Erythropoietic protoporphyria. Photoactivation of the complement system.

I Gigli, … , N A Soter, M A Pathak

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Research Article

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Erythropoietic Protoporphyria

PHOTOACTIVATION OF THE COMPLEMENT SYSTEM

IRMA GIGLI, *Department of Dermatology and the Irvington House Institute, New York University, New York 10016*
ALBERT A. SCHOTHORST, *Department of Dermatology, University of Leiden, The Netherlands*
NICHOLAS A. SOTER and MADHUKAR A. PATHAK, *Department of Dermatology, Harvard Medical School, Boston Massachusetts 02114*

ABSTRACT The complement system was analysed in 14 asymptomatic patients with erythropoietic protoporphyria. In the majority of the sera studied the levels of complement components C1, C4, C2, and C3 were within the normal range. Upon ultraviolet light (330–460 nm) irradiation of the serum samples in vitro, a marked decrease in total hemolytic activity accompanied by reduction of C1, C4, C2, and C3 levels was observed. The loss of total hemolytic activity can be directly correlated with the levels of protoporphyrin (PP) and similar changes can be obtained in normal serum upon addition of PP followed by ultraviolet light irradiation. It is postulated that after irradiation the excited PP develops the capacity to activate the complement sequence with the production of cleavage products, which may contribute to the skin changes observed in these patients upon sun exposure.

INTRODUCTION

Erythropoietic protoporphyria (EPP)[1] is a rare familiar disorder characterized biochemically by marked elevation of protoporphyrin concentration in erythrocytes (1) and a deficiency of ferrochelatase (2). Plasma and fecal protoporphyrin are frequently raised, but excretion of porphyrins in the urine is invariably normal (3). Although there is general agreement that increased protoporphyrin in circulating reticulocytes and erythrocytes originates in the bone marrow, controversy remains concerning the source of excessive hepatic and fecal protoporphyrin. An autosomal dominant pattern is evident in the transmission of this disease. However, there is variable penetrance, and isolated occurrence of EPP with no known affected relatives has been observed (4).

From early childhood persons with EPP experience a sensation of "burning" and "stinging" in the skin exposed to sunlight. Erythema and edema may appear within hours, sometimes followed by petechiae. Less frequently, vesiculation with crusting occurs, followed by superficial scarring (1, 5). Bullae are rare but purpura and urticarial reactions in fair-skinned individuals have been observed in a few cases. The skin of the knuckles and fingers, particularly over metacarpophalangeal joints, often presents as thickened, wrinkled, and waxy, characteristic of actinic-damaged skin.

Although photohemolysis of erythrocytes obtained from EPP patients has been described (6, 7) in vitro, severe anemia is a rare event (8). Photohemolysis is caused by light in the Soret spectrum between 380–420 nm wavelength, coinciding with the maximum absorption of protoporphyrins. With erythrocytes as a model for photohemolysis, Goldstein and Harber (9) have shown that photoexcitation of protoporphyrins in the presence of oxygen leads to the formation of lipid and hydrogen peroxides, both of which can be highly destructive to cell membranes. In addition, Allison et al. (10) have suggested that porphyrins function in the presence of light as initiators of free radical reactions that cause disruption of intracellular membranes such as lysosomes. Current studies also implicate a reactive moiety of oxygen, referred to as singlet oxygen, to be involved in the membrane damage associated with the photoactivated porphyrins (11).

Because a burning sensation of the skin, coupled with acute changes in vascular permeability, are prominent symptoms in EPP, it is possible that in addition

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[1] *Abbreviations used in this paper*: CH50, total hemolytic activity; DGVB++, dextrose-veronal-buffered saline; EA, sheep erythrocytes sensitized with rabbit antibodies; EPP, erythropoietic protoporphyria; PP, protoporphyrin-IX-dimethyl ester; UV, ultraviolet light.

to membrane alterations some of the humoral mediators of the inflammatory response may contribute to the production of clinical manifestations. In this study, the complement system in EPP patients has been investigated in an attempt to delineate the possible role of the complement system in the inflammatory reaction observed in the skin.

METHODS

Heparinized plasma (2.5 μg/ml = 0.24 USP U) or serum from 14 asymptomatic EPP patients and normal donors was collected and processed immediately in the cold without exposure to light to avoid injury of the erythrocytes in the blood. The sera and plasmas were stored at −70° in aliquots until used. The heparin concentration used was found not to affect the activity of the complement system (12).

The clinical diagnosis of EPP was confirmed by examining the fluorescence characteristics of erythrocytes and by demonstrating the presence of abnormal amounts of protoporphyrin in them and in plasma, and its absence in the urine. Protoporphyrin was measured by the method of Schwartz et al. (13) (Table I).

Ultraviolet light (UV) irradiation of the plasma samples was carried out in a specially designed Corning glass apparatus (Corning Glass Works, Science Products Div., Corning, N. Y.) capable of holding eight revolving test tubes (taper joint 14/35), each 15 × 1.75 cm in size. A 15-cm diameter flat-bottom flask (standard taper 24/40) holding eight glass-fused test tubes was motor driven to achieve uniform exposure of the samples to ultraviolet-visible radiation. The source of light to which the test samples were exposed was a 250 W, high pressure mercury arc lamp in a glass envelope (model 70; GLO Graft, Switzer Bros., Inc. Cleveland, Ohio) equipped with a removable Wood's light filter. For irradiation purposes, a Wood's filter was replaced with a Corning filter (Corning Glass Works) to effectively screen out wavelengths shorter than 330 nm. The samples attached to the rotating flask were irradiated with 330–460 nm radiation. The irradiance at the surface of the sample tube ranged from 6.0–6.93 mW/cm². The output of the lamp at 30 cm measured with a precalibrated spectroradiometer (IL783; International Light, Inc., Dexter Industrial Green, Newburyport, Mass.) at certain wave lengths was as follows: 333–338 nm = 14.6×10^{-5} mW/cm², 364–370 nm = 28.4×10^{-4} mW/cm², 404–409 nm = 13.0×10^{-4} mW/cm², and 435–440 nm = 25.25×10^{-4} mW/cm². The UV irradiation was carried out in a temperature-controlled cold room at 4°C (±1) and the samples were placed perpendicularly to the light source during irradiation.

Measurement of complement components: veronal-buffered saline, pH 7.5, ionic strength 0.15 M, containing 0.1% gelatin, 0.00015 M Ca^{++}, and 0.0005 M Mg^{++} (GVB++); dextrose-veronal-buffered saline, ionic strength 0.075 M (DGVB++); and 0.01 M or 0.04 M disodium EDTA were prepared as previously described (14). Sheep erythrocytes were coated with rabbit anti-sheep hemolysin to obtain sheep erythrocytes sensitized with rabbit antibodies (EA). $EAC\bar{1}$ were prepared by mixing EA with C1 guinea pig (gp) and diluted to provide 200 effective molecules per cell in the fluid phase. After incubation for 90 min at 0°C, the cells were washed once in DGVB++ and stored in the same buffer with penicillin and streptomycin (15). $EAC\bar{1}$ cells were mixed with 2.5 times their volume of pooled human serum diluted 1:10 in 0.01 M EDTA and incubated for 10 min at 0°C to yield $EAC\bar{4}$ (16); the $EAC\bar{4}$ cells were washed and incubated twice at 37°C for 30 min in 0.04 M EDTA and resuspended in DGVB++. $EAC\bar{1}\bar{4}$ cells were prepared by incubating $EAC\bar{4}$ cells at 5×10^8/ml at 30°C for 10 min with $C\bar{1}$gp to provide 200 effective molecules per cell in the fluid phase (17); the cells were resuspended in DGVB++ at 1×10^8 cells/ml before use. Effective molecule titrations with these cellular intermediates were used to measure C1 (16), C4 (15), C2 (17), C3 (14), and C9 (18). Whole serum complement activity was measured as described (19).

Immunoelectrophoresis in 1% agarose was performed according to Scheidegger (20).

TABLE I
Laboratory Data in 14 Patients with Erythropoietic Protoporphyria

Patient	Protoporphyrin levels	
	Plasma	Erythrocytes
	μg %	μg %
1	8.9	675
2	7.2	1,250
3	4.4	825
4	8.1	715
5	10.9	1,800
6	42.5	1,250
7	16.5	1,250
8	10.6	950
9	neg*	375
10	27.8	1,180
11	10.9	1,100
12	8.8	375
13	3.	94.5
14	9.4	1,800

* Neg, negative.

RESULTS

Complement levels in asymptomatic patients. The total hemolytic activity (CH50) and levels of C1, C4, C2, C3, and C9 in serum from 14 patients with EPP were measured. The CH50 of the majority of the sera studied were within the normal range (Fig. 1), indicating that the complement system in asymptomatic patients was normal. However, when the Mean±2SD were calculated, the levels of C2 and C3 were found to be slightly below the normal range. C9 levels were within the values of the normal population.

Effect of UV irradiation on the complement system. The effect of radiation on the complement system was studied by exposing the cold (4° ± 1°C), normal, and EPP plasma to varying doses of 330–460 nm radiation by increasing the exposure periods up to 120 min (50J/cm²). In sera from two normal individuals, <10% of the CH50 was lost in 120 min, whereas sera from two patients with EPP showed a marked drop in total complement activity when compared with the same nonirradiated sera. In serum samples obtained from a patient with polymorphic light eruptions exhibiting

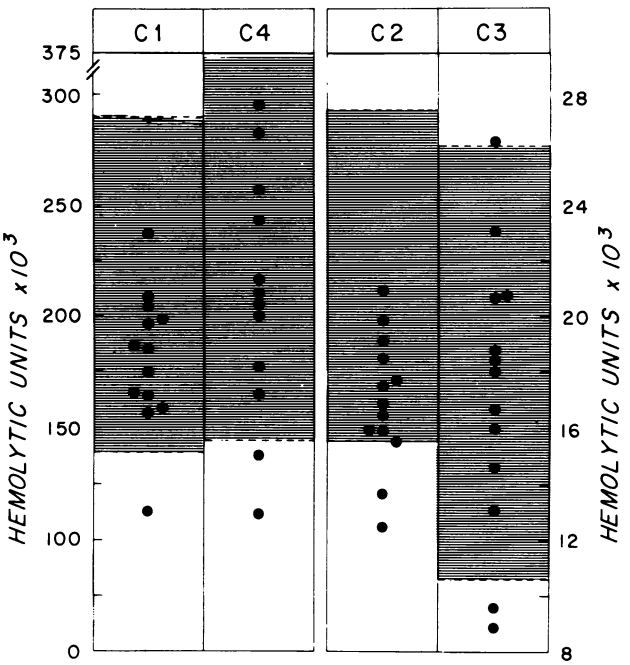

FIGURE 1 C1, C4, C2, and C3 levels in sera from asymptomatic EPP patients. The shaded areas represent the Mean±2 SD of the values in the normal population.

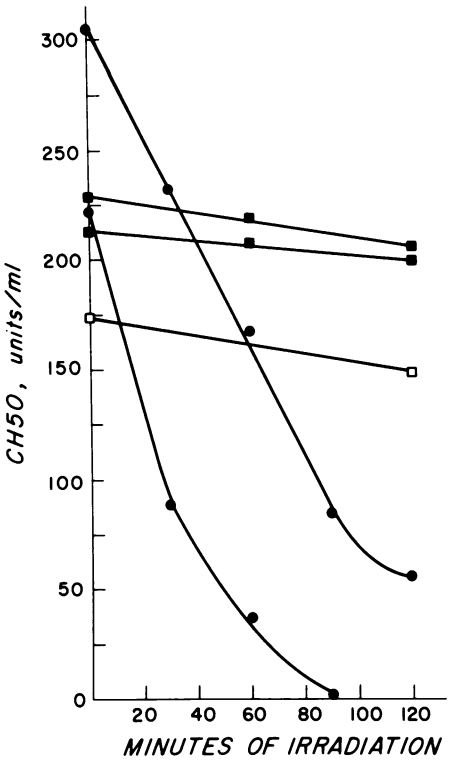

FIGURE 2 Time course of the effect of irradiation on the complement system in the sera of EPP patients (●), a normal individual, (■), and a patient with polymorphic light eruption (□).

several large papules and marked photosensitivity to UV-B (290–320 nm), UV-A (320–400 nm), and visible spectrum (400–560 nm), and in whom protoporphyrin was not detectable in the serum, no decrease in CH50 was observed upon irradiation (Fig. 2). The time course of the effect of irradiation in the levels of C1, C4, C2, and C3 are shown in Fig. 3. These components remained unchanged in the serum from a normal individual after 720 min of irradiation (Fig. 3). In contrast, there was a marked drop in the component levels within the first 30 min of irradiation in a serum of the EPP patients. The loss of activity continued as time of irradiation increased, and affected all four components tested. Table II shows the changes in levels of C1, C4, C2, C3, and CH50 in 10 additional patients studied. Although there are changes in all four components tested, the residual C2 and C3 were consistently lower than C1 and C4. Reduction of C4 and C3 hemolytic levels were accompanied by electrophoretic conversion in immunoelectrophoresis as shown in Fig. 4.

The relationship between levels of protoporphyrin and loss of hemolytic activity was examined in an experiment in which the CH50 of serum samples were measured before and after 60 min irradiation; the results correlated with the protoporphyrin levels. As shown in Fig. 5, there is a direct relationship between levels of protoporphyrin and the total number of hemolytic units lost after light exposure.

Effect of protoporphyrin in normal serum. To elu-

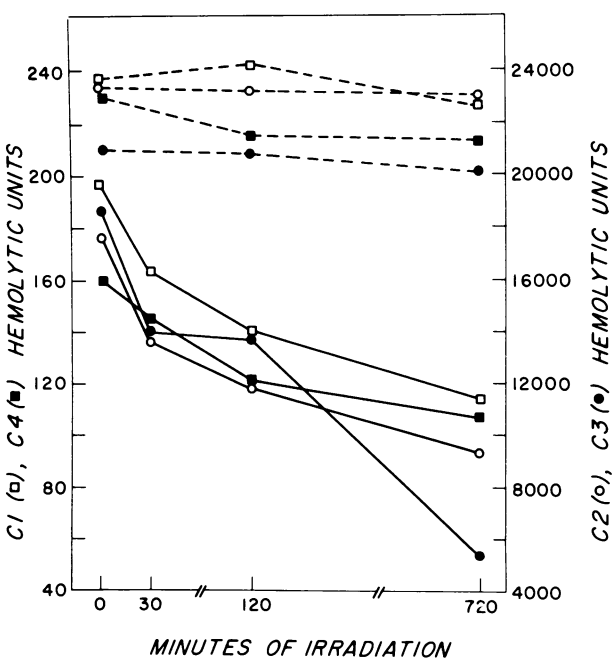

FIGURE 3 Time course of the effect of irradiation on C1, C4, C2, and C3 in normal serum (----) and serum from an asymptomatic EPP patient (——).

TABLE II

Residual Complement Activity in EPP Sera after 120 Min Irradiation*

Patient Number	C1	C4	C2	C3	CH50
1	70	75	68	66	64
2	70	55	50	38	59
3	50	68	30	34	2
4	82	96	65	61	66
5	89	75	52	47	43
6	74	71	65	58	5
7	64	60	34	68	25
8	74	62	41	34	53
9	82	82	100	60	93
10	40	59	36	33	25
Normal Serum	105	90	100	97	98

* Values expressed as percent of the values in nonirradiated serum.

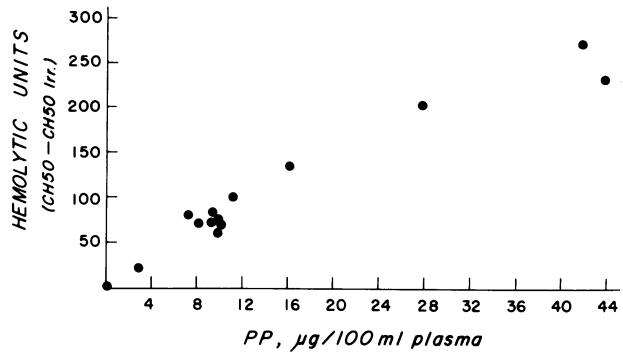

FIGURE 5 Correlation between levels of PP in the patient's plasma and loss of hemolytic activity in sera upon irradiation. The loss of hemolytic activity is calculated as the difference between the CH50 of nonirradiated and irradiated serum samples.

cidate whether the loss of CH50 was related to the protoporphyrin itself, an experiment was performed in which protoporphyrin-IX-dimethyl ester (PP) solution in acidified methanol (400 μg/ml) was added to normal human serum to final PP concentrations of 5, 2.5, and 1.25 μg/ml. Duplicate serum samples received acidified methanol alone. The hemolytic activity was measured in all samples in UV-irradiated and in nonirradiated specimens. The hemolytic activity in the absence of protoporphyrin was the same before and after UV irradiation, whereas addition of increasing concentrations of protoporphyrin followed by light exposure produced a loss of hemolytic activity proportional to the concentration of protoporphyrin added (Fig. 6). The greater amount of PP required to produce loss of hemo-

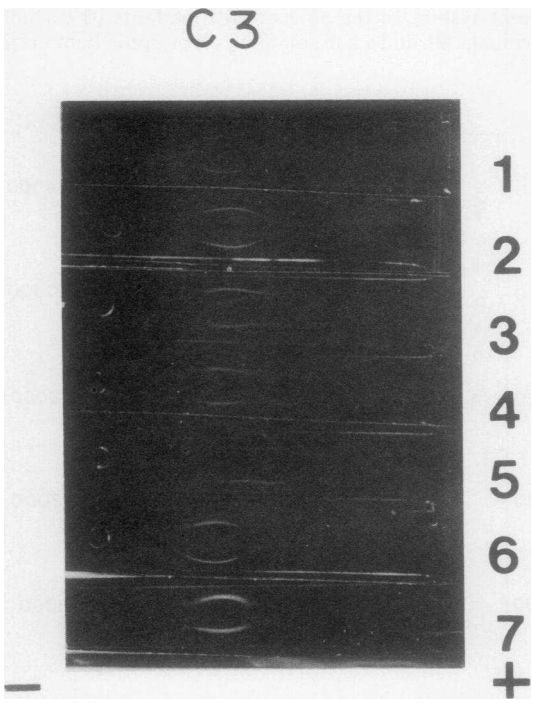

FIGURE 4 Immunoelectrophoresis using rabbit anti-human C3 of EPP and normal sera before and after 120 min UV irradiation. Sera from two individuals with EPP before irradiation: 1, 2; after UV irradiation: 3, 4; normal human serum activated with zymosan: 5; normal human serum: 6; and irradiated normal human serum: 7.

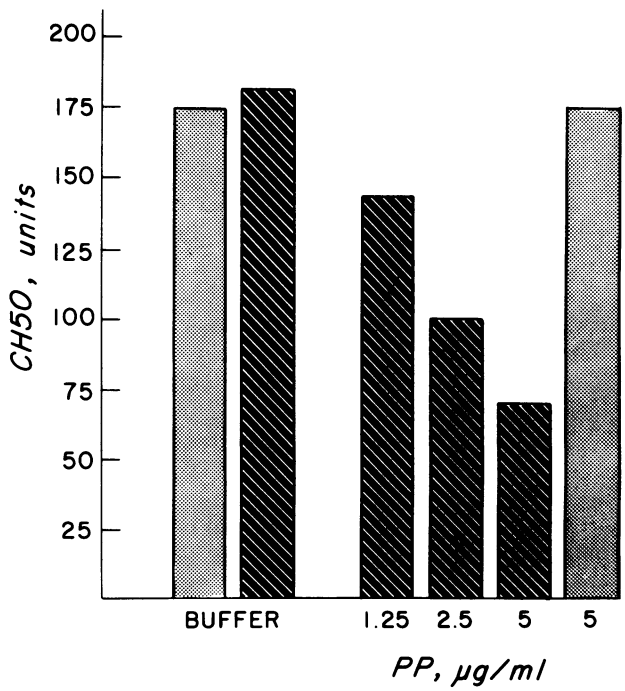

FIGURE 6 Effect of PP on normal serum. Residual complement activity of normal human serum to which different amounts of PP were added and were either kept in the dark (▨) or were irradiated (▧).

lytic activity may reflect the relatively poor solubility of the PP in the methanol solution when mixed with serum.

To evaluate the role of singlet oxygen and peroxide in the inhibition of complement activity observed in the previous experiment, human sera were treated with either 1 mM cysteine, 10 μg/ml bovine superoxide dismutase (Truett Laboratories, Dallas, Texas), or 10 μg/ml catalase (Sigma Chemical Co., St. Louis, Mo.). 2.25 μg/ml Protoporphyrin was added and the mixtures were exposed to UV irradiation for 30 min at room temperature. The results demonstrate that the inhibition of complement activity induced by porphyrins and UV was not affected by the simultaneous presence of scavengers of O_2 radicals.

DISCUSSION

This in vitro study describes the complement system in 14 photosensitive asymptomatic patients with EPP, before and after UV irradiation of serum samples. The CH50 and individual complement components in sera from these patients before light exposure were comparable with the values found in normal subjects (Fig. 1). After UV irradiation there was a marked decrease in C1, C4, C2, C3, and CH50; the latter could be correlated with the concentration of PP in the plasma (Fig. 5) and the dose of irradiation (Figs. 2 and 3). Normal sera and sera from patients with polymorphic light eruption, in whom PP is absent from the serum, exhibited a maximum of 10% decrease in complement levels during 120 min of exposure (Figs. 2 and 3), whereas a 30–90% decrease was observed in EPP plasma, except for patient No. 9 (Table II) who, at the time of the experiment, did not have detectable levels of PP in the serum. When the CH50 is compared with the levels of the individual complement components, there is no consistent relationship. Patient A.G., for example, has only 2% residual hemolytic activity, whereas C1, C4, C2, and C3 were 50, 68, 30, and 34% of the values found in nonirradiated serum, respectively. There is however, a direct correlation between the number of hemolytic units depleted upon irradiation and the level of PP in the sera (Fig. 5). Immunoelectrophoresis of the irradiated sera using monospecific antisera demonstrated changes in the electrophoretic mobility of C3, consistent with an enzymatic cleavage of this component (Fig. 4). That these findings are the result of a photodynamic action produced by PP in the blood is demonstrated by the observation that the addition of PP to normal serum, followed by irradiation, produces marked reduction in complement levels (Fig. 6). The role of singlet oxygen and peroxides in this reaction can be excluded based on the observation that the radical scavengers, cysteine, catalase, and superoxide dismutase fail to inhibit the reaction. Moreover, it has been demonstrated that cross-links between membrane proteins in erythrocyte ghosts subsequent to protoporphyrin-sensitized photooxidation is dependent on free amino groups and a particular intermediate of histidine photooxidation (21). Further, it could be shown that dityrosine formation, the crucial mechanism in oxidative cross-linking of proteins by peroxidase, H_2O_2 treatment (22), as well as the formation of disulfide bridges (23), plays no role in photodynamic cross-linking. Whether a similar mechanism is involved in complement activation by light in the presence of porphyrins remains to be determined.

In sun-exposed skin of patients with EPP, the major changes are seen in the dermis. There is often marked thickening of blood vessels as a result of the accumulation in and around the vessel walls of an amorphous, homogeneous, and slightly basophilic hyalinelike substance. Histochemical studies suggest that this material may be a neutral glycoprotein with smaller amounts of acid glycosaminoglycans and lipids (24–26). Electron microscopic examination shows that the amorphous material consists of a multilayered, partially fragmented basement membrane and finely fibrillar material of moderate density that permeates and surrounds the vessel walls (27). Direct immunofluorescence of light-exposed skin in these patients has demonstrated the deposition of immunoglobulins (28) and complement components (29, 30). The basement membrane zone and blood vessel walls showed the presence of IgG and IgM as well as C4 and C3. In three cases, C1q and C5 were detected around blood vessels. Moreover, IgA, IgM, and complement components C1q, C4, C3, and C5 were detected in back skin of EPP patients between 3 and 72 h after experimental irradiation with visible light ($\Lambda > 400$ nm), suggesting that the complement system may play a direct or indirect role in the blood vessel lesions seen in histological examinations of skin of EPP patients. The fact that as much as 44% of UV of wavelength > 320 nm can penetrate to the dermis when applied to caucasian skin (31), and the demonstration of complement activation in vivo in guinea pigs that received PP followed by UV exposure (32), supports the possibility of local complement activation in the presence of circulating PP.

The mechanism of complement activation either at tissue level or in vitro is not clear. Based on the deposition of IgG and IgM, it can be speculated that PP binds immunoglobulins in a manner similar to its binding to hemopexin or albumin (33), which upon irradiation could aggregate by cross-linking, thus providing the changes propitious for complement activation. Alternatively, a direct effect of PP on complement components could be responsible for activation of this system and subsequent generation of the ingredients for the development of the inflammatory response. The accumulation of hyaline-like substance in the blood ves-

sels of exposed skin (25) can be interpreted as an hypertrophic response of the endothelium resulting from a previous injury initiated by the photosensitizing action of PP on proteins capable of producing tissue injury.

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