Activation of the Alternative Complement Pathway by 405 nm Light in Serum from Porphyric Rat

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Sera from hexachlorobenzene-induced porphyric rats were examined for evidence of complement activation. The serum irradiated in vitro with 405 nm light resulted in a dose-dependent diminution of the total hemolytic activity of the complement. Further, such irradiated serum showed immunoelectrophoretical C3 conversion and chemotactic activity for rat polymorphonuclear leukocytes, and caused increased vascular permeability in vivo. C3 conversion was not induced in serum chelated with EDTA, but occurred in serum chelated with Mg^{++}-EGTA. By sephadex G-75 chromatography, the irradiated serum had potent chemotactic activity eluted near the cytochrome C marker. These studies indicate that the irradiation of porphyric rat serum with 405 nm light induces activation of the complement system via the alternative pathway, with the resultant development of anaphylatoxin. *Key words: Complement activation; C3 conversion; Anaphylatoxin; Porphyria.* (Received November 27, 1983.)

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Porphyria cutanea tarda (PCT) exhibits skin photosensitivity on exposed areas and is characterized by the excess excretion of uroporphyrin in urine. In 1961, Ockner & Schmid reported that hexachlorobenzene could induce experimental porphyria in rats (6). Because the metabolic pattern in rats resembles that of human PCT (8), it is possible to use rats as a model for PCT.

Recently, Lim et al. demonstrated that the irradiation of normal human serum containing uroporphyrin with 405 nm light resulted in the activation of the classical complement pathway and the generation of chemotactic activity for polymorphonuclear leukocytes (PMNs) (2). They extended these studies and obtained similar data from patients with PCT (3).

The object of this study was to determine whether similar mechanisms take place in serum from hexachlorobenzene-induced porphyric rats activated by 405 nm light in vitro.

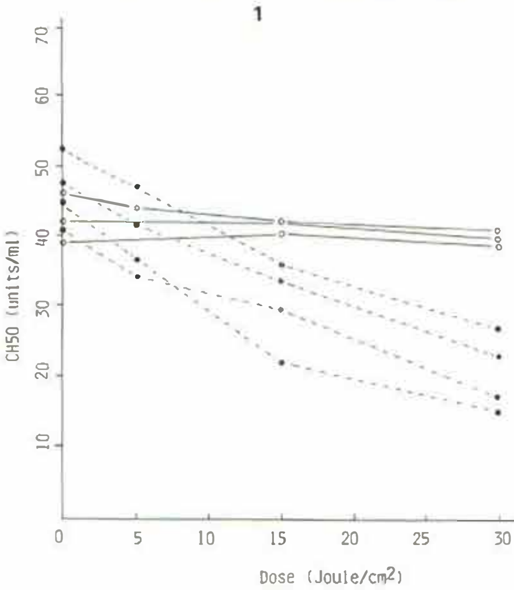
MATERIALS AND METHODS

Serum from porphyric rat

In order to induce porphyria in Sprague-Dawley female rats, they were fed a 0.25% hexachlorobenzene-diet for two months. Blood specimens were obtained by abdominal artery puncture and the serum was separated by centrifugation (3000 rpm) for 10 min at 4°C. Serum was stored at −70°C in aliquots until used. Serum uroporphyrin levels were measured by the procedure of Moore et al. (5).

In vitro irradiation

A grating monochromator with a xenon lamp (2 KW, SS-25NX type: Japan Spectroscopic Co. Ltd) was used as a light source for the experiments. The apparatus emitted a narrow wavelength of ultraviolet rays in a half-band width of 5 nm. A milliliter of serum samples was pipetted into a quartz cell and irradiated with 405 nm light at 0°C. After irradiation, the hemolytic titer of total complement activity (CH50) was measured as previously described (4).



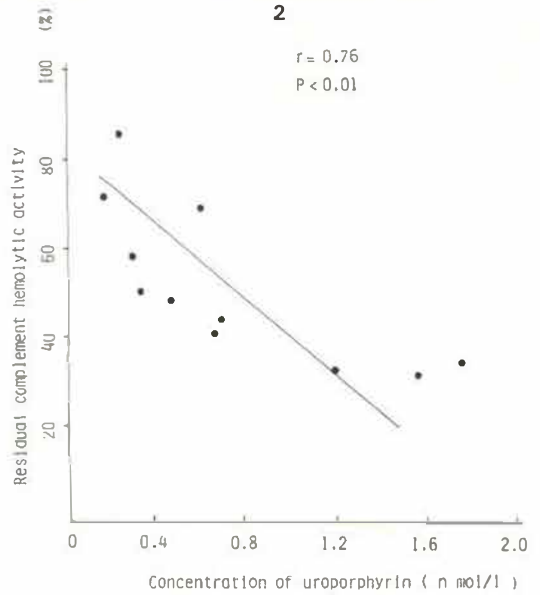


Fig. 1. Effect of 405 nm irradiation on the complement activity in the sera of 4 porphyric rats (●---●) and 3 normal rats (○—○).

Fig. 2. Effect of uroporphyrin level on total hemolytic complement activity in 30 Joule-irradiated serum from 11 porphyric rats.

C3 immunoelectrophoresis

Two-dimensional immunoelectrophoresis was performed on microscope slides using 1% agarose (Agarose-I; Wako Pure Chemical Co., Japan) in veronal buffer (pH 8.6, $\mu=0.05$), according to minor modifications of the methods of Crowle (1). Goat antiserum to rat C3 (Lot. 15072) was obtained from Cappel Laboratories, Inc., USA. In all chelator studies, porphyric rat serum was chelated by the addition of 2/10 volume of 0.1 M ethyleneglycol-bis(β-amino-ethyl ether)N,N'-tetraacetic acid (EGTA) supplemented with 10 mM Mg^{++} or 0.1 M ethylenediamine tetraacetic acid (EDTA) in veronal buffer (pH 7.4), before irradiation with 405 nm light. Each serum sample was incubated at 56°C for 30 min to prevent artifactual complement activation by the agarose film, before immunoelectrophoresis.

Neutrophil chemotaxis

In vitro chemotaxis was measured by use of a modified Boyden chamber (blind well chamber; BIO-RAD Lab., USA) assayed with a 3.0 µm polycarbonate filter (BIO-RAD Lab., USA) as previously reported (7). Neutrophils obtained from normal rat 3 h after intraperitoneal injection of 10 ml of 0.1% oyster glycogen in isotonic saline were suspended in Hanks solution with 0.5% bovine serum albumin (Hanks-BSA; pH 7.4) to a final concentration of 1×10^6 cells/ml. All serum samples were diluted with Hanks-BSA to a final concentration of 5% (vol/vol). We then placed 0.2 ml of the cell suspension into the top compartment of the chamber and 0.2 ml of the diluted serum into the bottom compartment, and the chamber was incubated at 37°C for 1 h in an atmosphere of 5% CO_2. The number of cells reaching the bottom surface of the filter was counted at a magnification of ×400. Chemotactic activity was expressed as the average number of cells per 10 fields for each filter.

Molecular-sieve chromatography

Sephadex G-75 in a column of 2.5×75 cm was equilibrated with 1/15 M phosphate-buffered saline (pH 7.2). It was calibrated with blue dextran, cytochrome C and vitamin B_{12}; 4 ml of irradiated serum from a porphyric rat was applied to the chromatographic column. The absorbancy of each fraction (6 ml) collected was determined at 280 nm. To measure chemotactic activity, 0.2 ml of ech fraction was placed in the test compartment without Hanks-BSA.

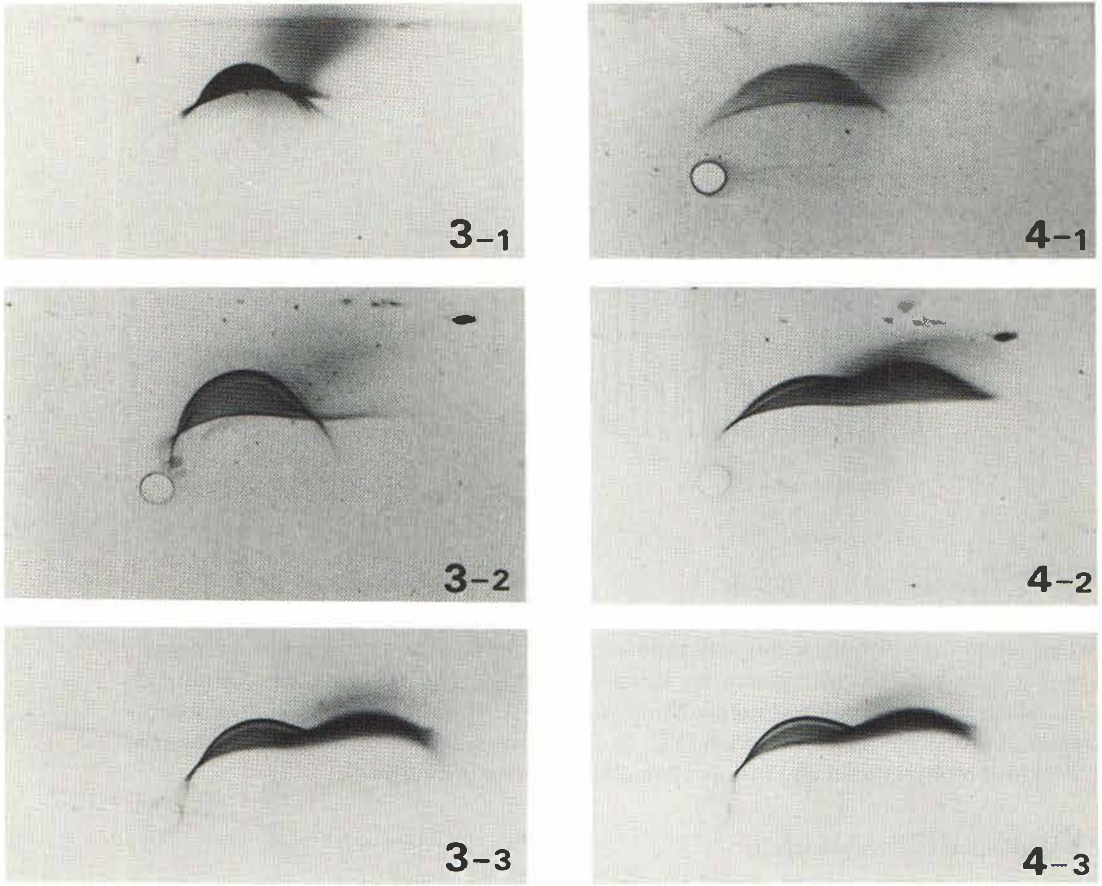


Fig. 3. Two-dimensional immunoelectrophoresis with anti-C3 antiserum. (1) Normal rat serum without irradiation; (2) non-irradiated serum from porphyric rat; (3) irradiated serum from porphyric rat.

Fig. 4. Two-dimensional immunoelectrophoresis of irradiated serum from porphyric rat with or without chelation. (1) EDTA-chelated serum; (2) Mg^{++}-EGTA-chelated serum; (3) non-chelated serum.

Vascular permeability

Normal rat was pretreated by an intravenous injection of 0.5 ml of 1% Evans blue in isotonic saline. We then intradermally injected 0.05 ml of 30 Joule/cm^2-irradiated serum from a porphyric rat into the depilated skin of the back of the rat with a 27-gauge needle. Non-irradiated serum from a porphyric rat and normal rat serum with or without irradiation were similarly injected into other areas of the skin as controls. The rat was killed 45 min later under ether anesthesia and the skin was excised. The intensity of the lesion as shown by extravasated Evans blue was estimated from the dermal side.

In vivo irradiation

The light source used consisted of a bank of four black lights (Toshiba FL-20BLB; Toshiba, Japan) which emit rays between 300 and 420 nm, with a maximum at 360 nm (mainly UV-A). The energy intensity was 3.9 mW/cm^2 at a target distance of 15 cm (UV radiometer UVR-305/365; Eisai Co., Japan). The depilated skin from the back of a porphyric rat was exposed to the black lights for 24 h. Skin biopsies were taken at various intervals: 1 h, 8 h, 16 h and 24 h after the initiation of the irradiation. After routine tissue processing, they were stained with hematoxylin-eosin and toluidine blue.

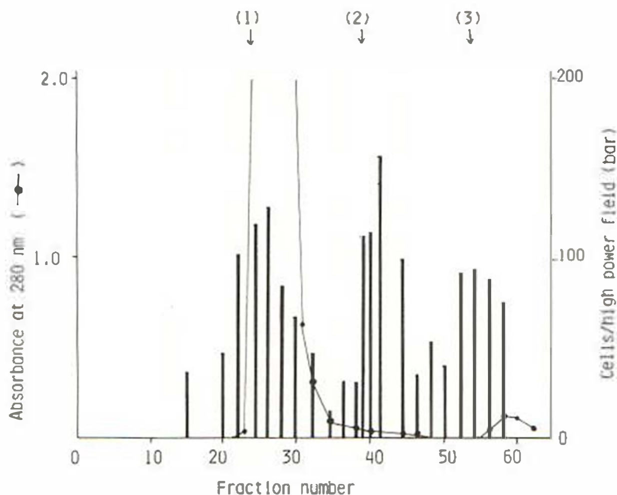


Fig. 5. Characterization of chemotactic activity in irradiated serum from porphyric rat by Sephadex G-75. (1) Blue dextran; (2) Cytochrome C; (3) Vitamin B_{12}.

RESULTS

Complement activation

When serum from porphyric rats was exposed to 405 nm irradiation, there was a progressive decrease in CH50 with increasing irradiation energy (Fig. 1). In the case of normal rats, no decrease in CH50 was noted. Diminution of CH50 was dependent on the concentration of uroporphyrin in the serum (Fig. 2). There was a linear correlation between the serum uroporphyrin level and residual hemolytic activity ($p<0.01$).

Crossed immunoelectrophoresis

Porphyric rat serum resulted in the conversion of C3 from β_1C to β_1A after 15 Joule-irradiation with 405 nm light (Fig. 3). Such an extra precipitin peak was absent from both non-irradiated serum from porphyric rat and normal rat serum with or without irradiation. Whereas the conversion was not observed in 20 mM EDTA-chelated serum, it occurred in serum chelated with 20 mM Mg^{++}-EGTA, indicating that 405 nm light activates the alternative pathway of complement in serum from porphyric rat (Fig. 4). The addition of Mg^{++} to the porphyric serum *per se* did not produce such C3 conversion.

Leukocyte chemotaxis

The irradiation (30 Joule) of serum from porphyric rat by 405 nm light resulted in the generation of highly potent chemotactic activity for rat PMNs as compared to both the non-irradiated serum from a porphyric rat and to the irradiated normal rat serum (data not shown). The chemotactic activity was characterized further by molecular-sieve chromatography (Fig. 5) The irradiated serum from porphyric rat revealed a triphasic pattern of highly potent chemotactic activity; i.e., the first peak was coincident with a void with a high protein content, the second was eluted with the cytochrome C marker which showed a low UV absorbance at 280 nm, indicating that there were very potent chemotactic factors, and the third was eluted with the low-molecular-weight fractions.

Increased vascular permeability

We observed the effect of irradiated serum from porphyric rat on vascular permeability. The rat, which was intraveneously injected with Evans blue dye beforehand, showed

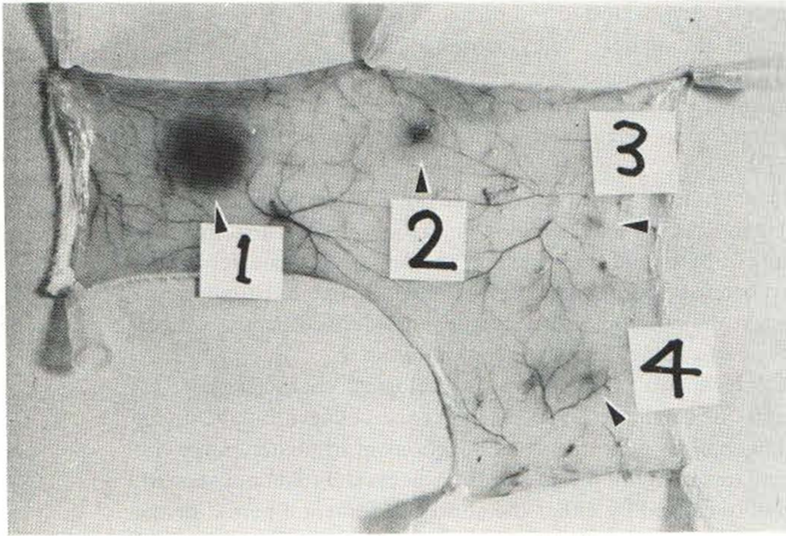


Fig. 6. Leakage of Evans blue dye after intradermal injection into normal rat skin. (1) Irradiated serum from porphyric rat; (2) non-irradiated serum from porphyric rat; (3) irradiated serum from normal rat; (4) non-irradiated serum from normal rat.

remarkable blueing at the site of the injection of irradiated serum from a porphyric rat (Fig. 6).

In vivo irradiation with UVA-visible light

Histologic studies in porphyric rat showed slight edema, dilation of blood vessels, and infiltration with PMNs and mast cells in the upper dermis 1 h after the initiation of irradiation. These histologic changes became exaggerated with increasing irradiation energy. In addition, there were a few dyskeratotic cells characterized by homogeneous, eosinophilic cytoplasm and hyperchromatic nucleus in the epidermis (data not shown).

DISCUSSION

In this report, we have presented data indicating that the irradiation of porphyric rat serum with 405 nm light induces activation of the complement system even after abrogation of classical pathway, which, in turn ,results in the development of anaphylatoxin with complement-derived chemotactic activity for rat PMNs.

Lim et al. recently demonstrated that the irradiation of normal human serum containing uroporphyrin with 405 nm light resulted in complement activation through the classical pathway, and the generation of C5-derived chemotactic activity (2). This report was based on the finding that chemotactic activity, which can be inhibited by incubation with anti-C5 antiserum, was not generated in Mg^{++}-EGTA-treated serum nor in C4-deficient guinea pig serum. In our experiment, complement conversion was not, however, abolished in the porphyric rat serum chelated by the addition of 20 mM EGTA supplemented with 2 mM $MgCl_2$, to effectively block activation of the classical pathway (Fig. 4). Thus, it can be concluded that 405 nm light activated the alternative complement pathway in the case of porphyric rat serum, although our data do not exclude the possibility of the involvement of the classical pathway activation in these rats. We could not perform other experiments to prove alternative pathway activation by analysis of split products of factor B or analysis of properdin because the antisera to these substances of rat were unavailable. At any rate, this difference between human patients (2, 3) and our experimentally induced porphyric animal model in rats is of great interest.

Molecular sieve chromatography demonstrated the presence of a peak of chemotactic activity with a low protein content eluting near the cytochrome C marker (12000 daltons), the same location as that of $C5_a$ found in zymosan-activated serum (data not shown). We also confirmed that the intradermal injection of the irradiated serum enhanced vascular permeability as measured by the extravasation of intravascular Evans blue dye. The presence of anaphylatoxins ($C3_a$ and $C5_a$), which mediate histamine release from mast cells and basophils, seems to account for this vascular change (9). Furthermore, irradiation of porphyric rat with UVA-visible light induced acute inflammation in vivo; histologically there were edema, infiltration of PMNs and mast cells in the upper dermis a few hours after irradiation. These data, taken together with the development of anaphylatoxin in the irradiated serum in vitro, suggest the possibility that complement-derived peptides mediate the early inflammatory phase of cutaneous lesions in porphyria.

Chronic skin lesions in PCT have two principal histopathological characteristics; (1) PAS-positive hyaline in the walls of capillaries, and (2) thickened collagen bundles. Our attempt to produce chronic skin changes by intradermal injections of irradiated porphyric serum at the same site of normal rats daily for 4 weeks was unsuccessful (data not shown). This lack of success was probably caused by either the short experimental period or the need for factor(s) other than complement-derived peptides in the production of actual lesions in vivo.

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