Suppressive Effects of Linoleic Acid on Neutrophil Oxygen Metabolism and Phagocytosis

Hirohiko Akamatsu, Jinro Komura, Yoshiki Miyachi, Yasuo Asada, and Yukie Niwa

Department of Dermatology, Kansai Medical University, Moriguchi, and Department of Dermatology (YM), Faculty of Medicine, Kyoto University, Kyoto, Japan

On the basis of recent reports that the proportion of linoleic acid (C_{18}:2Cis 9,12), a free fatty acid, is markedly decreased in acne comedones and that tetracycline is effective against acne comedones by acting directly as an antioxidant on infiltrating neutrophils, we investigated the effect of linoleic acid on several inflammatory parameters of neutrophils, including neutrophil chemotaxis, phagocytosis, and generation of reactive oxygen species (ROS).

Linoleic acid significantly decreased phagocytosis and the generation of O_2^-, H_2O_2, and OH · by neutrophils, whereas it did not significantly inhibit neutrophil chemotaxis or decrease the ROS levels generated in a cell-free, xanthine-xanthine oxidase system. The present study seems to suggest that decreased levels of linoleic acid in acne comedones contribute, in part, to the worsening of acne inflammation by the failure of low levels of linoleic acid to suppress neutrophil phagocytosis and ROS generation. *J Invest Dermatol 95:271–274, 1990*

The pathogenesis of acne is generally thought to consist of two stages: comedo formation and inflammation [1]. The most important events in acne inflammation are the disruption of the follicular epithelium followed by extrusion of intrafollicular material into the dermis, triggering a variety of inflammatory processes. At this stage, *Propionibacterium acnes* [2,3] produces important inflammatory mediators and chemoattractants. Neutrophils attracted to this inflammatory site by *P. acnes*–produced chemoattractants are thought to generate the most potent inflammatory mediators, reactive oxygen species (ROS), which have recently been found to induce subsequent tissue injury [4–11]. These neutrophil-generated ROS are also thought to induce chemical insult to the integrity of the follicular epithelium.

The mechanism of action of chemotherapeutic agents and tetracyclines in acne has been found to be the effective suppression of the production of these *P. acnes*–associated inflammatory mediators and therefore of neutrophil chemotaxis [12–14]. Furthermore, we have recently reported [4,5] that tetracyclines effectively inhibit the generation of ROS by neutrophils, which supports the hypothesis that these agents are effective in acne treatment not only by reducing the numbers of *P. acnes,* but also by inhibiting neutrophil-generated inflammatory mediators.

The proportion of linoleic acid, a free fatty acid, has been shown to be markedly decreased in acne comedones [15,16]. In the present study, we investigated the effect of linoleic acid on the inflammatory parameters of neutrophils, including neutrophil chemotaxis, phagocytosis, and ROS generation.

Manuscript received July 6, 1989; accepted for publication March 28, 1990.

Reprint requests to: Dr. Yukie Niwa, 4-4 Asahimachi, Tosashimizu, Kochi-ken, 787-03, Japan.

Abbreviations:
KMB: α-keto-methiolbutyric acid
KRP: Krebs Ringer phosphate buffer
PAF: platelet-activating factor
PBS: phosphate-buffered saline
PMN: polymorphonuclear leukocytes
ROS: reactive oxygen species

MATERIALS AND METHODS

Chemicals Linoleic acid (Nakarai Tesque Inc., Kyoto, Japan) was added to the following neutrophil function assay systems in concentrations of 0.05, 0.5, or 5 μg/ml.

Neutrophil Preparation Neutrophils were isolated from heparinized venous blood from seven healthy volunteers by a modification of a previously described method [6]. After centrifugation of the blood over a Ficoll-Hypaque gradient, the plasma-containing upper layer, mononuclear cell layer, and remaining cell pellet were each removed separately. The plasma was freed of platelets by centrifugation. The cell pellet, containing neutrophils and erythrocytes, was washed with saline solution and resuspended in plasma containing dextran 170 [molecular weight (MW) 170,000] at a final concentration of 1%. The neutrophils were recovered after sedimentation at unit gravity, and the few contaminating erythrocytes were lysed by treatment of the preparation with 0.876% NH_4Cl. By this procedure, the viability of the harvested neutrophils was always greater than 99% by the trypan blue exclusion test, and [^{14}C] inulin uptake, which measures phagocytic activity, was greater than 900 dpm [17]. The neutrophils were then resuspended in media appropriate for their subsequent use: RPMI for the assay of agarose plate chemotactic activity; Krebs Ringer phosphate (KRP) buffer [18] for phagocytosis; KRP containing glucose (5 mM) for OH · generation; and KRP buffer containing glucose (5 mM) and gelatin (1 mg/ml) for the assaying of O_2^- and H_2O_2 generation.

Viability and Phagocytic Function of Neutrophils Neutrophil viability after incubation with the agents was determined by trypan-blue exclusion; phagocytic functions were measured by zymosan-induced stimulation of [^{14}C] inulin uptake [17]. When over 2% of the neutrophils were stained by trypan-blue, or when neutrophils showed less than 600 dpm [^{14}C] inulin uptake/mg protein, their function was considered to have been impaired and the results were discarded.

0022–202X/90/$03.50

Neutrophil Chemotaxis Assay Neutrophil chemotactic activities were measured using an agarose plate [19]. Agarose plates were prepared by mixing 2.5 ml of 2.4% agarose solution with 2.5 ml of 2 × normal RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Three wells with a diameter of 3 mm were cut, on a straight axis, at 8-mm intervals. Ten microliters of neutrophil suspension with RPMI medium containing 1×10^8 cells/ml was added to the center well. To the outer well, 10 μl of 10^{-7} M or 10^{-6} M fMLP was added as a chemoattractant. To the inner well, 10 μl of the RPMI 1640 medium was added as a control. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 2 h. The distance traveled by the ten fastest-moving neutrophils toward the outer well (true chemotaxis) and the inner well (random migration) was measured with a microprojector. Agarose plate neutrophil chemotaxis was also assessed using 10^{-7} M platelet-activating factor (PAF) as a chemoattractant instead of fMLP.

Neutrophil Phagocytosis Assay Emulsions of paraffin oil containing oil red O were prepared as previously described [20], except that a lipopolysaccharide solution (endotoxin) was replaced with normal human serum. The emulsion was incubated with an equal volume of normal human serum at 37°C for 30 min for opsonization. Neutrophils (2×10^7 cells/0.9 ml KRP) were added with 0.1 ml of the opsonized emulsion, the mixture was incubated for 5 min at 37°C, and then 9 ml ice-cold KRP was added to the solution to stop the reaction. The cells were washed 3 times with ice-cold KRP to remove the paraffin oil droplets that had not been ingested. Paraffin oil containing oil red O was extracted from the cells by the method of Bligh and Dyer [21], using chloroform and methanol (v/v, 1:2) and the optical density of the chloroform layer was determined at a wavelength of 525 nm.

The mean optical density of oil red O extracted from 2×10^7 neutrophils incubated with opsonized paraffin oil droplets was 0.0332 ± 0.0082 (average ± SD of five experiments), and microscopic examination revealed that a majority of neutrophils were heavily loaded with oil droplets. On the other hand, when non-opsonized paraffin oil droplets were incubated with neutrophils, the optical density was less than 0.005, and under the microscope only a few neutrophils were loaded. These findings confirmed that most of the extracted oil red O represented droplets ingested by the neutrophils.

Neutrophil ROS Generation Assay In studies of O_2^- formation, 1×10^6 neutrophils were preincubated at 37°C for 10 min with 1 mg/ml opsonized zymosan (Sigma), then 0.1 mM ferricytochrome *c* (type III, Sigma) was added. The neutrophils were incubated for another 30 min. Immediately after sedimentation of the neutrophils and opsonized zymosan by centrifugation, 0.1 ml of the supernatant was assayed for reduced cytochrome c by measuring absorbance at 550 nm [22,23] in 2 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA (pH 7.8). The results were converted to nmole of reduced cytochrome *c*, using Δ E550 nm = 2.1×10^4/M/cm [24].

H_2O_2 generation was measured by quantifying the decrease in fluorescence intensity of scopoletin (Sigma) due to its peroxidase-mediated oxidation by H_2O_2 [24]. After incubation of 2.5×10^6 neutrophils for 10 min at room temperature in KRP containing 5 mM glucose and 0.1 mg/ml gelatin in the presence of 1 mg/ml opsonized zymosan, 0.1 ml of 50 mM scopoletin in KRP, and 0.05 ml of 1 mg/ml horseradish peroxidase (type II, Sigma) in phosphate-buffered saline (PBS) were added. The H_2O_2-plus-peroxidase–induced rate of decrease in fluorescence intensity of the scopoletin within 30 min was quantified using a fluorescence spectrophotometer (Hitachi Co. Ltd., Tokyo, Japan). To calculate H_2O_2 concentration, we assumed that 1 mole of H_2O_2 oxidized 1 mole of scopoletin [24].

OH · was quantitated by the amount of ethylene formed from α-keto-methiolbutyric acid (KMB) (Sigma) plus OH · generated by neutrophils [25]. Neutrophils (2×10^6) in 2 ml KRP containing glucose were preincubated with 1 mM KMB in a stoppered tube and gently mixed in a 37°C shaker bath for 5 min. Opsonized zymosan was then added, and the cells were incubated for 10 min. Thereafter, aliquots of gas in the tube were sampled using a gas-tight syringe, and the ethylene content was determined by a gas chromatograph (Hitachi). The total amount of ethylene formed during 10, 20, and 30 min served as the OH · value.

ROS Generation Assay in the Xanthine-Xanthine Oxidase System All ROS were also measured in the xanthine-xanthine oxidase system. Instead of adding neutrophils and opsonized zymosan, 0.1 mM hypoxanthine, 1.25 mM EDTA and 16.5 μM ferricytochrome *c* were mixed in a total volume of 2 ml (125 mM phosphate buffer). After the addition of the various concentrations of linoleic acid, approximately 0.006 U/ml of dialyzed xanthine oxidase was added to generate ROS [26].

Triplicate assays were performed in each experiment; the results are expressed as the mean ± SE of replicate assays. Statistical significance was ascertained by the Student t test.

RESULTS

Linoleic acid showed no significant effect on true or random neutrophil chemotaxis (both $p > 0.05$) (Table I).

Neutrophil phagocytosis was reduced in the presence of linoleic acid in a dose-dependent manner (at 0.05 μg/ml the p value was $0.05 > p > 0.01$, at 0.5 μg/ml, the p value was $0.01 > p > 0.001$, and at 5 μg/ml the p value was $0.001 > p$) (Fig 1).

Generation of all these ROS by neutrophils was significantly decreased in a dose-dependent fashion by linoleic acid (at 0.05 μg/ml, the p value for O_2^- and H_2O_2 was $0.01 > p > 0.001$, at 0.5 μg/ml, the p value for OH · was $0.05 > p > 0.01$, and the p value for O_2^- and H_2O_2 was $0.001 > p$; and at 5 μg/ml, the p value for OH · was $0.01 > p > 0.001$ and the p value for O_2^- and H_2O_2 was $0.001 > p$) (Fig 2). However, the generation of ROS in the cell-free, xanthine-xanthine oxidase was not significantly decreased in the presence of linoleic acid ($p > 0.05$) (Table II). H_2O_2 generation in the xanthine-xanthine oxidase system could not be correctly measured because the agents in the xanthine-xanthine oxidase system reacted with scopoletin and peroxidase in the presence of linoleic acid.

Neutrophil chemotaxis induced by 10^{-7} M PAF in the presence of linoleic acid was similar to that with fMLP; neutrophil chemotaxis by 10^{-7} M PAF was not affected by the addition of linoleic acid (data not shown).

DISCUSSION

In these experiments, it was found that the free fatty acid, linoleic acid, exhibits an inhibitory action on neutrophil functions. In some respects, this action was similar to that previously found in the tetracyclines [4,5]. Both ROS generation and phagocytosis by neutrophils were effectively inhibited in the presence of linoleic acid. In previous investigations of the action of tetracyclines [4,5], the effect of these drugs on neutrophil phagocytosis was not examined. However, we have recently found that tetracycline does not significantly decrease neutrophil phagocytosis (unpublished data). The effect of linoleic acid on ROS generation could not be explained by a sca-

Table I. Effect of Linoleic Acid on Neutrophil Chemotaxis[a]

Linoleic Acid (μg/ml)	True Chemotaxis (fMLP)		Random Chemotaxis
	10^{-6} M	10^{-7} M	
0	27.7 ± 1.15 (mm)	20.3 ± 1.15 (mm)	7.8 ± 0.45
0.05	27.7 ± 0.58	21.3 ± 2.31	7.9 ± 0.39
0.5	28.7 ± 2.08	20.0 ± 1.00	7.7 ± 0.42
5.0	28.7 ± 1.53	19.3 ± 0.58	7.6 ± 0.44

[a] The experiment was performed as described in *Materials and Methods*. The distance traveled by the ten fastest-moving neutrophils toward the outer well was measured with a microprojector, and expressed as mm.

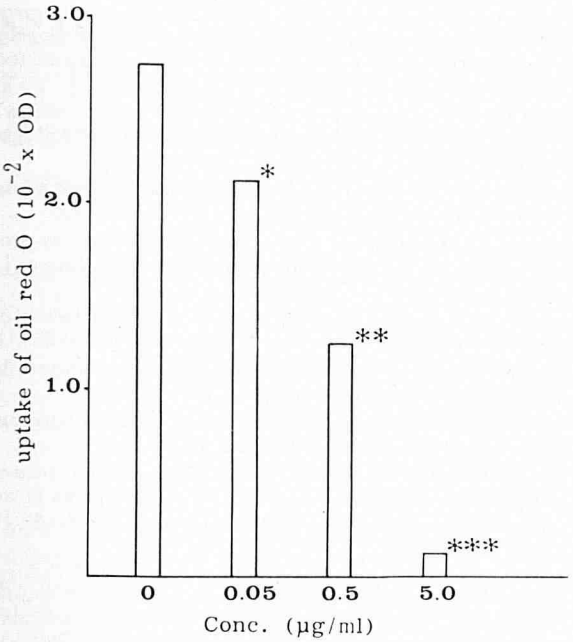

Figure 1. Effect of linoleic acid on neutrophil phagocytosis. * $p < 0.05$ vs control, ** $p < 0.01$, *** $p < 0.001$.

venging or quenching effect of this agent, because it had no effect on ROS levels generated in the xanthine-xanthine oxidase system. It thus appears that linoleic acid decreases ROS generation by acting on neutrophil metabolism.

Initially, we predicted that the ROS scavenging activity would be stronger in neutrophils from patients with *P. acnes* than in those from normal individuals. However, the results obtained from both patients and normals were comparable (data not shown). It thus appears that ROS generation by neutrophils is not abnormal in acne patients.

In the pathogenesis of acne inflammation, *P. acnes* seems to play an important initiating role by producing low MW chemotactic factors [2], resulting in the accumulation of neutrophils at the site of acne comedones. The attracted neutrophils, after phagocytosis, release inflammatory factors such as lysosomal enzymes, complement components, and ROS [4,5,27,28], all of which subsequently induce tissue damage. Because acne inflammation first occurs as a

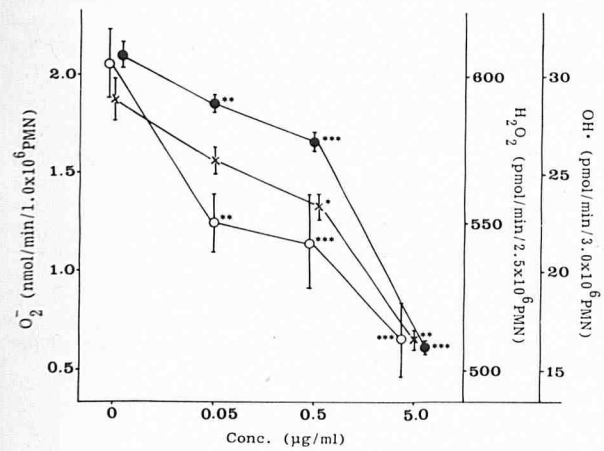

Figure 2. Effect of linoleic acid on ROS generation by neutrophils. *Closed circle,* O_2^-; *open circle,* H_2O_2; *cross symbol,* OH · levels. PMN, polymorphonuclear leukocytes. * $p < 0.05$ vs control, ** $p < 0.01$, *** $p < 0.001$.

Table II. Effect of Linoleic Acid on ROS Levels Generated by the Xanthine-Xanthine Oxidase System[a]

Linoleic Acid (μg/ml)	ROS		
	O_2^- (mnol/min)	H_2O_2 (pmol/min)	OH· (pmol/min)
0	1.166 ± 0.0050	n.d.[b]	102.9 ± 3.55
0.05	1.227 ± 0.0165	n.d.	118.1 ± 3.86
0.5	1.122 ± 0.0172	n.d.	114.8 ± 4.97
5.0	1.102 ± 0.0186	n.d.	114.0 ± 4.51

[a] H_2O_2 levels could not be correctly determined in this assay system because the agents in the xanthine-xanthine oxidase system reacted with scopoletin and peroxidase in the presence of linoleic acid.
[b] n.d., not done.

disruption of the integrity of the follicular epithelium leading to extrusion of intrafollicular material into the dermis, subsequently resulting in a variety of inflammatory processes, these inflammatory mediators are thought to increase the inflammatory state of acne and to aggravate the initial acne lesion.

In this study, it was found that linoleic acid suppresses neutrophil phagocytosis and ROS generation. Thus, the decrease in proportion of linoleic acid in acne comedones [15] may have the effect of indirectly enhancing these neutrophil functions in such lesions. In normal hair follicles, linoleic acid may serve to protect the skin from the insult of acne inflammation by inhibiting neutrophil phagocytosis and ROS generation.

The quantity of skin surface lipids on the human forehead has been estimated to be 155–310 μg/cm² [29]. Because fatty acids occupy 16.4% of these lipids [30], it can be estimated that fatty acid contact in situ ranges from 25.42–50.84 μg/cm². The concentration of 0.5 μg/ml linoleic acid, which was shown to inhibit both neutrophil phagocytosis and ROS generation in vitro in the present study, thus appears to be in the physiologic range and may therefore actually inhibit neutrophil phagocytosis and ROS generation in normal human hair follicles. A decrease in the proportion of linoleic acid, which has been documented in acne comedones [16], would provide more favorable conditions for an increase in neutrophil-mediated inflammation and oxidative injury in situ. In addition to acne inflammation, the important role of fatty acid metabolism in a variety of inflammatory dermatoses has recently been noted; the deficiency in unsaturated fatty acids is found in the atopic children, and the feeding with added-fat supplements produced clinical improvement [31]. Unsaturated fatty acids were also found to inhibit leukotrien B_4 generation, which suggests the beneficial effect for suppressing cutaneous inflammatory disorders such as psoriasis [32]. Further, linoleic acid is important for preserving epidermal integrity [33], and its deficiency in human or animal skins induces morphologic changes characterized by severe scaly dermatoses, extensive percutaneous water loss, and hyperproliferation of the epidermis [34].

Although *P. acnes* has been found to be closely related to the worsening of acne inflammation by attracting neutrophils in situ through release of low MW chemotactic factors [2], *P. acnes* generation of free fatty acids, though previously documented [35,36], has been considered to play a lesser role in the pathogenesis of acne inflammation [37]. However, our study suggests that a decreased proportion of linoleic acid in acne comedones, which effectively inhibits neutrophil function, contributes to the worsening of acne inflammation.

REFERENCES

1. Webster GF, Leyden JJ: Mechanisms of *Propionibacterium acnes* mediated inflammation in acne vulgaris. Semin Dermatol 1:299–304, 1982
2. Puhvel SM, Sakamoto M: The chemoattractant properties of comedonal contents. J Invest Dermatol 71:324–329, 1978
3. Webster GF, Leyden JJ: Characterization of serum-independent poly-

morphonuclear leukocyte chemotactic factors produced by *Propionibacterium acnes*. Inflammation 4:261–271, 1980

4. Miyachi Y, Yoshioka A, Imamura S, Niwa Y: Effect of antibiotics on the generation of reactive oxygen species. J Invest Dermatol 86:449–453, 1986
5. Miyachi Y, Yoshioka A, Imamura S, Niwa Y: Anti-inflammatory activities of tetracyclines are partly exerted by their anti-oxidant effect. Proceedings of the IVth International Congress of Pediatric Dermatology. In: Urabe H, Kimura M, Yamamoto K, Ogawa H (eds.). University of Tokyo Press, Tokyo, 1987, pp 291–294
6. Niwa Y, Sakane T, Shingu M, Yanagida I, Komura J, Miyachi Y: Neutrophil-generated active oxygens in linear IgA bullous dermatosis. Arch Dermatol 121:73–78, 1985
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 49:247–255, 1982
8. Niwa Y, Sakane T, Shingu M, Yokoyama MM: 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 3:228–240, 1983
9. Miyachi Y, Uchida K, Komura J, Asada Y, Niwa Y: Auto-oxidative damages in cement dermatitis. Arch Dermatol Res 277:288–292, 1985
10. Niwa Y, Somiya K: Enhanced neutrophilic functions in mucocutaneous lymph node syndrome, with special reference to the possible role of increased oxygen intermediate generation in the pathogenesis of coronary thromboarteritis. J Pediatr 104:56–60, 1984
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 9:163–172, 1985
12. Martin RR, Warr JA, Couch RB, Yeager H, Knight V: Effects of tetracycline on leukotaxis. J Infect Dis 129:110–115, 1974
13. Esterly NB, Furey NL, Flanagan LE: The effect of antimicrobial agents on chemotaxis. J Invest Dermatol 70:51–55, 1978
14. Esterly NB, Koransky JS, Furey NL, Trevisan M: Neutrophil chemotaxis in patients with acne receiving tetracycline therapy. Arch Dermatol 120:1308–1313, 1984
15. Perisho K, Wertz PW, Madison KC, Stewart ME, Downing DT: Fatty acids of acylceramides from comedones and from the skin surface of acne patients and control subjects. J Invest Dermatol 90:350–353, 1988
16. Wertz PW, Miethke MC, Long SA, Strauss JS, Downing DT: The composition of the ceramides from human stratum corneum and from comedones. J Invest Dermatol 84:410–412, 1985
17. Skosey JL, Damgaard E, Chow DC, Sorensen LB: Modification of zymosan-induced release of lysosomal enzymes from polymorphonuclear leukocytes by cytochalasin B. J Cell Biol 62:625–634, 1974
18. Johnson AR, Erdos G: Metabolism of vasoactive peptides by human endothelial cells in culture. J Clin Invest 59:684–695, 1977
19. Nelson RD, Quie PG, Simmons RL: Chemotaxis under agarose. A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J Immunol 115:1650–1656, 1977
20. Stossel TP: Evaluation of opsonic and leukocyte function with a spectrophotometric test in patients with infection and with phagocytic disorders. Blood 42:121–130, 1973
21. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917, 1959
22. Johnston RB Jr, Lehmeyer JE: Elaboration toxic oxygen by-products by neutrophils and a model of immune complex disease. J Clin Invest 57:836–841, 1976
23. Massey V: The microestimation of succinate and the extinction coefficient of cytochrome c. Biochim Biophys Acta 34:255–256, 1959
24. Root RK, Metcalf JA: H_2O_2 release from human granulocytes during phagocytosis. J Clin Invest 60:1266–1279, 1972
25. Klebanoff SJ, Rosen H: Ethylene formation by polymorphonuclear leukocytes. J Exp Med 148:490–505, 1978
26. Niwa Y, Kasama T, Miyachi Y, Kanoh T: Neutrophil chemotaxis, phagocytosis and parameters of reactive oxygen species in human aging: cross-sectional and longitudinal studies. Life Sci 44:1655–1664, 1989
27. Webster GF, Leyden JJ, Tsai CC, Baehni P, McArthur WP: Polymorphonuclear leukocyte lysosomal release in response to *Propionibacterium acnes* in vitro and its enhancement by sera from patients with inflammatory acne. J Invest Dermatol 74:398–401, 1980
28. Webster GF, Kligman AM: A method for the assay of inflammatory mediators in follicular casts. J Invest Dermatol 73:266–268, 1979
29. Greene RS, Downing DT, Pochi PE, Strauss JS: Anatomical variation in the amount and composition of human skin surface lipid. J Invest Dermatol 54:240–247, 1970
30. Downing DT, Strauss JS, Pochi PE: Variability in the chemical composition of human skin surface lipids. J Invest Dermatol 53:322–327, 1969
31. Burton JL: Dietary fatty acids and inflammatory skin disease. Lancet I:27–31, 1989
32. Ziboh VA: Implications of dietary oils and polyunsaturated fatty acids in the management of cutaneous disorders. Arch Dermatol 125:241–245, 1989
33. Wertz PW, Swartzendruber DC, Abraham W, Madison K, Downing DT: Essential fatty acids and epidermal integrity. Arch Dermatol 123:1381–1384, 1987
34. Ziboh VA, Chapkin RS: Biologic significance of polyunsaturated fatty acids in the skin. Arch Dermatol 123:1688–1690, 1987
35. Strauss JS, Pochi PE: Intracutaneous injection of sebum and comedones. Arch Dermatol 92:443–456, 1965
36. Freinkel RK, Strauss JS, Yip SY, Pochi PE: Effect of tetracycline of the composition of sebum in acne vulgaris. N Engl J Med 273:850–854, 1965
37. Puhvel SM, Sakamoto M: A re-evaluation of fatty acids as inflammatory agents in acne. J Invest Dermatol 68:93–99, 1977