Complement Activation by Pulsed Tunable Dye Laser in Normal Skin and Hemangioma

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Pulsed tunable dye laser (577 nm) (PTDL) therapy induces hemoglobin coagulation and tissue necrosis, which is mainly limited to blood vessels. To define whether this treatment activates complement in normal skin and senile hemangioma, we analyzed complement deposition in blood vessels by immunofluorescence.

C3 fragments, C8, and C9 were detected with specific polyclonal antibodies. The membrane attack complex of complement (MAC) was demonstrated with a monoclonal antibody which reacts only with a neoantigen of MAC. Amplification of C3 deposition by the alternative pathway was determined on cryostat sections by indirect immunofluorescence with use of C4 deficient guinea pig (GP) serum. Normal skin and hemangiomas from three individuals were studied.

In PTLD-irradiated normal skin, the main findings were as follows: 1) C3 fragments, C8, C9, and MAC were deposited in vessel walls; 2) these deposits were not due to denaturation of the proteins since they became apparent only 7 min after irradiation, contrary to immediate deposition of transferrin at the sites of erythrocyte coagulates; 3) the C3 deposits were shown to amplify complement activation by the alternative pathway, a reaction which was specific since tissue necrosis itself did not lead to such amplification; 4) these reactions preceded the local accumulation of polymorphonuclear leucocytes.

Tissue necrosis was more pronounced in the hemangiomas. The larger angiomatous vessels in the center of the necrosis did not fix complement significantly. By contrast, complement deposition in the vessels situated at the periphery was similar to that observed in normal skin with one exception: C8, C9, and MAC were detected in some blood vessels immediately after laser treatment, a finding consistent with assembly of the MAC occurring directly without the formation of a C5 convertase.

These results indicate that complement is activated in PTDL-induced vascular necrosis, and might be responsible for the ensuing inflammatory response. *J Invest Dermatol 94:426–431, 1990*

Following PTDL treatment, necrosis in the skin is mainly limited to the blood vessels [1]. This reaction has been well studied: erythrocytes are immediately coagulated because oxyhemoglobin absorbs the electromagnetic energy and transforms it to heat. Structures in the immediate vicinity are also damaged by the short but efficient elevation in the local temperature [2]. It is likely that many cells are destroyed and that proteins are denatured during this reaction. In particular, degenerative changes have been observed in endothelial cells and pericytes [3]. After a latent phase, polymorphonuclear leucocytes (PMN) localize around the blood vessels which are filled with aggregated erythrocytes; the whole picture resembles that of acute vasculitis [1]. Port wine stains have been treated with PTDL [4]. The histologic lesions induced did not differ: erythrocytes were agglutinated and the vessels walls showed signs of degeneration. This acute vascular injury was the center of an inflammatory reaction after 4 to 6 h, which was replaced at later stages by granulation tissue and finally healing without scarring. The mechanism responsible for the rapid influx of PMN has not been studied. Various mediators including complement might be involved.

Hypoxic cell necrosis, as observed after coronary artery occlusion, has been shown to trigger complement activation [5]. Various intracellular fragments are known to bind and activate C1 under such circumstances [6,7]. However, cell necrosis may activate complement by several other mechanisms. For example, various proteases released by damaged cells, or generated by activation of the coagulation system, cleave C3 directly [5,8,9]. Alternatively, the control mechanisms of the alternative pathway might be destroyed [10]. In addition, the lytic pathway can be triggered directly by the cleavage of C5 to C5b by proteases, or by partial denaturation of C5 by oxygen radicals [8,11]. The membrane attack complex of complement (MAC) has been detected on cells undergoing necrosis in myocardial infarction [12]. The activation of complement at the time of myocardial necrosis is responsible for the influx of PMN and the extent of tissue damage [13–15]. Similar reactions may occur at the time of vascular necrosis induced by PTDL.

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Abbreviations:
- BSA: bovine serum albumin
- EDTA: ethylene diamine tetraacetic acid
- EGTA: ethylene glycol tetraacetate
- FITC: fluorescein isothiocyanate
- GP: guinea pig
- MAC: membrane attack complex of complement
- PMN: polymorphonuclear leucocytes
- PTDL: pulsed tunable dye laser
- WD: working dilution

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The present study was designed to see whether complement was deposited at the site of laser treatment in normal and angiomatous skin, and to follow the sequence of complement deposition. We analyzed by immunofluorescence techniques the deposition of C3 and late components (C8 and C9), and the tissue fixation of MAC using a monoclonal antibody which recognizes a neoantigen of MAC [16]. In addition, the capacity of the C3 deposits to amplify C3 deposition by the alternative pathway was assessed by an indirect technique [17].

MATERIALS AND METHODS

PTDL Treatment Five normal individuals participated in the study; all had skin type III. Normal skin was studied in two, senile hemangioma in two, and both in the last subject. Normal skin and senile hemangioma (2–3 mm diameter cherry spots) were exposed to laser radiation from a Candela model SPTL-1a tunable dye laser operating at 577 nm with a mixture of two rhodamines (560 and 590), at a pulsewidth of 360 μs, over 4-mm-diameter area, and with 8 J/cm^2. The normal skin sites were on the arm, and the hemangioma were on the thorax.

Skin Biopsies These biopsies were obtained under local anesthesia (2% xylocaïne) before and at different time intervals (1 [two biopsies], 7 and 60 min). Biopsies were also taken from one individual (normal skin and hemangioma) 4 and 24 h after irradiation. They were immediately frozen in liquid nitrogen and stored at −85°C.

Reagents Different proteins and complement fragments were identified on cryostat section with specific antibodies: Fluorescein isothiocyanate-conjugated (FITC) rabbit antihuman C3c, and FITC goat IgG antiguinea pig (GP) C3 were obtained from Nordic (Tillburg, The Netherlands), and rhodamine-conjugated antihuman C1q from Behringwerke AG (Marburg, West Germany); working dilutions (WD) were 1/20, 1/10, and 1/30, respectively. FITC antihuman transferrin, haptoglobin, and fibrinogen were obtained from ICN (Lisle, UK) (WD: 1/20, 1/50, and 1/100). Polyclonal goat IgG against human C8 and against human C9 antibodies were obtained from Cappel Laboratories (West Chester, UK) (WD 1/200 and 1/50). The second antibody was FITC pork antigoat IgG (H + L) (The Binding Site, Birmingham, UK) diluted 1/50. PolyC9 of the human MAC was revealed with a specific mouse monoclonal antibody (MCaE11) [16], with the ascitic fluid diluted 1/500, followed by an FITC sheep antimouse IgG (H + L) (Serotec, Oxford, UK) diluted 1/50.

The different reagents used as sources of complement for the indirect assay were: (1) Reagents deficient in classical pathway function: C4-deficient GP serum used fresh or after storage at −85°C for less than 6 weeks. Normal and C4-deficient GP sera supplemented with 10 mM ethylene glycol tetraacetate (EGTA) and 2 mM Mg. (2) Reagents without complement function: C4-deficient GP serum was heat inactivated at 50°C for 20 min before used so as to destroy factor B as previously reported [17]; normal and C4-deficient GP sera were also depleted of complement by heat inactivation for 30 min at 56°C, or complement function was blocked by the addition of ethylene diamine tetraacetic acid (EDTA) 10 mM.

Immunofluorescence (IF) Studies Standard IF was performed on all skin biopsies. Briefly, 4 μm thick cryostat sections of frozen unfixed skin biopsies were cut, air dried, and washed for 30 min in phosphate-buffered saline (pH 7.4), supplemented with 0.1% bovine serum albumin (PBS-BSA). All incubations were performed at 22°C, and washes were done in PBS-BSA. The deposits were revealed by incubation with FITC (or rhodamine conjugated) antibodies (human C3, C1q, transferrin, haptoglobin, and fibrinogen) for 30 min, or by incubation for 30 min with the first antibody (C8, C9, polyC9), followed after a 15-min wash, by the second FITC antibody which was also incubated with sections for 30 min. The sections were washed again for 30 min, mounted in Fluoroprep (Biomérieux, Charbonnières-les-Bains, France) and examined on an Olympus microscope equipped with an episcopic UV light system. Micrographs were taken with high-speed Ektachrome ASA 400 film. Phase contrast microscopy allowed us to localize the vessels that contained erythrocyte coagulates: distinct brownish spheres were easily recognized in the hemangioma, and similar images were seen in blood vessels of normal skin.

Amplification of Complement Deposition by the Alternative Pathway (GP C3 Binding Assay) Indirect IF studies for the detection of GP C3 were performed as previously reported [17]. Briefly, we incubated cryostat sections with the various complement reagents diluted 1/10 in PBS-BSA for 15 min at 37°C to allow complement activation and C3 deposition. The sections were washed for 30 min at 22°C, and the GP C3 was revealed by IF as described above. To determine whether the goat antiGP C3 stained

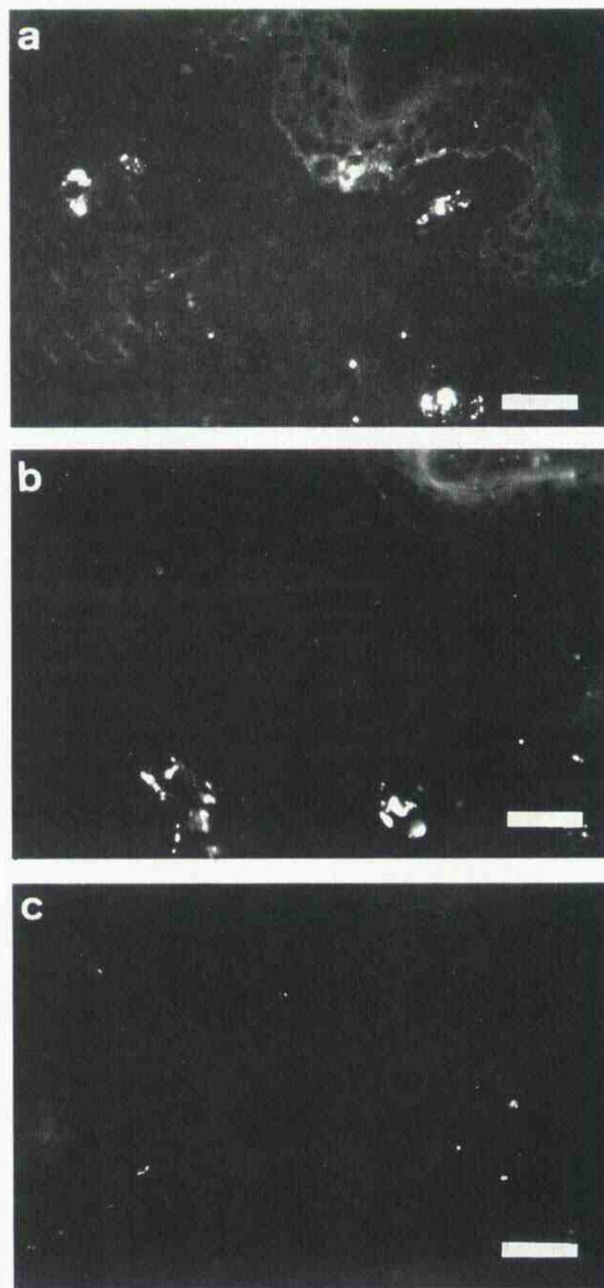

Figure 1. Immunofluorescence of normal skin. *a*: Direct fluorescence with antihuman C3 showing vascular deposits 1 h after irradiation with PTDL. *b*: The GP C3 binding test on a serial section. This section was first overlaid with C4-deficient GP serum, and GP C3 was revealed with specific antibody. Note GP C3 deposition in similar vascular sites. *c*: There was no GP C3 deposition when GP complement was blocked with EDTA. Bar: 62 μm.

any human cutaneous structure, we performed direct IF on normal skin and angioma, before and after laser therapy. The whole set of complement reagents were studied on normal skin before, and 60 min after, laser treatment to establish that the GP C3 binding assay was valid [17]. The other skin biopsies were studied with the C4-deficient GP serum and with at least one of the control reagents devoid of complement function.

Histology The cryostat sections were also processed for histology for determination of the influx of polymorphonuclear leucocytes (PMN) [18]. The dried sections were incubated for 30 min at 22°C in a naphtol-ASD chloracetate (Sigma, St. Louis, MO) in a Michealis buffer, pH 7.2. After a rinse in distilled water, coloration was performed with Hemalum of Mayer 0.1% for 8 min. The sections were washed in distilled water, dehydrated in ethanol and xylol, and mounted with Eukitt (Kindler GmbH). This coloration detects esterase activity of tissue macrophages, mast cells, and PMN [18].

RESULTS

Complement Deposits in Normal Skin One hour after PTDL treatment, complement deposits were obvious in most blood vessels which also contained erythrocyte coagulates. This was determined by switching from the UV light to phase-contrast microscopy. Erythrocyte coagulates were easily identified by this technique. The strongest deposits were those of C3 (Fig 1*a*) and C1q, although C8, C9, and MAC were all readily identified (Table I). Such deposits were not seen immediately after irradiation (at 1 min), indicating that they were not due to protein denaturation. They became visible only at 7 min or more, with C1q and C3 being visible first (Table I). This sequence of events is consistent with local complement activation. To determine if this occurs, we used the GP C3 binding assay (see *Materials and Methods*). After incubation of the sections with C4-deficient GP serum, it was possible to detect GP C3 deposits in the vessels containing human C3 fragments (Fig 1*b*). This reaction was specific, and due to complement activation by the alternative pathway as defined from the controls used: there was no fluorescence when the FITC antiGP C3 was used directly on the cryostat sections (i.e., no cross reactivity with human C3), and the GP C3 binding assay was negative when sera devoid of complement function were used (Fig 1*c*). Positive results were obtained with normal or C4-deficient GP sera supplemented with Mg EGTA. This assay was positive only for biopsies containing human C3 deposits (i.e., only at 7 min and thereafter), thus indicating that the immediate damage induced by irradiation was not, by itself, sufficient to trigger complement deposition by the alternative pathway.

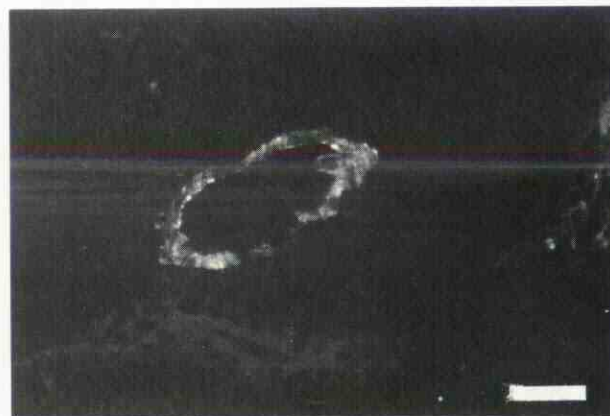

Figure 2. Immunofluorescence of angioma. Vacular deposits of C1q 7 min after irradiation: this vessel was located underneath the central area of necrosis. Bar: 62 μm.

Complement Deposits in Hemangioma We repeated the IF studies on hemangioma to see whether similar reactions occur in abnormal vessels. The deposition of complement was analyzed for two different zones of lesions. First, the central part of the angioma was filled with erythrocyte coagulates. Such coagulates presented a background autofluorescence that rendered the analysis difficult. By contrast to normal skin, none of these angiomatous lesions became strongly positive for any of the complement deposits tested, and the GP C3 binding assay remained doubtful. However, at the edges of this total necrosis, vascular deposits were easily identified. Vascular C3 and C1q (Fig 2) deposits were observed after 7 min. The C3 deposits activated the alternative pathway similarly to those seen in the normal skin (positive GP C3 binding assay). In this outer zone, the late components C8 and C9 and MAC were recognized already 1 min after irradiation in some blood vessels, a finding consistent with direct triggering of the assembly of the membrane attack complex without C3 activation (Fig 3, and Table I). At later stages (7 min and later), many angiomatous vessels around the central necrosis became positive for C8, C9, and MAC in parallel with the appearance of C1q and C3 deposits.

Deposition of Other Proteins To see whether other proteins localize in the lesions induced by laser irradiation, we analyzed the deposition of transferrin, haptoglobin, and fibrinogen. Fibrinogen

Table I. Complement Deposits After PTDL Treatment[a]

		After				
	Before	1 min	7 min	1 h	4 h	24 h
Normal skin						
1) C1q	∅	∅	+	+	n.d.[b]	n.d.
2) C3	∅	∅[c]	+	+	+	+
3) C8	∅	∅	±	+	n.d.	n.d.
4) C9	∅	∅	±	+	+	+
5) PolyC9	∅	∅	±	+	+	+
6) Indirect deposition of GP C3	∅	∅	+	+	+	+
Angiomatous lesions[d]						
1) C1q	∅	∅	+	+	n.d.	n.d.
1) C3	∅	∅	+	+	+	+
2) C8	∅	+	+	+	n.d.	n.d.
3) C9	∅	+	+	+	+	+
4) PolyC9	∅	+	+	+	+	+
5) Indirect deposition of GP C3	∅	∅	+	+	+	+

[a] Deposits were defined as: ∅, absent; ±, doubtful; or +, present. Total number of biopsies studied at the different time intervals for both types of skin: before, 3; after 1 min, 5; 7 min, 3; 60 min, 3; 4 and 24 h, 1.
[b] n.d., not done.
[c] Except for one vessel which was positive, out of the 5 skin biopsies studied.
[d] At the edges of the central necrosis.

was present throughout the upper dermis before the PTDL application, but there was no increase in the fluorescence in the vessel walls at any stage after treatment. Haptoglobin became weakly positive on the erythrocyte coagulate on normal and angiomatous skin, but only after 6 h. By contrast, transferrin was immediately apparent inside all irradiated vessels, in normal skin and angioma, and already at 1 min (Fig 4).

Histology: PMN Influx After PTDL Treatment The irradiated zone was immediately identified by the erythrocyte coagulates in the small superficial blood vessels of the normal skin and in the angiomatous lesions. Due to their oxyhemoglobin content, the angioma evidently absorbed more energy: keratinocyte vacuolization and small intraepidermal blisters were seen in the biopsies taken at 7 min and at all later stages.

The sequence of PMN infiltration in normal skin and hemangioma was analyzed by esterase coloration of the biopsies taken at different time intervals. Although the technique also reveals mast cells and tissue macrophages, the PMN could be easily recognized. No increase in the baseline number of PMN was observed in the biopsies taken in the minutes following irradiation. In normal skin, some isolated PMN could be seen in superficial vessels at 1 h. The vessels at the edges of the irradiated hemangiomatous lesion already contained many marginated PMN at that time. The PMN infiltration was pronounced at 4 and 24 h in both types of skin.

DISCUSSION

The main finding of this work was that the vascular necrosis induced by PTDL irradiation activated complement. This observation may explain the distinct histologic inflammation that follows PTDL treatment.

In normal skin the sequence of complement activation may be explained by the following events. First, cell membranes and intercellular structures in the blood vessels are damaged directly by PTDL irradiation. Second, complement activation is initiated on denatured structures either by the classical and/or the alternative pathway, thereby leading to the deposition of some C3b molecules, which in turn are responsible for the amplification of C3 deposition by the alternative pathway and the formation of the MAC.

Many intracellular structures are capable of binding C1q and activating the classical pathway of complement [6,7,19,20]. Such structures are exposed after cell necrosis, and they are responsible for in situ complement fixation [5,12,21]. Whether the initial C3 deposited was due exclusively to classical or alternative pathway activation was not investigated here; however, both might participate in the reaction. The fact that C1q was present in the lesions is consistent with direct triggering of the classical pathway. Vascular C3 deposits were also observed in PTLD-irradiated skins in guinea pigs (Polla et al, unpublished); such deposits were also present in C4-deficient animals, indicating that complement may be activated independently of the classical pathway. Complement activation

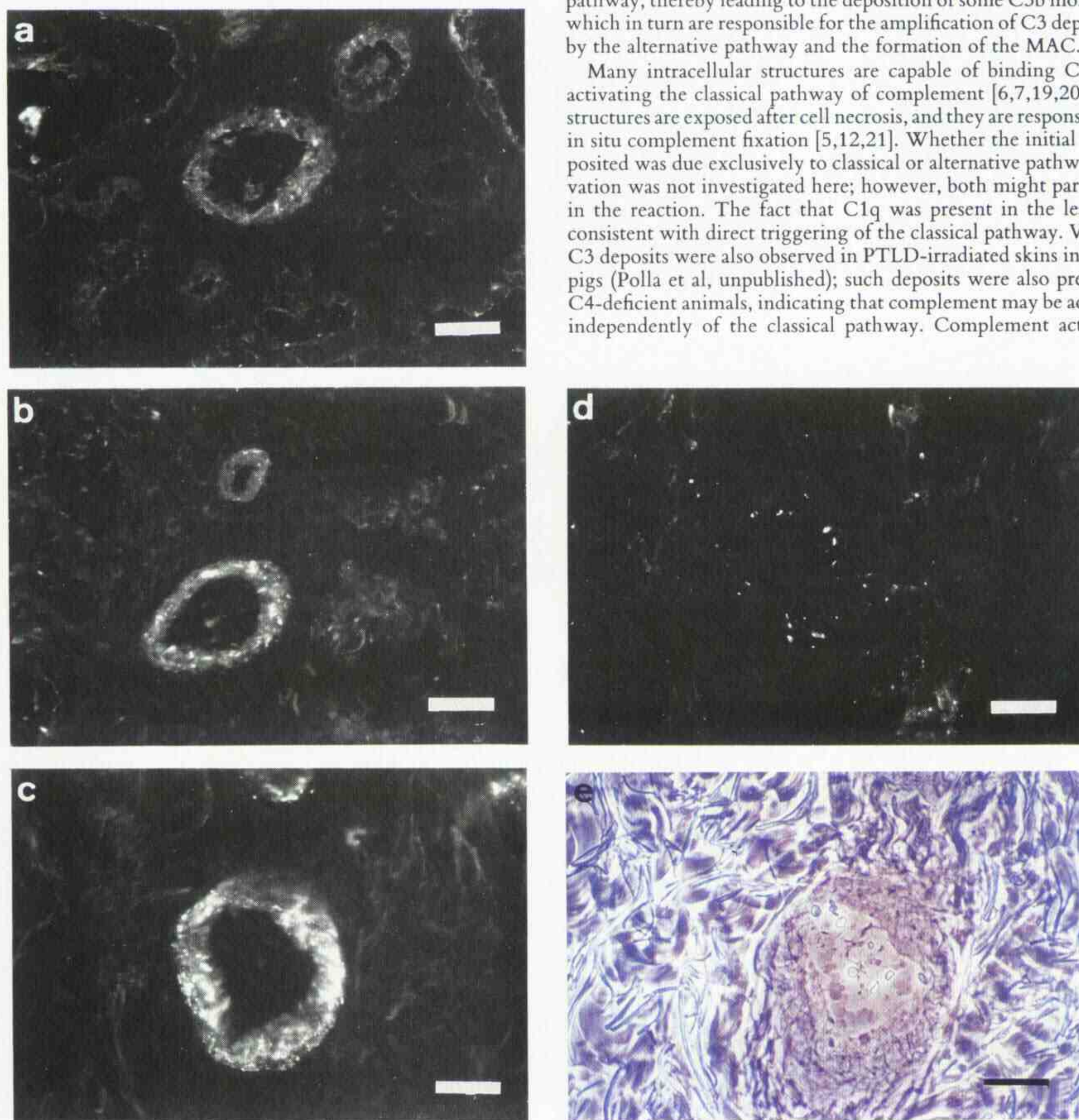

Figure 3. Immunofluorescence of angioma. Deposits of C8 *(a)*, C9 *(b)*, and MAC *(c)*, in the wall of an angiomatous vessel at the edge of the central necrosis (1 min after irradiation). There was no significant C3 deposition *(d)* in this vessel shown to contain coagulated erythrocytes by phase contrast microscopy *(e)*. Bar: 62 μm.

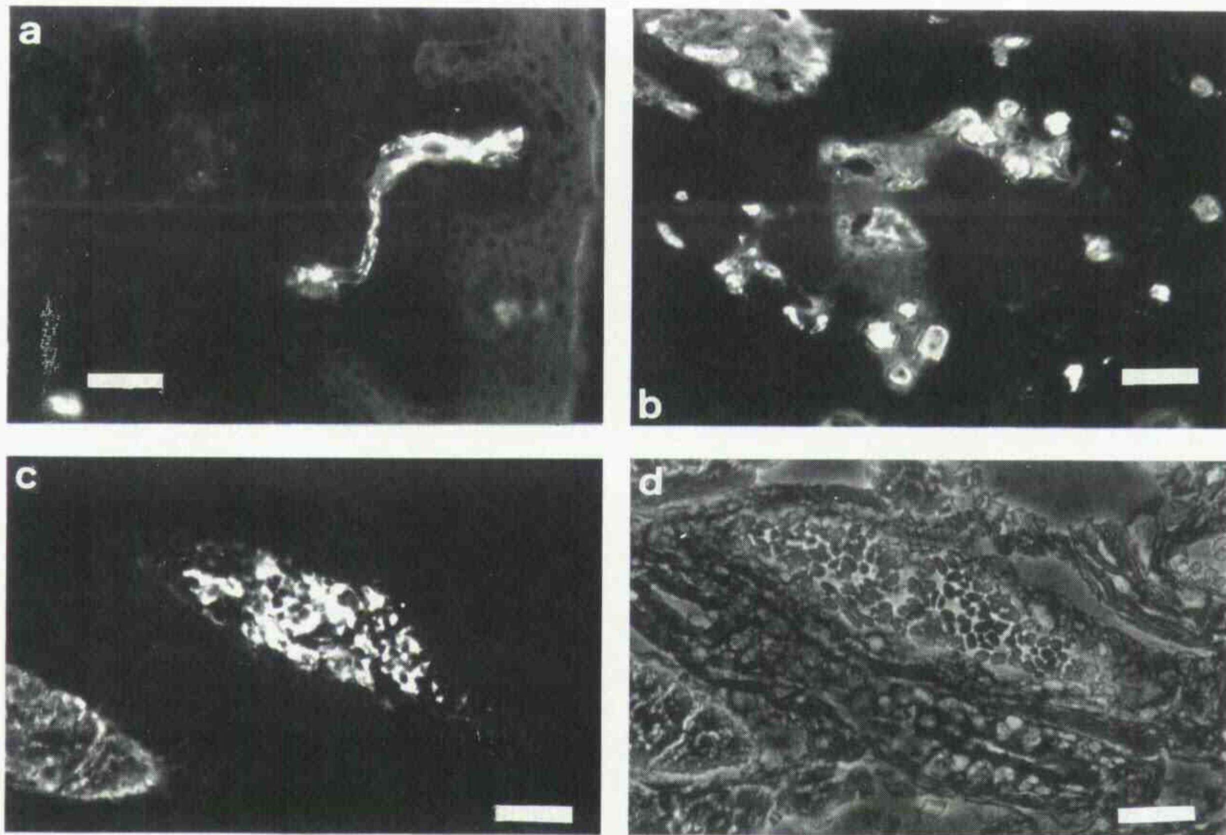

Figure 4. Transferrin deposits. Such deposits filled blood vessels in normal *(a)* and angiomatous *(b)* skin (1 min after irradiation). A comparison between IF *(c)* and phase-contrast microscopy *(d)* illustrates this type of intravascular deposits with negative vessel wall fluorescence (7 min after irradiation). Bar: 62 μm.

might also be initiated by the direct cleavage of C3 by other enzymes released locally after the irradiation. However, to be of biologic significance, the activation reaction has to be more powerful than inhibitory mechanisms present in the soluble phase as well as on cell membranes [10]. Decay-accelerating factor (DAF) is a regulatory protein which dissociates the alternative pathway C3 convertase on cell surfaces and in the fluid phase [22,23]; it is found on epithelial and endothelial cells [24,25], and attached to elastic fibers in the extracellular matrix of the dermis [26]. Vitronectin (S-protein), which prevents the membrane insertion of the MAC, is also found associated with microfibrils on elastic fibers in the dermis [27]. These proteins might prevent complement activation under physiologic conditions. The deposition of C3 in the damaged blood vessels was shown to be amplified by the alternative pathway and to result in the assembly of MAC, which perhaps would indicate that control of complement activation was lost. Complement activation did not extend beyond the vessel wall; this finding might be related to lack of irradiation damage, but, in addition, it is possible that DAF and vitronectin associated with the elastic fibers limited complement activation to the structures that have been directly damaged. The presence of MAC in the vessel walls indicated also that C5a had been formed. Seifert et al have shown that C5a is generated in serum exposed to damaged endothelial cells [28]. The local release of C5a is responsible for PMN influx [29]. Finally, the formation of MAC on cellular membranes may enhance necrosis and/or inflammation directly [12,30].

In angioma, the lesions differed from those observed in normal skin probably because the absorption of the electromagnetic energy was more pronounced and because the tissue destruction was more extensive. The center of the necrosis consisted of coagulated blood vessels. It is likely that the absence of efficient diffusion of complement proteins to this site prevented significant complement activation. By contrast, complement activation was prominent on the edges of this necrosis. The deposition of C3 and the amplification of C3 deposition were similar to those seen in normal skin. The finding of some vessel walls containing C8, C9, and MAC deposits immediately after irradiation, before appearance of C3, suggested that MAC formed immediately upon irradiation. C5 may have been cleaved by a protease; however, it is more likely that C5 was directly altered by the laser treatment. Indeed, C5 has been shown to be modified under specific physicochemical conditions (oxydative burst, pH, freezing and thawing, etc:.), and to become C5b-like, i.e., capable of initiating directly the assembly of the MAC [11,31–33]. Why was direct activation of MAC evident only on the edges of the central necrosis? Recently, Tan et al [34] have shown that many variables, including the laser spot size, determine the severity of the damage in the outer lesional zone. The specific conditions for direct activation of C5 may have been reached only in this zone in angiomatous skin lesions.

Other proteins are altered directly upon laser treatment. Hemoglobin is coagulated immediately [1]. Hemoglobin and/or erythrocyte stroma has been reported to activate the alternative pathway of complement [35–37]; we could not identify complement deposits on erythrocyte coagulates or demonstrate that they bound GP C3 in the indirect assay. By contrast, such coagulates immediately fixed transferrin very strongly. This might have been due to direct protein denaturation, or to specific binding of transferrin to erythrocyte coagulates. Erythrocyte stromata have been reported to bind transferrin [38]. Of relevance to the present work was that this reaction provided a clear IF image of the vessel lumen, contrasting with the IF pictures obtained with complement proteins, which were localized essentially in the vessel wall.

Many other biologic systems might be activated at the time of PTDL treatment. Inflammation is unlikely to be initiated only by complement fixation. However, the results obtained here indicate that complement participates in the triggering of the inflammatory

reaction. Inhibitors of complement might be helpful to reduce damage in tissue necrosis whether such necrosis involves vessels only or whole tissues.

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