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In situ Localization of Chloroquine and Immunohistological Studies in UVB-irradiated Skin of Photosensitive Patients

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Chloroquine can prevent photosensitivity reactions, but its mechanism of action is poorly understood. To investigate if the drug may interfere with inflammatory or immunological mechanisms of the UV-induced erythema of photosensitive patients, we studied the localization of chloroquine in the skin and its effect on the epidermal/dermal expression of IL-1, TNF-α, IL-6 and ICAM-1 and the occurrence of different lymphoid cells in normal skin and UVB-induced erythema in 8 patients with photosensitive discoid and systemic lupus erythematosus and 4 patients with polymorphic light eruption (PMLE), before and during chloroquine treatment. Using a specific monoclonal antibody against chloroquine, we found a strong granular staining pattern of mainly keratinocytes in all biopsy specimens from normal and erythematous skin during chloroquine treatment. In non-irradiated skin, T lymphocytes, macrophages and HLA-DR expressing cells were sparsely distributed within the dermis in similar amounts before and during chloroquine treatment. In UVB-induced erythema an increase in the number of these cells, mainly located in the dermal perivascular area, was seen before medication. During chloroquine treatment such cellular infiltration was reduced. ICAM-1 expression was detected on the endothelium of dermal vessels but not on keratinocytes. The accumulation of chloroquine in the epidermis and the decreased cellular infiltration in erythematous skin during chloroquine treatment indicate a local anti-inflammatory effect. This effect may be due to either unspecific UV-protective properties of the drug or to some specific downregulating action by chloroquine on keratinocyte function. ***Key words: anti-inflammatory; erythema.***

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Erythema is the most conspicuous acute cutaneous response to ultraviolet radiation (UVR). The erythema normally resolves within a week, but in patients with photosensitive disorders it may initiate more long-standing cutaneous reactions.

Twenty-four hours after UVB irradiation of normal skin, a mixed dermal perivascular infiltrate consisting mainly of T lymphocytes but also including monocyte-macrophages and polymorphonuclear leukocytes is observed (1). A number of inflammatory mediators appear to be involved in the UV-induced erythematous response, including serotonin, prostaglandins, lysosomal enzymes and kinins. UV radiation is also a potent stimulus for keratinocyte release of cytokines, including IL-1, TNF-α and IL-6 (2). Aberrant UVR-induced release of such pro-inflammatory factors and directly or indirectly induced aberrant expression on the keratinocytes of certain adhesion molecules such as intercellular adhesion molecule (ICAM-1), which is not seen in erythematous skin of healthy subjects (3), have therefore been suggested as pathogenetic mechanisms of the subacute and chronic cutaneous reactions that may develop after sun exposure in predisposed patients (4,5). In a study by Norris et al. (6), keratinocyte ICAM-1 expression was seen in UV-induced polymorphic light eruption (PMLE) lesions, and in a recent study (7) of patients with photosensitive lupus erythematosus and PMLE, similar ICAM-1 expression on keratinocytes was found in lesions induced by repeated UVA or UVB irradiation.

Long-term treatment with chloroquine is often successful in preventing photosensitivity in, for example, systemic and discoid lupus erythematosus (8) and PMLE (9), although its mechanism of action in this respect is poorly understood. Since the drug binds strongly to melanin and accumulates in the skin (10,11,12), some kind of local protective effect of the drug could be a possibility. Anti-inflammatory and direct immunosuppressive properties have also been proposed as underlying mechanisms (13).

To gain a better understanding of possible local immunological mechanisms of UV-induced erythema in patients with photosensitive disorders and of the way in which chloroquine may interfere with these mechanisms, it seemed of value to investigate by immunohistochemistry the in situ localization of chloroquine in the skin in such patients, and the expression of IL-1, TNF-α, IL-6 and ICAM-1 and the occurrence of T lymphocytes, macrophages and HLA-DR-expressing cells in normal-appearing skin and UVB-induced erythema prior to and during chloroquine treatment of the patients.

MATERIAL AND METHODS

Subjects

Twelve patients, 1 man and 11 women (mean age 38, range 18–56 years), participated in the study, which was approved by the local ethical committee of the University Hospital, Uppsala. All the patients had disorders including photosensitivity: 6 had discoid lupus erythematosus, 4 had PMLE, 1 had systemic lupus erythematosus and 1 had a subacute cutaneous lupus erythematosus. They were all on the point of starting treatment with chloroquine phosphate at a daily dose of 250 mg with the aim of reducing sun sensitivity. None of them were having any concomitant treatment or had been exposed to UV radiation during the last 8–9 months. Skin samples were collected during the months of April-May (before treatment) and August-September (at steady state, after at least 2 months of chloroquine treatment).

Erythema induction

In each patient a 16 cm^2 area of non-involved skin on the back was irradiated with UVB in nine successively increasing doses from 0.05 to 1.5 J/cm^2 in order to produce erythema. Identical irradiations were performed before chloroquine treatment and at steady state during this treatment. The irradiation source was a monochromator (Applied Pho-

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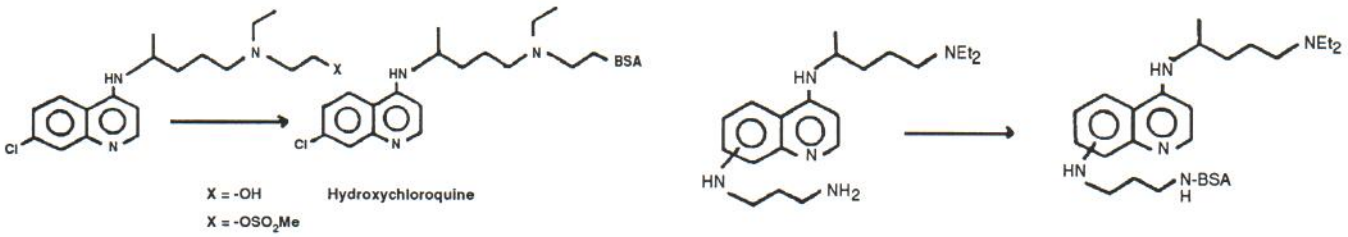

Fig. 1. (*a*) F-73 monoclonal chloroquine antibody. Hydroxychloroquine coupled to bovine serum albumin (BSA) by converting the hydroxy group into a methanesulphonyl group and coupling the mesylate to the protein. (*b*) F-157–7 monoclonal chloroquine antibody. A chloroquine-protein immunogen in which chloroquine is linked at a ring position to the carrier protein.

tophysics Clinical Photoirradiator, UK), emitting a narrow band of UVB at 313 nm (slit width 3 mm, corresponding to a band width of ±4–6 nm) through a 1-m liquid light guide with an aperture diameter of 0.9 cm. The irradiance, measured with a thermopile before each treatment session, was 14–17 mW/cm^2.

Skin biopsies

Before chloroquine treatment and at steady state during this treatment, 3-mm punch biopsy specimens were obtained under local anaesthesia with 1% lidocaine. Two biopsies were taken on each occasion, one from normal dorsal forearm skin and one from the irradiated back skin site 24 hours after irradiation, when the erythema was maximal. The erythematous skin had been irradiated with the highest dose of UVB (1.5 J/cm^2; 4–7 MED) which in all patients before and during treatment had yielded marked erythema and slight to moderate oedema. With the highest UVB dose there was no significant difference in the erythema or oedema reaction before or during chloroquine treatment. The specimens were placed in Histocon medium (Histo-Lab; Betlehem Trading Ltd., Gothenburg, Sweden) and kept at +4°C until quick-frozen in −70°C isopentane within 4 h. They were then stored at −70°C until analysed.

Antibodies and other reagents

Two different monoclonal antibodies against chloroquine (originally developed for use in ELISA inhibition tests) were produced by immunization of balb/c mice as follows (14, 15):

1) Hydroxychloroquine was coupled to bovine serum albumin (BSA) by converting the hydroxy group into a methanesulphonyl group and coupling the mesylate to the protein, producing a type F-73 antibody, which reacts not only with chloroquine but also with its metabolites and amodiaquine (Fig. 1a).

2) A chloroquine-protein immunogen was made in which chloroquine was linked at a ring position to the carrier protein, thereby producing a type F 157–7 antibody with much less cross-reactivity with the metabolites of the drug than the F-73 antibody (Fig. 1b).

The murine monoclonal antibodies (MoAbs) denoted anti-Leu4 (CD3, T lymphocytes), anti-LeuM3 (macrophages) and anti-HLA-DR were all purchased from Becton-Dickinson (Sunnyvale, CA). Anti-IL-1, -TNF-α, -IL-6 and -ICAM-1 MoAbs were purchased from Serotec Ltd (Oxford, UK). Rabbit anti-mouse immunoglobulin and the peroxidase-antiperoxidase mouse monoclonal complex used for immunoperoxidase staining were purchased from Dakopatts (Glostrup, Denmark).

Immunohistochemical staining

Acetone-fixed 6 μm thick frozen biopsy specimens were stained by the immunoperoxidase method (16). Subsequent incubations were carried out sequentially for 30 minutes at room temperature and in a humid atmosphere, followed by washing for 5 minutes in phosphate-buffered saline (PBS) between each step. Endogenous peroxidase was blocked by incubation in H_2O_2 (0.3%) for 15 minutes. The primary antibodies anti-Leu4, anti-Leu-M3 and anti-HLA-DR were used at concentrations of 0.8, 0.4 and 0.6 μg/ml, respectively. Anti-ICAM-1, anti-TNF-α and anti-IL6 were all used at a concentration of 10 μg/ml. Incubation with primary MoAbs was followed by incubation with rabbit anti-mouse Ig. Finally, the PAP complex was layered on the slides. The peroxidase reaction was developed for 15 minutes using a 3-amino-9-ethylcarbazole-containing buffer. The slides were counterstained with haematoxylin and mounted in glycerine-gelatin. In controls the MoAbs were omitted and the samples were then stained as described above. This only generated the counterstaining.

The extent and intensity of the expression of chloroquine, IL-1, TNF-α, IL-6, ICAM-1, M3 and HLA-DR-positive cells were graded as absent, weak, moderate or strong. The total number of CD3-positive cells in each tissue section was counted and expressed as number of cells per high-power field (x400) and finally graded as absent (0), few (0–20), moderate (21–50), and high (>50) number of CD3-positive cells. Three consecutive sections were assessed in random order by two observers (AKP and GSF) independently of each other.

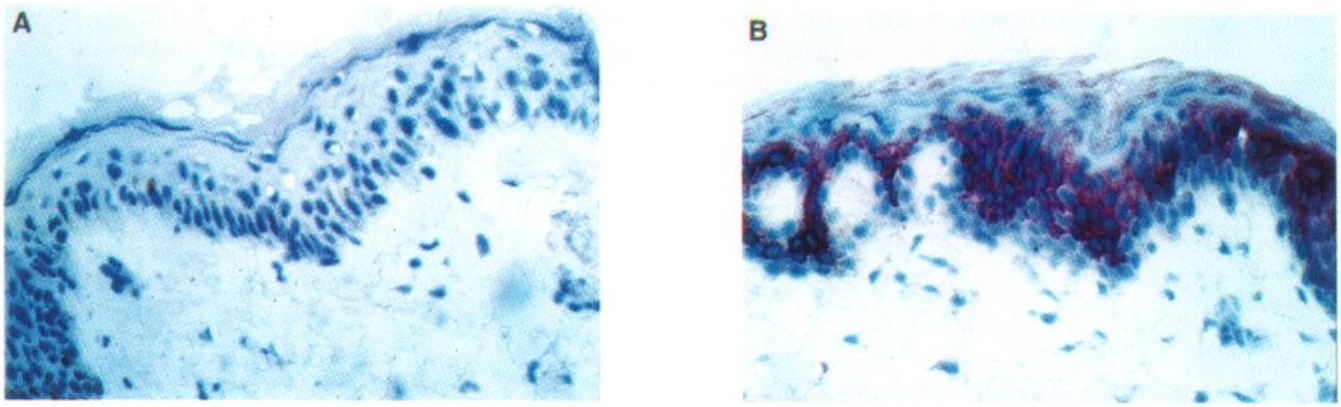

Fig. 2. Immunoperoxidase staining with the F-157–7 monoclonal antibody against chloroquine. (*a*) Normal dorsal forearm skin prior to chloroquine treatment. (*b*) Skin from the same site in the same patient during steady state of chloroquine treatment, exhibiting a strong granular staining pattern of the epidermis except for the basal cell layer.

RESULTS

Localization of chloroquine in the skin

A strong granular staining pattern of epidermal cells, i.e. mainly keratinocytes, was found in all the biopsy specimens taken during chloroquine treatment when the F-157–7 monoclonal antibody against chloroquine was used. The staining was similar in normal and erythematous skin. Absent or weak staining was generally observed in the basal layers, except for local aggregations interpreted as melanocytes. In the stratum corneum the intensity of the staining varied between the different patients and reflected the staining intensity in the lower parts of the epidermis (Fig. 2a,b). The cytoplasm of keratinocytes was strongly stained while the intercellullar space showed a moderate staining. A weak staining of the inner epithelium of the hair follicles was also seen. There was no staining of other tissue components except for a weak staining of occasional smooth muscle bundles. When the F-73 monoclonal antibody, which was developed from hydroxychloroquine, was used, no staining was observed in any of the biopsy specimens.

In 2 of the patients there was also positive staining with the F-157-7 antibody in the specimens taken before the start of chloroquine treatment. These patients had been taking chloroquine for 4–5 months every summer for the last 8 to 10 years, and 1 of them had used the drug continuously for 1 year with a wash-out period of 2–3 months before the specimens were taken. In these 2 patients the staining was equally strong before and during chloroquine medication. No difference in staining pattern was found between normal and erythematous skin.

Cellular infiltration, cytokines and ICAM-1 expression

Non-irradiated skin: In normal-appearing skin of the forearm, dermal T lymphocytes (Leu 4) were few in number. A weak staining of macrophages (LeuM3) and HLA-DR-expressing cells was found in the dermis. All cell types were found in similar amounts before and during chloroquine treatment. HLA-DR-expressing cells were also seen scattered in the epidermis in all non-irradiated skin specimens.

Irradiated, erythematous skin: The number of T lymphocytes within dermis increased from few before to a high number after irradiation. During chloroquine treatment this amount of T lymphocytes decreased to a moderate number. In the irradiated skin the extent and intensity of macrophages and HLA-DR-expressing cells, mainly located in the dermal perivascular area, was moderate and decreased to weak during chloroquine treatment.

ICAM-1 expression of similar magnitude could be detected in the endothelium of the dermal vessels in all biopsies from non-irradiated and erythematous skin both before and during treatment. No ICAM-1 expression was found on keratinocytes in any of the subjects; nor was there any expression of IL-1, TNF-α or IL-6.

DISCUSSION

In this study a monoclonal antibody against chloroquine, initially developed for the ELISA inhibition test, has been used for the first time to investigate the distribution of chloroquine in the skin by application of immunohistochemical staining of skin biopsy specimens. With this technique an accumulation of the drug was found within the epidermis. These data corroborate our previous findings (12), using the HPLC technique on skin suction blister fluid, that chloroquine accumulated in the skin and that it remained there much longer than in the plasma.

As in healthy subjects (1), the early (24 h) UVB-induced erythema was characterized by an increase in mononuclear cells in the dermis. During chloroquine treatment this cellular infiltration in irradiated skin was reduced. ICAM-1 was found to be expressed on the vascular endothelium, as reported earlier (3). The expression was strong and was not affected by irradiation or chloroquine treatment. No ICAM-1 expression could be detected on the keratinocytes in any of the specimens taken before or during chloroquine treatment in either normal or erythematous skin. This finding is in accordance with in vitro observations by two groups (4,17) of a biphasic effect of UVR on ICAM-1 expression on normal human keratinocytes, with an initial suppression at 24 hours. Norris (6) discussed the possibility that UVB-induced ICAM-1 expression could differ between patients predisposed to photosensitivity and healthy subjects. He demonstrated that the keratinocytes from UVB-induced skin lesions in patients with PMLE, in contrast to irradiated skin of healthy subjects, expressed ICAM-1 beginning at 5 hours and persisting 6 days after UV irradiation. It was not clearly stated, however, whether the lesions were induced by a single or by repeated irradiations. In a recent study (7), expression of ICAM-1 on keratinocytes was observed in skin lesions induced by repeated UV irradiations in patients with lupus erythematosus and PMLE, but the time of sampling was not stated. In contrast to these latter studies, where irradiation was given in order to induce skin lesions, the present study was designed to investigate the initial erythema reaction, and the samples were taken 24 hours after a single irradiation with UVB. This difference may therefore explain our negative results as to ICAM-1 expression on keratinocytes.

The mechanism of action of chloroquine in the prevention of photosensitivity in predisposed patients is not known. The finding in the present study of an accumulation of chloroquine in the epidermis during chloroquine treatment, together with a reduced cellular infiltration of UVB-induced marked erythema, indicates that the drug has an anti-inflammatory effect. This effect may either be due to unspecific UV-protective properties by the accumulation of chloroquine in the epidermis or to some specific downregulating action by chloroquine on keratinocyte function.

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