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STUDIES ON THE MECHANISM OF RETINAL NEOVASCULARIZATION

ROLE OF LACTIC ACID*

BY

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ACCORDING to Michaelson (1948, 1954), neo-vascularization is due to an unknown vasoproliferative factor that appears in the tissues in hypoxic conditions. This factor is supposed to be responsible for the development of embryonic retinal vessels and for pathological neovascularization.

The role of hypoxia in vascularization is confirmed by observations that areas of the retina well supplied with oxygen, such as the outer layers, macula lutea, and the neighbourhood of arteries, contain no vessels and that at the beginning of vascularization hypoxic conditions prevail in the inner retinal layers. At that stage of development the retina thickens and its inner layers become insufficiently supplied with oxygen. Campbell (1951) and Ashton and Cook (1954) showed in animal experiments that the width of the avascular zone in the neighbourhood of retinal arteries depends on the oxygen tension. At higher oxygen concentrations a wide zone (and, alternatively, at lower oxygen concentrations a narrow zone) developed. With high oxygen tension, Ashton, Ward, and Serpell (1953, 1954), Michaelson, Herz, Lewkowitz, and Kertesz (1954), and Ashton and Blach (1961) were able to cause obliteration of the vessels in the immature retina of newborn animals. Ashton and others (1954) found that high concentrations of oxygen caused obliteration in the retinal arteries, while the subsequently applied lower oxygen tension induced revascularization. Michaelson (1954), Ashton (1957), and Eliasoph (1957) described an association between the formation of a vasoproliferative factor and the increase of anaerobic metabolic products. Ashton summarized the conditions for neovascularization as follows:

(1) Active metabolism;

(2) Lowered oxygen tension resulting in the predominance of anaerobic metabolism;

(3) Inadequate venous flow causing the retention of anaerobic metabolic products and vasoproliferative factor.

Under hypoxic conditions anaerobic glycolysis predominates in the retina. As anaerobic glycolysis yields lactic acid from glucose, in hypoxia and decreased venous flow, this substance, as well as other endogenous substrates oxidized through the citrate cycle, will appear in higher concentrations. In this study an attempt was made to assess the role of lactic acid in neovascularization.

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Materials and Methods

Experimental Animals.—In the experiments 7- to 9-day-old kittens were used, and reliable results were obtained with 31 out of 36 animals.

Intravitreal Injections.—Aliquots of 0·05, 0·1, and 0·5 per cent. lactic acid, 0·1 per cent. pyruvic acid, and 0·1 and 0·5 per cent. acetic acid were injected intravitreally in the region of the retina. The injections were always given into the right eye; into the left eye only diluents of the above substances were injected. As diluent, in order to facilitate the diffusion of the substances, a hyaluronidase solution ("Hyason", Organon Oss, Netherlands) was generally used. In some cases physiological saline, distilled water, or adenosine triphosphate ("Atriphos", Richter, Hungary) were employed. After local anaesthesia the kittens were given five to eight intravitreal injections. A hypodermic needle was introduced at the upper part of the ora serrata and 0·05 ml. aliquot of the solutions was injected into the posterior pole of the vitreous. The first injection was given when the kittens were 7 to 9 days old and the injections were repeated at 2 to 3 day intervals.

Filling the Vessels with Indian Ink and Preparation of the Retina.—3 days after the last intravitreal injection the kittens, then aged 22 to 29 days, were anaesthetized with 1 ml. Evipan intraperitoneally. Then the thoracic cavity was opened and the descending aorta was ligatured. The right auricle was incised and 20 ml. Indian ink were slowly injected into the left ventricle. Both eyes were removed and, after being frozen in carbon dioxide snow, were opened transversely through the ora serrata. The posterior portions were placed in 4 per cent. formalin for 45 minutes. The vitreous was then removed and the retina cut radially, placed flat on a slide, and treated with 75 per cent. alcohol for 5 minutes, 95 per cent. alcohol for 5 minutes, absolute alcohol for 1 minute, and finally with xylene for 1 minute. The finished preparations were covered with Canada balsam.

For normal controls, the vessels of two untreated kittens (aged 8 and 25 days) were filled with Indian ink.

Results

Kittens were used in these studies because their retinal vascularization is similar to that of man. As the normal vascularization in kittens is completed only in the third week of life, it was in a stage of active development at the time of the intravitreal injections. It was assumed that substances injected into the vitreous at that immature stage were absorbed more slowly.

Fig. 1 shows the normal vasculature of an 8-day-old kitten, and Fig. 2 (opposite) that of a 25-day-old kitten.

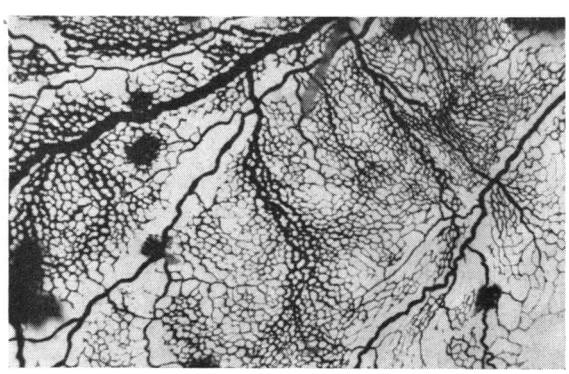

FIG. 1.—Part of the retina of an 8-day-old kitten. Characteristic primitive capillary network. ×52.

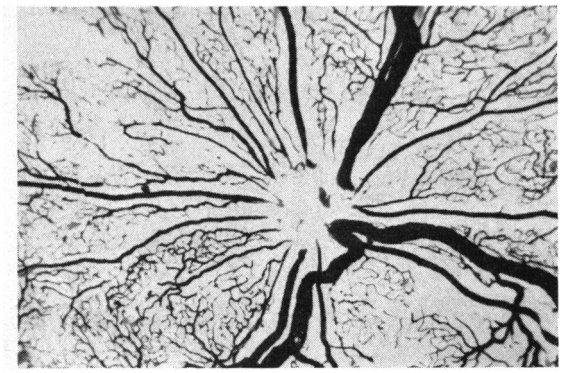

FIG. 2.—Normal retinal vasculature of a 25-day-old kitten. ×52.

Intravitreal injections of 0·1 per cent. lactic acid were given into the right eyes of sixteen animals, and pathological neovascularization was seen in eight out of the sixteen eyes. The ingrowth of new vessels into the vitreous was regarded as pathological. These vessels grew into the vitreous generally from the surface or region of the disc. Definite peripheral neovascularization was encountered in only one case. In two cases, only one or two vascular loops extended into the vitreous (Fig. 3). Three eyes showed several newly formed vessels or the glomerular-type of vasoproliferation (Figs 4 and 5).

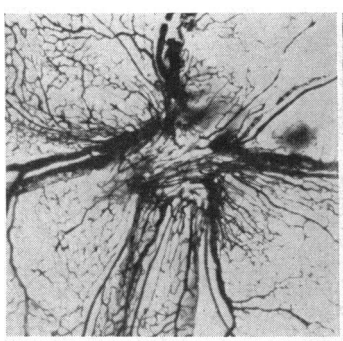

FIG. 3.—Kitten No. 14, 26 days old, after seven intravitreal injections of 0·05 ml. 0·1 per cent. lactic acid. Note newly formed vascular loops on surface of disc. ×52.

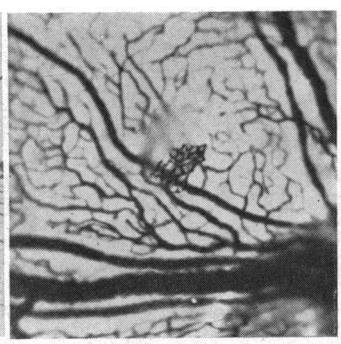

FIG. 4.—Kitten No. 15, 26 days old, after seven intravitreal injections of 0·05 ml. 0·1 per cent. lactic acid. Note glomerular-like vasoproliferation in neighbourhood of disc in front of retina. ×112.

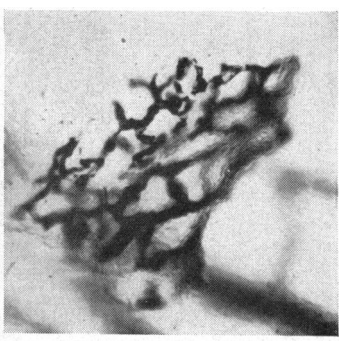

FIG. 5.—Glomerular-like vasoproliferation shown in Fig. 4 at higher magnification. ×460.

In two cases neovascularization towards the vitreous was even more evident (Figs 6 and 7, overleaf).

In one animal an extensive dense preretinal vascular network was observed (Fig. 8 overleaf).

Two groups of three kittens were injected with 0·05 and 0·5 per cent. lactic acid. These eyes showed no pathological neovascularization. In three animals treated with 0·5 per cent. lactic acid, a thick avascular fibroplastic proliferation occurred in the vitreous, where microscopic examination revealed large numbers of fibroblasts.

Six and three eyes were injected with 0·1 and 0·5 per cent. acetic acid respectively. Pathological neovascularization was not observed, but the acetic acid at 0·5 per cent. concentration caused fibroplastic proliferation.

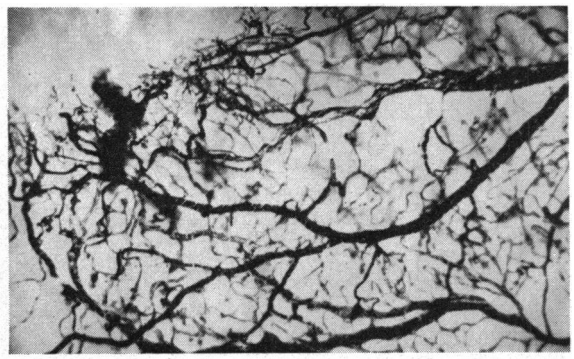

FIG. 6.—Kitten No. 10, 22 days old, after five intravitreal injections of 0·05 ml. 0·1 per cent. lactic acid. At the periphery there is a larger area with intravitreal ingrowth of loops. ×52.

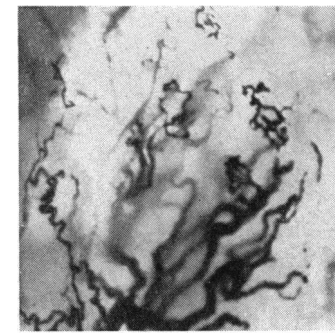

FIG. 7.—Kitten No. 18, 26 days old, after eight intravitreal injections of 0·05 ml. 0·1 per cent. pyruvic acid. Note intravitreal ingrowth of loops in front of disc. ×52.

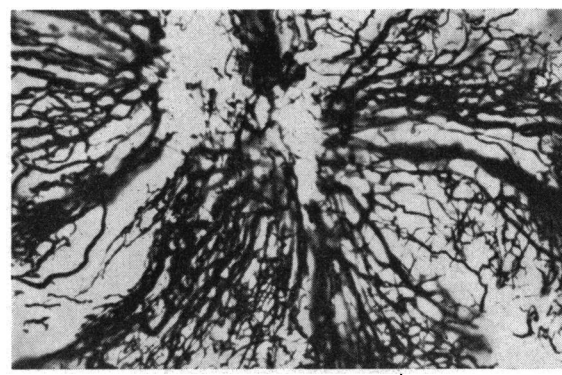

FIG. 8.—Kitten No. 4, 23 days old, after five intravitreal injections of 0·05 ml. 0·1 per cent. lactic acid. Note profuse preretinal neovascularization in front of posterior pole. ×52.

In the 31 left eyes injected with the diluents, no pathological neovascularization took place. It should be noted that the retinal vasculature of kittens of the same age varies considerably. In many animals the retinal vascular network was complete as early as 22 days after birth, but in some 26-day-old kittens the periphery of the retina contained only a primitive capillary network.

The experimental results are summarized in the Table (opposite).

Discussion

In the present experiments we attempted to establish the well-known condition for neovascularization, decreased absorption, by injecting the examined substances into the vitreous. Injections of 0·1 per cent. lactic acid produced the ingrowth of new vessels into the vitreous in 50 per cent. of the animals. As to the mechanism of neovascularization, the following points should be considered:

(*i*) The finding that none of the 31 control eyes showed pathological changes, excluded the possibility that injuries caused by the injections *e.g.* vitreal haemorrhages were responsible for the neovascularization.

TABLE

RESULTS IN 31 ANIMALS

Animal No.	Age (days)	Intravitreal Injections (0·05 ml.)			Age when Killed (days)	Vaso-proliferation	Hyperplastic Proliferation	Vaso-proliferation in Control Eyes
		Substance (per cent.)	Diluent	No.				
1	9	0·05	"Hyason"	6	22	0	0	0
2	9	Lactic		6	22	0	0	0
3	9	acid		6	22	0	0	0
4	8	0·1	"Hyason"	5	23	+ + + +	+	0
5	8	Lactic		5	23	+	0	0
6	8	acid		5	23	0	0	0
7	8	0·1	"Hyason"	6	23	+	0	0
8	8	Lactic		6	23	0	0	0
9	8	acid		6	23	0	+	0
10	8	0·1	Saline	5	22	+ + +	0	0
11	8	Lactic		5	22	0	0	0
12	8	acid		7	24	+	0	0
13	8	0·1	"Atriphos"	7	26	0	0	0
14	8	Lactic		7	26	+ +	0	0
15	8	acid		7	26	+ +	0	0
16	8			7	26	0	0	0
17	8	0·1	Distilled	7	25	0	0	0
18	8	Pyruvic	Water	8	26	+ + +	0	0
19	8	acid		8	26	0	0	0
20	7	0·5	"Hyason"	7	25	0	+ + + +	0
21	7	Lactic		7	26	0	+ + + +	0
22	7	acid		5	23	0	+ + + +	0
23	8	0·1	Saline	7	29	0	0	0
24	8	Acetic		7	29	0	0	0
25	8	acid		7	29	0	0	0
26	8	0·1	Distilled	7	25	0	0	0
27	8	Acetic	Water	8	26	0	0	0
28	8	acid		8	26	0	0	0
29	7	0·5	"Hyason"	6	24	0	+ + + +	0
30	7	Acetic		6	24	0	+ + + +	0
31	7	acid		6	24	0	+ + + +	0

Degrees of retinal neovascularization:
+ 1 or 2 newly formed loops in the vitreous;
+ + Several newly formed loops or some glomerular vessels;
+ + + Definite neovascularization into the vitreous;
+ + + + Profuse preretinal vasoproliferation.

(*ii*) Injuries to the vitreous may, however, give rise to neovascularization. In our experiments a coagulation of vitreal proteins might have occurred. In proliferative retinitis due to haemorrhages, large numbers of fibroblasts enter the vitreous, where their anaerobic metabolic products are retained because of the lack of circulation. These substances then induce neovascularization. In our experiments, however, only one eye showed a mild fibroplastic proliferation into the vitreous. In eyes

treated with 0·5 per cent. lactic or acetic acid, there was marked fibroplastic proliferation at the end of the experiments, but no new vessels developed. These concentrations were undoubtedly injurious to the vitreous. It is obvious that for the mentioned types of neovascularization longer periods of time are needed.

(*iii*) The aetiological role of the changes in pH should also be considered. This factor cannot be responsible for neovascularization, because none of the six eyes treated with 0·1 per cent. acetic acid developed pathological new vessels. Acetic acid was chosen for control purposes, as this subtance is an important metabolic product of fatty acid breakdown and synthesis, and is a weak acid similar to lactic acid.

(*iv*) Accordingly, neovascularization is to be considered as the specific effect of lactic acid.

Before explaining this theory, one must review the previous histological studies on development of retinal vessels. Michaelson (1948) found that the normal development of the vasculature started with dense vascular buds from the disc, and that canalization occurred subsequently. These hyperplastic foci of endothelial cells were always encountered before the development of vessels; thus in the early stages of retrolental fibroplasia they were found within the inner layers of the retina. In addition to these, the proliferation of spindle-cells described by Friedenwald, Owens, and Owens (1951) and Reese, Blodi, and Locke (1952) should also be considered. According to Ashton (1954), these cells are situated directly ahead of the endothelial vascular buds, are not of glial or endothelial origin, and are not present in the retina before vascularization commences. Ashton claims that these cells are mesenchymal precursors of the muscular and connective components of the retinal vascular system. Ward (1954) and Serpell (1954) demonstrated the presence of PAS-positive granules in these mesenchymal cells. Serpell (1954) showed histochemically that the granules consisted of glycogen, and brought evidence that, in the inner layers of the retina of new-born rabbits, premature infants, and cases of retrolental fibroplasia, mesenchymal cells with such formations were always associated with developing vessels. The retinae of adults or of normal new-born infants lack glycogen granules and foci of mesenchymal cells as well as retinae that are in a pre-vascular stage of development. After the commencement of vascularization these formations are never found outside the edge of the developing capillary network. Serpell assumed that the accumulation of glycogen is important for the metabolism of developing vessels, and cited the theory of Vastarini-Cresi (1924) associating the canalization of vessels especially with glycogen metabolism.

In our opinion it is probable that mesenchymal cells synthesize glycogen from lactic acid. It is known that, in addition to the liver, other tissues like mesenchymal muscles, are also capable of synthesizing glycogen from lactic acid. For the synthesis of glycogen from lactic acid, ATP is required, and this is produced by using a part of lactic acid. It is possible that mesenchymal cells that are regarded as precursors of developing vessels synthesize their glycogen in a similar manner. These cells are presumably able to carry out oxidative processes, since they multiply directly ahead of the developing vessels. This concept explains why the vessels always grow in the direction of highly hypoxic areas, where the substances needed for their

growth especially lactic acid are found in higher concentrations. Accordingly, the vasoproliferative factor is lactic acid itself. The present experiments confirmed the active part of lactic acid in vascularization. That neovascularization took place only in 50 per cent. of the eyes treated with 0·1 per cent. lactic acid, may be attributed to technical reasons. If the solution was not always injected exactly into the region of the posterior pole, prolonged high lactic acid concentrations could not be always attained at that particular site. Lactic acid in a concentration of less than 0·1 per cent. caused no neovascularization, probably because the physiological lactic acid concentration in the vitreous increased only slightly. In our experiments ATP exerted no influence on neovascularization. Adult vessels undoubtedly also contain cells capable of inducing the formation of new vessels. It is justifiable, therefore, to assume that, when these cells are exposed to higher concentrations of lactic acid, neovascularization commences in a manner similar to that described for glycogen synthesis. In this connexion the observation of Cogan (1962) seems to be important. He found at autopsy of a case of glycogenosis that glycogen accumulated in glial and vascular mural cells of the retina. Mesenchymal cells growing ahead of the developing vessels may take up lactic acid from the vitreous. This could happen in every vascular ingrowth directed towards the vitreous, and probably happened also in our experiments. The ability of the retina to take up substances from the vitreous has been described by Nakashima (1926) and Adler (1930).

It is probable that the mesenchymal precursors of developing vessels use, in addition to lactic acid, other substances present in hypoxic tissues, or other endogenous substrates that are usually oxidated through the citrate cycle. Our experiments indicate, however, that lactic acid accumulated in hypoxic tissues may cause vascular proliferation *per se*. Glycogen needed for the metabolism of developing vessels is synthesized from the accumulated lactic acid. This explains why the vessels always grow towards hypoxic tissues where the venous flow is decreased. Accordingly, by promoting normal vascularization, anaerobic glycolysis, which is a less developed, primitive energy-yielding process, assures the normal development of tissues.

Summary

Injections of 0·1 per cent. lactic acid into the vitreous body of kittens' eyes caused neovascularization in 50 per cent. of the animals. Control experiments showed that the ingrowth of new vessels into the vitreous was attributable to the specific effect of lactic acid and not to injuries or pH alterations caused by the injections. In hypoxic tissues lactic acid, which is formed in anaerobic glycolysis and is retained in consequence of a decreased venous flow, is synthesized into glycogen by mesenchymal cells of developing vessels. That vascularization is probably closely associated with glycogen metabolism is indicated by the observation that vessels tend to grow in the direction of areas rich in lactic acid. Thus, by promoting normal vascularization, anaerobic glycolysis, which is a more primitive energy-yielding process, assures the normal development of tissues.

REFERENCES

ADLER, F. H. (1930). *Trans. Amer. ophthal. Soc.*, **28**, 307.
ASHTON, N. (1954). *Brit. J. Ophthal.*, **38**, 385.
——— (1957). *Amer. J. Ophthal.*, **44**, No. 4, pt 2, p. 7.

ASHTON, N., and BLACH, R. (1961). *Brit. J. Ophthal.*, **45**, 321.
——— and COOK, C. (1954). *Ibid.*, **38**, 433.
——— WARD, B., and SERPELL, G. (1953). *Ibid.*, **37**, 513.
——— ——— ——— (1954). *Ibid.*, **38**, 397.
CAMPBELL, F. W. (1951). *Trans. ophthal. Soc. U.K.*, **71**, 287.
COGAN, D. G. (1962). *Amer. J. Ophthal.*, **54**, 347.
ELIASOPH, I. I. (1957). *A.M.A. Arch. Ophthal.*, **58**, 495.
FRIEDENWALD, J. S., OWENS, W. C., and OWENS, E. U. (1951). *Trans. Amer. ophthal. Soc.*, **49**, 207.
MICHAELSON, I. C. (1948). *Trans. ophthal. Soc. U.K.*, **68**, 137.
——— (1954). "Retinal Circulation in Man and Animals". Thomas, Springfield, Ill.
———, HERZ, N., LEWKOWITZ, E., and KERTESZ, D. (1954). *Brit. J. Ophthal.*, **38**, 577.
NAKASHIMA, C. (1926). *v. Graefes Arch. Ophthal.*, **116**, 403.
REESE, A. B., BLODI, F. C., and LOCKE, J. C. (1952). *Amer. J. Ophthal.*, **35**, 1407.
SERPELL, G. (1954). *Brit. J. Ophthal.*, **38**, 460.
WARD, B. A. (1954). *Ibid.*, **38**, 445.
VASTARINI-CRESI, J. (1924). *Arch. ital. Biol.*, **73**, 97.

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Notes