receptor is centered on 12 residues which are mostly located on the same side of the neurotoxin molecule, leaving a large part of the toxin accessible from outside the membrane (*9*). Therefore, ACh receptor–bound toxin may be a target for one or more neurotoxin immunoglobulins. To determine whether such interactions between bound toxin and specific antibodies occur and, if they do, whether they affect the extremely stable neurotoxin-receptor complex, we studied the effect of horse antiserum to *Naja nigricollis* venom and of monoclonal neurotoxin–specific immunoglobulin on the dissociation of the neurotoxin–ACh receptor complex in vitro. We found that the dissociation of the complex was significantly accelerated by the neurotoxin-specific immunoglobulins we studied.

Toxin α isolated from *N. nigricollis* venom (*10*) and heavily labeled with tritium (*11*) was incubated with ACh receptor–rich membranes isolated from *Torpedo marmorata* (*12*) until equilibrium was reached. The amount of ^{3}H-labeled toxin bound to ACh receptor was determined by a filtration assay and the kinetics of dissociation was followed under a variety of conditions. To observe the dissociation of the [^{3}H]toxin-receptor complex it is necessary to prevent reassociation of [^{3}H]toxin molecules on the receptor—for instance, by trapping the free receptor or [^{3}H]toxin molecules or by diluting the solution. The results obtained are shown in Fig. 1. Addition of an excess of nonradioactive toxin saturates free receptor molecules and thus a slow decrease of bound [^{3}H]toxin is observed (curve 2 in Fig. 1). A great dilution (1:100) of the solution produces similar results (data not shown), indicating that there is no cooperative effect involving toxin molecules during the dissociation process. Addition of an excess of horse antiserum to *N. nigricollis* venom results in a significant acceleration of the dissociation, with 40 percent compared to 5 percent dissociated after 1/2 hour (curves 3 and 2, respectively, in Fig. 1). This effect of immunoglobulins against venom components is specific, since it does not occur when a normal horse serum is added (data not shown). Moreover, prior incubation of the specific serum with a large excess of nonradioactive neurotoxin prevents the accelerating effect (curve 2), indicating that the immunoglobulins responsible for this phenomenon are neurotoxin-specific. This effect cannot be explained by simple trapping of free [^{3}H]toxin molecules because, in that case, one would expect to observe dissociation kinetics similar to curve 2. Therefore, there may be an additional process which involves a direct effect of immunoglobulins on [^{3}H]toxin molecules bound to the receptor, inducing destabilization of the complex.

Recently, a monoclonal antibody against *N. nigricollis* toxin α was produced (*13*). The antigenic site at which this immunoglobulin binds has been identified and is distinct from the ACh receptor binding site of the toxin. As shown in Fig. 1, addition of this homogeneous toxin-specific immunoglobulin population accelerates the dissociation in a manner similar to that observed with the antiserum to the venom. This effect is inhibited when the antibody combining site is initially blocked by unlabeled toxin, confirming the importance of specific recognition of the antibody for the toxin molecule. The same effect is observed with Fab fragments (*14*) (Fig. 1).

The results presented here show that the neurotoxin–ACh receptor complex is appreciably destabilized in vitro by neurotoxin-specific immunoglobulins. This effect probably results from a direct interaction between at least one accessible antigenic determinant of the ACh receptor–bound toxin molecule and the corresponding antibody combining site. Whether the effect is dependent on the induction of a conformational change in the bound toxin remains to be determined. If the destabilizing effect is confirmed in vivo, it may be important for establishing an adequate serotherapy.

JEAN-CLAUDE BOULAIN
ANDRÉ MÉNEZ
Service de Biochimie, Département de Biologie, Centre d'Études Nucléaires, 91191 Gif-sur-Yvette Cedex, France

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15. We thank P. Fromageot for helpful discussions and J. Couderc and P. Liacopoulos for their collaboration in the preparation of monoclonal antibodies.

2 March 1982

Psoralen Phototoxicity: Correlation with Serum and Epidermal 8-Methoxypsoralen and 5-Methoxypsoralen in the Guinea Pig

Abstract. *Serum and epidermal concentrations of 8-methoxypsoralen and 5-methoxypsoralen 2 hours after oral administration to guinea pigs were determined by high-performance liquid chromatography. A linear relation was found between the serum and epidermal concentrations of 8-methoxypsoralen. In addition, a relation was found between serum concentrations of 5-methoxypsoralen and 8-methoxypsoralen and the appearance of phototoxicity. The lower phototoxicity of orally administered 5-methoxypsoralen as compared to 8-methoxypsoralen in the guinea pig appears to be due to its reduced concentrations in the epidermis, the primary site of the phototoxic events.*

Psoralens are a class of compounds present in many plants. These naturally occurring psoralens, such as 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and unsubstituted psoralen (Fig. 1), have been identified as phytoalexins; they are part of the plant's complex defensive response against fungal and insect (*1*) challenges. Furthermore, psoralens are powerful phototoxic agents in animals and humans. Humans can be exposed topically by contact with psoralen-containing fruits and vegetables (*2*) or cosmetics (*3*) and orally by ingestion of certain common fruits and vegetables (*4*) or the use of psoralen-containing drug formulations (*5*).

Psoralens are increasingly used in photochemotherapy for management of such disorders as vitiligo, psoriasis, and mycosis fungoids (*6*). This application involves oral administration of the chosen psoralen, typically 8-MOP, followed by irradiation of the patient with ultraviolet A (UVA) light (*7*). A well-designed photochemotherapy regimen, which produces the maximum therapeutic effect while eliciting the minimum phototoxic response, requires that the individual patient be tested for photosensitivity before treatment. In efforts to establish tests that reliably predict a clinical response, numerous investigators have tried to correlate blood concentrations of

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8-MOP with phototoxicity or clinical effectiveness. No such correlation was found by Thune and Volden (*8*) with 31 photochemotherapy patients. The 8-MOP plasma concentrations 2 hours after an oral dose varied between 2 and 167 ng/ml. Similar results were obtained by Steiner *et al.* (*9*), who found an even wider range of serum levels (10 to 930 ng/ml). These investigators concluded that skin type is a major factor in the phototoxic response to 8-MOP. However, Ljunggren *et al.* (*10*) recently reported a correlation between psoralen dose and serum levels measured repeatedly in the same individuals.

Thus it has not yet been clearly demonstrated that blood psoralen concentrations predict the appearance or severity of phototoxicity. Concern has been expressed that blood psoralen concentrations may not represent the amount of psoralen that has diffused from the superficial dermal capillaries into the epidermis (*8*). This concern may be particularly justified for short intervals after an oral dose corresponding to realistic irradiation times in photochemotherapy. The possibility remains that psoralens, as used in photochemotherapy, are among the drugs whose blood levels are not adequately predictors of toxic or therapeutic effects (*11*).

The use of psoralens in photochemotherapy has raised another question concerning their oral phototoxicity. The structurally similar psoralens 5-MOP and 4,5′,8-trimethylpsoralen (TMP) have similar phototoxicity when administered topically. However, when administered orally, they are much less phototoxic than 8-MOP (*12, 13*). Recently, two European groups have introduced 5-MOP as an alternative to 8-MOP in the photochemotherapy of psoriasis (*13, 14*). Although 5-MOP and 8-MOP were comparable in their ability to clear psoriatic lesions, acute side effects (including phototoxicity) were significantly reduced with 5-MOP. As more becomes known about the biotransformations of psoralens (*12*), it appears that metabolism may play a central role in determining the relative oral phototoxicity of substituted psoralens. However, it has not been established that reduced delivery of the phototoxic psoralen to the epidermis, due to metabolism or lack of absorption, is the basis for the observed differences in oral phototoxicity.

We report determinations of serum and epidermal concentrations of 5-MOP and 8-MOP in guinea pigs. To our knowledge, psoralen concentrations in the epidermis, the primary target organ for phototoxicity, have not previously been reported for humans or animals. The guinea pig has been shown by us and others (*15*) to be a reliable animal model for predicting phototoxicity in humans.

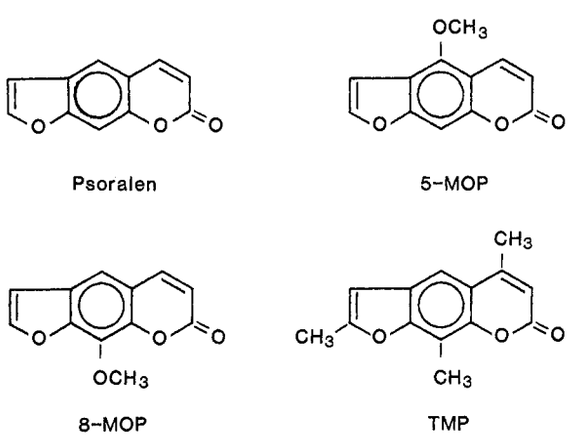

Fig. 1. Structures of psoralen and substituted derivatives.

Table 1. Phototoxicity and serum and epidermal concentrations of 8-MOP and 5-MOP in guinea pigs. Concentration values are means ± standard errors. Phototoxicity is indicated by erythemal grade after 72 hours. When applied topically at 100 and 10 μg/cm^2, both 8-MOP and 5-MOP gave erythemal grade ++++. At 1 μg/cm^2 both psoralens failed to evoke a phototoxic response.

Psoralen	Dose (mg/kg)	Serum concentration (μg/ml)	Epidermal concentration (μg/g)	Phototoxicity
8-MOP (N = 4)	15	1.85 ± 0.24	1.19 ± 0.21	++++
8-MOP (N = 5)	10	0.99 ± 0.13	0.66 ± 0.16	++++
8-MOP (N = 5)	5	0.30 ± 0.04	0.22 ± 0.04	+++
8-MOP (N = 5)	3	0.05 ± 0.01	*	0
5-MOP (N = 5)	15	0.35 ± 0.03	0.41 ± 0.05	+++
5-MOP (N = 5)	10	0.07 ± 0.01	*	0

*Detectable but not significant.

The backs of young adult albino guinea pigs of the Hartley strain were epilated (*16*). Each group of five animals to be used for phototoxicity studies was given either an oral dose or a topical application in acetone (*17*). Two hours after the oral dose or 15 minutes after the topical application, the guinea pigs were immobilized on a wooden board and irradiated with UVA at 3 J/cm^2 (*18*). At 24, 48, and 72 hours after irradiation, the severity of erythema was graded as 0, +, ++, +++, or ++++ (*19*). To determine serum and epidermal psoralen levels, additional groups of five guinea pigs were given oral doses. Blood samples, taken by heart puncture 2 hours after dosing, were allowed to clot at 0°C to obtain serum. Immediately after blood samples were drawn, the guinea pigs were killed by CO_2 asphyxiation. Skin was excised from the epilated portion (approximately 40 cm^2) of the back. To obtain epidermis, skin samples were immersed in distilled water at 60°C for 30 seconds and epidermis was gently peeled off with forceps. This treatment resulted in no measurable loss of either 5-MOP or 8-MOP.

The weighed epidermis was homogenized in distilled water with a Polytron homogenizer. Serum and epidermal homogenates were extracted (*20*). Organic layers from the serum extraction were combined and evaporated to dryness under N_2 at 60°C. Elution of epidermal extracts through a silica-gel cleanup column (*21*) was necessary to eliminate impurities in the final high-performance liquid chromatographic (HPLC) analysis. The residues from each serum and epidermis extraction were reconstituted in 0.2 ml of methanol and analyzed by HPLC (*22*).

The serum and epidermal concentrations for each guinea pig receiving an oral 8-MOP dose of 5, 10, or 15 mg/kg are shown in Fig. 2. There is a high correlation between serum and epidermal concentrations of 8-MOP over a wide range of serum concentrations. Table 1 presents data organized with respect to dose and observed phototoxicity. A phototoxic response was first observed in animals receiving an 8-MOP dose of 5 mg/kg. The appearance of phototoxicity is accompanied by an appreciable increase in serum and epidermal 8-MOP concentrations. It is important to note that the guinea pig serum concentrations marking the onset of phototoxicity (0.05 to 0.3 μg/ml) are within the range of serum con-

centrations accompanying the appearance of phototoxicity in humans receiving therapeutic doses (0.6 mg/kg) of 8-MOP (*8*, *9*). When given 8-MOP at either 10 or 15 mg/kg, all animals showed a severe phototoxic reaction with elevated serum and epidermal levels. These results in our model system indicate that 8-MOP serum levels are correlated with epidermal levels and may be used to predict phototoxicity.

As indicated in Table 1, the topical phototoxicity of 5-MOP in the guinea pig was similar to that of 8-MOP. However, as in humans (*13*), 5-MOP administered orally was less phototoxic than 8-MOP. The onset of phototoxicity occurs between oral doses of 10 and 15 mg/kg for 5-MOP but between 3 and 5 mg/kg for 8-MOP (Table 1). This may indicate that less 5-MOP than 8-MOP reaches the epidermis (due to lack of absorption or rapid metabolism) or that the efficacy of 5-MOP in eliciting a phototoxic response in the epidermis is reduced. We found (Table 1) that the serum and epidermal concentrations required for a phototoxic response are similar for both 5-MOP and 8-MOP. Thus we conclude that the chief reason for the lower oral phototoxicity of 5-MOP is decreased delivery to the epidermis.

The molecular events initiating the phototoxic reaction for both 5-MOP and 8-MOP are thought to be intercalation of the unmetabolized psoralen into DNA followed by formation of a covalent monoadduct (on absorption of one photon) or diadduct (absorption of two photons) (*23*). The latter leads to cross-linked complementary strands of DNA. Similar abilities of 5-MOP and 8-MOP to intercalate and form adducts in vitro have been reported (*24*). Therefore, once in the epidermis, 5-MOP and 8-MOP should be similarly efficacious in initiating the molecular events leading to a phototoxic reaction.

The fact that psoralens, as used in photochemotherapy, react covalently with DNA indicates a potential risk of mutagenicity and oncogenicity. Indeed, in an in vitro study, 8-MOP and 5-MOP showed essentially the same activity in inducing chromosome damage in human cells (*25*). Furthermore, topical 5-MOP in combination with UVA induced carcinogenesis in mice comparable to that induced by 8-MOP (*26*). The results of these two studies suggest that 5-MOP and 8-MOP have a similar oncogenic potential. Our results indicate that, when equivalent oral doses are given, 5-MOP is present at lower epidermal concentrations than 8-MOP. Therefore, by orally administering 5-MOP it should be possible to maintain epidermal drug concentrations at lower levels than in an 8-MOP regimen. Extrapolating these findings to clinical applications in photochemotherapy, a 5-MOP regimen may involve less damage to epidermal DNA and thus a reduced risk of carcinogenesis which is strongly suspected in 8-MOP photochemotherapy (*27*), and yet be effective in inhibiting rapidly proliferating psoriatic cells. The European experience with 5-MOP therapy shows that phototoxicity is not required for a therapeutic effect. For this reason, and because of the reported reduced acute side effects with a 5-MOP regimen (*13*, *14*), we recommend that 5-MOP be further tested, along with other psoralen derivatives, as an alternative to 8-MOP in photochemotherapy.

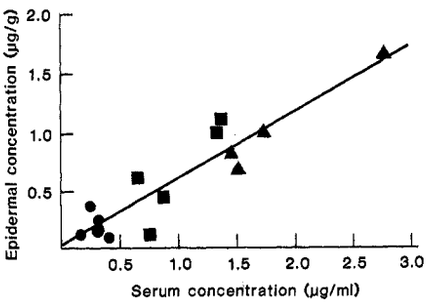

Fig. 2. Relation between 8-MOP serum and epidermal concentrations in guinea pigs receiving (●) 5, (■) 10, and (▲) 15 mg of 8-MOP per kilogram. Values from guinea pigs obtaining 3 mg/kg are omitted since epidermal levels were too low to quantitate. The least-squares fitted line is $y = 0.579x + 0.034$, with standard deviations in slope and intercept of 0.066 and 0.080, respectively.

ANDRIJA KORNHAUSER

Food and Drug Administration, Bureau of Foods, Division of Toxicology, Washington, D.C. 20204, and *Harvard School of Dental Medicine, Boston, Massachusetts 00115*

WAYNE G. WAMER

ALBERT L. GILES, JR.

Food and Drug Administration, Bureau of Foods, Division of Toxicology

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16. A depilatory agent composed of one part beeswax, two parts rosin powder (with diluent), and two parts of purified rosin lumps (Fisher Scientific) at 40° to 48°C was uniformly spread over the clipped area of the back of anesthetized guinea pigs. The depilatory agent was removed after about 5 minutes. Three to five days after epilation, the skin returned to normal appearance, without signs of inflammation or abrasions.
17. 5-MOP (Memphis Chemical, Cairo, Egypt) and 8-MOP (Paul Elder Co., Bryan, Ohio) were checked for identity and purity by nuclear magnetic resonance, gas chromatography, HPLC, and mass spectrometry. Oral dose levels are listed in Table 1. 5-MOP and 8-MOP were applied topically, in acetone, at 100, 10, and 1 $\mu g/cm^2$. Control guinea pigs received no oral dose or a topical application of acetone and were irradiated as described.
18. Light was measured with an IL 700 research radiometer (International Light), using UVA detector 338 and diffuser 698, and was from a Sylvania sunlamp filtered through glass (cutoff ≤ 315 nm).
19. Grading system: +, definite positive but weak erythema; ++, moderate erythema; +++, strong erythema; and ++++, strong erythema with necrosis.
20. The method of J. Kreuter and T. Higuchi [*J. Pharm. Sci.* **68**, 451 (1979)] was modified for extraction of serum and epidermal homogenates. A 1-ml portion of serum, diluted to 5 ml with distilled water, and a 15-ml portion of epidermal homogenate were each spiked with an appropriate amount of internal standard, TMP. Concentrated HCl was added to each spiked sample to a final concentration of 0.1*N* and samples were heated at 90°C for 1 hour. Samples were then extracted three times with equal volumes of 20 percent (by volume) CH_2Cl_2 in heptane.
21. Epidermal extracts were combined and loaded onto a column containing 1.5 g of silica gel (5 to 30 μm; Supelcosil 12A, Supelco Inc.). The column was eluted, under N_2 pressure, with CH_2Cl_2. Psoralen-containing fractions were obtained by collection of fluorescent bands eluting from the column.
22. The chromatographic system included a Waters model 440 absorbance detector, model U6K injector, model 6000A solvent delivery system, data module, and model 720 system controller. A Waters C_{18}-μBondapak column (3.9 mm by 30 cm) was eluted with 65 percent (by volume) methanol in distilled water at 2 ml/min.
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