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Enhancement by Clofazimine and Inhibition by Dapsone of Production of Prostaglandin E_2 by Human Polymorphonuclear Leukocytes in Vitro

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The effects of the antileprosy agents clofazimine and dapsone (1 to 10 μg/ml) on the spontaneous and stimulated release of prostaglandin E_2 (PG E_2) by human polymorphonuclear leukocytes (PMNL) in vitro have been investigated. PMNL were obtained from normal adult volunteers and three patients with leprosy (two borderline lepromatous and one subpolar lepromatous leprosy). The synthetic chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) at a concentration of 10^{-7} M was used as the stimulant of PG E_2 synthesis. None of the test agents at the concentrations used inhibited the binding of radiolabeled FMLP to PMNL. However, dapsone at 5 and 10 μg/ml inhibited the spontaneous and FMLP-induced release of PG E_2 by PMNL. Clofazimine, on the other hand, significantly increased both the spontaneous and the FMLP-induced synthesis of PG E_2 by PMNL. The enhancing effects of clofazimine on FMLP-mediated synthesis of PG E_2 were particularly striking and were observed at concentrations of 1 to 10 μg of the drug per ml. Measurements of PMNL spontaneous and FMLP-induced synthesis of PG E_2 in the presence of both clofazimine and dapsone (5 μg/ml) indicated that the two drugs are mutually antagonistic. PMNL from both normal control subjects and patients with leprosy were equally sensitive to these effects of clofazimine and dapsone. The immunostimulatory and immunosuppressive properties of dapsone and clofazimine, respectively, may be related to the opposite effects of these agents on PG E_2 synthesis in human leukocytes.

The antileprosy agent clofazimine by virtue of its anti-inflammatory properties is a useful agent in the treatment of patients with the lepromatous form of the disease since it prevents or controls erythema nodosum leprosum (ENL; immune complex) reactions (10, 19, 20). Dapsone, on the other hand, possesses both antimicrobial and nonspecific immunostimulatory properties and this agent is likely to precipitate or exacerbate ENL reactions (10, 20). The opposite effects of clofazimine and dapsone on ENL reactions are probably related to inhibition and potentiation, respectively, by these agents of polymorphonuclear leukocyte (PMNL) migration and lymphocyte proliferation (2, 18).

The occurrence of reversal immunity reactions (cell-mediated type) in some borderline cases is also influenced by antimicrobial chemotherapy (5, 11, 12). Reversal immunity reactions are characterized by recovery of cell-mediated immunity to *Mycobacterium leprae* antigens, an acute inflammatory reaction in the lesions with an influx of lymphocytes, and increased numbers of epithelioid cells and giant cells. Direct and indirect enhancement of cell-mediated immunity by dapsone may lead to the development of reversal immunity reactions (2). Clofazimine inhibits lymphocyte proliferation (8, 18) and may be useful in controlling reversal immunity reactions (13, 17). We have suggested previously that the anti-inflammatory, immunosuppressive properties of clofazimine and the nonspecific immunostimulatory effects of dapsone are related to the respective pro- and anti-oxidant activities of these agents (2, 18). Nevertheless, the pharmacological mechanisms by which clofazimine and dapsone modulate leukocyte migration and proliferation have not been identified. In this study I have investigated the effects of both of these agents as well as that of rifampin on the synthesis of the immunomodulatory prostaglandin, prostaglandin E_2 (PG E_2), by human PMNL in vitro.

MATERIALS AND METHODS

Drugs. Dapsone and indomethacin were obtained from Lennon Pharmaceuticals Ltd., Port Elizabeth, and Merck, Sharp and Dohme Ltd., Johannesburg, Republic of South Africa, respectively. Clofazimine and rifampin were provided by CIBA-GEIGY Ltd., Basel, Switzerland. Dapsone, indomethacin, and rifampin were dissolved in 30% ethanol in 0.15 M phosphate-buffered saline to give a stock solution of 3 mg/ml. The drugs were diluted further in phosphate-buffered saline to 1 mg/ml and thereafter in 4.2 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, Mo.)-buffered Hanks balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, N. Y.), pH 7.2. These agents were used at final concentrations of 1, 2.5, 5, and 10 μg/ml. The corresponding ethanol concentrations of 0.01 to 0.1% without drugs were included.

As previously stated by Cline (6), aqueous solutions of clofazimine are particularly difficult to prepare. It was found, however, that clofazimine was readily soluble in an aqueous mixture of 10% acetic acid and 30% dimethyl sulfoxide, an observation that led to preparation of stock solutions of 10 mg/ml. Subsequent dilutions could be made in distilled, deionized water to give a clofazimine concentration of 100 μg/ml. The drug was then diluted to 20 μg/ml in HBSS and the pH was brought to 7 with 1 N NaOH. Under these conditions the drug remained completely in solution. Nevertheless, this solution was centrifuged at 12,000 × *g* for 3 min in a microcentrifuge (model KM-15200; Kubota Corp., Tokyo, Japan), and residual particulate material was measured by laser nephelometry (helium-neon laser; Behring Institute, Frankfurt, Federal Republic of Germany). Relative to identically processed control systems containing the final concentrations of acetic acid and dimethyl sulfoxide without

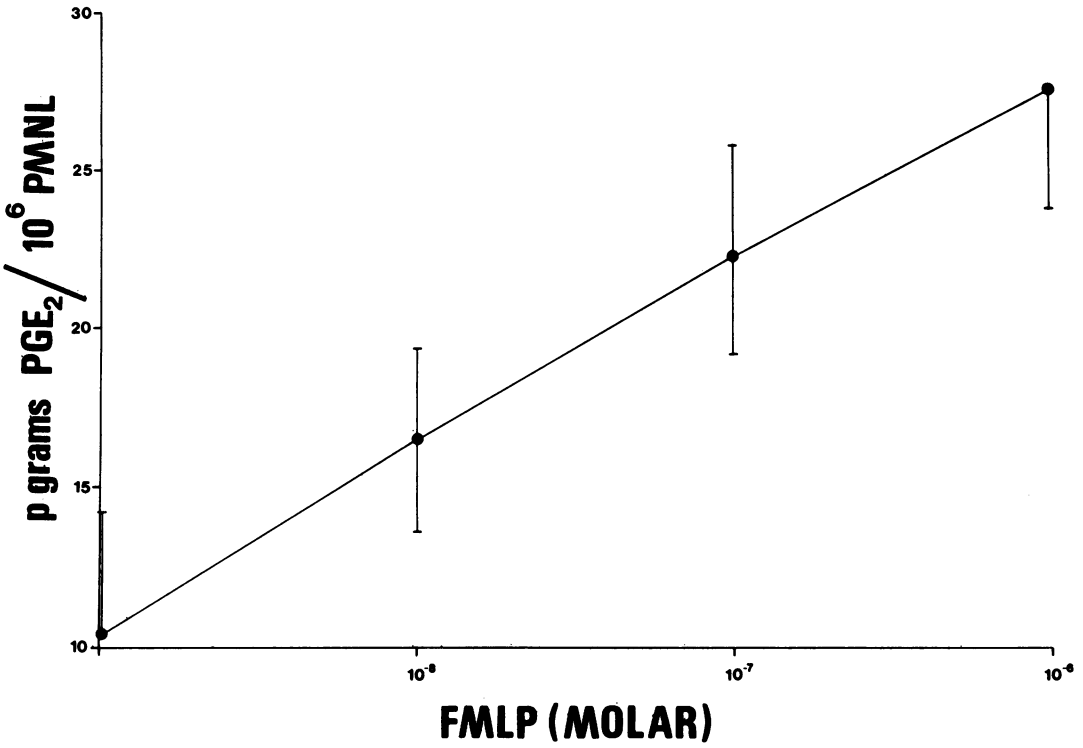

FIG. 1. Effects of FMLP (10^{-8} to 10^{-6} M) on synthesis of PG E_2 by human PMNL. Results are expressed as the mean value ± standard error in picograms of PG E_2 per 10^6 PMNL per 30 min of three different experiments.

clofazimine, no insoluble particulate material was detectable.

PMNL preparation. Purified PMNL were used for these investigations. Heparinized venous blood (5 U of preservative-free heparin per ml) was obtained from healthy male and female laboratory workers and from three untreated, newly diagnosed patients with leprosy admitted to Westfort Hospital, Pretoria. According to the clinical and histopathological criteria of Ridley and Jopling (16), two patients were classified as borderline and one was classified as subpolar. PMNL were separated from mononuclear leukocytes by centrifugation of whole blood for 15 min at 400 × *g* on Ficoll-Hypaque cushions (9% Ficoll–12% Hypaque; specific gravity, 1.072). The resultant pellet was suspended in phosphate-buffered saline and sedimented with a 3% volume of 25% gelatin for 30 min. The PMNL-enriched layer was decanted and centrifuged at 250 × *g* for 10 min, and the resultant pellet was treated with 0.85% ammonium chloride at 4°C for 10 min to lyse residual erythrocytes. The remaining cells, which after washing were suspended in HBSS, contained >90% viable PMNL as determined by trypan blue (0.1%) dye exclusion.

Measurement of PG E_2 release by PMNL. PMNL were incubated at a final concentration of 2×10^6 per ml with the test drugs (clofazimine, dapsone, indomethacin, rifampin) at concentrations of 1 to 10 μg/ml or with the appropriate solvent controls in the presence or absence of the synthetic chemotactic tripeptide *N*-formyl-L-methionyl–L–leucine-L-phenylalanine (FMLP; Miles Laboratories, Elkhart, Ind.) at 37°C for 30 min in a final volume of 1 ml of HBSS. FMLP was used at a final predetermined concentration of 10^{-7} M. After incubation, reactions were terminated by the addition of 1 ml of ice-cold HBSS, and the samples were placed in an ice bath. PMNL were subsequently removed by centrifugation at 4°C, and PG E_2 in the cell-free supernatants was assayed by a radioimmunoassay method (New England Nuclear Corp., Boston, Mass.). This is a competitive binding radioimmunoassay based on the differential precipitation by 16% polyethylene glycol of immune complexes formed between a standard amount of added ^{125}I-labeled PG E_2 and specific antiserum to PG E_2 after an 18-h incubation period at 4°C. The amount in the polyethylene glycol precipitate is inversely proportional to the amount of competing, nonradiolabeled PG E_2 in the test (supernatant) samples. The concentration of PG E_2 in the supernatants was calculated by using a standard curve with added pure PG E_2 in the range of 1 to 250 pg/ml. The results are expressed as picograms of PG E_2 per 10^6 PMNL.

No extraction steps were required with this method since serum- and protein-free HBSS was used throughout and 100% recovery of added radiolabeled PG E_2 was consistently obtained. To exclude the possibility of false reactions due to interference of reagents with the PG E_2 assay system or cross-reactivity with PG E_2 antibody, the following control systems were included: (i) HBSS only, (ii) HBSS + 10^{-7} M FMLP, and (iii) HBSS + various drugs and solvents at the final concentrations used.

Effects of simultaneous exposure of PMNL to clofazimine and dapsone. In these experiments PMNL from normal control persons and leprosy patients were coincubated with 5 μg each of both clofazimine and dapsone, with and without 10^{-7} M FMLP. The kinetics of spontaneous and stimulated PG E_2 release by PMNL were measured after 15, 30, 60, and 90 min of incubation. Control systems contained no drugs or 5 μg of clofazimine or dapsone only.

Effects of clofazimine and dapsone on binding of radiolabeled FMLP to PMNL. PMNL were incubated at 37°C for 10 min in the presence or absence of the antileprosy agents (10

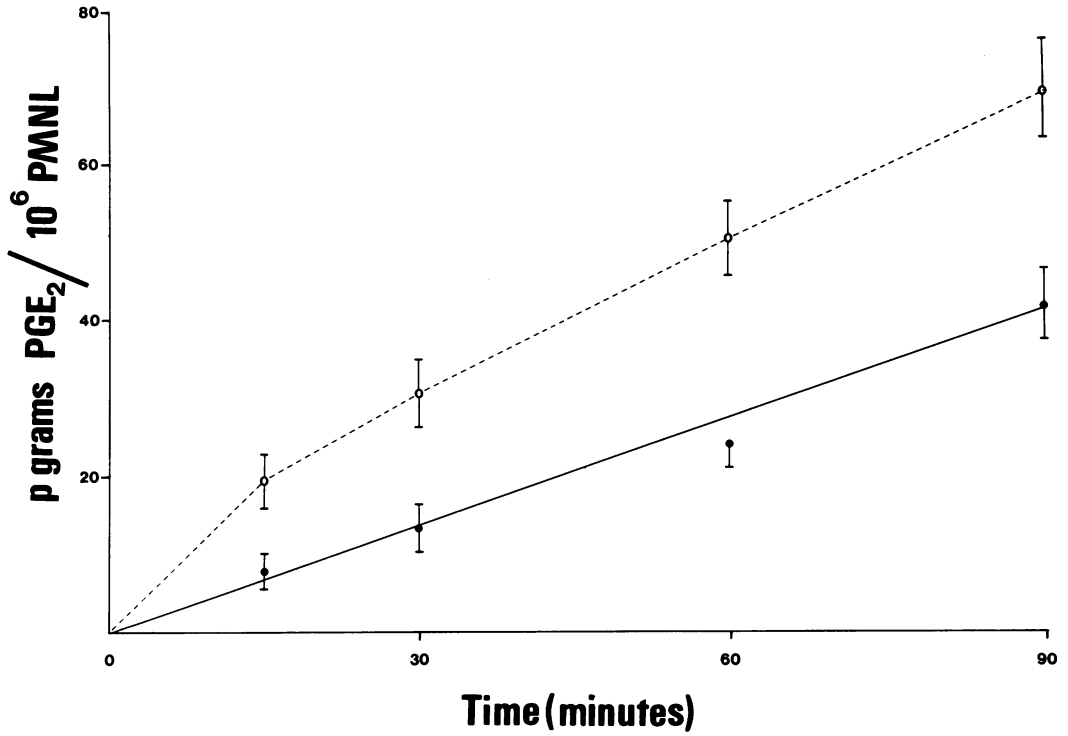

FIG. 2. Kinetics of production of PG E_2 by control (●) and FMLP-stimulated (○) PMNL. Results are expressed as the mean values ± standard error of three different experiments.

μg/ml) and then exposed (10^6 PMNL) to various amounts (1 to 50 fmol) of tritiated FMLP [*N*-phe-*ring*-2,6-^{3}H(*N*), 47 Ci/mmol; New England Nuclear] in the presence or absence of a 100-fold excess of unlabeled FMLP (15 min at 37°C in a final volume of 200 μl of HBSS). Incubation was carried out over 150 μl of silicone oil as described by Prokesch and Hand (14). PMNL were pelleted at 12,000 × *g* for 3 min and the tubes were frozen at −70°C overnight. The PMNL pellets and supernatants were isolated by cutting the tubes at the oil-HBSS interface. The solubilized cell pellets and supernatants were assayed for radioactivity in a liquid scintillation counter. Specific binding is defined as the total amount of ^{3}H-labeled FMLP bound to PMNL minus the amount bound in the presence of a 100-fold excess of unlabeled FMLP. Regression analysis of Scatchard plots was performed to determine receptor number and K_d (nanomolar).

Expression and analysis of results. Results are expressed as the mean value with standard error of the mean for each series of experiments. Statistical analyses of results were performed by using Student's *t* test (paired *t* statistic).

RESULTS

Effects of FMLP on PG E_2 synthesis by human PMNL. The effects of FMLP at concentrations of 10^{-8} to 10^{-6} M on PG E_2 release by PMNL from healthy control persons after 30 min of incubation at 37°C are shown in Fig. 1. Statistically significant potentiation ($P < 0.05$) was observed with 10^{-7} M FMLP. This concentration of FMLP is also leukotactic by comparison with 10^{-6} M, which is a supraleukotactic concentration, and was therefore used for all subsequent experiments.

Kinetics of PG E_2 release by FMLP-treated PMNL. The spontaneous and FMLP (10^{-7} M)-induced syntheses of PG E_2 by normal PMNL after 15, 30, 60, and 90 min of incubation are shown in Fig. 2. Both the spontaneous and the FMLP-mediated release of PG E_2 by PMNL increased significantly with time.

Effects of dapsone and rifampin on PG E_2 release by PMNL. Rifampin did not affect either the spontaneous or the stimulated release of PG E_2 by normal PMNL. At 10 μg of rifampin per ml, the resting and stimulated values were 8.5 ± 2.1 and 27.6 ± 10 pg of PG E_2 per 10^6 PMNL per 30 min, respectively, and the corresponding control values were 7.7 ± 1.4 and 25.7 ± 8.8. Dapsone, on the other hand, was found to inhibit both the spontaneous and the FMLP-mediated synthesis of PG E_2 by PMNL. Statistically significant inhibition was observed with dapsone concentrations of 5 and 10 μg/ml (Fig. 3). Ethanol (0.01 to 0.1%) did not affect either the spontaneous or the stimulated release of PG E_2. At 0.1% ethanol the resting and stimulated values were 7.9 ± 1.7 and 29.4 ± 14 pg of PG E_2 per 10^6 PMNL per 30 min, which are not significantly different from the control values shown above. Relative to indomethacin, dapsone is a weak inhibitor of PG E_2 synthesis. At 5 μg/ml, indomethacin inhibited spontaneous and FMLP-induced synthesis of PG E_2 in PMNL by 100 and 85 ± 13%, respectively. The corresponding inhibitions by dapsone at 5 μg/ml were 32 ± 10 and 35 ± 7%.

Effects of clofazimine on PG E_2 release by PMNL. Clofazimine at concentrations of 5 μg/ml and greater significantly increased ($P < 0.005$) the spontaneous synthesis of PG E_2 by normal human PMNL (Fig. 3). This drug at all concentrations also markedly increased FMLP-induced synthesis of PG E_2 by PMNL (Fig. 3). At 2.5 and 5 μg/ml, clofazimine potentiated the FMLP stimulation of PG E_2 synthesis in PMNL by approximately three- and fivefold, respectively. The acetic acid-dimethyl sulfoxide solvent system alone did not influence PG E_2 release by PMNL.

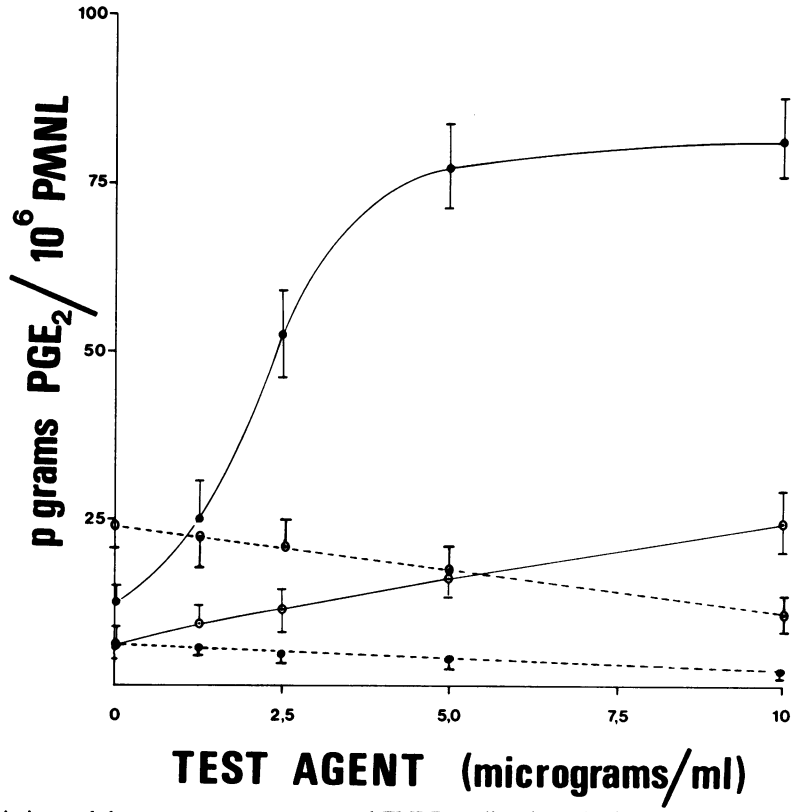

FIG. 3. Effects of clofazimine and dapsone on spontaneous and FMLP-mediated synthesis of PG E_2 by human PMNL: clofazimine and PMNL only (⊙——⊙); clofazimine, FMLP, and PMNL (●——●); dapsone and PMNL only (●---●); dapsone, FMLP, and PMNL (⊙---⊙). Significant potentiation of spontaneous synthesis was observed with clofazimine at concentrations of 5 and 10 μg/ml ($P < 0.005$) and of FMLP-mediated synthesis at 1, 2.5, 5, and 10 μg/ml ($P < 0.005$). Dapsone at 5 ($P < 0.005$) and 10 ($P < 0.005$) μg/ml inhibited both the spontaneous and the FMLP-mediated synthesis of PG E_2 by PMNL.

Effects of simultaneous coincubation of dapsone and clofazimine on PG E_2 synthesis by PMNL. The effects of simultaneous addition of 5 μg each of clofazimine and dapsone per ml on the kinetics of spontaneous and FMLP-stimulated release of PG E_2 by normal human PMNL are shown in Fig. 4. The two drugs were found to be mutually antagonistic in relation to their effects on both spontaneous and FMLP-stimulated PG E_2 synthesis in that dapsone diminished the potentiating effects of clofazimine and clofazimine partly neutralized the inhibitory effects of dapsone. Similar effects were found with PMNL from leprosy patients (Fig. 4). PMNL from leprosy patients behaved identically to normal control PMNL with respect to the effects of clofazimine and dapsone individually and in combination on spontaneous and FMLP-stimulated synthesis of PG E_2.

Effects of dapsone and clofazimine on binding of radiolabeled FMLP to PMNL. At the maximal concentration used (10 μg/ml), the antileprosy agents did not affect the binding of radiolabeled FMLP to PMNL. For dapsone the mean receptor number and K_d value (three different experiments) were 81,103 ± 4,933 and 28.9 ± 18.4, respectively, and the corresponding values for the solvent control system were 99,973 ± 13,322 and 28.9 ± 7.9. The inhibitory effects of dapsone on PG E_2 synthesis are therefore not due to interference with FMLP binding to PMNL. For clofazimine the mean receptor number and K_d value (three different experiments) were 83,529 ± 3,261 and 31.3 ± 17.6, respectively, and the corresponding values for the solvent control system were 78,889 ± 6,613 and 36 ± 12. The enhancing effects of clofazimine are not caused by increased binding of FMLP to PMNL.

DISCUSSION

Treatment of leprosy with antimicrobial drugs is frequently complicated by the development of ENL and reversal immunity reactions. This is particularly evident with dapsone monotherapy and often necessitates reductions in the dosage and frequency of administration of this agent, leading to dapsone resistance (20). Chemotherapy-associated disintegration of bacilli with immune-complex formation is the probable cause of ENL. Reversal immunity reactions, on the other hand, occur as a consequence of chemotherapy-related elimination of bacteria from tissues with loss of immunological tolerance to *M. leprae* antigens and recovery of cell-mediated immunity. There is a second mechanism, however, distinct from the antimicrobial activity of dapsone, by which this agent may potentiate adverse immunological reactions in patients with leprosy. Dapsone increases PMNL migration and lymphocyte proliferation in vitro and in vivo (2) and may exacerbate ENL and reversal immunity reactions by these immunostimulatory mechanisms. Clofazimine, however, has been reported to inhibit leukocyte migration and mitogen-induced proliferation (8, 18) and has been reported to be useful, in high dosage, in controlling both ENL (10, 20) and reversal immunity reactions (13, 17) while continuing to function chemotherapeutically. The value of clofazimine in the treatment of patients with lepromatous leprosy and ENL is probably related to the

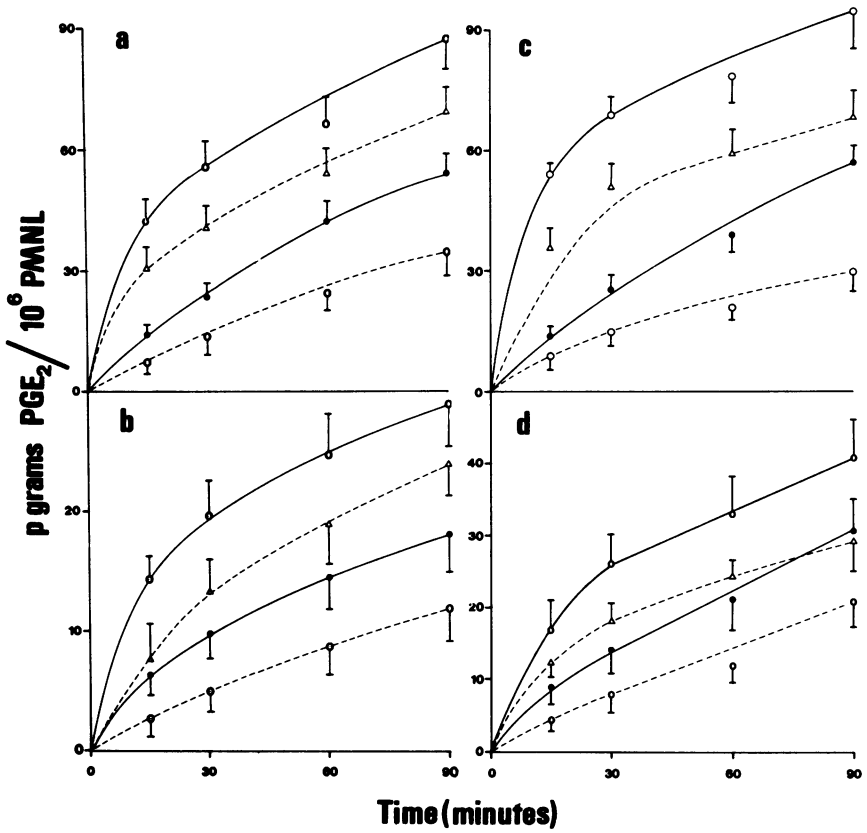

FIG. 4. Kinetics of spontaneous (b, d) and FMLP-stimulated (a,c) production of PG E_2 by normal human PMNL (a, b) and PMNL from patients with lepromatous leprosy (c, d) in (a) the absence of clofazimine or dapsone (●), (b) with 5 μg of clofazimine per ml only (○—○), (c) with 5 μg of dapsone per ml only (○---○), and (d) with both clofazimine and dapsone (△). Results are expressed as the mean values ± standard error for PMNL from three different individuals in each group.

inhibitory effects of the drug on PMNL migration (8, 18). In borderline cases with residual specific cell-mediated immunity and reversal immunity reactions, the beneficial immunosuppressive effects of clofazimine are probably attributable to inhibition of leukocyte migration and proliferation (8, 18).

In this study, the effects of clofazimine, dapsone, and rifampin on PG E_2 synthesis by human PMNL in vitro were investigated. PG E_2 derived from phagocytes is a potent inhibitor of lymphocyte proliferation (7, 9) and may modulate 5-lipoxygenase, which produces chemotactic and proinflammatory products. Rifampin at concentrations of 1 to 10 μg/ml did not influence PG E_2 synthesis. Dapsone, however, at concentrations of >1 μg/ml inhibited both the spontaneous and the FMLP-stimulated synthesis of PG E_2 by PMNL. After ingestion of a single 100-mg oral dose of dapsone, peak serum levels occur at approximately 4 to 6 h, with an average serum value of 2 μg/ml (1). Since dapsone did not affect the binding of radiolabeled FMLP to PMNL, it is likely that the drug is an inhibitor of the enzyme prostaglandin synthetase, albeit a weak one relative to indomethacin. Inhibitors of this enzyme, such as aspirin and indomethacin, increase the proliferative responses of lymphocytes to mitogens by preventing PG E_2-induced immunosuppression (7, 9) and may enhance PMNL responses also. Interestingly, dapsone and acedapsone administration to patients with the borderline tuberculoid and tuberculoid forms of leprosy is associated with significant enhancement of skin reactivity to lepromin (15). By inhibition of PG E_2 synthesis in PMNL, monocytes, and macrophages, dapsone may potentiate cell-mediated immunity and reversal immunity reactions in susceptible borderline cases.

Clofazimine at all concentrations tested increased the spontaneous and FMLP-stimulated synthesis of PG E_2 by both control and leprosy PMNL. The effects on FMLP-stimulated release of PG E_2 were especially striking. It is probable that clofazimine potentiates the synthesis and release of PG E_2 by phagocytes, which suppresses antigen-induced lymphocyte proliferation, thereby controlling reversal immunity reactions. Ingestion of 200 mg of clofazimine daily gives peak serum levels of 0.7 to 1 μg of the drug per ml (21), indicating that the effects on PG E_2 synthesis described here may occur in vivo. Enhancement of PG E_2 synthesis by clofazimine is probably due to the redox properties of the drug. Clofazimine, which readily penetrates cell membranes, has a redox potential of −0.18 V at pH 7 (3) and increases both the phagocytic activity and the membrane-associated oxidative metabolism of PMNL and macrophages (4, 6, 18). By insertion into the cell membrane of the phagocyte, clofazimine, by acting as an electron acceptor in the FMLP-activatable NADPH oxidase complex, may enhance oxygen consumption and PG E_2 synthesis. In support of this mechanism, we have recently found that clofazimine at the concentrations used in this study markedly stimulates the spontaneous and FMLP-induced chemiluminescence of PMNL (unpublished data). The opposite effects of clofazimine and dapsone on PG E_2 synthesis in human PMNL may be due to the respective pro-oxidant (6, 18) and anti-oxidant (2) properties of these agents. Alternatively, inhibition by clofazimine of a competing step,

e.g., inhibition of thromboxane synthetase or 5-lipoxygenase, could also increase production of stable prostaglandins.

It seems paradoxical that a nonsteroidal anti-inflammatory agent such as clofazimine potentiates the activity of PMNL prostaglandin synthetase in response to pro-inflammatory stimuli such as leukoattractants and antigens. However, ingestion of pharmacological amounts of PG E_2 or its poorly metabolizable analogs has been reported to relieve nephritis and adjuvant arthritis in animals and to eliminate immune-complex arthritis (22, 23). Enhancement of PG E_2 production by phagocytes, if operative in vivo, is therefore a likely mechanism of clofazimine-mediated anti-inflammatory and immunosuppressive activity.

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