areas of the same retina appear to have been healthy at the time of sacrifice. Retinal detachments were observed in all rats that had extended post-exposure periods of 38, 45, or 56 days in room air (12 of 12). No detachment was seen in age-matched rats that had not received oxygen (0 of 12).

The earliest time at which a detachment was observed was a single instance in which a 21-day old rat (14 days oxygen, 7 days room air) exhibited a transition between subretinal edema adjacent to a retinal fold and full blown detachment some 200 μm distant (Figure 9). Red blood cells that had not yet been cleared were found beneath the fold (arrow in A and inset). Such cases, where retinal folds and/or hemorrhages were found spatially coincident with edema or full blown detachment, provide evidence of an association between the phenomena.

Retinal folds and rosettes were still prevalent at the long post-exposure times, most often found beneath areas of retinal detachment. Occasionally, clustered or scattered erythrocytes were also found beneath large areas of retinal detachment at long post-exposure times (Figure 10). In the case of Figure 10, the cluster of erythrocytes was associated with additional unidentified cells (arrow) that may serve to clear the blood by phagocytosis.

DISCUSSION

Subretinal hemorrhages and retinal dysplasias are promoted by hyperoxic rearing of newborn rats. Each of these manifestations of oxygen rearing leads to an abnormal separation of the retina from the underlying epithelium. We emphasize the difference between this type of separation (or *exudative* detachment) and *tractional* detachment such as that occurring in infants suffering from severe cases of ROP where the primary



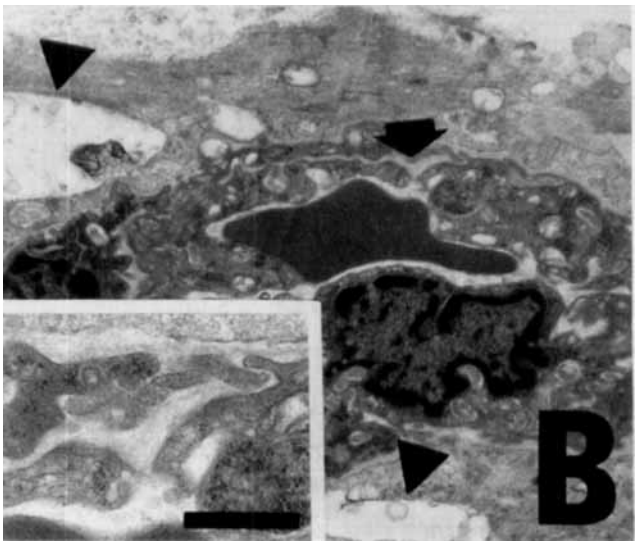


Figure 6. Electron micrographs of cross-sections of two retinal capillaries, both of which have red blood cells in the lumen, proving their maturity. In the capillary of a rat raised in room air for 16 days (A), the tight junctions (arrows) between adjacent endothelial cells are intact. In the capillary of the rat raised in 80% oxygen for 14 days followed by 2 days in room air (B) these junctional complexes are lost (arrow), and there is additional cytoplasmic vacuolization of surrounding tissue (arrow heads). Inset is higher magnification of the cell boundary in B. Scale bar is 1μm for A and B, and 0.5 μm for inset.

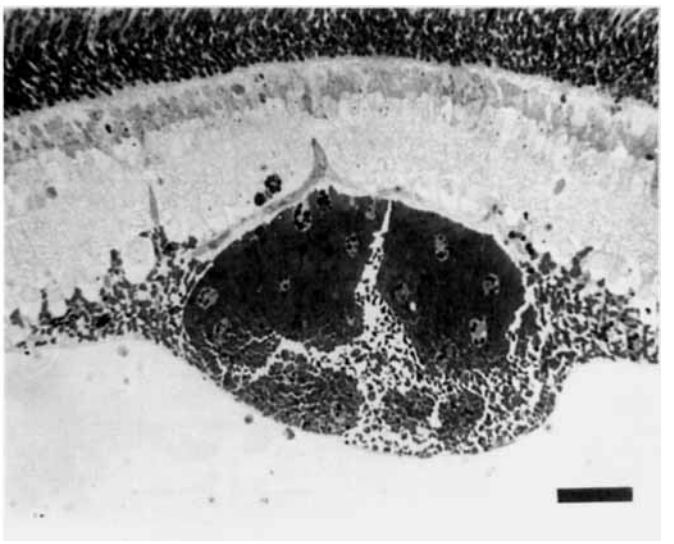


Figure 7. Blood has pooled at the vitreal interface of this retina, but the inner limiting membrane has remained intact. The rat was exposed to 10 days of 80% oxygen, followed by two days of room air. There were only two occurrences of superficial hemorrhages; the other was in an animal exposed to oxygen for 14 days, followed by two days in room air. Scale bar is 50 μm.

force is believed to be a tractional draw on the retina resulting from pre-retinal vascular and glial growth. Still, if hemorrhages and folds similar to those observed in rats occur in neonatal infants who receive oxygen therapy, they may combine with pre-retinal traction (or, if the rat is indicative, they may act alone) to increase the likelihood of full-blown detachment. One might theorize that, in a case of relatively mild traction from pre-retinal vessels, the additional presence of subretinal hemorrhages and/or retinal folds might promote severe retinal detachment in an infant where pre-retinal traction alone would have been otherwise inoffensive.

Irreversible degeneration of photoreceptors underlying subretinal hemorrhages has been shown to occur in as little as 24 hours (14). Studies employing intraocular ferric, ferrous, or metallic iron have shown that each has a direct toxic effect on the retina (15-17). More noteworthy, iron released from intraocular whole blood and hemoglobin causes a similar toxic effect (16, 17). In all cases, iron causes a selective destruction of photoreceptors, often sparing secondary neurons. In addition, subretinal blood forms a severe barrier to metabolic exchange between the retinal epithelium and the neuroretina (14). The metabolism of photoreceptor

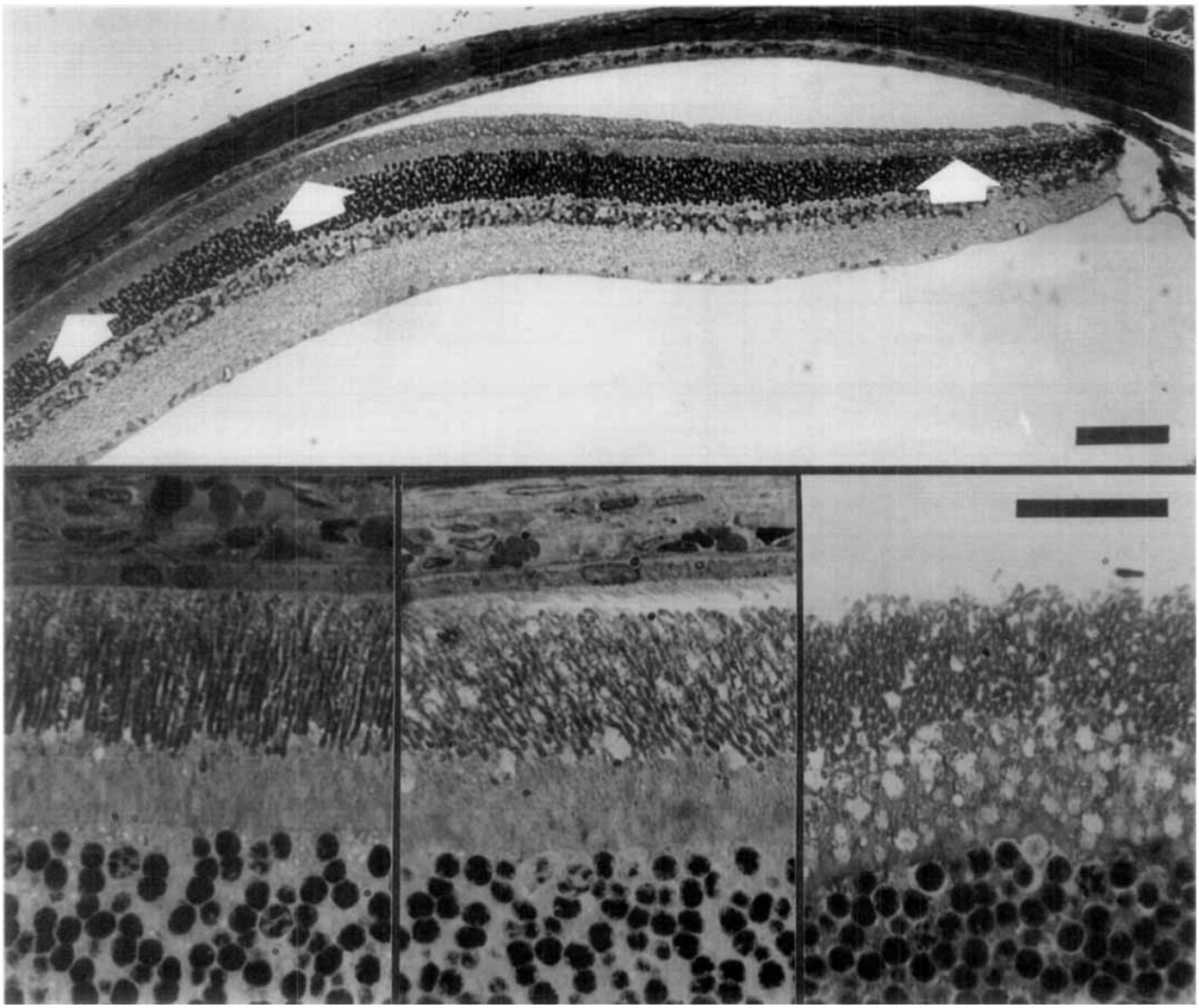


Figure 8. A non-rhegmatogenous detachment has occurred in the far periphery of a retina from a rat exposed to 80% oxygen for 14 days, followed by 56 days in room air. There is a transition from healthy photoreceptors beneath areas of attached retina, to photoreceptors with swollen and vacuolated outer segments, to autolytic photoreceptors with degeneration of both inner and outer segments beneath areas of detachment (left to right). Arrows on the top panel represent the retinal locations from which the lower panels were photographed on the same or adjacent sections. Scale bar is 100 μm for the top panel and 25 μm for the bottom three panels.

cells is integrally linked to that of the epithelium. Processes of photoreceptor renewal, visual pigment regeneration and nutrient supply to the retina all require spatial intimacy and interaction between the two cell types. A hemorrhagic barrier in the subretinal space might be expected to cause retinal destruction irrespective of the inherently toxic nature of interstitial blood.

It is impossible to determine from the data obtained in this study if the apparent degeneration of

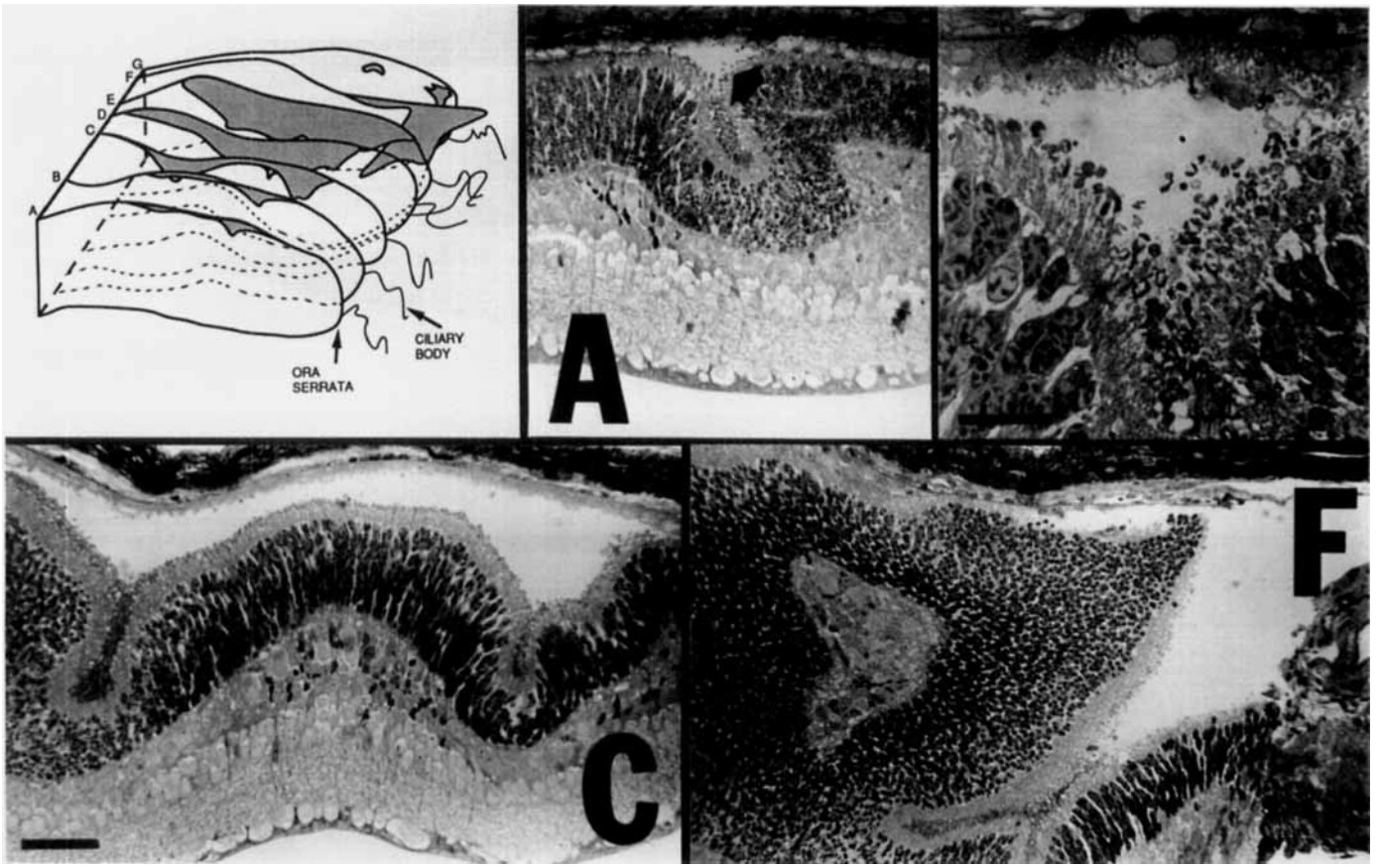


Figure 9. The top left-hand panel is a 3-dimensional reconstruction of seven retinal cross-sections taken at 50 μm intervals. Labels on the other panels refer to the location on the schematic from which the section was taken. All photographs depict the retinal region near the ora serrata. Section G (not photographed) transected a portion of the optic nerve head in the retinal vertical meridian. Section A contains a retinal fold (arrow) with debris in the edemic subretinal space that includes erythrocytes (scale bar of inset in the upper right-hand corner is 20 μm). Sections C and F exhibit full-blown detachments. A large retinal rosette is evident beneath the detachment in Section F. Note signs of pyknosis in the inner nuclear layer of sections A and C and in the rosette in section F. Scale bar for A, C and F is 50 μm.

photoreceptors seen under areas of detachment after long post-exposure times was due to the toxicity of blood or to the separation of photoreceptors from their nutrient source, or if it was caused by a combination of these two things. More importantly, the separation of retina and epithelium caused by hemorrhages may predispose the retina to detach under the influence of additional factors that often exist in the context of ROP. In fact, serous detachments alone can cause the breakdown of two important mechanisms of retinal adhesion: 1) interdigitation of outer segments with RPE microvilli, and 2) the metabolic activity of the RPE which drives the active transport of fluid and ions that normally keeps the subretinal space dehydrated (18).

As is the case in many infants, retinal hemorrhages apparently resolve in rats if allowed a long enough post-oxygen exposure period in room air. Koshibu (19), studying the effect of experimentally induced subretinal hemorrhages in adult rats, found that red blood cells in the subretinal space were phagocytized by macrophages (of unspecified origin), retinal epithelial cells, and Muller cells. The cells observed intermingled with erythrocytes in Figures 5 and 10 have many of the features of macrophages. In Koshibu's experiments,

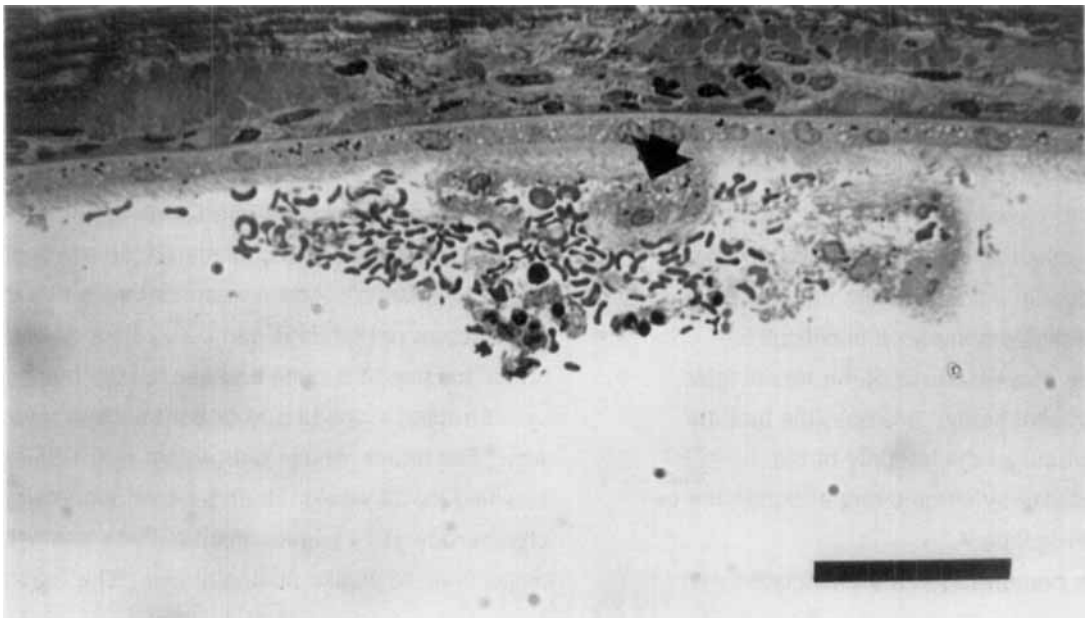


Figure 10. On one occasion, a cluster of erythrocytes and other unidentified cells (arrow) remained adjacent to the epithelial layer beneath an area of retinal detachment. The animal was exposed to oxygen for 14 days, followed by 56 days in room air. More often at long post-exposure times, scattered (rather than clustered) erythrocytes were found beneath detached retina. Scale bar is 50 μm.

blood was completely "resorbed in 21 to 60 days". Our experiments imply a more rapid clearing may occur initially in very young rats, leaving only scarce clumps of red blood cells remaining at extended post-exposure times.

In our attempt to locate the source of subretinal hemorrhages, we have resorted to high magnification electron micrographs of cross-sectioned retinal capillaries. Our photographs are reminiscent of those published by Ashton and Pedler (20). In addition to exhibiting many of the signs of oxygen damage reported by Ashton and Pedler for their kitten retinas, our rats' retinal capillaries completely lacked endothelial cell tight junctions in many instances (such as the oxygen-treated retina depicted in Figure 6B). While this loss of tight junctions was not true of every capillary sectioned from oxygen-treated rats, it occurred in a substantial number of cases. We are certain this lack of tight junction integrity was not due to vessel immaturity since, in the rat, tight junctions are formed early, at the time of cord formation by spindle-shaped precursor cells (21). According to past reports junctional complexes should certainly be in place before vessel patency, but more telling, the capillary depicted in Figure 6B contained a red blood cell in its collapsed lumen, proving its maturity. We suspect that this absence of tight junctions may explain the leaks from which extra-cellular blood originated before migrating to the subretinal space. The absence of any pathological sign in the choroid, Bruch's membrane, or the retinal epithelium, together with the presence of red blood cells throughout the area between the deep retinal capillaries and the subretinal space, point to the deep retinal vessels as the source of the hemorrhages. And, in fact, the direction of migration of these erythrocytes is to be expected, given what is known about the direction of fluid flow in the retina (18, 22). On three occasions superficial vessels probably were the source of hemorrhages that accumulated at the inner limiting membrane but did not penetrate into the vitreous (Figure 7).

Oxygen-induced retinal folds can take the form of partial or total "rosettes" – a term applied to dysplasias in

which one or more of the retinal layers make a complete loop (Figure 4). Retinal folds and rosettes are a particularly common abnormality of canine retinal development (23). It may be that the reported occurrence of "falciform retinal folds" in newborn beagle puppies exposed to elevated oxygen and supplemented with aspirin (4) is linked to a developmental anomaly, rather than to tractional influence from neovascular growth as the investigators imply. It is difficult to speculate as to the specific cause of the retinal folds observed in the present study. It is possible that the oxygen has compromised the integrity of the developmental process by some direct effect on the cell population(s) that regulate it.

In many cases hemorrhages are associated with retinal folds and rosettes. The presence of the two phenomena simultaneously in one retinal location in the rat is identical to an occurrence depicted years ago in oxygen-reared mice (ref. 5, fig. 15). In this earlier work, mice were subjected to a combination of oxygen (98-100%) and subsequent room air, as were many of the rats in the present study. Retinal folds were found in over half of the oxygen-exposed mice examined in the previous study. The authors described these "irregularities" in some detail, but placed little emphasis on their possible role in the context of ROP. Furthermore, it is important to note that retinal folds were found in a quarter of all non-exposed mice in the study. Ashton, et al., (2) also reported "acute-angled folds" of the outer retinal layers in an unspecified percentage of oxygen-reared kittens, again with little emphasis beyond photographic description. These authors did not compare the incidence of folds in oxygen-exposed and non-exposed kittens. Therefore, a spontaneously occurring genetic anomaly cannot be ruled out in either of the two previously published cases. In our opinion, this report marks the first instance in which oxygen induction of retinal dysplasia is well supported by the evidence provided.

The retinal folds and rosettes can also appear in the absence of bleeding and are predominately found in the far periphery of our meridional sections (i.e., very near the ora serrata). This is a common location of retinal folds in infants with ROP (24). In the case of infants, the folds are presumably caused by a buckling of retinal tissue under tractional force by extra-retinal vasoproliferative growth. However, at least one case of a retinal rosette that was almost certainly not caused by traction has been documented in an infant with acute ROP (25). The authors considered the retina of this infant "dysplastic". And we concur that, while the presence of retinal folds can easily be explained by pre-retinal traction, it is quite another matter to account for uninterrupted loops of one or both nuclear layers in this way. The infant, whose birthweight was 1,350 gm, survived for 24 weeks. It underwent unsuccessful cryotherapy at 14 weeks postnatal age and scleral buckling at 16 weeks postnatal age. The retinal rosette illustrated in the published description of this infant was formed by an entire loop of the photoreceptor nuclei, and was not immediately associated with a hemorrhage. The rosette was located in the retinal mid-periphery.

It should be emphasized that hemorrhages were never seen in rats that were sacrificed *immediately* after oxygen exposure, but by only 2 days post-exposure in room air the majority of rats had developed retinal bleeding in the vertical meridian. It appears that a normoxic period was necessary for hemorrhages to develop. This fact is one of two pieces of evidence which suggest that hemorrhages arose from new vessels, rather than from previously formed vessels damaged during oxygen exposure. The other is the typical location of the hemorrhages – the deep plexus in the far retinal periphery. This is an area of the retina that was essentially avascular at the time of removal from oxygen, even in rats exposed for 14 days. Therefore, any leakage in this region must have come from vessels that formed after revascularization had commenced upon return to room air.

By contrast, retinal folds occurred in 54% of all rats that were sacrificed immediately after oxygen exposure. Hence, a normoxic period was not necessary for their formation. Usually, the intermediate post-exposure periods of four and five days were those at which hemorrhages and folds were found spatially coincident in our experiments. Our speculation is that soon after

return to room air the hemorrhages commenced, but it took some time for the blood to migrate to the subretinal space. Once there, the blood induced the formation of folds, or (more likely) it was predisposed to migrate to locations where folds already existed – perhaps because these were the paths of least resistance. The folds then remained for some period after the blood was cleared by phagocytosis in addition to other possible mechanisms. Of course, the existence of folds in rats immediately after removal from oxygen (a time at which no hemorrhages were observed) would argue that the blood migrated to preexisting folds for those cases in which the two phenomena were observed to occur simultaneously.

Whether infants frequently develop such dysplastic folds and rosettes as a typical consequence of hyperoxic maintenance irrespective of tractional forces is a question worth pondering. If so, the possible contribution of these dysplasias to eventual detachment should be addressed. Because our observation of retinal hemorrhages and folds and that of subsequent retinal detachment most often are displaced in time, it is difficult to apply a cause and effect relationship. However, although the sample is small, we have no better explanation for our finding of 100% incidence of detachment in rats with long post-exposure periods (38 days or more), especially since there is no sign of tractional influence in their eyes. It is possible, although unlikely, that the detachments are artifactual, induced at the time of enucleation. However, if this is the case, oxygen-exposed rats must be far more susceptible to this artifact than their room air counterparts in which no detachments were observed. This scenario would indicate that something was fundamentally different regarding retinal attachment in the two groups — no surprise given the separation of retina and epithelium caused by subretinal hemorrhages and retinal folds. In any case, proliferation of pre-retinal vessels may not be the only factor contributing to retinal detachment in animals in alleged cases (4, 6), or in humans in any case. Other conditions may exist that reduce the retina's adherence to the underlying tissue and enhance the likelihood of detachment.

SUMMARY

The following original observations were made concerning the occurrence of retinal hemorrhages, folds and detachments in rats reared in the various oxygen conditions:

1. This marks the first case in which a high incidence of retinal detachment has been reported in oxygen-reared animals. We believe these to be oxygen-induced detachments for two reasons: a) the underlying photoreceptors have had time prior to enucleation to undergo partial autolysis implying that the detachment cannot be due to enucleation or fixation artifact and b) eyes from age-matched control rats were enucleated and processed simultaneously, and no detachments were seen. This marks a significant advance in the development of an animal model of ROP.

2. The origin of oxygen-induced hemorrhages most often appears to be the deep capillary bed, with rare instances of leaky superficial vessels. The temporal and regional characteristics of the hemorrhages point to newly formed vessels as the source of the leaks.

3. While the hemorrhages appeared to originate primarily in the deep vascular net, the bulk of the exudate moved quickly to the subretinal space. There is no evidence of any compromise in the integrity of the tissue between the choroid and the retina, and therefore there is no reason to credit the choroid as the source of the hemorrhages.

4. Retinal vessels of oxygen-exposed rats often displayed a lack of endothelial cell tight junction integrity, implying a breakdown of the blood retina barrier and indicating a possible explanation of hemorrhage origin.

5. This report establishes the incidence of retinal hemorrhages and dysplastic folds in an oxygen-reared animal. Included are the following observations:

a) Rats sacrificed immediately after oxygen exposures of various durations had not developed retinal hemorrhages, but in all exposure groups combined, 54% exhibited folds.

b) For all rats exposed to combinations of oxygen and brief room air, 40% suffered retinal hemorrhages and 50% developed retinal folds.

c) No rats raised in room air alone developed retinal hemorrhages, retinal folds or detachments.

d) In rats exposed to 10 days of elevated oxygen or longer followed by 2 days of room air, hemorrhages occurred 87% of the time. Extending the room air period to 7 days reduced the incidence to 33%, indicating a spontaneous resolution with time.

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