Effect of Antibiotics on the Generation of Reactive Oxygen Species

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The relative antioxidant efficacy, in vitro, of several antibiotics was examined by studying their effects on the generation of reactive oxygen species (ROS) using zymosan-stimulated polymorphonuclear leukocytes (PMNL) and the cell-free, xanthine-xanthine oxidase system. The species investigated are superoxide radical anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$). Three tetracyclines (tetracycline HCl, oxytetracycline HCl, and minocycline HCl), erythromycin, cephalexin, penicillin G, chloramphenicol, and streptomycin were used as test drugs.

At concentrations comparable to therapeutic blood levels, tetracycline HCl, oxytetracycline HCl, minocycline HCl, and erythromycin inhibited some of the ROS production by PMNL. In the xanthine-xanthine oxidase system, only minocycline HCl suppressed the H_2O_2 level. Cephalexin, penicillin G, chloramphenicol, and streptomycin did not affect any of the ROS examined at the concentrations tested. The capacity of some of these agents to inhibit ROS generation by PMNL may account, in part, for their efficacy in inflammatory skin diseases such as acne vulgaris. The antioxidant effect of these antibiotics does not stem from their capability to scavenge ROS, but originates rather from their effect on PMNL cell function directly with resultant anti-inflammatory effects on the inflammatory processes. *J Invest Dermatol 86:449–453, 1986*

Systemic administration and topical application of certain antibiotics have been shown to be effective in the treatment of acne vulgaris. The efficacy of acne therapy has been monitored by noting the decrease in inflammatory activity of acne lesions. Acne can be conceptualized as a 2-stage process, i.e., comedo formation and inflammation [1]. Inflammation first occurs as the disruption of the integrity of the follicular epithelium, and then extrusion of intrafollicular material into the dermis results in a variety of inflammatory processes. *Propionibacterium acnes* (*P. acnes*) appears to play an important role in the inflammatory process. Although *P. acnes* generation of free fatty acids may play a lesser role in the pathogenesis of acne inflammation [2] than previously believed [3,4], it has been reported that *P. acnes* produces low-molecular-weight chemotactic factors [5] that can diffuse through intact follicular epithelium and attract polymorphonuclear leukocytes (PMNL). Once PMNL have reached the sebaceous follicles, they are considered to release lysosomal enzymes in the presence of *P. acnes*, antibody to *P. acnes*, and complement [6,7] with resultant damage of follicular epithelium and extrusion of follicular contents into the dermis. Successful treatment with antibiotics has been attributed to the reduction in number of *P. acnes* as well as the inhibitory effects on the production of *P. acnes*-associated inflammatory mediators [1]. However, inflammation often begins to resolve before a drop in *P. acnes* number is noted [1] and direct anti-inflammatory effects for antibiotics have been postulated [8]. Martin et al [9] have demonstrated that tetracycline, in concentrations comparable to common drug therapy, markedly depressed migration of human leukocytes in vitro, and Esterly et al [10] confirmed the suppressive effects of several chemotherapeutic agents on the chemotaxis of human leukocytes as well as in patients with acne receiving oral tetracycline therapy [11]. Since inflammatory acne lesions are the result of an influx of PMNL [12], it is reasonable to ask whether these antibiotics exert their anti-inflammatory effects by affecting other inflammatory factors produced by the attracted PMNL at the site of inflammation.

In the present study, we have examined the effects of several antibiotics on the generation of reactive oxygen species (ROS) both by zymosan-stimulated PMNL and in the cell-free, xanthine-xanthine oxidase system, because we have already demonstrated that dapsone, which is also effective in acne therapy [13,14] without affecting *P. acnes* density or free fatty acid ratio to triglyceride [15], has an antioxidant action [16,17] with subsequent protection against the autooxidative tissue damage in some inflammatory skin diseases [18,19].

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Abbreviations:
- CL: chemiluminescence
- H_2O_2: hydrogen peroxide
- KMB: α-ketomethiol butylic acid
- KRP: Krebs-Ringer phosphate buffer
- $O_2^{\cdot -}$: superoxide
- $OH^{\cdot}$ hydroxyl radical
- PBS: phosphate-buffered saline
- PMNL: polymorphonuclear leukocyte(s)
- ROS: reactive oxygen species

MATERIALS AND METHODS

Chemicals Tetracycline HCl, oxytetracycline HCl, erythromycin, cephalexin, and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, Missouri. Minocycline HCl was a generous gift from Japan Lederle Ltd., Tokyo. Penicillin G and streptomycin were purchased from Meiji Seika Co., Tokyo.

Preparation of PMNL for ROS Assays PMNL were prepared from the heparinized peripheral venous blood using a previously described method [20]. After centrifugation of the blood over a Ficoll-Hypaque gradient, the cell pellet, containing PMNL

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and erythrocytes, was washed with saline solution and resuspended in plasma containing dextran 170 (M_r = 170,000) at a final concentration of 1%. PMNL were recovered after sedimentation at unit gravity, and the few contaminating erythrocytes were lysed by treatment of the preparation with 0.876% NH_4Cl. The PMNL were then resuspended in Krebs-Ringer phosphate buffer (KRP), containing 5 mM glucose and 1 mg/ml gelatin, for assays of ROS. Gelatin was added in order to prevent PMNL from adhering to the test tubes, but was excluded from the medium for hydroxyl radical ($OH^{\cdot}$) generation assay on account of its inhibitory effect.

ROS Generation Assay Opsonized zymosan was prepared freshly each time before experiments by incubating 11 mg of zymosan (Sigma) with 1 ml of autologous serum at 37°C for 30 min. Superoxide ($O_2^{\cdot -}$) formation was determined according to Johnston and Lehmeyer [21]. PMNL (4 × 10^6) were preincubated for 10 min with 1 mg/ml opsonized 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 PMNL and opsonized zymosan by centrifugation, the supernatants were assayed for reduced cytochrome c by measurement of the absorbance at 550 nm using a spectrophotometer (Hitachi Co., Tokyo). Generation of hydrogen peroxide (H_2O_2) was measured by quantitating the decrease in fluorescence intensity of scopoletin (Sigma) due to its peroxidase-mediated oxidation by H_2O_2 [22]. After incubation of 2.5 × 10^6 PMNL in KRP with 1 mg/ml opsonized 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 quantitated in a fluorescence spectrophotometer (Hitachi). $OH^{\cdot}$ was quantitated by the amount of ethylene gas formed from α-ketomethiol butylic acid (KMB, Sigma) plus $OH^{\cdot}$ generated by PMNL [23]. PMNL (2 × 10^6) in 2 ml KRP were preincubated with 1 mM KMB at 37°C for 5 min. After the addition of 1 mg/ml opsonized zymosan, incubation was continued for a further 10 min. The amount of $OH^{\cdot}$ gas formed was assayed at 30 min on a gas-chromatograph (Hitachi). Chemiluminescence (CL) was measured in a scintillation spectrometer (Packard, Downers Grove, Illinois), according to Allen and Loose [24] with slight modification. PMNL (5 × 10^6) in 3 ml colorless Hanks' solution containing gelatin were incubated at 37°C for 10 min with opsonized zymosan in the absence of luminol. CL was monitored on the spectrometer which was operated in out-of-coincidence summation mode. All procedures were performed in the dark.

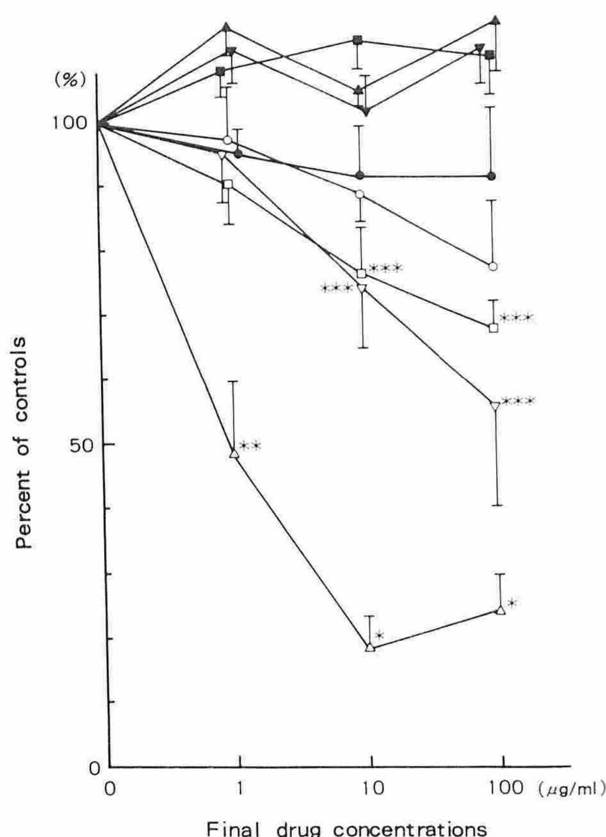

Figure 2. Effects of antibiotics on hydrogen peroxide (H_2O_2) generation by PMNL. The addition of minocycline HCl, oxytetracycline HCl, or erythromycin resulted in a significant reduction of H_2O_2 by PMNL. Tetracycline HCl, cephalexin, penicillin G, streptomycin, and chloramphenicol did not affect the H_2O_2 levels at the concentrations examined. Results are expressed as percent of controls of experiments. Each point denotes mean ± SD. For symbols, see the explanation in Fig 1.

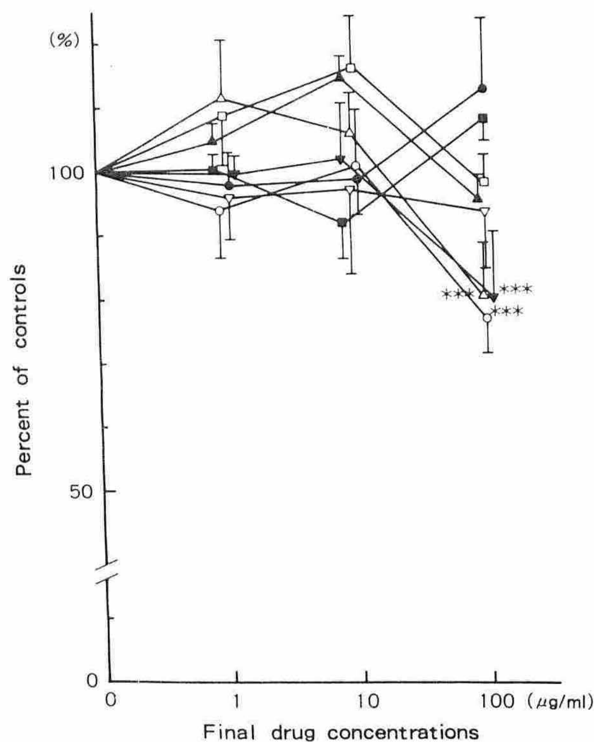

Figure 1. Effects of antibiotics on superoxide ($O_2^{\cdot -}$) generation by PMNL. No antibiotics tested had inhibitory effects on $O_2^{\cdot -}$ production by PMNL at concentrations of 1 or 10 μg/ml. Minocycline HCl, tetracycline HCl, and chloramphenicol decreased the $O_2^{\cdot -}$ generation slightly at 100 μg/ml. Results are expressed as percent of controls of experiments. Each point denotes mean ± SD. ○——○, tetracycline HCl; □——□, oxytetracycline HCl; △——△, minocycline HCl; ▽——▽, erythromycin; ●——●, cephalexin; ■——■, penicillin G; ▲——▲, streptomycin; ▼——▼, chloramphenicol. *, $p < 0.01$; **, $p < 0.05$; ***, $p < 0.1$.

Effects of Antibiotics on the Generation of ROS from PMNL Penicillin G and streptomycin were dissolved in PBS and other antibiotics were dissolved in a solution of 50% DMSO and 50% ethanol. The solution was added to the PMNL-suspended medium of each ROS assay system to make the final concentrations of 1, 10, and 100 μg/ml (1, 10, and 100 U/ml for penicillin G). The final concentration of DMSO was 0.05% and that of ethanol was also 0.05%. Although these 2 agents are potent radical scavengers, this system is considered to be representative of the environment, in vivo, of these antibiotics. The same volume of DMSO/ethanol-containing buffer was added to the con-

trol to determine its effect on ROS production. The same volume of PBS was added to the control in experiments for penicillin G and streptomycin. Since the sensitivity of PMNL to the zymosan stimulation varies from donor to donor, the effects of antibiotics on ROS generation were expressed as percent of controls of experiments and compared statistically.

Effects of Antibiotics on ROS Generation in the Xanthine-Xanthine Oxidase System In separate experiments, the effects of antibiotics on ROS generation were also examined in the cell-free, xanthine-xanthine oxidase system. Instead of adding PMNL and opsonized zymosan, 0.1 ml of 13.5 mg hypoxanthine (Sigma) in 50 ml of physiologic saline plus 0.05 ml of 50 mM EDTA were diluted in 2 ml of KRP (pH = 7.2–7.4). Then, the antibiotic in DMSO/ethanol solution or PBS was added to the medium to make the final concentrations of 1, 10, and 100 μg/ml (1, 10, and 100 U/ml for penicillin G). The same volume of the solution (DMSO/ethanol or PBS) was added to the control. Thereafter, 0.1 ml of 0.1 unit/ml dialyzed xanthine oxidase (Sigma) was added to generate ROS, and each ROS was determined as described above. Since the same doses of hypoxanthine plus xanthine oxidase did not necessarily produce the same amounts of ROS, the effects of antibiotics on ROS levels were expressed as percent of controls of experiments and compared statistically.

RESULTS

Effects of Antibiotics on ROS Generation by PMNL As shown in Fig 1, none of antibiotics tested had any inhibitory effect on $O_2^{\cdot -}$ production by PMNL at the concentrations comparable to common drug therapy (1–10 μg/ml) [25]. Minocycline HCl and tetracycline HCl and chloramphenicol decreased the $O_2^{\cdot -}$ generation slightly at 100 μg/ml. H_2O_2 generation assays (Fig 2) showed a remarkable decrease by minocylcine HCl at concentrations comparable to therapeutic blood levels. The addition of oxytetracycline HCl or erythromycin resulted in a significant dose-dependent reduction of H_2O_2 by PMNL above 10 μg/ml. The decreasing trend of H_2O_2 production observed with tetracycline HCl was not statistically significant. Cephalexin, penicillin G, streptomycin, and chloramphenicol did not affect H_2O_2 levels at any concentration examined. Remarkably reduced $OH^{\cdot}$ generation by PMNL was observed by the addition of tetracycline HCl, oxytetracycline HCl, and minocycline HCl (Fig 3). Erythromycin also decreased $OH^{\cdot}$ generation in a dose-dependent manner; however, only 100 μg/ml gave a statistically significant value. Cephalexin, penicillin G, streptomycin, and chloramphenicol had no influence on $OH^{\cdot}$ production by PMNL. Fig 4 demonstrates that only minocycline HCl (10, 100 μg/ml) and tetracycline HCl (100 μg/ml) had suppressive effects on CL by PMNL, the other antibiotics, oxytetracycline HCl, erythromycin, cephalexin, penicillin G, streptomycin, and chloramphenicol having no effect on CL at the concentrations tested.

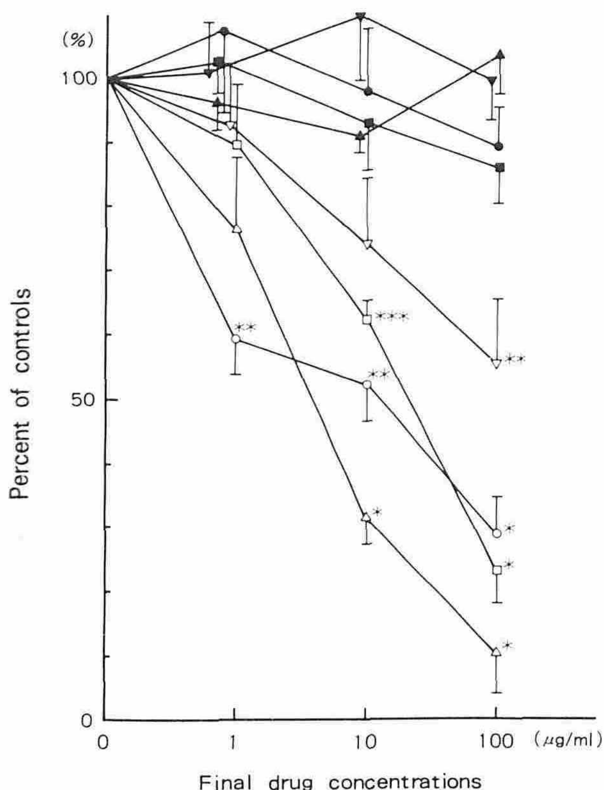

Figure 3. Effects of antibiotics on hydroxyl radical ($OH^{\cdot}$) generation by PMNL. Remarkably reduced $OH^{\cdot}$ generation by PMNL was observed by the addition of tetracycline HCl, oxytetracycline HCl, and minocycline HCl. Erythromycin decreased $OH^{\cdot}$ generation in a dose-dependent manner, although significantly only at 100 μg/ml. Cephalexin, penicillin G, streptomycin, and chloramphenicol had no influence on $OH^{\cdot}$ production. Results are expressed as percent of controls of experiments. Each point denotes mean ± SD. For symbols, see the explanation in Fig 1.

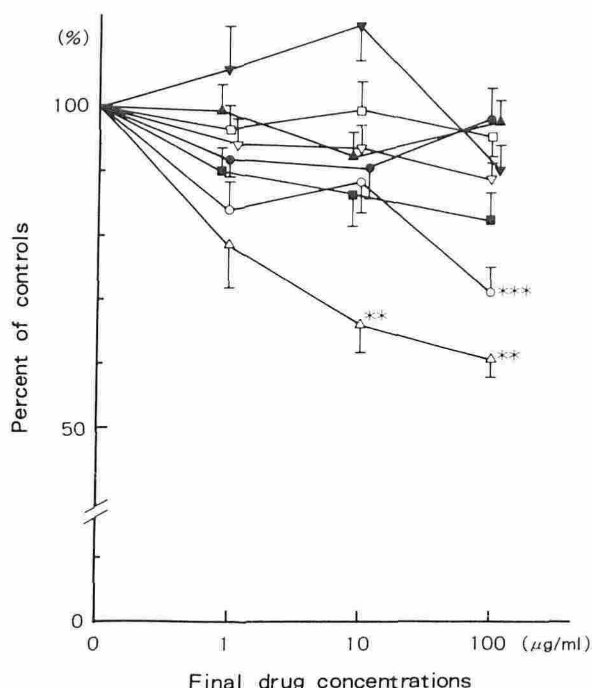

Figure 4. Effects of antibiotics on chemiluminescence (CL) in a PMNL system. Only minocycline HCl (10, 100 μg/ml) and tetracycline HCl (100 μg/ml) had suppressive effects on CL by PMNL. Other antibiotics, oxytetracycline HCl, erythromycin, cephalexin, penicillin G, streptomycin, and chloramphenicol did not affect CL at the concentrations tested. Results are expressed as percent of controls of experiments. Each point denotes mean ± SD. For symbols, see the explanation in Fig 1.

Effects of Antibiotics on ROS Levels in the Xanthine-Xanthine Oxidase System (Table I) In the cell-free, xanthine-xanthine oxidase system, no antibiotic tested affected the $O_2^{\cdot -}$ levels at any concentration examined. Decreased H_2O_2 levels were noticed when minocycline HCl (1–100 μg/ml), tetracycline HCl (100 μg/ml), or oxytetracycline HCl (100 μg/ml) was added. This inhibitory effect of minocycline HCl on H_2O_2 level was dose dependent, and thus seems to be due to its H_2O_2 scavenging effect. $OH^{\cdot}$ levels were little affected by these antibiotics, and only a high concentration of oxytetracycline HCl (100 μg/ml) significantly reduced the $OH^{\cdot}$ level. Since no significant CL was ob-

Table I. Effects of Antibiotics on ROS Levels in Xanthine-Xanthine Oxidase System[a]

Antibiotic[b]	Final Concentration ($\mu g/ml$)	Superoxide ($O_2^{\cdot -}$)	Hydrogen Peroxide (H_2O_2)	Hydroxyl Radical ($OH^{\cdot}$)
	1	111 ± 9.8	99 ± 1.6	104 ± 5.0
Tetracycline HCl	10	112 ± 9.4	97 ± 2.0	102 ± 7.0
	100	114 ± 10.6	15 ± 1.0[c]	86 ± 5.5
	1	103 ± 4.4	99 ± 0.5	99 ± 8.0
Oxytetracycline HCl	10	107 ± 5.0	93 ± 6.9	64 ± 4.0[d]
	100	114 ± 12.2	15 ± 3.3[c]	56 ± 4.0[e]
	1	104 ± 2.8	69 ± 5.6[d]	99 ± 3.0
Minocycline HCl	10	102 ± 3.8	13 ± 2.5[c]	88 ± 12.0
	100	109 ± 5.2	0 ± 0[c]	92 ± 13.0

[a] Data are expressed as percent of controls (mean ± SD) of triplicate assays.
[b] No significant suppression was observed by erythromycin, cephalexin, penicillin G, streptomycin, or chloramphenicol.
[c] $p < 0.01$, by Student's t-test.
[d] $p < 0.1$.
[e] $p < 0.05$.

served in this ROS-producing system, the effects of antibiotics on CL are not shown in Table I. No significant effect was observed by any other antibiotic tested on any kind of ROS production in this system.

DISCUSSION

The most important event of acne inflammation is considered to be the disruption of the integrity of the follicular epithelium. In the early phase of this process, PMNL are found within intact follicular epithelium [26], probably attracted by a PMNL chemotactic factor produced by *P. acnes* [5,27]. One may expect that, in contact with and/or after ingestion of *P. acnes*, these PMNL are activated to secrete a variety of inflammatory products at the site of inflammation. PMNL-mediated tissue injury has been thought to be a consequence of lysosomal degranulation [28], however the possibility exists that other factors contribute to the development of inflammation.

Recently, the role of ROS produced by PMNL in mediating tissue injury has been studied. It was reported that ROS generated from stimulated PMNL can exert tissue injury, called *autooxidative damage*, at the site of inflammation. These oxidants can attack DNA and/or membrane lipids, resulting in a chemical insult to the surrounding healthy tissues [29].

We found that several inflammatory skin diseases are mediated in part by enhanced production of ROS [18,19,30–33]. Furthermore, some anti-inflammatory agents interfere with the PMNL-derived ROS generation, thus conferring protection against autooxidative tissue injury [16,17,34–37].

In view of these findings, it is reasonable to ask whether certain antibiotic agents suppress skin inflammation by affecting ROS production which may account for the therapeutic effect. We found that antibiotics commonly used in acne therapy, such as tetracycline HCl, oxytetracycline HCl, minocycline HCl, and erythromycin reduced some of the ROS generation from zymosan-stimulated PMNL. Especially, minocycline HCl remarkably suppressed H_2O_2 production, which is one of the most potent oxidants capable of causing tissue damage. This marked reduction of minocycline HCl in H_2O_2 level is partly ascribable to its H_2O_2 quenching activity as observed in Table I. On the contrary, cephalexin, penicillin G, streptomycin, and chloramphenicol did not have any inhibitory effect on ROS level at the concentrations tested. These findings suggest that certain antibiotics have the capacity to inhibit ROS generation by PMNL, accounting for their efficacy in inflammatory skin diseases such as acne vulgaris. This antioxidant effect may relate to alterations in PMNL metabolism, because no inhibitory effects were noted in the cell-free, xanthine-xanthine oxidase system except 3 tetracyclines for H_2O_2 levels. However, before drawing the conclusion that alterations in PMNL metabolism are responsible for the protective effects of these antibiotics rather than their quenching/scavenging properties, it is necessary to consider the following possibilities: within the cellular system, the localization of the antibiotics and local concentrations are such that scavenging and/or quenching are operational so that we are just looking at differences in relative rates of various ROS-related processes, and in comparing it to the cell-free xanthine-xanthine oxidase system, one might be looking at altered relative rates of various ROS-related processes and direct effects on PMNL.

Since inflammatory acne lesions contain PMNL, which are considered to play an important role in the pathologic dynamics of acne, anti-inflammatory aspects of drug therapy should be taken into consideration. Wong et al [38] reported that the combination of ibuprofen and tetracycline therapy is effective in the treatment of moderately severe acne. Ibuprofen is an antioxidant [39] as well as a cyclooxygenase inhibitor, a pivotal enzyme in the arachidonic acid cascade [37]. Beneficial effects of retinoids on acne lesions are partly attributed to the inhibition of $O_2^{\cdot -}$ production and lysosomal enzyme release as well as to the prevention of the PMNL accumulation [40]. We recently found that retinoids have inhibitory effects on other more potent ROS such as $OH^{\cdot}$ and H_2O_2 (Yoshioka, Miyachi, Imamura, Niwa, unpublished observation).

From these considerations, we speculate that these antibiotics have favorable effects in resolving inflammatory acne lesions not merely by reducing the *P. acnes* density but also by directly acting on infiltrated PMNL as antioxidants when applied topically or systemically. These antibiotics are reasonably the first-line drugs of choice in the treatment of acne and, in severe cases, other anti-inflammatory agents such as nonsteroidal anti-inflammatory drugs, dapsone, or retinoids may be combined to enhance the inhibitory action on PMNL-mediated inflammatory processes.

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