EXPERIMENTAL APPROACH TO THE PATHOGENESIS OF RETROLENTAL FIBROPLASIA*

III. CHANGES IN THE EYE INDUCED BY EXPOSURE OF NEWBORN MICE TO GENERAL HYPOXIA

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A VAST literature has accumulated on the strong correlation between exposure to high concentrations of oxygen during the postnatal care of premature babies and the development of retrolental fibroplasia (for reviews of the literature see François and others, 1954; Gordon and others, 1954; Henry, 1954; Ingalls, 1954; Lanman and others, 1954; Patz, 1954). Changes in the eye, similar to those seen in human retrolental fibroplasia, have also been produced experimentally by exposing young animals to high concentrations of oxygen (Gyllensten and Hellström, 1952, 1954, 1955; Ashton and others, 1953, 1954; Patz and others, 1953).

The role of oxygen, however, is not yet clear, and oxygen cannot be the only cause of the disease. Exline and Harrington (1951), Lelong and others (1952), and Zacharias and others (1954) found no obvious correlation between oxygen treatment and subsequent retrolental fibroplasia. A few cases are known of retrolental fibroplasia in stillborn infants (Reese and others, 1952) and in children who were never given any extra oxygen (Coxon, 1951; Bembridge and others, 1952).

These objections to the theory of oxygen poisoning as the only cause of retrolental fibroplasia would be explained if it could be demonstrated that oxygen is not the immediate cause of the disease but acts by way of an intermediate mechanism that can be provoked in exceptional cases by other agents.

Clinical observations (Szewczyk, 1951) tended to demonstrate that in the early stages of retrolental fibroplasia oxygen-induced changes in the eye develop after the transfer from a high concentration of oxygen to normal air, and that the changes regress if the child is given oxygen again. Szewczyk (1951, 1952, 1953) suggested that the disease depended on relative anoxia, which is supposed to occur as a sort of adaption disease when the child is rapidly transferred to normal air. Similar views have been propounded by many workers, including Jefferson (1952), Crosse and Evans (1952),

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Bedrossian and others (1954), François and others (1954), Ingalls (1954), and Manschot (1954). The fact that the retinal vessels contract when the animal or human being is breathing concentrated oxygen (Huerkamp and Rittinghaus, 1950; Huggert, 1953; Ashton and others, 1954) apparently supports the theory of "hyperoxic hypoxia". A retarding of the development of retinal vessels in growing mice incubated in concentrated oxygen was observed by Gyllensten and Hellström (1954), and an obliteration of retinal vessels in other animals in similar conditions was demonstrated by Ashton and others (1954). These changes in the retinal vessels may cause a local hypoxia in the retina when the subject is transferred to normal air after a period in oxygen. Experimentally, the rapid withdrawal from a high oxygen atmosphere has been shown to be highly provocative of changes in the eye akin to human retrolental fibroplasia (Gyllensten and Hellström, 1954; Ashton and others, 1954). It is, however, also possible that a hypoxic condition of the retina develops during the stay in oxygen, which could be explained by an oxygen-induced interference with the enzymatic systems, especially in the oxidation of carbohydrates (Himwich, 1953). The breathing of pure oxygen produces severe oedema and other changes in the lungs (Gyllensten and Hellström, 1954), which may interfere with normal respiration and cause a general hypoxia.

Further evidence of the possible effect of hypoxia on the development of retrolental fibroplasia are experimental anomalies of the eye in mice and rats produced by exposure to hypoxia during the foetal period (Werthemann and others, 1950; Ingalls and others, 1952). These changes are produced at earlier stages of development than those corresponding to viable premature human babies, and the changes are not the same as those of retrolental fibroplasia, though certain similarities exist.

The early stages of human retrolental fibroplasia consist mainly in abnormal overgrowths of retinal vessels. It is well known that hypoxia stimulates the growth of vessels in many organs (for review see Stickney and van Liere, 1953) and also in the eyes (Huerkamp and Opitz, 1950; Huerkamp, 1952; Opitz, 1952). The growth of the retinal vessels is designated not hyperplasia but hypertrophy (Opitz, 1952), which implies a difference from the most characteristic vascular changes in retrolental fibroplasia.

The effect of general hypoxia on the growing retina of experimental animals has been studied by Ashton and others (1954) and Patz (1954). These series, however, included only a very small number of young (six in the experiments of Ashton and others, and two in those of Patz), and the oxygen concentrations were not sufficiently low (10-15 per cent.) to have much effect on the oxygen saturation of haemoglobin, especially having regard to the high saturation coefficient of the haemoglobin in foetal and newborn animals compared with that in adult animals (Barcroft, 1946). Ashton and Patz and their colleagues found no changes in the retinal vessels related to the changes of retrolental fibroplasia.

The aim of the present work was to study the influence of severe general hypoxia on the postnatal development of the eyes of young mice with special regard to the eye changes previously found in young mice exposed to high concentrations of oxygen (Gyllensten and Hellström, 1954).

Material and Methods

Newborn litters of inbred black mice (stock C 57 BL/6 Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine) were used. This strain has a low frequency of spontaneous eye anomalies (Gyllensten and Hellström, 1955). The animals were fed on a mixture of dry milk, yeast, oats, wheat, rye, corn, and bread, with cod liver oil, ferric citrate, potassium iodide, calcium carbonate, sodium fluoride, manganese sulphate, and added salt.

The exposure to hypoxia took place in air-tight cages of 27 l. volume. The exposure was initiated with a flow of pure nitrogen, diminishing the oxygen content of the cage down to 5 to 6 per cent. within 20 to 30 minutes. A mixture of 3 per cent. oxygen in nitrogen was then streamed through the cage at a flow of 0.5 l./min., lowering the oxygen content in the cage down to close upon 3 per cent. The oxygen concentration was thereafter checked once or twice every hour with a Beckman oxygen analyser. The relative humidity was 80 to 90 per cent., the temperature about 24°C. The duration of the exposure was measured from the start of the pure nitrogen flow. At the end of the exposure, air was rapidly introduced, bringing the oxygen content to normal atmospheric values within a few minutes. The duration of a period of exposure ranged from 3 to 10 hrs. On an average 5 to 7 hrs' exposure continuously or divided into two periods was administered daily. The total duration of hypoxia varied between 3 and 144 hrs. The mother animals were separated from the litter during the exposures as their survival in the low oxygen concentration was limited to 2 hrs.

Altogether 249 newborn animals from 32 litters were used, but only a small number of them survived the intended exposure. The mice were killed between the first and 22nd day of life with 0.5 ml. 20 per cent. urethane intraperitoneally. The middle part of the head with the eyes was fixed, decalcinated, embedded, sectioned, and stained as previously described (Gyllensten and Hellström, 1954).

Results

Only 44 animals survived the intended duration of hypoxia, which points to the fact that the oxygen concentrations used were, as a rule, not tolerable for any longer period of time. In addition, 28 animals, which died spontaneously during the hypoxic conditions but were prepared immediately after death, were included in the material. The duration of hypoxia in these 72 animals is set out in the Table.

During hypoxia the young mice were cyanotic and dyspnoeic. Their general activity was reduced to a minimum and the animals were as a rule lying motionless. Some of those which died developed convulsions. Those surviving seemed to recover within a few minutes of the introduction of air, without any persisting neurological symptoms. The older animals subjected to repeated periods of hypoxia suffered to some extent in their nutrition, at least partly because of the separation from the mother.

Microscopic Examination of the Eyes.—The regression of the hyaloid vessels seemed to occur to a normal extent and at a normal rate in the experimental animals. The vascularization of the nerve fibre layer, as judged by the outgrowth of angioblasts and vessels from the disc and by the

occurrence of a marginal vessel close to the ora serrata, was not accelerated, as compared with the control animals (Gyllensten and Hellström, 1954). The separation of the nuclear layers by the development of the outer plexiform layer seemed to be completed a little later (11 to 14 days) than in the control animals (10 to 11 days).

The most conspicuous changes were haemorrhages originating from partly dilated vessels in the nerve fibre layer and occasionally breaking through the internal limiting membrane into the vitreous body (Fig. 1). Small single haemorrhages in the nerve fibre layers were rarely found in control animals (Gyllensten and Hellström, 1954), but the high incidence in these experimental animals subjected to hypoxia (22 out of 72) and their multiple localization is definitely pathological. Their incidence was not higher in animals which died spontaneously in hypoxia. The haemorrhages were frequently bilateral and could also be seen bulging down into the inner plexiform layer with scattered red cells in the deeper layers of the retina (Fig. 2). The shortest duration of hypoxia, after which a retinal haemorrhage was observed, was $18\frac{1}{2}$ hrs. No obvious correlation could be found between a longer total duration of hypoxia and the incidence of haemorrhages (Table, opposite).

FIG. 1.—Retina of mouse, 8 days old. Exposure to 3 per cent. oxygen since birth for 3 to 6 hrs every day (total 42 hrs). Haemorrhage in nerve fibre layer breaking into vitreous body. $\times 133$.

FIG. 2.—Retina of mouse, 11 days old. Exposure to 3 per cent. oxygen for 6 hrs every day since birth. Haemorrhage in nerve fibre layer bulging into inner plexiform layer. $\times 133$.

In no instance were hyperplastic changes of the vessels in the nerve fibre layer, or the formation of capillary tufts budding into the vitreous body, observed.

TABLE
DURATION OF HYPOXIA AND INCIDENCE OF HAEMORRHAGE

Total Duration of Hypoxia (hrs)	Number of Animals with Haemorrhages from Retinal Vessels in Nerve Fibre Layer	Total Number of Animals
- 24	4	12
25 - 48	9	22
49 - 72	5	16
73 - 96	4	13
97 - 120	0	0
121 - 144	0	9
Total	22	72

In addition to these findings minor irregularities of the nuclear layers and small sharp folds of the outer layers of the retina were observed in some cases, but this is a frequent finding in control animals (Gyllensten and Hellström, 1954).

Discussion

These microscopic changes in the eyes of the experimental animals essentially consisted in haemorrhages originating from frequently dilated retinal vessels. They occurred in from one-third to one-fourth of the cases. The leakage from these retinal capillaries is apparently a result of hypoxic injury to the capillary wall. Capillary bleeding in various tissues and organs is a frequent finding in both human and animal pathology in severe hypoxic conditions. In the present investigation no attempt was made to find bleeding in organs other than the eye.

The absence of any signs of hyperplasia of the retinal vessels, as judged by the outgrowth of newly-formed vessels into the vitreous body or into the deeper layer of the retina, seems to be significant. Such vascular hyperplasia is a basic finding in the early pathology of retrolental fibroplasia. Thus the theory that this hyperplasia is caused only by general hypoxia is not supported by the present findings. This is in accordance with the experimental findings of Patz and others (1953). Ashton and others (1954) described the vascular engorgement of the retinal vessels in kittens as a result of continuous hypoxia (10 to 15 per cent. oxygen) for periods of 4 to 13 days. In one animal a localized area of increased angioblastic activity was found, which was probably outside the upper limits of normality.

On the whole, in our experiments, the changes in the eyes after hypoxia were much less conspicuous than those seen after hyperoxia. The latter include, in addition to the retinal vascular changes, abnormal persistence of hyaloid vessels, vitreous bleedings, and thickening and irregularities of the retinal layers (Gyllensten and Hellström, 1954, 1955). In hypoxia, in contrast, the histological picture of the eye is normal apart from the bleeding.

The animals were exposed to the low oxygen concentrations in periods of

about 5 to 7 hrs daily. The possibility remains that a more continuous exposure would have given a different result. On the other hand, the hypoxia provoked was intense as reflected in the high mortality and the poor condition of the surviving animals during the exposure. It is also possible that the poor condition of the mice had a depressing influence on the formation of new vessels.

The morphological picture of the retina during these hypoxic conditions revealed no signs of impaired nutrition or of insufficient oxygenation which would have been severe enough to cause any gross cellular damage. *In vitro* studies have demonstrated anaerobic glycolysis in the retina exposed to low oxygen concentrations (Craig and Beecher, 1943). This mechanism might also be responsible *in vivo* for the relatively unimpaired metabolism during hypoxic conditions.

These considerations do not justify the conclusion that hypoxia plays no role in the pathogenesis of retrolental fibroplasia. It is true that the animal experiments so far presented favour hyperoxia as the most important environmental factor. According to Gyllensten and Hellström (1954) and Ashton and others (1954), however, the proliferation of retinal vessels does not occur while the animals are still in the high oxygen environment. The vaso-proliferation occurs as a second phase, when the animals are withdrawn from the abnormal oxygen atmosphere. A conceivable theory that hypoxia should accelerate or intensify this vaso-proliferative phase is not supported by the findings in Ashton's experiments. In spite of the lack of positive experimental evidence that postnatally induced hypoxia plays a role in the development of the disease, several significant clinical observations seem to indicate such a connexion. Thus the occurrence of conditions known to be able to interfere with normal oxygenation of the premature infant (anaemia, interstitial pneumonia and other infections, and hyaline membranes) and simultaneous progression of the disease have been described (Szewczyk, 1951, 1952, 1953; Ingalls, and Purshottam, 1954; Bedrossian and others, 1954; Manschot, 1954). Although the child may still be under treatment with oxygen when progression of the retrolental fibroplasia occurs, there is no guarantee that the child is well oxygenated. That general hypoxia should be the *only* factor of importance in the aetiology of retrolental fibroplasia is, on the other hand, not probable, in view of the frequency with which clinical signs of insufficient oxygenation occur in premature babies who do not develop the disease.

Thus evidence from the present and previous animal experiments indicates that the experimental disease in animals is a complex mechanism wherein neither pure hyperoxia nor pure hypoxia is solely responsible. The same is probably true of the retrolental fibroplasia in premature babies. It is, however, possible that a local hypoxia caused by retinal ischaemia may be more intense than the general one and thus influence the development of retrolental fibroplasia.

Summary

(1) Newborn mice were exposed to 3 per cent. oxygen for 3 to 10 hrs a day during 1 to 22 days, and the eyes were examined histologically.

(2) In 22 out of 72 animals haemorrhages from retinal vessels occurred in the nerve fibre layer.

(3) Besides an apparent dilatation of vessels in the nerve fibre layer, no vaso-proliferative or other changes were produced similar to those found in human retrolental fibroplasia or in mice exposed to high concentrations of oxygen with a subsequent stay in air.

(4) It is concluded that *general* hypoxia is not a sufficient factor in the genesis of the oxygen-induced disease of the mouse eye, which has previously been described and which exhibits similarities to human retrolental fibroplasia.

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