VASCULAR CHANGES IN THE BULBAR CONJUNCTIVA ASSOCIATED WITH SICKLE-CELL DISEASE: SOME OBSERVATIONS ON FINE STRUCTURE*

BY *Austin I. Fink*, M.D.

THE MECHANISM OF PAINFUL CRISIS in patients with sickle-cell disease remains obscure. In 1959 an investigation[1] was undertaken to determine whether it was possible to visualize, within the small vessels of the conjunctiva, an increase in the number of sickle cells at time of crisis. The technique developed confirmed that the bulbar conjunctiva was an excellent "window" for the *in vivo* observation of a vascular bed; however, the investigators encountered difficulties with the optical resolution and methodology, which prevented the visualization of individual circulating erythrocytes.

At this time, several investigators[1-3] reported the presence of comma-shaped capillary and venular microaneurysms which appeared to be specific for sickle-cell disease. These microaneurysms, which could be seen in the unexposed area of the bulbar conjunctiva, were concentrated in the inferior temporal quadrant. It was noted that in patients under eleven years of age these ectasias seemed to disappear when exposed to the heat of the examining lamp, but reappeared when a mild vasoconstrictor (Cyclomydril, Schiefflin) was instilled into the eye. It was noted also that this ability to reverse decreases with age, while the number of visible microaneurysms increases. However, older patients with a history of relatively few crises usually presented ectasias that maintained some degree of reversibility.

Light microscope studies[4] of these microaneurysms seemed to indicate some swelling and proliferation of the capillary endothelial cells.

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Based on these findings, the hypothesis was proposed that this swelling and proliferation resulted from repeated irritation by the rigid sickle cell. It was suggested that the trapped sickle cells thus contributed to the development of the "reversible" conjunctival microaneurysms and that the endothelial cell change affected the reversibility.

Realization of the possible misinterpretation of histologic findings in a 6-μ section examined by light microscopy suggested the need to examine with the electron microscope a 1/40-μ section of conjunctival biopsy material. Preliminary viewing under relatively low magnification (3000×) failed to elicit evidence of capillary endothelial cell swelling and proliferation,[5] and indicated the necessity for a more comprehensive study with the electron microscope. To my knowledge, this paper represents the first, earnest utilization of a fine-structure technique (the electron microscope) to examine the vasculature of the bulbar conjunctiva of a patient affected with a hematologic disease in order to understand better the mechanism of the disease.

By chance, conjunctival biopsy material was obtained from a young patient with diagnosed sickle-cell disease and diabetes mellitus. It was then decided to obtain biopsy material from a few patients with diabetes mellitus without sickle-cell disease, as well as from patients with sickle-cell disease without diabetes mellitus. Several subjects with no evidence of either disease were used as controls.

HISTORICAL REVIEW

In 1753, Boerhaave[6] published what appears to be the original study of conjunctival circulation. He described the separation of blood into an axial stream of erythrocytes and a marginal stream of serum. In addition, he noted the aggregation of erythrocytes during episodes of fever, and he believed that this aggregation explained many clinical symptoms. There was no further report in the literature until similar descriptions of conjunctival blood circulation appeared approximately one hundred years later.[7–9] A description of bulbar conjunctival varicosities by Bajardi[10] in 1892 is, apparently, the first study in clinical biomicroscopy. In 1902, Schleich[11] noted the different rates of flow in vessels of different size, as well as the changeability in the direction of flow in the small conjunctival vessels.

The first publication in this country relating to conjunctival circulation appeared in 1911 and described an improved illuminator for studying conjunctival vessels.[12] Clinical papers followed with comment on the rate of capillary blood flow and its alteration after the

local instillation of Dionin and epinephrine,[13] and a study of conjunctival biopsy material with the light microscope.[14] The clinical diagnostic value of miliary microaneurysms, venular dilatation, marked granular flow, and variations in caliber of erythrocytes was discussed by Streiff[15] in an attempt to relate variations in these findings to many systemic diseases. Dennis[16] studied the conjunctival circulation to determine early signs of arteriosclerosis. He misinterpreted physiologic intravascular erythrocytic aggregation as pathologic, but he did describe the minute aneurysms attributed to arteriosclerosis, and he studied conjunctival biopsy material with the light microscope. In addition, he assumed correctly, as Bloch[17] was to confirm later, that the conjunctival circulation was a representative index of the general vascular system. Zeller[18] confirmed earlier observations[11] on the velocity of blood flow in the conjunctival arteries, veins, and capillaries. He also noted marked venous distention and an increase in capillary loop formation in patients with diabetes mellitus.

The phenomenon of intravascular erythrocyte aggregation was first earnestly considered by Fähraeus.[19–22] In a series of comprehensive investigations concerned with variations in the stability of blood suspensions, he suggested that this aggregation was essentially rouleaux formation; he confirmed Nasse's[23] earlier findings of a strong positive relationship between increased sedimentation rate and increased aggregation of erythrocytes. Fähraeus stated that the rouleaux formation resulted from a reversible interaction between the erythrocyte surfaces and the plasma proteins, and while this interaction is usually harmless, he suggested that the resulting decrease in blood flow and increase in blood viscosity may act as a factor in the formation of thrombi in capillaries and veins.

The views of Fähraeus remained uncontested until Knisely and others,[24–27] studying the composition of blood in monkeys infected with *Plasmodium knowlesi* (malaria), stressed the importance of what they called "sludged blood." They maintained that "sludged blood" was fundamentally different from erythrocyte aggregation ascribed to rouleaux formation. The sludge was caused by sticky, sometimes microscopically visible, particles which caused clumping of the blood into irregular masses. Knisely maintained that sludged blood was inconsistent with normal health. This assumption has been contested, reports having been made of (*a*) intravascular erythrocyte aggregation during menstruation,[28] (*b*) the presence of sludged blood in many normal children and adults (where its increase was said to be related to advancing age),[29,30] and (*c*) blood sludge formation as

a product of rouleaux formation and stasis within the capillaries because of the high correlation between the degree of blood sludge formation and erythrocyte sedimentation rate.[31] All investigators agree however that, whether due to rouleaux formation or to a sticky substance being present on the erythrocyte surface, this intravascular erythrocyte aggregation may lead to the development of thrombosis.

Sludged blood may be present in the vessels of the bulbar conjunctiva as a result of systemic disease or from changes in the local conditions of flow. One group of investigators noted that systemic vasodilators produced sludging in normal subjects for one or two hours.[32] Harders[33] placed a piece of ice on the bulbar conjunctiva of patients demonstrating cold precipitable agglutinins and produced marked vasodilatation with granularity of flow. Other investigators obtained a similar result by immersing the patient's arm in icy water.[34]

The systemic conditions of a patient will contribute to sludging in the conjunctival vasculature when the erythrocyte sedimentation rate rises over 30 mm in the first hour.[31] This phenomenon is not disease-specific[35] and has been noted in hypertension,[36] macroglobulinemia, myeloma,[37] and cryoglobulinemia.[38–40] The capillary alterations that have been noted in patients with sickle-cell disease[5,41–43] will be discussed in connection with capillary microaneurysms and ectasias.

The past fifteen years have witnessed a resurgence of interest among investigators of conjunctival microcirculation in the incidence, nature, and significance of conjunctival microaneurysms. The areas of the conjunctiva most suitable for study are those covered by the lids and thus protected from dust, wind, and so forth. The blood vessels in these areas are a good index of the systemic circulation.

Davis *et al.*[44] have provided a very satisfactory classification of conjunctival microaneurysms or, as they call them, "micropools." They are divided into three types: Type I, the micropool which is seen on only one side of the venule (referred to by some investigators as a saccular microaneurysm); Type II, the micropool which is symmetrical around the venule (occasionally referred to by others as fusiform in shape); and Type III, as described by Davis, "where the micropool at first sight may seem to be without vascular connection but on careful focusing, a fine capillary can be seen to enter and leave the vascular formation" (often referred to as the "berry" type). They are noted more often on the nasal side of the bulbar conjunctiva than on the temporal side.

It has been noted in older patients that microaneurysms may change in appearance within a period of one week.[45] (It is important to mention that, with the exception of sickle-cell disease, no one type or

combination of types of micropools (microaneurysms) can be correlated with any specific disease.[44]) This point is further emphasized by the fact that similar vascular changes have been observed in apparently normal individuals.[46] The incidence of conjunctival microaneurysms in a normal population seems to vary with the investigator. One study noted no "true" microaneurysms in 90 normal adults;[47] another reported an incidence of 10 per cent in 500 normal subjects;[48] still another study reported an incidence of 13.7 per cent in 161 normal subjects.[44] Perhaps the difference in incidence rate lies in how the individual investigator defines a microaneurysm.

Although it is generally recognized that conjunctival microaneurysms may be related to local conditions such as rosacea or mustard gas, the majority of studies have been concerned with the systemic etiology of these vascular irregularities. The systemic diseases which have attracted the attention of the majority of investigators include arteriosclerosis, diabetes mellitus, hypertension, and, more recently, sickle-cell disease. The earlier investigators concerned themselves with changes induced by arteriosclerosis, a disease responsible for the greatest incidence of conjunctival microaneurysms. It was proposed that vessel caliber alteration might be attributed to arteriosclerosis,[8,13,14] and a detailed description of the conjunctival microaneurysms related to this disease appeared.[16] More recent investigators of the subject have noted that the conjunctival signs of arteriosclerosis may antedate ophthalmoscopic findings;[49] they have quantitated the incidence of micropools in this disease[50] and have observed such changes more often in men than in women.[51] Recently, a relationship has been established between arteriosclerotic changes in the vessels of the bulbar conjunctiva and the incidence of coronary thrombosis.[52,53]

Patients suffering from benign hypertension often do not exhibit changes in the small vessels of the bulbar conjunctiva, although they will show capillary narrowing when the diastolic pressure exceeds 120.[54] When arteriosclerosis is associated with hypertension, the vessel changes are often quite marked. Extreme vaso-obliteration of the limbal capillaries has been observed in cases of severe hypertension,[55] and it has been proposed that limbal microaneurysms in these patients may indicate kidney pathology.[56] Variations in vessel caliber (microaneurysms, increased vessel tortuosity with narrowing) have been recorded by many investigators.[57–59] General telangiectasis, multiple microaneurysms, and areas of marked convolution and coiling have been observed in extreme cases of hypertension.[60] The precapillary sphincters show an increased sensitivity to topical epinephrine,[61] a phenomenon that is

observed in gestation[62] and exaggerated with the toxemia of pregnancy.[63] Administration of an antihypertensive agent (Ansolysen, Wyeth) will dilate the conjunctival capillaries in patients with known hypertension.[64]

Bajardi[10] was the first investigator to mention conjunctival microaneurysms occurring in diabetic patients, and this observation was confirmed somewhat later.[13] Light microscope studies of these conjunctival microaneurysms have demonstrated capillary endothelial proliferation and basement membrane changes similar to those observed in retinal vessels.[67] Further studies have indicated a difference between the findings in known diabetic patients and in those with arteriosclerosis and without diabetes.[73] A review of the literature indicates that some controversy exists as to the incidence of these capillary alterations in diabetic patients. McCulloch and Pashby[65] have reported a high incidence of 55 per cent (55 out of 100 patients), confirming the results of earlier studies.[45] Other investigators also have reported a high incidence of capillary microaneurysms: 62 per cent (25 out of 40 patients),[66] 64 per cent (14 out of 22),[67] 35 per cent (88 out of 250),[68] and 27 per cent (20 out of 75).[69] Some studies, however, have reported a significantly lower incidence of observed conjunctival micropools in diabetic patients: Friedenwald, 5 per cent;[70] Ditzel, 2 per cent;[71] and Labram and his associates, 5–10 per cent.[72] The marked variations in these figures would indicate that some confusion exists as to whether these vessel changes are specific for diabetes. Cook[68] was first to discount the significance of these conjunctival changes and questioned a significant relation to the diabetic process. This opinion (most current) has been seconded by other observers.[72,74,75] It has been suggested recently that prolonged arteriolar and capillary constriction with erythrocyte aggregation need not be accompanied by conjunctival microaneurysms, but may offer an explanation for the origin of diabetic microangiopathy.[76] The ultimate answer for clarification might be found in Cogan's suggestion of a double-blind study.[77]

The capacity of sickle-cell hemoglobin under conditions of low oxygen tension to form "tactoids,"[90] to change the shape of the erythrocyte, and thus to alter blood viscosity has attracted the attention of investigators of microcirculation. In 1952, deQuevedo,[41] a student of Knisely, described venular sacculations of the bulbar conjunctiva, which he believed to be characteristic of sickle-cell disease. He noted their concentration in the inferior temporal quadrant on the unexposed portion of the bulbar conjunctiva. These findings were confirmed by a number of investigators [1–3,42,43,78,79] who also observed the comma and

corkscrew shapes of these capillary and venular alterations. Although unaffected by oxygen inhalation,[80] microaneurysms or ectasias in children under eleven years of age seemed to disappear when exposed to heat, and to reappear after instillation of a mild vasoconstrictor.[5]

Similar vascular changes were reported for some of the variants of sickle-cell (Hb S-S) disease. Paton[43] described some capillary microaneurysms in patients having S-C and S-D disease, as well as in those with certain types of sickle thalassemia (S-Thal.). The highest incidence and the greatest number of conjunctival microaneurysms, however, have been observed in patients with homozygous (Hb S-S) sickle-cell disease.

The number and degree of these capillary alterations increased during crisis and decreased with transfusion.[5] However, limitations in methodology did prevent the visualization of individual circulating erythrocytes. Similar difficulties were encountered when a 6-μ section of the conjunctival vessel was studied by light microscopy.[4] This has encouraged me to examine similar biopsy material with the electron microscope to understand better the mechanism and symptomatology of painful crisis.

MATERIALS AND METHODS

Material for biopsy was obtained from the bulbar conjunctivas of seven patients with sickle-cell anemia who, on slit-lamp examination, had demonstrated evidence of the disease-specific corkscrew- and comma-shaped microaneurysms and venous sacculations.[1,2] These ectasias were observed throughout the entire bulbar conjunctiva but were concentrated in the unexposed portion of the inferior temporal area. A complete physical examination with laboratory studies ruled out the presence of intracurrent infection. The diagnosis of S-S hemoglobin was confirmed by sickle-cell preparations and paper electrophoresis. The group ranged in age from ten to forty-six years. Seven patients with no evidence of sickle-cell or other disease were included in the control group and ranged in age from two to ninety-two years. One 28-year-old patient with sickle-cell disease was known also to have diabetes and this prompted the acquisition of conjunctivas from three patients (aged sixty-six to eighty-two years) with known diabetes who showed evidence of conjunctival microaneurysms.

The conjunctival vessels and ectasias were photographed prior to biopsy. The optics included a 63-mm Leitz Photar lens attached to a bellows extension which was, in turn, connected to a Leitz III Visoflex housing and a Leica M-2 camera. The combined apparatus was mounted on the base of a Leitz ophthalmic microscope. Illumination

was provided by a microflash unit with a capacity of 25 watt-seconds. Agfa Agepe FF orthochromatic film (ASA 12) was used to photograph the vessels. The Photar lens gave a magnification of 7× on the negative. Suitable portions of the negative were enlarged. The area photographed was also the site of biopsy.

The conjunctivas of all patients and all controls were anesthetized with Ophthaine (Squibb) eye drops. A Haag-Streit slit lamp or a Zeiss operating microscope, with magnifications of 16× and 25× respectively, was used to visualize and to excise the superficial bulbar conjunctiva. The blood vessels in question and the adjacent conjunctiva were grasped with a Bonn forceps. Excision was completed with straight iris scissors and the tissue was immediately placed in fixative.

The acquisition of conjunctivas from patients with diabetes proved more difficult, because the vascular changes (including microaneurysms) were localized in the deep episcleral layer. Blood vessels in the superficial layer that seemed to demonstrate increased venous dilatation were used for biopsy.

FINE-STRUCTURE TECHNIQUE

After biopsy, the material was placed immediately into a Dalton's chrome-osmic solution (*p*H, 7.4) or phosphate-buffered osmic acid (*p*H, 7.4) and kept at room temperature.[81] After fixation (one to four hours) the tissue was rinsed in a number of changes of formalin until the leaching of chromate was completed. The tissue, after being post-fixed overnight in 10 per cent neutral or buffered (*p*H, 7.4) formalin, was cut in formalin under a dissecting microscope and placed in a solution of warm (48°– 52° C) agar. The tissue and agar were kept at this temperature for ten to fifteen minutes, and then the agar was allowed to harden. Alcohol (70 per cent) was added to harden further the agar, which was cut into small cubes, each containing a piece of tissue. After dehydration in graded alcohols and three changes of 100 per cent alcohol, the alcohol was replaced with methacrylate monomer composed of 50 per cent methylmethacrylate and 50 per cent butylmethacrylate without catalyst, and the agar cubes were allowed to stand overnight at 4° C.

The next day, after changing the monomer without catalyst to monomer containing 1.5 per cent azodi-isobutyronitrile (ADIB)[82] as catalyst, the tissue was placed in capsules containing lightly prepolymerized plastic to which 1.5 per cent ADIB and 0.07 per cent uranyl nitrate[83] had been added. Polymerization was completed at 47° C.

Additional conjunctival biopsy material, after dehydration in graded

alcohols followed by three changes in propylene oxide, was allowed to stand overnight in a 1 : 1 mixture of propylene oxide and the Epon-Araldite embedding mixture No. 1 of Mollenhauer.[84] The tissue was then placed in flat dishes in fresh Epon-Araldite and polymerized overnight at 80° C.

A preliminary survey of the biopsy material was made by examination with the light microscope of thick sections (0.5–1.0 μ) which were cut on a Porter-Blum MT 1 microtome, mounted on glass slides, and stained with toluidine blue. For more detailed study, the biopsy material was examined with the electron microscope. Silver to light-gold sections approximately 1/40 μ in thickness were picked up on copper grids whose Formvar had been stabilized with a thin film of evaporated carbon. The sections were stained with either uranyl acetate, or uranyl acetate followed by lead citrate. Electron miscroscope observations were made with Siemens Elmskop I or IA at direct magnifications of 1000 to 20,000×. Photographic enlargements were made to the desired magnification.

OBSERVATIONS

The blood vessels of the bulbar conjunctiva are divided into a superficial and a deep vascular network. The two systems anastomose through vessels which course in a potential space containing loose connective tissue that lies between the conjunctiva and the episclera. The superficial vessels lie in a connective tissue framework beneath an epithelium four to five cells deep and are composed of arterioles, capillaries, and venules. Gliding movements of the loosely attached bulbar conjunctiva are reflected in a similar shifting of the superficial vessels. The deep episcleral vessels are fixed, more regular in course, and embedded in a denser connective tissue. The visibility of the deep vessels depends upon the transparency of the superficial layer.

IN VIVO OBSERVATIONS

A microaneurysm has been defined by Ashton[85] as either a "diverticulum in the capillary basement membrane" or "a result of nothing more specific than stasis or engorgement of the capillaries." Conjunctival microaneurysms in the superficial vascular network that have been reported as specific for sickle-cell disease were seen in all the biopsy material obtained. I have observed, over a period of many years, that 90 per cent of the cases studied have demonstrated these capillary microaneurysms. It is not unreasonable to assume that similar

ectasias may be present elsewhere in the body where the capillary bed is localized within a framework of loose connective tissue. The comma- or corkscrew-shaped microaneurysms were visualized within the superficial layers of the bulbar conjunctiva toward the venous side of the capillary circulation and concentrated in that portion of the inferior temporal quadrant that is protected by the lower lid (Figure 1). Reactions of the microaneurysms to crisis, local stimuli, and aging have already been described.[1,5] One patient with sickle-cell disease and diabetes demonstrated a great many microaneurysms, all typical of sickle-cell disease (Figure 2). This observation encouraged some extension of the study to include bulbar conjunctival tissue from the inferotemporal site of three other diabetic patients with no evidence of sickle-cell disease but with evidence of conjunctival microaneurysms.

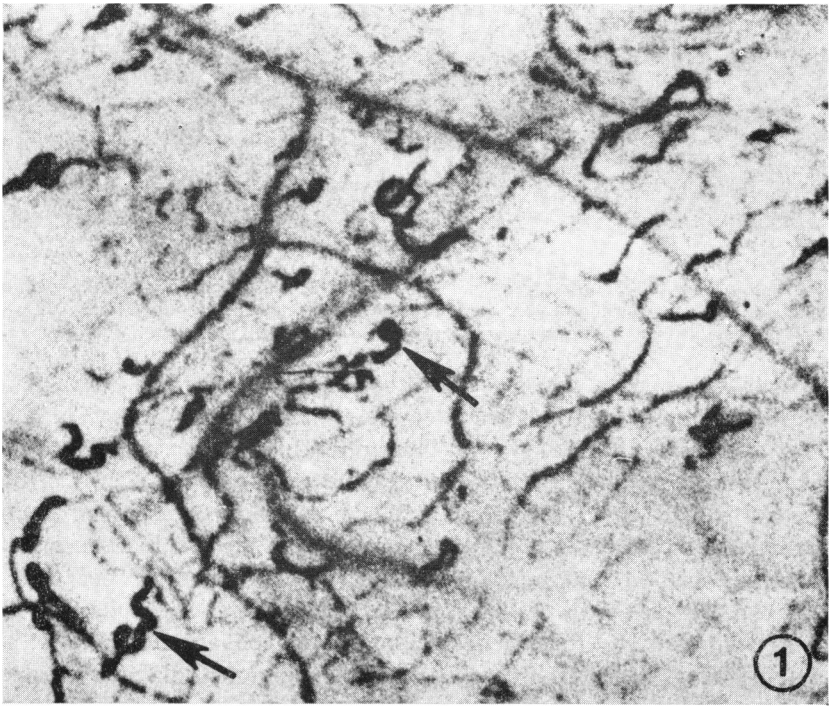

FIGURE 1

Photograph of conjunctival blood vessels of a 33-year-old Negro female with a history of sickle-cell anemia and crises since eight years of age. The disease-specific corkscrew- and comma-shaped microaneurysms (arrows) are observed in the postcapillary venules (×125).

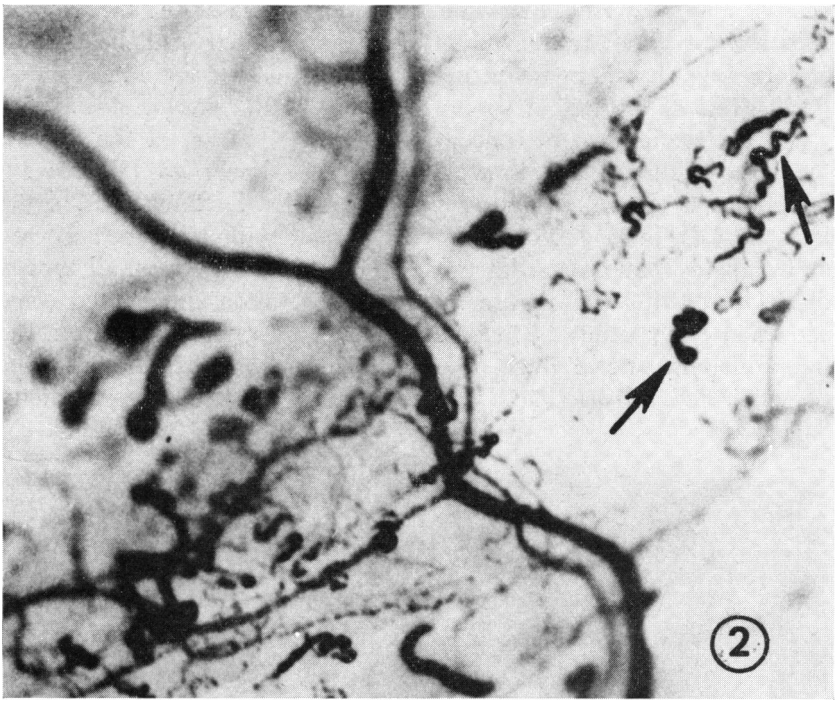

FIGURE 2

Photograph of conjunctival vessels from a 28-year-old Negro with a history of sickle-cell disease and diabetes (see Figure 10). Corkscrew- and comma-shaped microaneurysms (arrows) specific for sickle-cell anemia are noted (×125).

In patients with diabetes mellitus capillary microaneurysms are more commonly fusiform in appearance but may also be saccular or berry-shaped[44] (Figure 3), and most often are located nasally in the deep, fixed episcleral layer of the conjunctiva. Elderly patients in the control group often exhibited capillary microaneurysms similar to those seen in diabetic patients. Patients with sickle-cell disease will generally exhibit conjunctival microaneurysms (over 90 per cent), whereas patients with diabetes mellitus will exhibit such changes far less frequently.[68,71,77] When present, these changes may not be pathognomonic for the disease. In a recent study of 50 diabetic patients examined with the slit lamp, the author noted that the incidence of microaneurysms was only 24 per cent (12 cases). It was observed that, regardless of the duration of the diabetes, the number of microaneurysms increases with age, and *in vivo* differentiation between diabetes mellitus and arteriosclerosis could not be established.

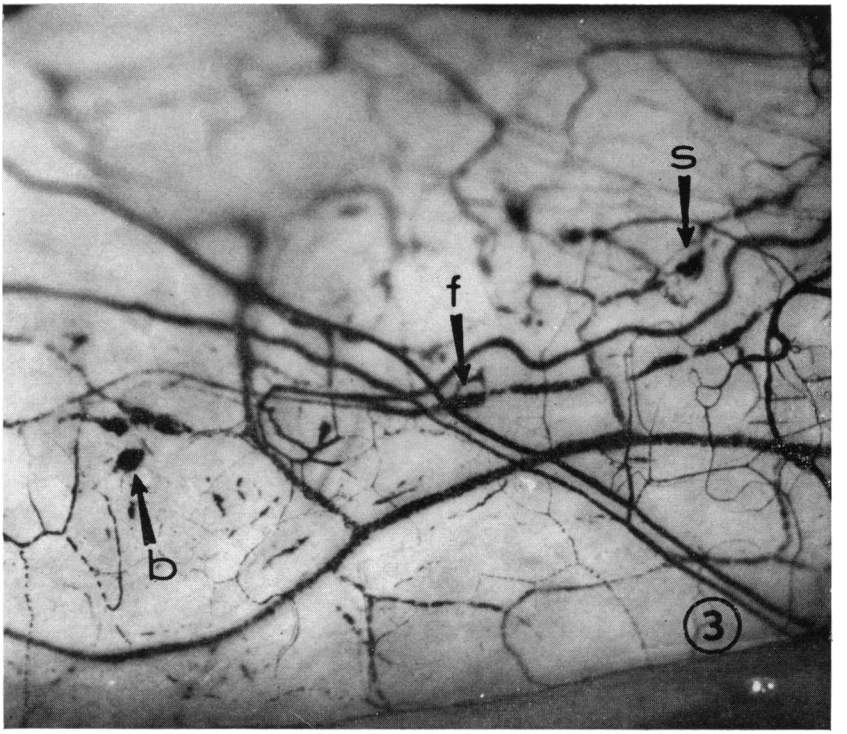

FIGURE 3

Photograph of conjunctival vessels from a 66-year-old female with a history of diabetes mellitus. Fusiform (f), saccular (s), and berry (b) microaneurysms are observed in the small venules (×75).

ELECTRON MICROSCOPIC OBSERVATIONS

GENERAL DESCRIPTION OF VASCULAR BED. Electronmicroscopic examination of the biopsy material of the bulbar conjunctiva reveals blood vessels in the superficial layer of connective tissue located beneath the epithelium and surrounded by collagen and cells (plasma cells, mast cells, macrophages, and fibroblasts). The blood vessels studied included arterioles with a single layer of smooth muscle (8–9μ), capillaries with a single or partial pericyte layer (4–8μ), and venules which were distinguished by the fact that they acquired more layers of pericytes than the capillaries as they increased in diameter (9–15μ).

MICROANEURYSMS. The microsurgical technique for conjunctival biopsy described in Materials and Methods permitted electron microscopic demonstration of conjunctival microaneurysms in two patients with sickle-cell disease. In one patient a microaneurysm was identified

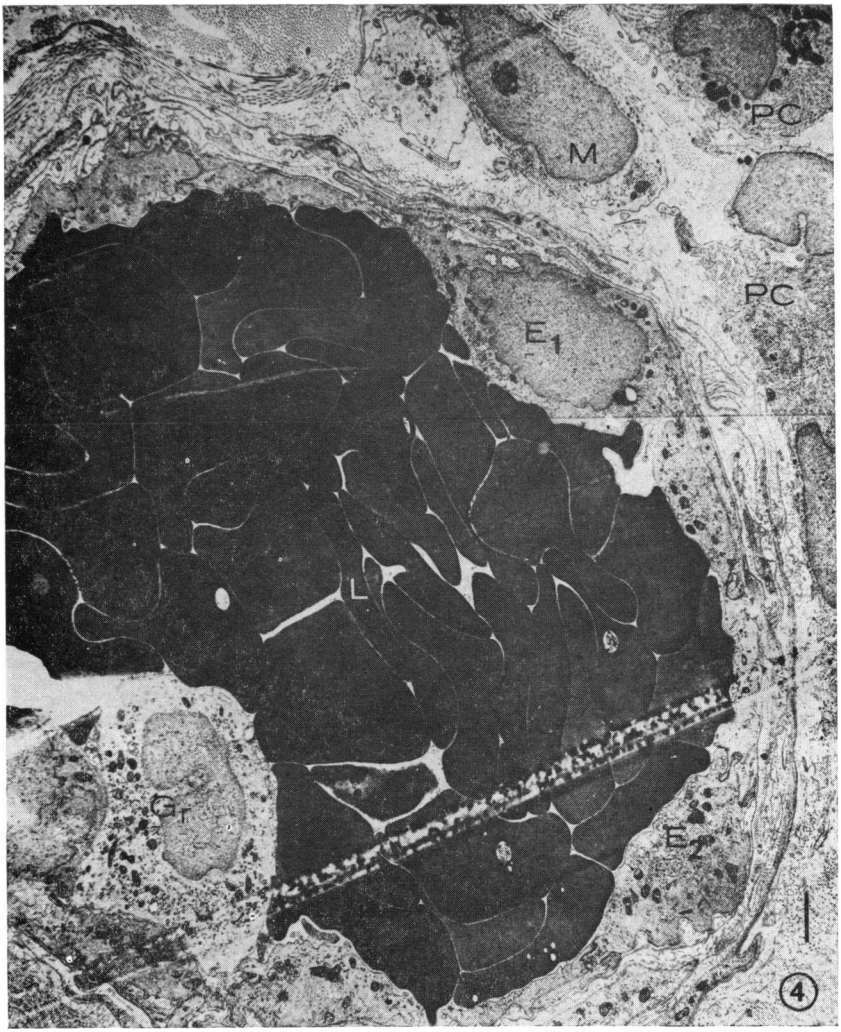

FIGURE 4

Venule packed with erythrocytes, presumably an aneurysm, from the bulbar conjunctiva of a 10-year-old Negro male. History of sickle crisis began at two years of age. Red blood cell packing is so tight that the erythrocytes and endothelial cells conform to each other at point of contact. Endothelial cells (E_1, E_2) show no change from controls (Figure 7). The only plasma visible is noted outside the lumen in proximity to the neutrophilic granulocyte (Gr) which is shown in detail in Figure 5. Plasma cells (PC) and a macrophage (M) are observed in the connective tissue and in proximity to the blood vessel. Chrome-osmium fixation (×8,750, reduced by ⅓).

by the large size of its lumen in comparison to wall thickness and by tight packing of erythrocytes; these findings thus parallel the *in vivo* observations (Figure 4). Pressure from erythrocyte packing was so marked in one instance that two endothelial cells were forcefully separated. The only plasma noted was localized under these cells, and its outer limit was defined by a portion of a pericyte (Figure 5). In the second patient, a microaneurysm revealed a mass of erythrocytes that seemed to have a sickle shape. A clump of lysing neutrophilic granulocytes was observed within the lumen of this ectasia (Figure 6). In diabetic patients the localization of conjunctival microaneurysms within the deeper (episcleral) layer of the bulbar conjunctiva made biopsy impossible; bleeding from the superficial vascular bed obscured the deep conjunctival microaneurysms and precluded satisfactory biopsy. Excision of biopsy material from the superficial layers and examination of small engorged venules failed to demonstrate erythrocyte packing. In this respect, the findings in the diabetic patient were no different from those in the control group.

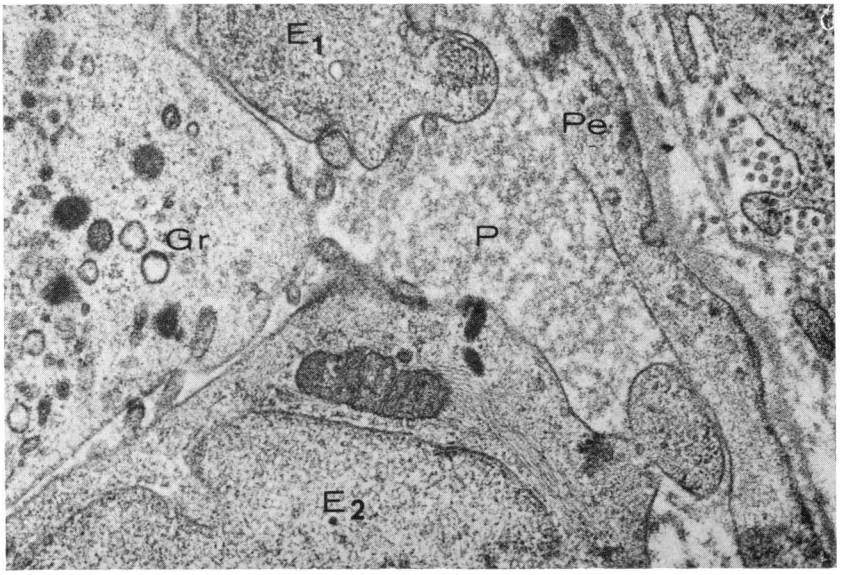

FIGURE 5

A detail of Figure 4. The plasma (P) is observed outside the lumen and between the two endothelial cells (E_1, E_2) which have an opening between them. A portion of a pericyte (Pe) defines the outer limit of plasma extension. Chrome-osmium fixation (×52,500, reduced by ⅓).

Gr
6

ENDOTHELIAL CELLS. Blood vessels are lined by a single layer of flattened endothelial cells (Figure 7). The thin extremities interdigitate with similar processes from adjacent endothelial cells. The inner wall is less regular and bulges into the lumen at the nuclear region, in contrast with a smoother outer cell wall whose limit is defined by a basement membrane. The cytoplasm contains mitochondria, ergastoplasm, a few free ribosomes, juxtanuclear Golgi's apparatus, pinocytotic vesicles, bundles of filaments coursing in various directions, and the more recently described thin, tapering cylindrical bodies of unknown origin[86] (Figure 8). Specimens obtained from patients with sickle-cell disease showed no alteration of the cells; in this respect, there was no difference between the sickle-cell disease patients and the control group (Figure 9). There seemed to be no evidence of endothelial cell swelling or proliferation regardless of the patient's age and whether or not the area studied contained a microaneurysm (Figure 4). Examination of biopsy material from diabetic patients did not reveal any positive findings.

PERICYTES. Patients with sickle-cell disease failed to exhibit any significant pericyte alteration when compared with controls. These mural cells—which are enclosed by the basement membrane of the vessel wall that splits to surround them—did not seem to exhibit a structural change with age and had a structural integrity that seemed unaffected by the presence of a microaneurysm (Figures 4 and 5). Subjects with diabetes, including the patient with sickle-cell disease and diabetes, often exhibited a vacuolation of the cytoplasm (Figure 10). Technical difficulties which prevented excision of the deeply situated conjunctival microaneurysms in diabetic patients similarly precluded evaluation of the direct relation between pericyte cytoplasmic vacuolation and capillary microaneurysm in diabetes mellitus. Vacuoles within the pericyte cytoplasm were found also in a 50-year-old normal male. The conjunctiva of this patient, however, seemed to demonstrate a tendency toward the formation of vacuoles in many of the cells (endothelial, pericyte, and connective tissue) (Figure 11).

BASEMENT MEMBRANE. The basement membrane, which forms the outer limit of endothelial cells and surrounds the pericytes of small

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FIGURE 6

Venule, presumably an aneurysm, bulbar conjunctiva, in a 21-year-old Negro male with a history of sickle crises since eight years of age. Last crisis fifteen days prior to biopsy. Bizarre-shaped, sickle-shaped red blood cells at varying stages of maturation are noted throughout the lumen. A mass of lysing granulocytes (Gr) are seen within the lumen. Osmium fixation (×2,700).

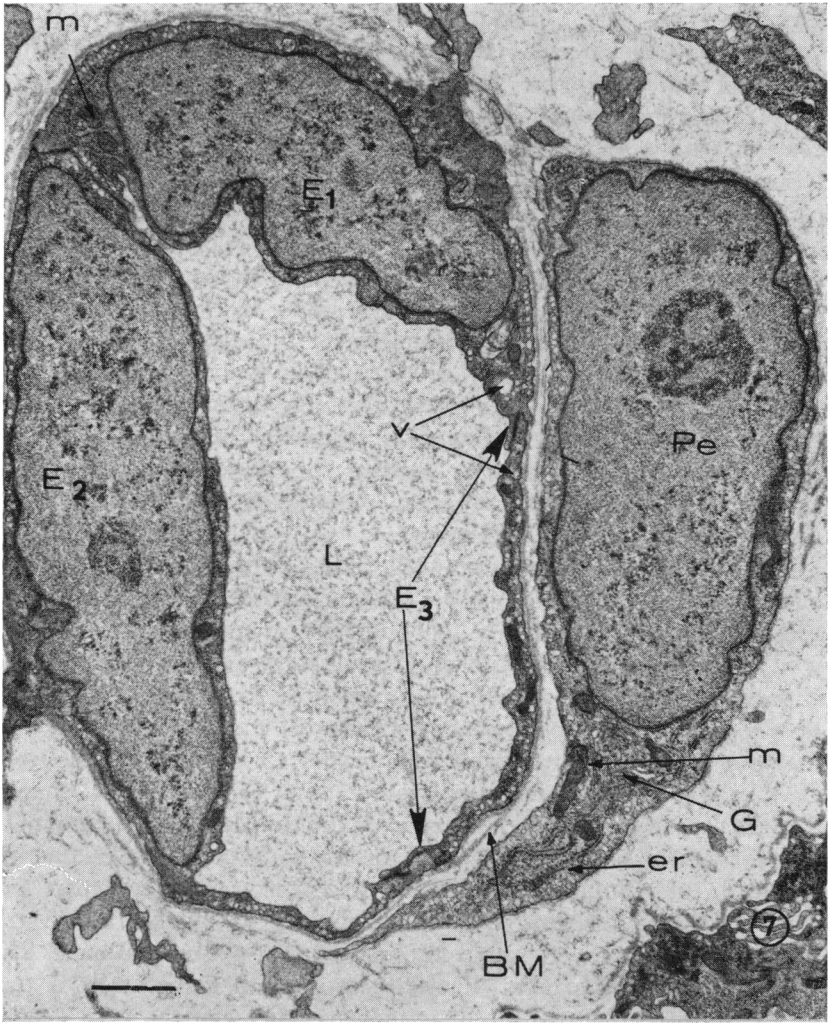

FIGURE 7

Capillary, bulbar conjunctiva in a 92-year-old White female. The flocculant material in the lumen (L) is plasma. Portions of three endothelial cells (E_1, E_2, E_3) line the lumen. The nuclear region of E_1 and E_2 bulge into the lumen in contrast to the very thin non-nucleated region of E_3 which presents a more irregular inner cell surface. A fine felt-like basement membrane (BM) lies beneath the endothelial cells and surrounds the pericyte (Pe). The cytoplasm of the pericyte demonstrates a small quantity of ergastoplasm (er) and Golgi's apparatus (G). Mitochondria at (m). Chrome-osmium fixation (×16,600, reduced by ⅓).

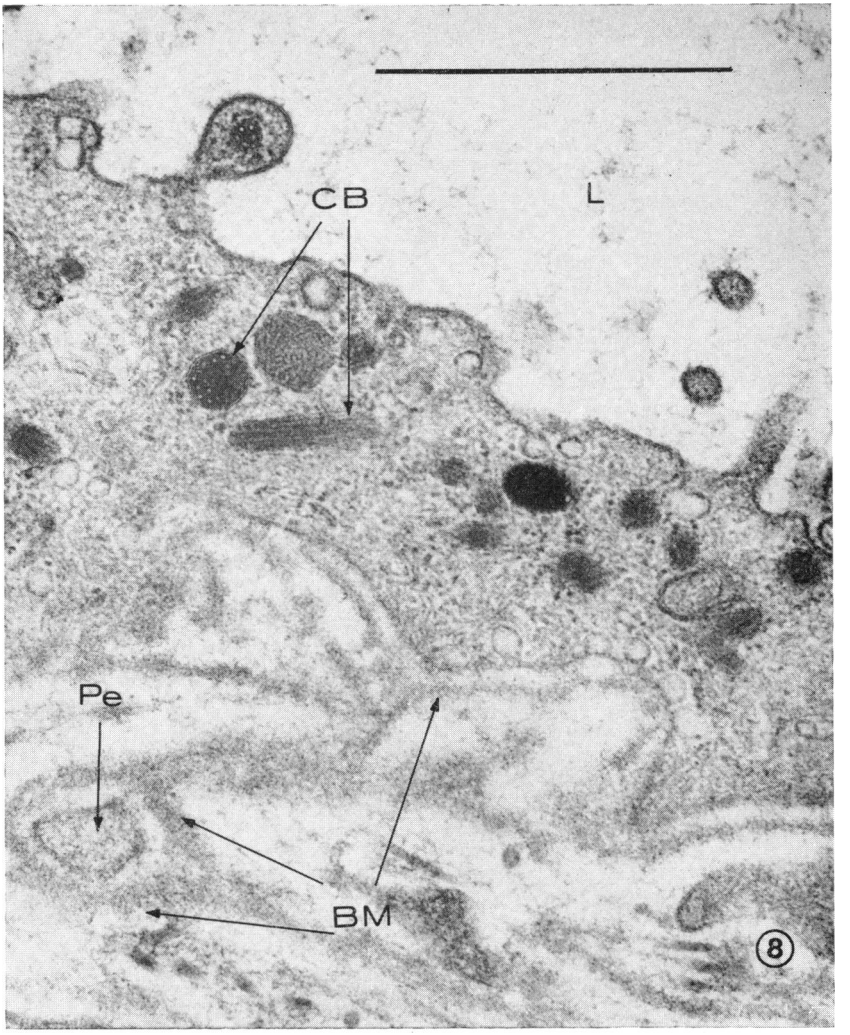

FIGURE 8

Conjunctiva in 28-year-old Negro with sickle-cell disease, and diabetes mellitus of two years' duration. History of sickle-cell crises since early childhood. A detail of a portion of endothelial cell cytoplasm with the underlying felt-like fibrillar basement membrane (BM) which branches to enclose a portion of a pericyte (Pe). Cylindrical bodies (CB) are noted. On cross section they appear round, with a central area containing rather evenly spaced "tubules" of uniform diameter. Their function is unknown. Chrome-osmium fixation ($\times$70,000, reduced by ⅓).

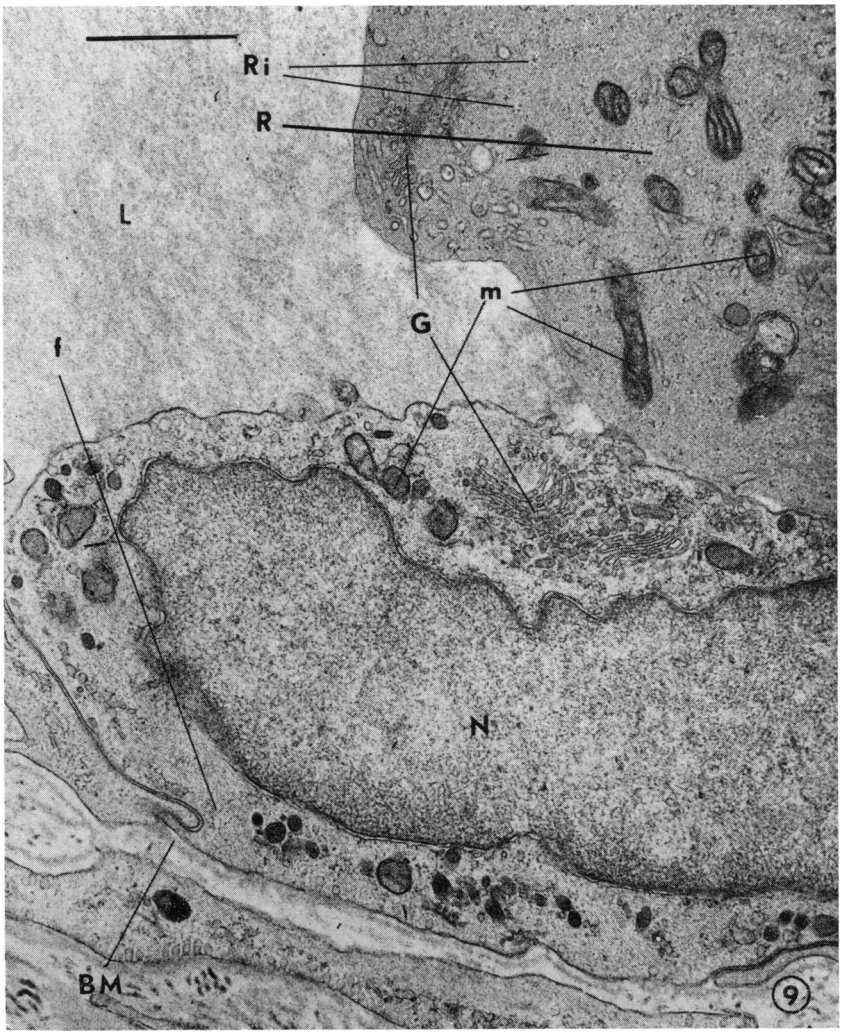

FIGURE 9

Conjunctiva, 33-year-old Negro female with a history of sickle-cell crises since seven years of age. The lumen (L) of the small venule contains a reticulocyte which, in turn, contains mitochondria (m), Golgi (G), and scattered ribosomes (Ri). The endothelial cell shows no change from controls (Figure 7). The nucleus (N) is clearly defined, and in the cytoplasm of the endothelial cell are a mitochondrion (m), Golgi's apparatus (G), and bundles of filaments (f). Basement membrane (BM); pinocytotic vesicle (v). Chrome-osmium fixation (×30,000, reduced by ⅓).

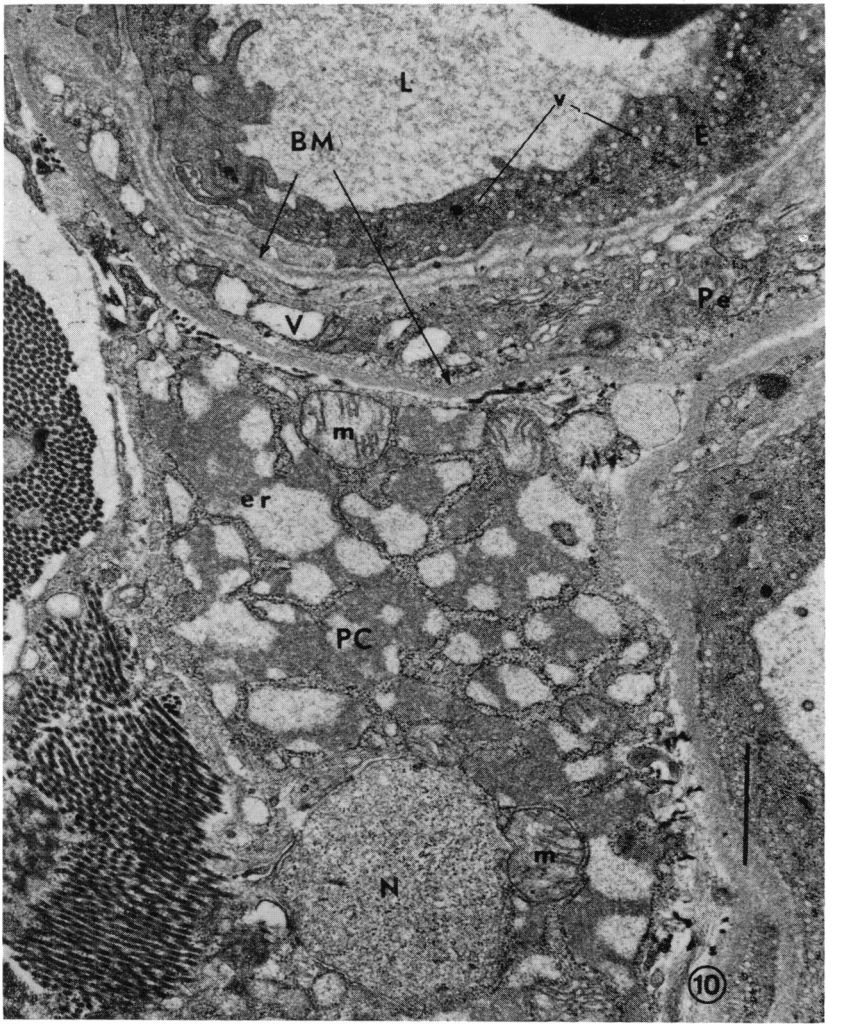

FIGURE 10

Conjunctiva in 28-year-old Negro male with a history of sickle-cell crises since early childhood and of diabetes mellitus of two years' duration. A portion of the wall of a venule is noted that demonstrates an endothelial cell in the cytoplasm of which appear many pinocytotic vesicles (v). The underlying basement membrane divides to enclose a pericyte (Pe) with vacuolation (V) of its cytoplasm. Adjacent to the outer venular wall a large, possibly dying, plasma cell is noted. The ergastoplasmic sacs (er) are dilated and filled with material of two densities. Swollen mitochondria (m) are present. Lumen of blood vessel (L); nucleus (N). Chrome-osmium fixation (×24,000, reduced by ⅓).

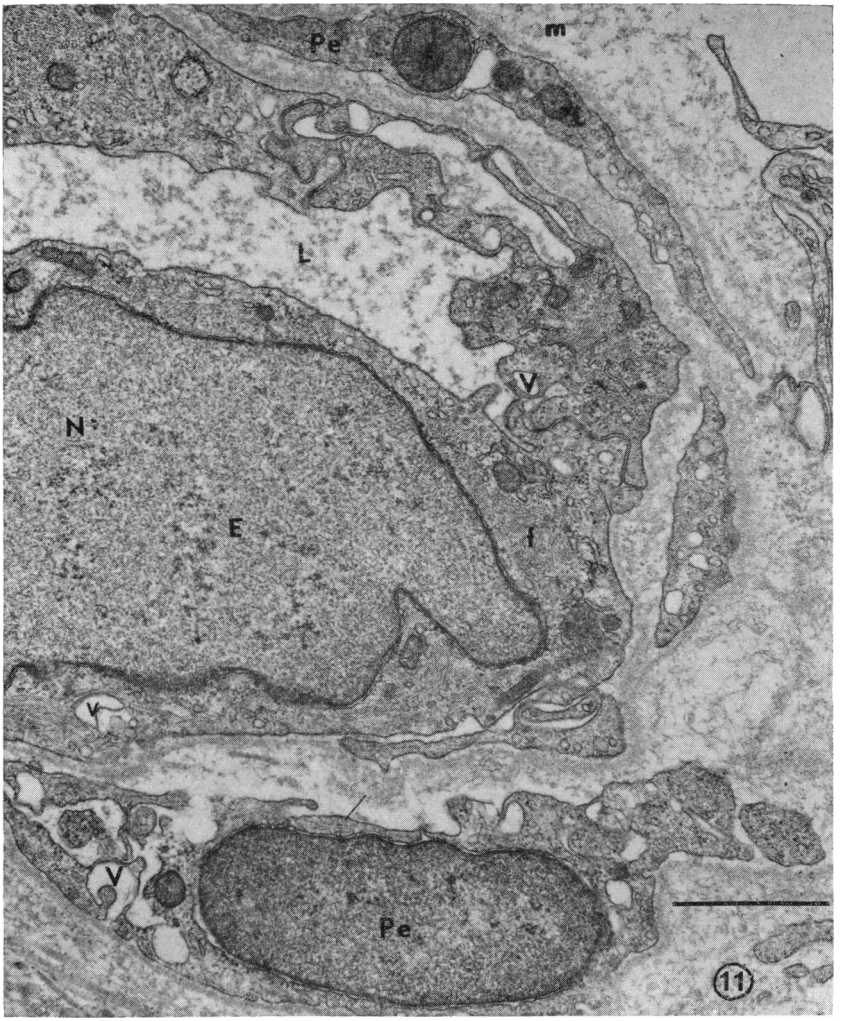

FIGURE 11

Conjunctiva in a 50-year-old White male. Section through a venule. Many vacuoles (V) are observed in the pericytes (Pe), and a few in the endothelial cells. Lumen (L); bundles of filaments (f); nucleus (N); mitochondrion (m). Chrome-osmium fixation (×30,000, reduced by ⅓).

blood vessels, is usually seen as a thin, felt-like structure. People apparently free from conditions which generally affect this membrane may demonstrate variations in this structure. At times it may appear as a thin lamella that branches to enclose the pericytes (Figure 4); in other instances, it may be a broad homogeneous structure (Figure 12). In this study, examination of tissue obtained from patients with sickle-cell disease did not reveal any evidence of basement membrane change except for occasional pleating or folding of the lamella (Figure 13). Biopsy material obtained from patients with diabetes mellitus often illustrated thickening and increased lamination of the basement membrane which surrounds the capillaries (Figure 14), although controls in the same age group demonstrated changes that were not dissimilar (Figure 15).

CELLS OF THE CONNECTIVE TISSUE. Patients with sickle-cell disease, when compared with controls, revealed an apparent increase in the number of plasma cells in proximity to the small vessels of the conjunctiva (Figure 16). This gamma globulin-producing cell, like all cells producing protein for export, demonstrated a large amount of ergastoplasm. A similar increase in the number of plasma cells was found in the patient with both sickle-cell disease and diabetes. Macrophages were encountered occasionally in the vicinity of plasma cells (Figure 16). Other cellular components of loose connective tissue, such as fibroblasts and mast cells, were observed. The observation of diapedesis with the presence of neutrophilic granulocytes in the tissue near small blood vessels in biopsy material obtained from patients with sickle-cell disease indicated an inflammatory reaction (Figure 17).

ERYTHROCYTE DESTRUCTION. Accelerated destruction of the fragile sickle cell was evidenced by the frequency with which reticulocytes and immature red blood cells were observed at all stages of maturation (Figure 18). Observation of an apparent increase in the phagocytosis of erythrocytes by endothelial cells in patients with sickle-cell disease reconfirms an earlier observation[5] and lends support to the impression of an increase in local red cell destruction. Biopsy material acquired from patients with diabetes mellitus offered no evidence of increase in the hemolysis or fragmentation of red blood cells.

DISCUSSION

Ashton[85] has noted that microaneurysms may result from "nothing more specific than stasis and engorgement of the capillaries." It is not surprising to have found them so frequently and so numerously

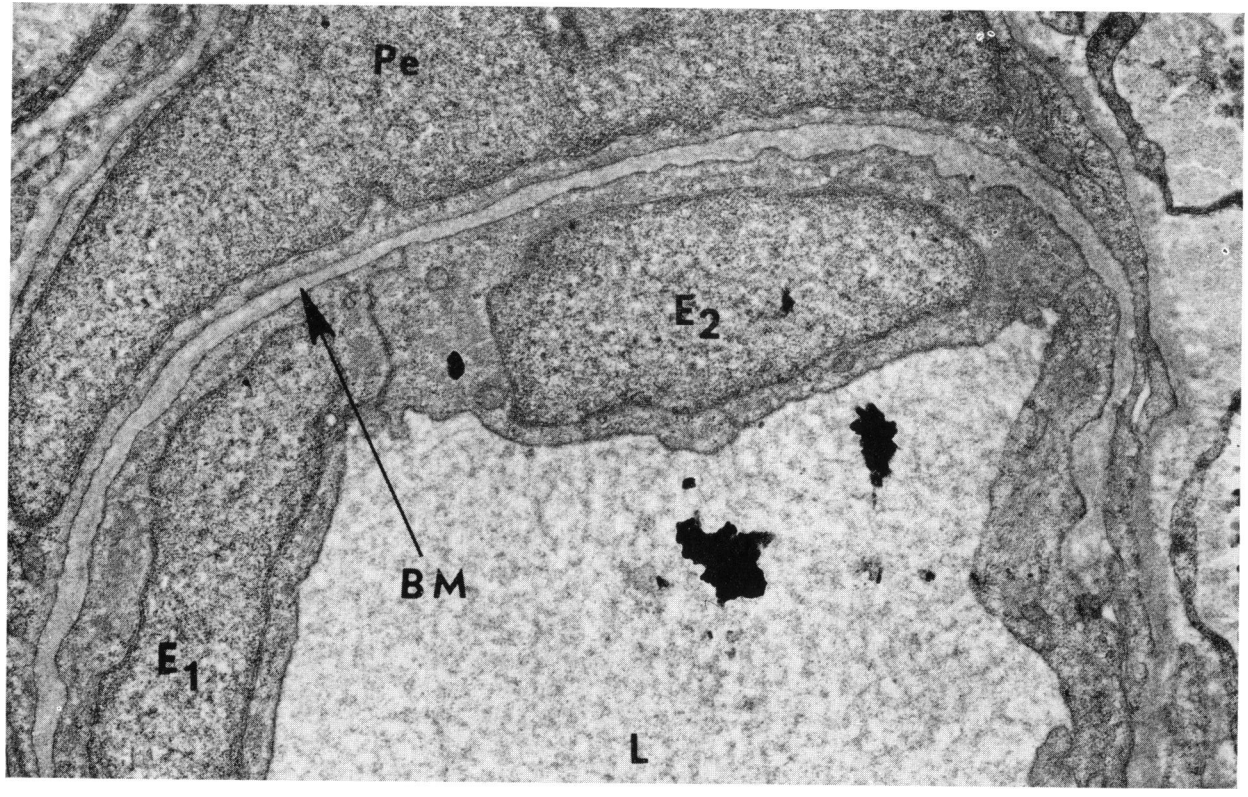

FIGURE 12

Conjunctiva in a 2½-year-old Negro with AA hemoglobin. Portions of five endothelial cells, two with nuclei (E_1, E_2) comprise the lining. A broad homogeneous basement membrane (BM) lies beneath them and surrounds the pericyte layer (Pe). Chrome-osmium fixation (×20,000).

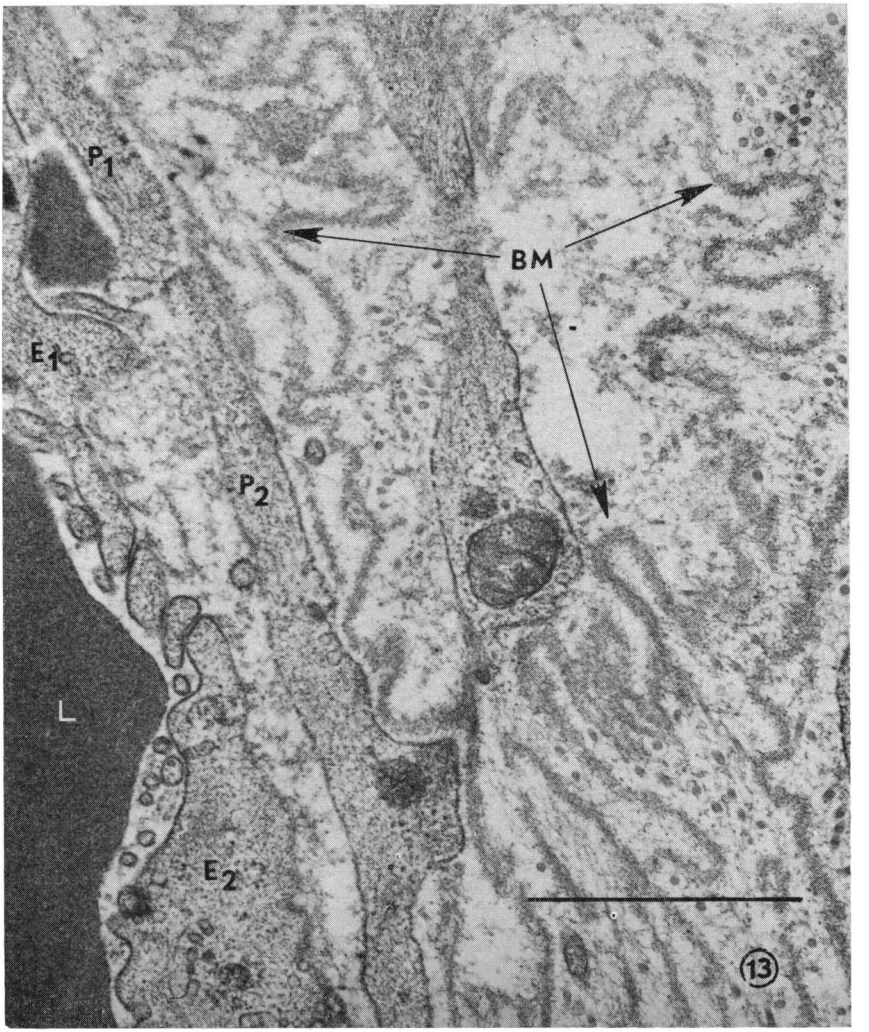

FIGURE 13

Conjunctiva in a 10-year-old Negro male (a detail of same vessel as in Figure 4). Lumen of venule (L) is packed with erythrocytes and is bordered by portions of endothelial cells (E_1, E_2) and pericytes (Pe_1, Pe_2). Pleating and folding of the thin felt-like basement membrane (BM) is noted (at arrows). Chrome-osmium fixation (×55,000, reduced by ⅓).

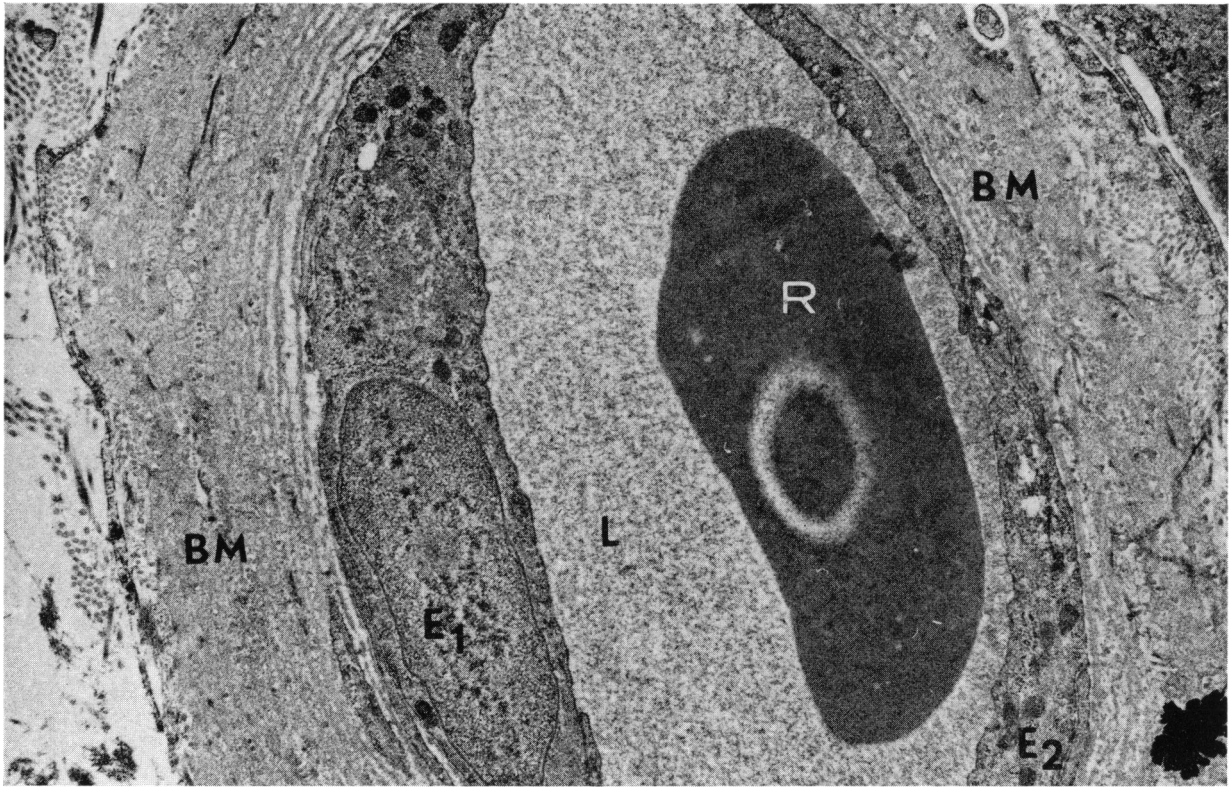

FIGURE 14

Capillary of conjunctival vessel obtained from an 82-year-old female with a twenty-year history of diabetes mellitus. A single erythrocyte (R) is observed in the vessel lumen (L), lined by two endothelial cells (E_1, E_2). The basement membrane is thickened and shows an increase in lamination. Chrome-osmium fixation ($\times$14,500).

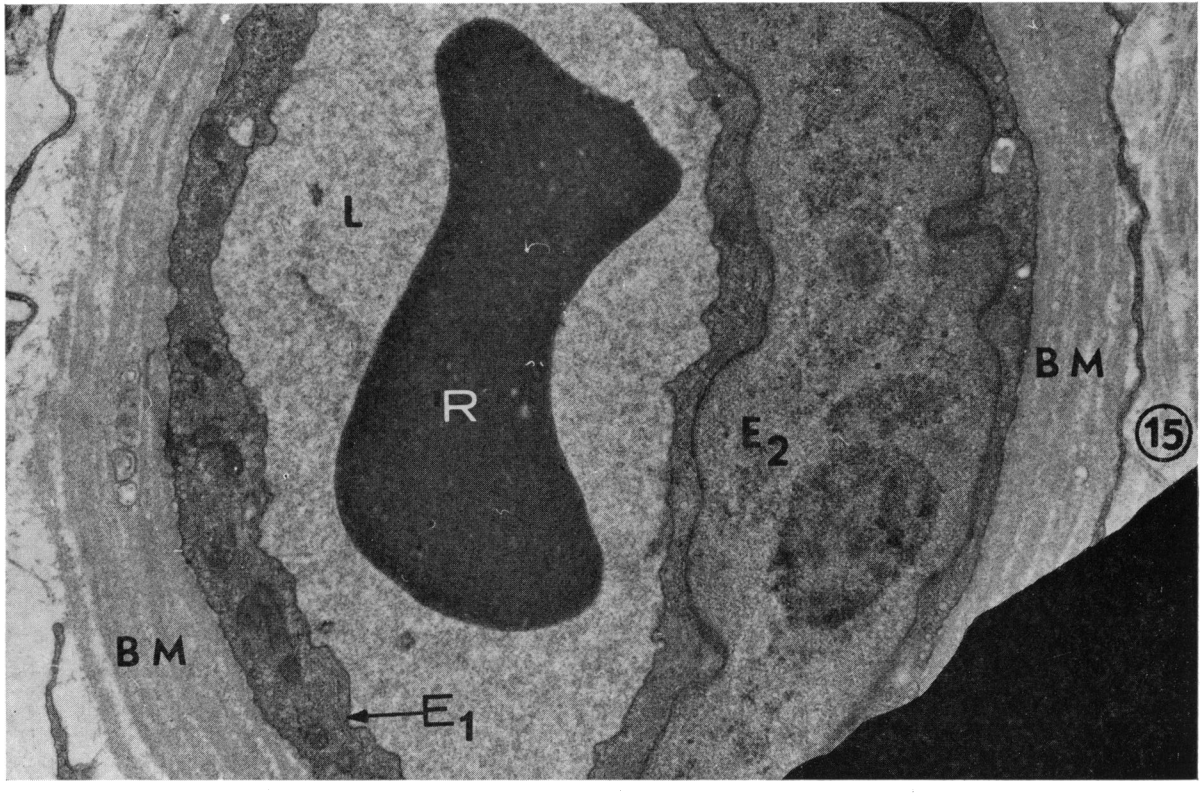

FIGURE 15

Capillary of conjunctival vessel obtained from a 92-year-old female in apparent good health. A single erythrocite (R) is observed in the lumen (L) of a capillary lined with endothelial cells (E_1, E_2). A thickening and increased lamination of the basement membrane (BM) is noted. Chrome-osmium fixation ($\times$16,500).

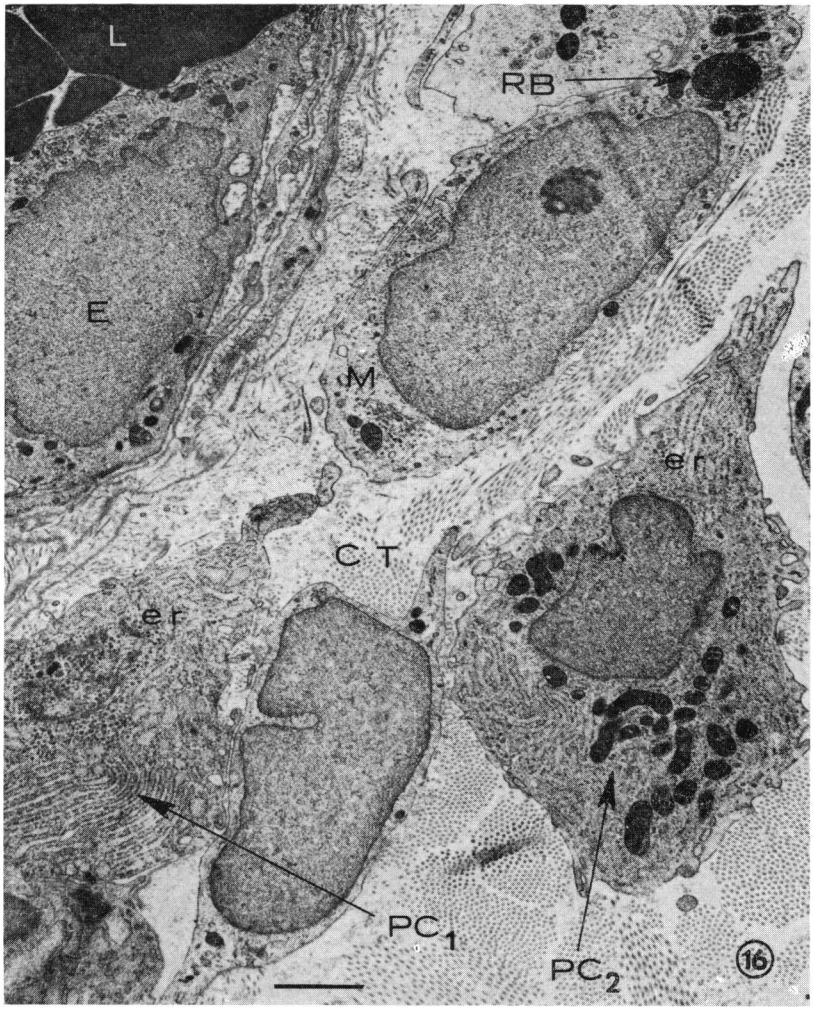

FIGURE 16

A detail of Figure 4. The lumen (L) in the upper, left-hand corner is packed with erythrocytes and bordered by an endothelial cell (E). A macrophage (M) is observed in the connective tissue (CT), whose cytoplasm contains a residual body (RB). Two plasma cells (PC_1, PC_2) are also noted in the connective tissue. Ergatoplasm (er). Chrome-osmium fixation ($\times$17,500, reduced by ⅓).

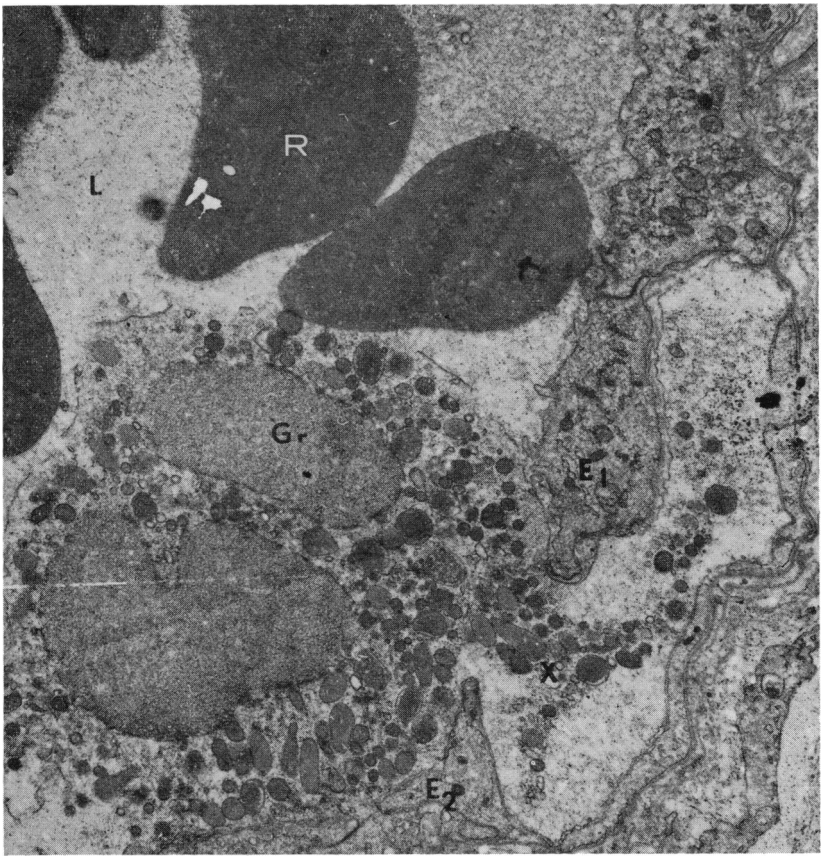

FIGURE 17

Detail of venular aneurysm in a 28-year-old Negro male, same patient as Figure 6. Neutrophilic granulocyte (Gr) in the process of diapedesis. Cell process has pushed between two endothelial cells (E_1, E_2) and can be seen between pericyte processes and endothelial cells. Chrome-osmium fixation (×22,000, reduced by ⅓).

(especially in crisis) in the bulbar conjunctiva of patients with sickle-cell disease. These microaneurysms had been observed (and confirmed by the present study) to be located in the postcapillary venules, which have been described as the site of slowest blood flow and erythrocyte aggregation.[87] The sickle hemoglobin is insoluble in the deoxygenated state[88] and polymerizes to form crystals[89] or "tactoids"[90] as a result of the aggregation of the hemoglobin molecules into tubules.[91,92] This results in a distortion of the erythrocyte into a rigid, fragile, elongated

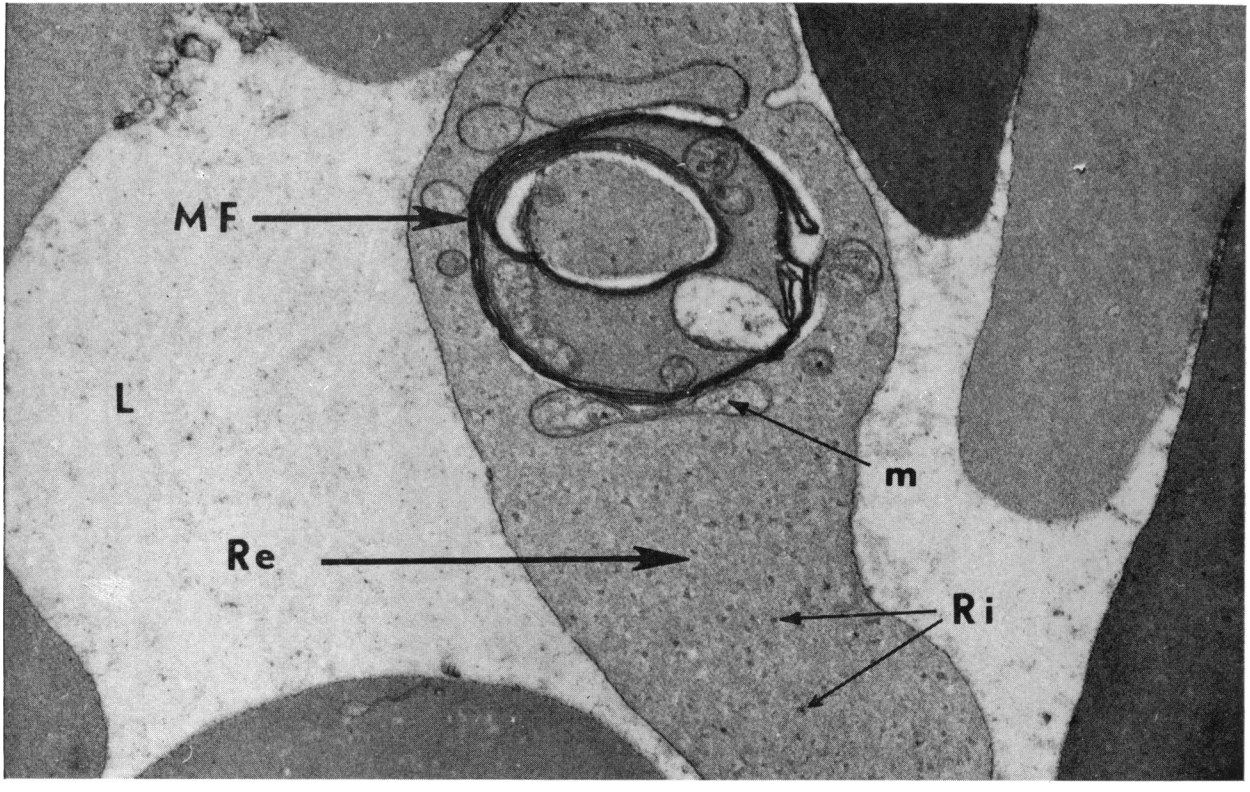

FIGURE 18

Reticulocytes at varying degrees of maturation. Same patient as in Figure 6. Darker cells contain more hemoglobin. One reticulocyte (Re) has numerous ribosomes (Ri), a myelin figure (MF), and mitochondrial remains (m). Osmium-fixation (×30,500).

sickle cell which, in turn, increases the blood viscosity and its capacity to occlude capillary flow. These adverse conditions may reduce further the oxygen tension and lead to hyperosmolality and a lowered *p*H that encourages additional sickling.[93] The marked packing of erythrocytes noted in a section through a microaneurysm (Figure 5), where pressure of this packing had forced a separation between two adjacent endothelial cells and the plasma had appeared outside the lumen, indicates the degree of local pressure due to sickling with aggregation. The pale conjunctiva, so often observed in patients with this disease,[94] suggests a vasoconstriction which could further accelerate the process just noted. In patients with diabetes, it was not possible to relate directly the conjunctival microaneurysms to their metabolic disturbance. The one patient with both sickle-cell disease and diabetes mellitus demonstrated conjunctival microaneurysms that were typical of sickle-cell aggregations.

A preliminary report[1] on conjunctival microaneurysms among patients with sickle-cell disease commented on their reversibility in children subjected to the heat of the examining lamp. Reversibility decreased after the patient was eleven years old. Light microscope studies[4] of conjunctival biopsy material suggested evidence of capillary endothelial cell swelling and proliferation in limited areas and seemed to demonstrate a "trapping" of sickled erythrocytes between the endothelial cells. Alteration in the endothelial cell structure was thought to result from mechanical injury by the rigid, sickled red blood cells. The fact that no evidence of endothelial cell change could be elicited by electron microscope studies of the biopsy material from conjunctiva of patients with sickle-cell disease supports the findings of a preliminary investigation.[5] This indicates a need for the abandonment of the hypothesis that endothelial cell swelling and proliferation[4] may result from, and influence, erythrocyte aggregation and sickle-cell crisis. Proliferation of the endothelial cells has been reported in light microscope studies of the conjunctiva[67] and retinal capillaries[95] of diabetic patients, although subsequent studies of retina[96] and skin[97] offered no evidence of such change. When the trypsin digestion technique is employed, it has been noted that the endothelial cells may "migrate"[85] and this observation may, in part, help to explain some of the confusion attributed to the role played by the endothelial cell in the formation of diabetic capillary microaneurysms.

The ectasias demonstrated by patients with sickle-cell disease are not static, but come and go and appear to change position in a matter

of seconds if the patient is under eleven years of age; it may take hours or days if he is older. The loose connective tissue surrounding the vessels may afford space for local dilatation and stretching of the vessel walls resulting in an enlarged lumen. New basement membrane may be synthesized as the lumen increases in size. When the ectasia disappears or moves to another site, the vessel may return to its original diameter; the basement membrane might become folded or pleated, rather than being immediately desynthesized. Therefore, basement membrane plications may offer some indication of the possible dynamics of capillary microaneurysm reversibility observed in patients with sickle-cell disease. Numerous S-shaped curves noted in this structure (Figure 13) suggest that the capillary may be stretched and contracted from time to time as the microaneurysm reappears and disappears. Thickening and increased lamination of the basement membrane observed in patients with diabetes mellitus supports the findings of an earlier study of conjunctiva with the light microscope.[67]

A possible role of the pericyte in the control of capillary blood flow and microaneurysm formation has been suggested in a number of recent articles. These intramural cells which are surrounded by a basement membrane were first described by Rouget[98] in 1873, named for him by Vimtrup[99] in 1922, and called "pericytes" by Zimmerman[100] one year later. Recent studies of these cells with the electron microscope[101-103] indicate that they are the same structure referred to by Kuwabara and Cogan[104] as "mural cells," and that, rather than being peculiar to the retinal blood vessels, they may be observed in the vasculature of any tissue. Cogan *et al.*[105] suggested that these cells may act as a support for the capillary wall and that their degeneration in diabetic patients, sometimes complete (ghost cells), might contribute to vessel-wall weakening with the formation of a retinal microaneurysm. An additional publication by Cogan and Kuwabara[106] advised that "the specific loss of mural cells from diabetic capillaries [in trypsinized material] [retina] removes the tonic control which normally regulates blood flow. . . . the result is both a diffuse and microaneurysmal distension of some capillaries." More recently, however, Oliveira[107] has presented evidence to indicate that a degeneration of intramural pericytes may be present in many diseases, including diabetes mellitus, and noted further that it was not possible to establish a significant correlation between ghost cells and microaneurysm formation. He concluded that "the function and pathology of intramural pericytes remain unknown, and the concept that the capillary pathology of diabetic retinopathy is dependent upon hemodynamic

changes resulting from selective degeneration of intramural pericytes is unsubstantiated."

Electron microscope observations of blood vessels of the bulbar conjunctiva from patients with sickle-cell disease, with diabetes mellitus, and with both sickle-cell disease and diabetes seem to lend support to Oliveira's contentions. Biopsy material acquired from patients with sickle-cell disease showed no alteration in the pericyte structure regardless of the patient's age or the presence of microaneurysms (Figures 4 and 6). Vacuolation of the pericyte cytoplasm was demonstrated in the vasculature of most patients with diabetes; however, it was seen also in the vasculature of one of the control subjects (Figure 11). The one patient with both sickle-cell disease and diabetes exhibited similar pericyte cytoplasmic vacuolation (Figure 10) but with a conjunctiva that demonstrated capillary microaneurysms typical of sickle-cell disease (Figure 2). The observation that pericyte change was not limited to an area of capillary microaneurysm would seem to lend further support to Oliveira's contention that these cells are not necessarily related to microaneurysm formation.

Local effects of obstruction in blood flow in the microvasculature through the localized aggregations of the fragile, elongated, rigid, and multipointed sickle cells have been discussed. The sickled erythrocytes are mechanically more fragile, more easily phagocytosed, and more rapidly destroyed in the circulation than are non-sickled cells.[108] These destructive processes are responsible for the anemia and are reflected in the peripheral blood as evidenced by the presence of reticulocytes and immature red blood cells (Figure 18). Massive and generalized sickling and aggregation may result in death.[109] Patients with this disease therefore have a shortened life expectancy, although intensive therapy recently has enabled many of the patients to live past the age of 30. Recurrent attacks of painful crisis plague patients suffering from this disease and are attributed to a sudden increase in sickling and aggregation in the small vessels. Although many precipitating causes have been listed[108]—infection, cold, hypoxia from blood loss, mountain climbing, and high-altitude flying—no one has been able to explain adequately a mechanism of crisis that might apply generally.

Examination with the electron microscope of the small blood vessels and microaneurysms of the bulbar conjunctiva has revealed no substantial evidence to explain the mechanism of precipitation of the enigmatic sickle crisis. One interesting and consistent finding, however, is the observation of an increase in the number of plasma cells close to the small blood vessels and microaneurysms as compared with control

subjects. A detailed study of the connective tissue surrounding the microaneurysm revealed a residual body contained in a macrophage accompanied by plasma cells (Figure 16). Macrophages may stimulate antibody production[110] in plasma cells which have been shown to produce gamma globulin antibodies.[111,112] Patients with sickle-cell anemia may demonstrate elevated gamma globulin levels.[113] This evidence suggests the possibility of an auto-immunity factor which may, perhaps, offer a mechanism to explain the precipitation of the painful sickle crisis. Although the literature supplies only sparse evidence to suggest the antigenicity of sickle hemoglobin,[114] many of the observations when grouped together seem to lend emphasis to the creditability of some auto-immune mechanism. The pale conjunctiva noted in patients with sickle-cell disease[1,94,108] suggests vasoconstriction and lends support to Kimmelstiel's[115] concept of a vasospastic process. The inflammatory reaction observed in many patients was characterized by diapedesis of neutrophilic granulocytes near small blood vessels (Figure 17). Lastly, an increase in the destruction by fragmentation of red blood cells, with their phagocytosis by endothelial cells and pericytes, partially justifies the contention that an auto-immune response may be present.

Lack of evidence of endothelial swelling or proliferation in the capillaries and small venules of the conjunctiva examined with the electron microscope forces the abandonment of the hypothesis that massive capillary aggregation and microaneurysm formation may lead to endothelial cell change, which in turn may lead to sickle crisis. An auto-immune mechanism is possible, but further investigation will be required to test its reasonableness.

SUMMARY

In an attempt to understand better the mechanism of crisis and symptomatology of sickle-cell disease, the vasculature of the bulbar conjunctivas of seven patients thus afflicted has been studied with the quantitation offered by the electron microscope. For comparative purposes, seven normal subjects were used as controls. A chance biopsy of conjunctiva from a patient with sickle-cell disease and diabetes mellitus encouraged the acquisition of similar material from three patients wih diabetes mellitus and without sickle-cell disease.

In vivo slit-lamp examination of the conjunctiva facilitated the localization of areas which would be suitable for detailed study with the electron microscope. Most patients with sickle-cell disease will

demonstrate conjunctival microaneurysms specific for this disease. Patients with diabetes mellitus may reveal microaneurysm formations in the bulbar conjunctiva which may or may not be related to diabetes.

Fine-structure studies of a conjunctival microaneurysm obtained from a patient with sickle-cell disease demonstrated severe erythrocyte packing and indicated clearly the high degree of pressure which may exist in the patient's capillary and postcapillary venules.

A pleating of the basement membrane was noted in the wall of a microaneurysm. This basement membrane change caused by repeated expansion and contraction of the vessel wall, supports the contention that microaneurysms may, as previously assumed, disappear and reappear from time to time.

The absence of evidence of endothelial cell swelling and proliferation in the material obtained from all patients with sickle-cell disease indicates the need for the abandonment of the hypothesis that endothelial cell change may result from, and influence, erythrocyte aggregation and sickle-cell crisis.

Pericyte vacuolation was not observed in patients with sickle-cell disease but was noted among patients with diabetes. However, one control subject without evidence of disease, revealed vacuolation of many different cells in the vessel wall, including pericytes.

Study of conjunctival biopsy material from all patients with sickle-cell disease indicated an apparent increase in the number of plasma cells in the connective tissue surrounding the microaneurysm as compared to the controls. Macrophages were occasionally found in the company of plasma cells. The presence of both of these cells raises the question of the possibility of an auto-immune mechanism: indications of vasospasm (pale conjunctiva), presence of an inflammatory reaction (diapedesis of neutrophilic granulocytes), and an increase in the destruction by fragmentation of erythrocytes with phagocytosis reinforce the impression that an auto-immune mechanism may be operating. It is possible that this auto-immunity, if present, may help to explain the onset of the enigmatic, painful sickle crisis that considerably shortens the life of the patient.

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