

Antioxidant action of antimalarials

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SUMMARY The effects of antimalarials, chloroquine and quinacrine, on the generation of reactive oxygen species were examined both in polymorphonuclear leucocytes and in the xanthine-xanthine oxidase system. Antimalarials showed inhibitory effects on the production of reactive oxygen species probably by affecting cell functions, such as membrane phospholipid methylation. It is suggested that antimalarial agents can work as antioxidants at the site of inflammation protecting against auto-oxidative tissue damage with resultant anti-inflammatory effects.

Key words: polymorphonuclear leucocytes, reactive oxygen species.

The antimalarials, chloroquine and quinacrine, are used primarily for malaria. However, they can be beneficial for various cutaneous and rheumatic diseases, including discoid and systemic lupus erythematosus and rheumatoid arthritis.^{1,2} Although antimalarial drugs have a number of effects both *in vitro* and *in vivo*,^{3,4} the precise reasons for their efficacy have not been determined.

We have recently shown that reactive oxygen species (ROS) are involved in the pathogenesis of several inflammatory disorders⁵⁻¹¹ and that the mechanism of action of some anti-inflammatory agents is partly ascribable to their antioxidant effects on the inflammatory process.¹²⁻¹⁴

In the present study we asked if antimalarials have inhibitory effects on ROS generation by polymorphonuclear leucocytes (PMNs) and in the xanthine-xanthine oxidase system.

Materials and methods

CHEMICALS

Chloroquine and quinacrine were purchased from Sigma Chemical Co., St Louis, MO, USA.

PREPARATION OF PMNS FOR ROS ASSAYS

PMNs were prepared from the heparinised peripheral venous blood by a previously described

method.¹⁵ After centrifugation of the blood over a Ficoll-Hypaque gradient the cell pellet containing PMNs and erythrocytes was washed with saline solution and resuspended in plasma containing dextran 170 (mol. wt 170 000) at a final concentration of 1%. PMNs were recovered after sedimentation at unit gravity, and the few contaminating erythrocytes were lysed by treatment of the preparation with 0.876% NH₄Cl. The PMNs were then resuspended in Krebs-Ringer phosphate buffer (KRP), containing 5 mM glucose and 1 mg/ml (g/l) gelatin, for assays of ROS. Gelatin was added in order to prevent PMNs from adhering to the test tubes but was excluded from the medium for OH⁻ generation assay on account of its inhibitory effect.

ROS GENERATION ASSAYS

Oposonised zymosan was prepared freshly each time before experiments by incubating 11 mg of zymosan with 1 ml of autologous serum at 37°C for 30 min. O₂⁻ formation was determined according to Johnston and Lehmeier.¹⁶ PMNs (4 × 10⁶) were preincubated for 10 min with 1 mg/ml oposonised zymosan. After the addition of 0.1 mM ferricytochrome *c* (type III, Sigma) they were further incubated at 37°C for 30 min. Immediately after sedimentation of the PMNs and oposonised zymosan by centrifugation the supernatants were assayed for reduced cytochrome *c* by measurement of the absorbance at 550 nm with a spectrophotometer (Hitachi Co., Tokyo, Japan). Superoxide dismutase was added to the PMN medium at a concentration of 400 U/ml,

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and the inhibition of the reduction of cytochrome *c* was confirmed. The enzyme denatured by autoclaving for 30 min at 130°C served as a control. Generation of H₂O₂ was measured by quantitative measurement of the decrease in fluorescence intensity of scopoletin (Sigma) due to its peroxidase-mediated oxidation by H₂O₂.¹⁷ After incubation of 2.5 × 10⁶ PMNs in KRP with 1 mg/ml opsonised zymosan for 10 min at room temperature 0.1 ml of 50 mM scopoletin in KRP and 0.1 ml of 1 mg/ml horseradish peroxidase (type II, Sigma) in phosphate-buffered saline (PBS) were added. The rate of decrease in fluorescence intensity of the scopoletin within 30 min was quantified in a fluorescence spectrophotometer (Hitachi). Incubation of supernatants with an excess of 600 U/ml catalase inhibited fluorescence reduction. Catalase heated at 130°C for 30 min served as a control. OH[•] was quantified by the amount of ethylene gas formed from α-ketomethylbutyric acid (KMB, Sigma) plus OH[•] generated by PMNs.¹⁸ PMNs (2 × 10⁶) in 2 ml KRP were preincubated with 1 mM KMB at 37°C for 5 min. After 1 mg/ml opsonised zymosan was added incubation was continued for a further 10 min. The amounts of OH[•] gas formation were assayed at 30 min on a gas chromatograph (Hitachi). The simultaneous addition of 10 mM benzoate into the PMN medium reduced peak OH[•] generation by 75%. Chemiluminescence was measured in a scintillation spectrometer (Packard, IL, USA), according to Allen and Loose¹⁹ with slight modification. PMNs (5 × 10⁶) in 3 ml colourless Hanks's solution containing gelatin were incubated at 37°C for 10 min with opsonised zymosan in the absence of luminol. Chemiluminescence was monitored on the spectrometer, which was operated in out-of-coincidence summation mode. All procedures were performed in the dark. Incubation of the PMNs plus opsonised zymosan with 2 mM xanthine reduced peak chemiluminescence by 70%.

EFFECTS OF ANTIMALARIALS ON THE GENERATION OF ROS FROM PMNs

Both chloroquine and quinacrine were dissolved in the solution of PBS, and this solution was added to the PMN-suspended medium of each ROS assay system to final concentrations of 1, 10, 100, and 1000 μg/ml (mg/l). The same volume of PBS was added to the control.

EFFECTS OF ANTIMALARIALS ON ROS GENERATION IN XANTHINE-XANTHINE OXIDASE SYSTEM

In separate experiments the effects of antimalarials on ROS generation were also examined in the xanthine-xanthine oxidase system. Instead of adding

PMNs and opsonised zymosan, 0.1 ml of 13.5 mg hypoxanthine in 50 ml physiological saline plus 0.05 ml of 50 mM ethylenediaminetetra-acetic acid were diluted in 2 ml of KRP (pH 7.2–7.4). Then antimalarials in PBS were added to the medium to final concentrations of 1, 10, 100, and 1000 μg/ml. The same volume of PBS was added to the control. Thereafter 0.1 ml of 0.1 unit/ml dialysed xanthine oxidase was added to generate ROS, and each ROS was determined respectively as described above.

Results

EFFECTS OF ANTIMALARIALS ON SUPEROXIDE (O₂⁻) GENERATION (Fig. 1)

As shown in Fig. 1 both chloroquine and quinacrine showed inhibitory effects on PMN derived O₂⁻ production in a dose dependent manner. Suppres-

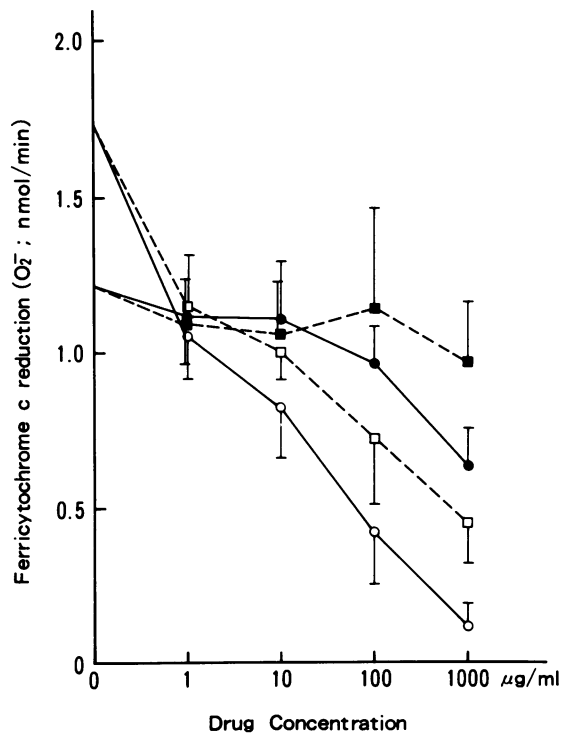


Fig. 1 Effects of antimalarials on superoxide (O₂⁻) generation. Both chloroquine (□- - □) and quinacrine (○—○) showed inhibitory effects on PMN derived O₂⁻ production. In the xanthine-xanthine oxidase system the levels of O₂⁻ were little affected by either chloroquine (■- - ■) or quinacrine (●—●) in drug concentrations lower than 100 μg/ml. Each point denotes mean ± SD of the triplicate assays. (μg/ml = mg/l).

sion was readily observed at the lowest concentration (1 $\mu\text{g/ml}$). However, in the xanthine-xanthine oxidase system the levels of O_2^- were not affected by the addition of antimalarials in drug concentrations of 1, 10, or 100 $\mu\text{g/ml}$. Quinacrine showed a significant reduction in O_2^- level only at 1000 $\mu\text{g/ml}$, which is an extremely high drug concentration.

EFFECTS OF ANTIMALARIALS ON HYDROGEN PEROXIDE (H_2O_2) GENERATION (Fig. 2)

Addition of quinacrine resulted in a significant decrease in H_2O_2 generation by PMNs dose dependently, and almost negligible H_2O_2 was detected at drug concentrations higher than 100 $\mu\text{g/ml}$. This inhibitory effect was not observed in the xanthine-xanthine oxidase system at 1 or 10 $\mu\text{g/ml}$, though a dose related decrease in H_2O_2 production was shown at 100 and 1000 $\mu\text{g/ml}$. On the other hand,

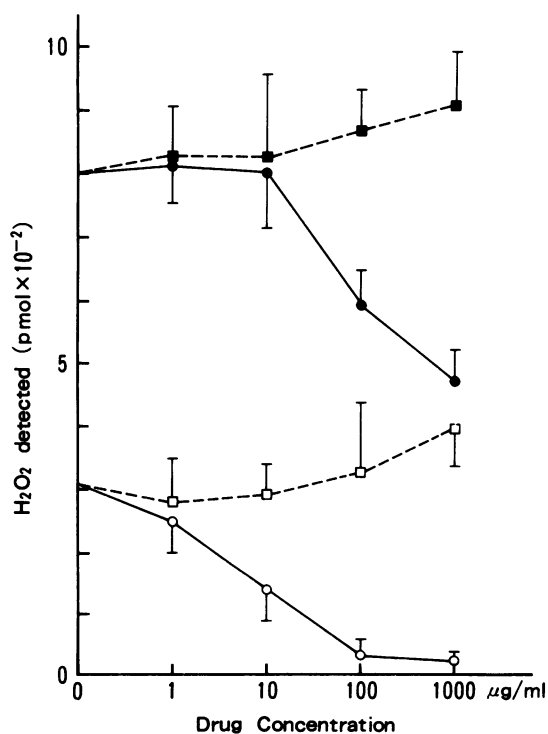


Fig. 2 Effects of antimalarials on hydrogen peroxide (H_2O_2) generation. Addition of quinacrine resulted in a significant decrease in H_2O_2 generation by PMNs (\circ — \circ) and in the xanthine-xanthine oxidase system (\bullet — \bullet). Chloroquine showed no inhibition of H_2O_2 generation either in PMN (\square — \square) or in the xanthine-xanthine oxidase system (\blacksquare — \blacksquare). Each point denotes mean \pm SD of the triplicate assays. ($\mu\text{g/ml}=\text{mg/l}$).

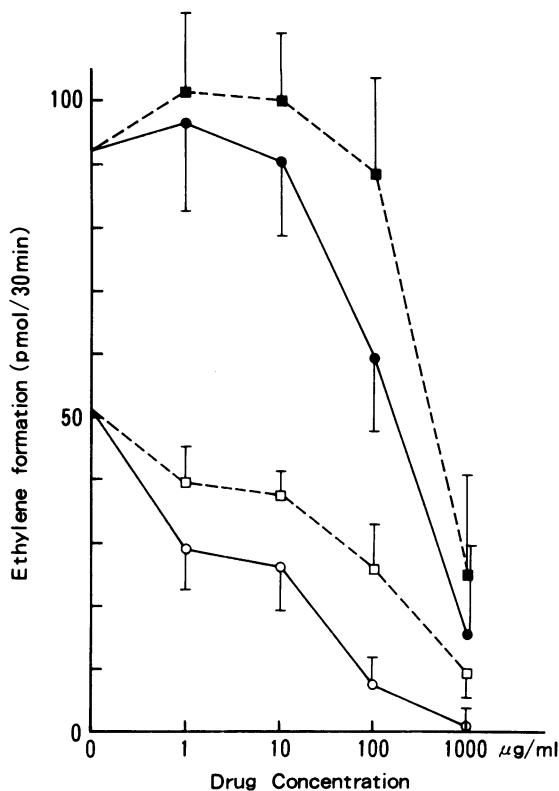


Fig. 3 Effects of antimalarials of hydroxyl radical (OH^\bullet) generation. Both chloroquine (\square — \square) and quinacrine (\circ — \circ) showed a suppressive effect on OH^\bullet production from zymosan stimulated PMNs. In the xanthine-xanthine oxidase system no significant inhibition was observed at lower concentrations either by chloroquine (\blacksquare — \blacksquare) or quinacrine (\bullet — \bullet). Each point denotes mean \pm SD of the triplicate assays. ($\mu\text{g/ml}=\text{mg/l}$).

chloroquine showed no inhibition of H_2O_2 generation at any doses examined either in PMN or in the xanthine-xanthine oxidase system.

EFFECTS OF ANTIMALARIALS ON HYDROXYL RADICAL (OH^\bullet) generation (Fig. 3)

Both chloroquine and quinacrine had a suppressive effect on OH^\bullet production from zymosan stimulated PMNs in a dose dependent manner. In the xanthine-xanthine oxidase system no significant inhibition was observed at lower drug concentrations (1, 10 $\mu\text{g/ml}$), though remarkable decrease in OH^\bullet level was seen at the drug concentration of 1000 $\mu\text{g/ml}$.

EFFECTS OF ANTIMALARIALS ON CHEMILUMINESCENCE (Fig. 4)

Both antimalarial agents showed significantly decreased chemiluminescence at drug concentrations

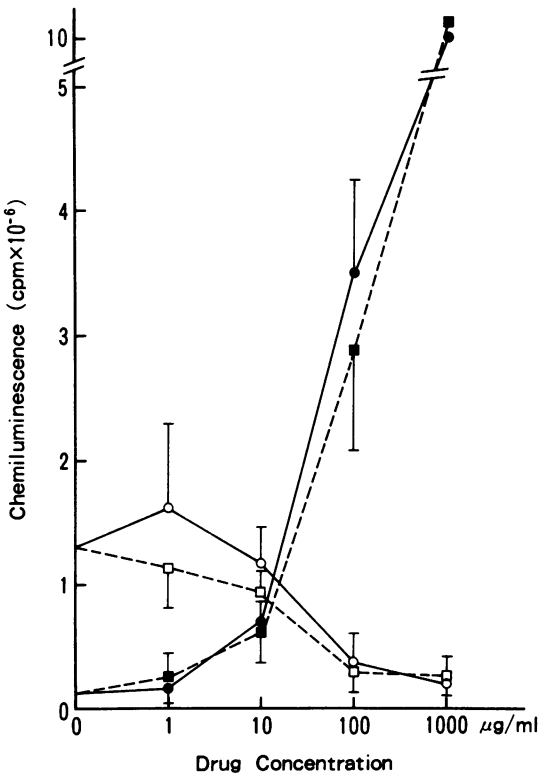


Fig. 4 Effects of antimalarials on chemiluminescence. Both chloroquine (\square — \square) and quinacrine (\circ — \circ) showed significantly decreased chemiluminescence in the PMN system. Unexpectedly, remarkably enhanced chemiluminescence was observed by the addition of chloroquine (\blacksquare — \blacksquare) or quinacrine (\bullet — \bullet), presumably due to the reaction with xanthine oxidase or hypoxanthine, or both. Each point denotes mean \pm SD of the triplicate assays. ($\mu\text{g/ml} = \text{mg/l}$).

higher than 100 $\mu\text{g/ml}$ in the PMN system. Unexpectedly, remarkably enhanced chemiluminescence was observed in a dose dependent fashion, presumably due to the reaction of antimalarials with xanthine oxidase or hypoxanthine, or both.

Discussion

The antimalarials have a wide and overlapping range of action.^{20–23} As for the anti-inflammatory mechanism of action, chloroquine has been shown to be a lysosomal stabiliser.^{20–21} It thus retards the release of hydrolytic enzymes. Chloroquine and quinacrine can both function as competitive prostaglandin antagonists.^{22–23} Other possible anti-

inflammatory mechanisms that have been proposed to account for the effects of antimalarials are chemotaxis inhibition,²⁴ and the antihistamine and antiserotonin effect.¹

In the present investigation we have shown the antioxidant action of antimalarials in a zymosan stimulated PMN system. Quinacrine reduced the generation of all ROS examined in a dose dependent way. Chloroquine also had inhibitory effects on O_2^- and OH^\bullet production, and on chemiluminescence. In contrast these antimalarial drugs showed no suppressive effect on ROS generation in the xanthine-xanthine oxidase system at the concentrations expected after the administration of therapeutic doses, while they decreased ROS levels at higher concentrations. In view of these facts it is suggested that antimalarials inhibit ROS generation by affecting PMN functions at therapeutic doses and exert a ROS scavenging effect at higher concentrations. Although the plasma levels of antimalarials remain low, in the range of 90–170 $\mu\text{g/l}$, after the administration of therapeutic doses,²⁵ the tissue concentrations are considered to be extremely high.^{26–27} Thus it is possible that these agents can work as antioxidants at the site of inflammation, though the potential of the metabolic products of antimalarials to influence the ROS systems are unknown.

Of great interest is the recent finding that quinacrine is a phospholipase A_2 inhibitor,²⁸ and that it blocked the release of arachidonic acid and chemotaxis in PMNs in a dose dependent manner.^{24–29} Recently, it has been reported that antimalarials inhibit membrane phospholipid methylation.³⁰ Taking these reports into consideration, it is quite reasonable that antimalarials are both antioxidants and prostaglandin antagonists. This mode of action seems to be quite similar to that of glucocorticoids.³¹

Antimalarials have numerous side effects, especially of ophthalmologic aspect,³² which make antimalarials not the first line drugs of choice. However, recent articles have indicated that antimalarial retinal toxicity can be avoided by proper monitoring and dosage limitation without sacrificing the therapeutic efficacy of these agents.^{32–36} In this context these drugs still remain useful in dermatological and rheumatological treatments.

References

- 1 Dubois E L. Antimalarials in the management of discoid and systemic lupus erythematosus. *Semin Arthritis Rheum* 1978; 8: 33–51.
- 2 Koranda F C. Antimalarials. *J Am Acad Dermatol* 1981; 4: 650–5.

- 3 Tanenbaum L, Tuffanelli D L. Antimalarial agents: chloroquine, hydroxychloroquine, and quinacrine. *Arch Dermatol* 1980; **116**: 587-91.
- 4 Issacson D, Eligart M, Turner M L. Anti-malarials in dermatology. *Int J Dermatol* 1982; **21**: 379-95.
- 5 Miyachi Y, Yanase K, Imamura S, Niwa Y. Increased hydroxyl radical generation by normal polymorphonuclear leukocytes incubated in sera from patients with leukocytoclastic vasculitis. *Arch Dermatol Res* 1982; **274**: 65-71.
- 6 Miyachi Y, Niwa Y. Effects of psoriatic sera on the generation of oxygen intermediates by normal polymorphonuclear leukocytes. *Arch Dermatol Res* 1983; **275**: 23-6.
- 7 Niwa Y, Miyake S, Sakane T, Shingu M, Yokoyama M. Auto-oxidative damage in Behçet's disease—endothelial cell damage following the elevated oxygen radicals generated by stimulated neutrophils. *Clin Exp Immunol* 1982; **49**: 247-55.
- 8 Niwa Y, Sohmiya K. Enhanced neutrophilic functions in mucocutaneous lymph node syndromes, with special reference to the possible role of increased oxygen intermediate generation in the pathogenesis of coronary thromboarteritis. *J Pediatr* 1984; **104**: 56-60.
- 9 Niwa Y, Sakane S, Shingu M, Yokoyama M. Effect of stimulated neutrophils from the synovial fluid of patients with rheumatoid arthritis on lymphocytes—a possible role of increased oxygen radicals generated by the neutrophils. *J Clin Immunol* 1983; **3**: 228-40.
- 10 Niwa Y, Sakane T, Shingu M, Yokoyama M. Role of stimulated neutrophils from patients with systemic lupus erythematosus in disturbed immunoreactivity, with special reference to increased oxygen intermediates generated by the neutrophils. *J Clin Lab Immunol* 1984; **14**: 35-43.
- 11 Niwa Y, Sakane T, Shingu M, Miyachi Y. Role of stimulated neutrophils from patients with systemic lupus erythematosus in tissue injury, with special reference to serum factors and increased active oxygen species generated by neutrophils. *Inflammation* 1985; **9**: 163-72.
- 12 Miyachi Y, Niwa Y. Effects of potassium iodide, colchicine and dapsone on the generation of polymorphonuclear leukocyte-derived oxygen intermediates. *Br J Dermatol* 1982; **107**: 209-14.
- 13 Miyachi Y, Ozaki M, Uchida K, Niwa Y. Effects of thalidomide on the generation of oxygen intermediates by zymosan-stimulated normal polymorphonuclear leukocytes. *Arch Dermatol Res* 1982; **274**: 363-7.
- 14 Niwa Y, Sakane T, Miyachi Y. Dissociation of the inhibitory effect of dapsone on the generation of oxygen intermediates—in comparison with that of colchicine and various scavengers. *Biochem Pharmacol* 1984; **33**: 2355-60.
- 15 Skosey J L, Chow D C, Damgaard E, Sorensen L B. Effects of cytochalasin B on the response of human polymorphonuclear leukocytes to zymosan. *J Cell Biol* 1981; **57**: 237-40.
- 16 Johnston R B Jr, Lehmeier J E. Elaboration of toxic oxygen by-products by neutrophils in a model of immune complex disease. *J Clin Invest* 1976; **57**: 836-41.
- 17 Root R K, Metcalf J A. H₂O₂ release from human granulocytes during phagocytosis. *J Clin Invest* 1977; **60**: 1266-79.
- 18 Klebanoff S J, Rosen H. Ethylene formation by polymorphonuclear leukocytes. *J Exp Med* 1978; **148**: 490-506.
- 19 Allen R C, Loose L D. Phagocytic activation of a luminol-dependent chemiluminescence in rat alveolar and peritoneal macrophages. *Biochem Biophys Res Commun* 1976; **69**: 245-52.
- 20 Weissmann G. Lysosomes. *N Engl J Med* 1965; **273**: 1143-9.
- 21 Lie S O, Schofield B. Inactivation of lysosomal function in normal cultured human fibroblasts by chloroquine. *Biochem Pharmacol* 1973; **22**: 3109-14.
- 22 Manku M S, Horrobin D F. Chloroquine, quinine, procaine, quinidine, tricyclic antidepressants, and methylxanthines as prostaglandin agonists and antagonists. *Lancet* 1976; **ii**: 1115-7.
- 23 Horrobin D F, Manku M S, Karmazyn M, Ally A I, Morgan R O, Karmali R A. Quinacrine is a prostaglandin antagonist. *Biochem Biophys Res Commun* 1977; **76**: 1188-93.
- 24 Hirata F, Corcoran B A, Venkatasubramanian K, Schiffmann E, Axelrod J. Chemoattractants stimulate degradation of methylated phospholipids and release of arachidonic acid in rabbit leukocytes. *Proc Natl Acad Sci USA* 1979; **76**: 2640-3.
- 25 Alving A S, Eichelberger L, Craige B Jr. Studies on the chronic toxicity of chloroquine. *J Clin Invest* 1948; **27**: 60-5.
- 26 Shaffer B, Cahn M M, Levy E J. Absorption of antimalarial drugs in human skin: spectroscopic and chemical analysis in epidermis and corium. *J Invest Dermatol* 1958; **30**: 341-5.
- 27 McChesney E W, Nachod F C, Tainter M L. Rationale for treatment of lupus erythematosus with antimalarials. *J Invest Dermatol* 1957; **29**: 97-104.
- 28 Yorio T, Bentley P J. Phospholipase A and the mechanism of action of aldosterone. *Nature* 1978; **271**: 79-81.
- 29 Hirata F, Axelrod J. Phospholipid methylation and biological signal transmission. *Science* 1980; **209**: 1082-90.
- 30 Hurst N P, French J K, O'Donnel M L. Inhibition of membrane phospholipid methylation [Abstract]. *XVIIth International Congress of Rheumatology* 1985: 224.
- 31 Hiarata F, Schiffmann E, Venkatasubramanian K, Salomon D, Axelrod J. A phospholipase A₂ inhibitory protein in rabbit neutrophils induced by glucocorticoids. *Proc Natl Acad Sci USA* 1980; **77**: 2533-6.
- 32 Portnoy J Z, Callen J P. Ophthalmologic aspects of chloroquine and hydroxychloroquine therapy. *Int J Dermatol* 1983; **22**: 273-8.
- 33 Rynes R I, Krohel G, Falbo A, Reinecke R D, Wolfe B, Bartholomew L E. Ophthalmologic safety of long-term hydroxychloroquine treatment. *Arthritis Rheum* 1979; **22**: 832-6.
- 34 Coblyn J S, Taylor P. Treatment of chronic Lyme arthritis with hydroxychloroquine. *Arthritis Rheum* 1981; **24**: 1567-9.
- 35 Zuehlke R L, Lillis P J, Tice A. Antimalarial therapy for lupus erythematosus: an apparent advantage of quinacrine. *Int J Dermatol* 1981; **20**: 57-61.
- 36 Olansky A J. Antimalarials and ophthalmologic safety. *J Am Acad Dermatol* 1982; **6**: 19-23.