Effects of Monomethylfumarate on Human Granulocytes

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Monomethylfumarate (MMF) is the most active metabolite of the new antipsoriasis drug Fumaderm. Because granulocytes play an important role in the pathophysiology of psoriasis, the effects of this drug on the functional activities of these cells were investigated. MMF stimulated polarization and elastase release, and enhanced the intracellular killing of bacteria by granulocytes. This compound suppressed the formyl-Met-Nle-Phe (FMLP)-stimulated respiratory burst in these cells. MMF and dimethylfumarate but not its stereoisomer dimethylmaleate, fumaric acid, or dimethylmalate stimulated polarization of and elastase release by granulocytes, indicating that methylated fumarate derivatives interact with granulocytes in a specific fashion. MMF did not affect the binding of formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein isothiocyanate to the FMLP receptor on granulocytes. This compound induced an increase in the intracellular Ca++ ([Ca++]I) and cyclic adenosine monophosphate concentration.

The agonistic effects of MMF on granulocytes are thought to be mediated by the rise in the [Ca++]I, and the antagonistic effects by the increase in the cyclic adenosine monophosphate concentration. These effects of MMF on granulocytes may in part explain the beneficial action of methylated fumarate derivatives on psoriatic skin lesions. J Invest Dermatol 101:37–42, 1993

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soriasis, a chronic inflammatory skin disease affecting up to 3% of the population, is characterized by epidermal hyperplasia and infiltration of lymphocytes and granulocytes into the skin [1,2]. Treatment is based on inhibition of the proliferation of epidermal cells and interference in the inflammatory process. In addition to the present therapeutic regimen a new systemic antipsoriasis drug, which consists of dimethylfumarate (DMF) and monomethylfumarate (MEF), has been introduced with successful results [3,4]. Monomethylfumarate (MMF), which is formed in the circulation by hydrolysis of DMF, is believed to be the most potent metabolite of this drug. Granulocytes are retained at the epidermis by means of a number of factors [5–7]. The exact role of granulocytes in the pathophysiology of psoriasis is not known. IL-8 in combination with TNF-α, which is also present in psoriatic skin lesions [2,5], stimulates granulocytes to produce reactive oxygen intermediates and proteolytic enzymes [8,9], which can damage epidermal cells and probably influence the growth and differentiation of keratinocytes. Because granulocytes play an important role in the pathophysiology of psoriasis the beneficial effect of treatment with fumaric acid derivatives may be caused by its effects on granulocytes. The present study concerns the effects of MMF on granulocytes from healthy donors.

MATERIALS AND METHODS

Compounds Used to Stimulate Granulocytes Cells were incubated with the following compounds: MMF (Sigma Chemical Co., St. Louis, MO), dimethylfumarate (DMF; Merck, Darmstadt, Germany), dimethylmaleate (DM; Merck), fumaric acid (FA; Merck), and S- or R-dimethylmaleate (S-DMM and R-DMM; Jansen Chimica, Geel, Belgium). In all experiments the pH of the stock solutions of these compounds was adjusted to about 7.2 with 0.1 N NaOH. At the concentrations and exposure time used in this study the stimuli did not affect cell viability, as determined by trypan blue exclusion and the lactate dehydrogenase release assay. For comparison, cells were stimulated with 10 to 100 nM formyl-Met-Leu-Phe (FMLP) (Sigma) or 1 nM rIL-8 (a gift from Dr. I. Lindley, Department of Immunostimulation, Sandoz Forschungsinstitut Gesellschaft H.B.H., Vienna, Austria). Because both FMLP and rIL-8 were dissolved in dimethylsulfoxide (Serva, Heidelberg, Germany) and diluted in PBS, cells were also exposed to PBS with an equivalent concentration of dimethylsulfoxide. Phorbol myristate acetate (PMA) and dibutyryl(dBCAMP)–cyclic adenosine monophosphate (cAMP) were obtained from Sigma.

Preparation of Human Leukocytes Buffy coats prepared from blood from healthy donors were diluted in PBS (pH 7.4) and then subjected to Ficoll-amidotrizoate (p = 1.077 g/ml; Pharmacia Inc., Uppsala, Sweden) density centrifugation at 440 X g for 20 min [10]. Granulocytes were purified from the Ficoll-Amidotrizoate pellets by dextran 1 X g sedimentation (Plasmatiril, Fresenius A.G., Bad Homberg, Germany) at 37°C for 10 min followed by removal of the remaining erythrocytes by a single hypotonic lysis.

Abbreviations: db-cAMP, dibutyryl–cyclic adenosine monophosphate; DMF, dimethylfumarate; DMM, dimethylmaleate; FA, fumaric acid; FITC, fluorescein isothiocyanate; FLPEP FITC, formyl-Nle-Leu-Phe-Nle-Tyr-Lys–FITC; FMLP, formyl-Met-Leu-Phe; PURA2/AM, pentakis (acetoxyethyl)–cyclic adenosine monophosphate (cAMP)–cyclic adenosine monophosphate (cAMP); Tw, Tween 8.


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The final cell suspensions consisted of 97 ± 2% granulocytes. The viability of the cell suspensions exceeded 95%, as determined by trypan blue dye exclusion.

**Measurement of Polarization of Granulocytes** The assay to determine polarization of granulocytes was performed as described [11] with minor modifications. Briefly, 400-μl aliquots of a suspension of 1 × 10⁶ granulocytes/ml RPMI 1640 (Flow Laboratories Ltd., Irvine, UK) were incubated with 100 μl of the stimulus diluted in RPMI 1640 at 37°C for 15 min. Incubation was stopped by the addition of 500 μl 9% v/v formaldehyde in PBS; the cells were maintained in this fixative until light microscopic determination of the percentage of cells that were not spherical. Two different investigators each analyzed 200 cells from each preparation.

**Chemotaxis Assay** Chemotaxis of granulocytes was measured with the 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD) assay as described [12]. In short, chemotaxis or PBS were placed in the lower compartments. Two filters were adjusted between the lower and upper compartments. The lower filter had a pore width of 0.45 μm and the upper filter of 8 μm. Granulocytes were placed in the upper compartment and, after incubation for 1.5 h instead of 2.5 h at 37°C, the filters were fixed, stained, and cleared with xylene. The number of granulocytes that had passed the upper filter was determined by light microscopy (magnification ×400). The results are expressed as the number of cells per 10 high-power fields (hpf).

**Measurement of Elastase Release by Granulocytes** Granulocytes suspended in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 0.9 mM CaCl₂, 0.49 mM MgCl₂, 1.5 mM KH₂PO₄, and 2.5 mg bovine serum albumin (BSA)/ml at a concentration of 5 × 10⁶ cells/ml were incubated with the indicated stimulus at 37°C for 30 min. The reaction was stopped by transferring the tubes to crushed ice. After centrifugation at 500 × g for 10 min, samples of the cell-free supernatants were assayed for elastase activity utilizing substrate 2-SAP (KabiVitrum, Stockholm, Sweden) as described [13]. The absorbance of the reaction product after 60 min was read at 405 nm using a Titertek multiscan (EFLAB, Helsinki, Finland). Granulocytes were incubated with 5 μg cytochalasin E (Serva)/ml PBS under slow rotation (4 rpm) at 37°C for 5 min, washed, and suspended to a concentration of 5 × 10⁶ cells/ml.

**Measurement of Intracellular Killing of Mycobacterium fortuitum by Granulocytes** Preparation of M. fortuitum (ATCC 12790, American Type Culture Collection, Rockville, MD) and the assay to determine the intracellular killing of this mycobacterium by granulocytes has been described [14]. Briefly, 5 × 10⁶ granulocytes/ml Hanks’ balanced salt solution (HBSS) (Oxoid Ltd., Basingstoke, UK) containing 0.01% gelatin and 0.01% Tween 80, further called HBSS-gel-Tw, and 5 × 10⁶ M. fortuitum/ml HBSS-gel-Tw were mixed and incubated in the presence of 10% v/v human serum at 37°C for 10 min. The supernatant containing extracellular bacteria was discarded, the granulocytes washed, and then 5 × 10⁶ granulocytes containing bacteria were stimulated with 10% serum with or without 200 μM MMF at 37°C. One and two hours after stimulation samples were taken and, after lysis of the granulocytes, the number of viable intracellular bacteria was determined microbiologically. As control, granulocytes containing bacteria were incubated with MMF or no stimulus.

**Measurement of the Oxygen Consumption by Granulocytes** Oxygen consumption by granulocytes at rest and after stimulation was determined polarographically [15]. In short, 300 μl of a suspension of 2.5 × 10⁶ leukocytes/ml RPMI 1640 were added to a thermostated vessel (37°C), and the oxygen tension in the medium was measured with a Clark electrode. The rate of oxygen consumption was calculated from the slope of the oxygen tension trace over a period of 5 to 10 min. To discriminate between oxygen consumption by the NADPH oxidase complex and the mitochondrial respiration, cells were also stimulated in the presence of 1 mM NaN₃, an inhibitor of mitochondrial respiration.

**Measurement of Superoxide Anion Production by Granulocytes** Superoxide anion (O₂⁻) production by cytochalasin E–incubated granulocytes at rest and after stimulation was assessed by the superoxide dismutase-inhibitable reduction of ferricytochrome c (type IV; Sigma) as described [16] with minor modifications. Briefly, the stimulus was added to 1 × 10⁶ cells/ml PBS supplemented with 1.2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 20 mM Hepes, and 0.1 mM ferricytochrome c at 37°C and the absorbance at 550 nm was measured every 30 seconds during a 10-min period. The production of superoxide anion by the cells was calculated from a standard curve obtained with reduced cytochrome.

**Measurement of the NADPH Oxidase Activity in a Cell-Free System** NADPH oxidase activity was measured by means of a Clark oxygen electrode at 27°C as the rate of oxygen consumption by the cytosolic and membranous components of NADPH oxidase that were isolated from granulocytes as described [17]. Briefly, 360 μl 10 mM Hepes (pH 7.0) containing 0.17 M sucrose, 75 mM NaCl, 0.5 mM ethyleneglycol-bis-(amino-ethylther)N,N’,N’’,N’’-tetra-acetic acid (EGTA), 1 mM MgCl₂, 2 mM NaN₃, and 10 μM GTPγS (CalBiochem Corp, La Jolla, CA) was mixed and 2 μl 10 membranes and 10 μl cytosolic components, each of which is an equivalent of 1 × 10⁶ granulocytes. Assembly of the NADPH oxidase complex was initiated by adding sodium dodecylsulfate at a final concentration of 100 μM and closing the reaction vessel. After 3 min, NADPH at a final concentration of 250 μM was added and the oxygen consumption measured.

**Determination of the Binding of FLPEP-FITC to Granulocytes by FACS Analysis** The fluorescent probe formyl–Nle-Leu-Phe-Nle-Tyr-Lys–fluorescein isothiocyanate (FLPEP-FITC, Molecular Probes Inc., Eugene, OR) was used to determine binding to the receptor as described [19]. Briefly, granulocytes at a concentration of 2 × 10⁶ cells/ml buffer (pH 7.4) were incubated with various concentrations of FLPEP-FITC in the dark at 4°C for 60 min and then the fluorescence of the cells was measured on a FACScan (Becton and Dickinson, Mountainville, CA) equipped with a 15 W argon-ion laser. For assessment of the non-specific binding of this probe, cells were incubated with 1 μM FLMP at 4°C for 15 min prior to addition of FLPEP-FITC. The results expressed as mean fluorescence intensity values are in arbitrary units (AU).

**Competition Binding Assay for cAMP** At selected intervals after stimulation, 50 μl of a suspension of 1 × 10⁶ granulocytes/ml were stimulated at 37°C for various intervals and then 450 μl ice-cold 1-propanol (Merck) were added to disrupt the cells. After sonication for 5 min this material was centrifuged at 7000 × g for 1 min and then the supernatant was lyophilized. The residue was dissolved in 100 μl distilled water and the cAMP content of these samples was determined as described previously [19]. In short, cAMP in the samples was allowed to compete with 5',8'-dIcAMP–specific activity 40–60 Ci/mmol; Amersham International, Amersham, Bucks, UK) for binding to sites in the bovine microsomal preparation. The cAMP content of these samples was calculated from a standard curve constructed in parallel with unlabeled cAMP (Amersham). The cAMP concentration was calculated from this value and the mean cell volume of the granulocytes.

**Measurement of the [Ca++]i, in Granulocytes** For measurement of the [Ca++]i, 2 × 10⁶ leukocytes/ml 20 mM Hepes supplemented with 138 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.1 mM CaCl₂, 1 mM NaH₂PO₄, 5.5 mM D-glucose, 0.1 mM EGTA, and 0.1% BSA (Ca++ buffer, pH 7.4) were incubated with 2 μM of the acetoxyethyl ester of FURA-2 (FURA-2/AM; Sigma) in the dark at 37°C for 30 min. Next, the cells were washed with Ca++ buffer and then stimulated in either Ca++ buffer or Ca++ buffer without CaCl₂ but supplemented with 2 mM EGTA, further called Ca++–free buffer. Two millimeter aliquots of 5 × 10⁶ cells/ml Ca++–free buffer or Ca++–free buffer were transferred to a quartz cuvette with magnetic stirring. The cell suspensions were excited alternately at 340, 360, and 380 nm (slit width 2.5 nm) and emission was

**Measurement of the Calcium Oxidase Activity in Granulocytes**...
part of the chambers than when no stimulus was applied (Table I). The effect on the number of cells in the filter did not depend on the concentration of MMF (range 2 nM to 2 mM; results not shown). These results indicate that MMF and DMF are hardly chemotactic for granulocytes; FMLP was a potent chemotaxin for these cells (Table I). Addition of MMF to the upper compartment of the microchemotaxis chamber did not affect this response to FMLP. 

Granulocytes incubated with MMF for 5 min responded to FMLP as well as cells incubated with PBS (results not shown). MMF but not DMF, DMM, FA, S-DMM, or R-DMM stimulated granulocytes to release elastase (Table I). Stimulation by MMF was considerably less than that by rIL-8 or FMLP (Table I). MMF-incubated granulocytes released significantly less elastase after rIL-8 or FMLP stimulation than PBS-incubated cells.

**Intracellular Killing of M. fortuitum by Granulocytes**

The phagocytosis of M. fortuitum by granulocytes and the growth of this bacterium in HBSS – gel-Tw were not affected by MMF (results not shown). The intracellular killing of M. fortuitum by granulocytes stimulated with serum was limited but addition of MMF enhanced the killing process significantly; the percentage of M. fortuitum killed 2 h after stimulation with and without MMF was 60 ± 4.5% and 29 ± 3.5% (means ± SEM; n = 3), respectively. In the absence of serum, intracellular killing of M. fortuitum by granulocytes did not occur and MMF had no effect. The cell-free medium, obtained after incubation for 2 h at 37°C of 5 X 10⁶ granulocytes and 5 X 10⁶ M. fortuitum in 1 ml HBSS – gel-Tw containing 10% serum with MMF, did not affect the growth of M. fortuitum, indicating that no extracellular killing of bacteria occurred (results not shown).

**Stimulation of Oxygen Consumption and O₂ Production by Granulocytes with MMF and FMLP**

Oxygen consumption and O₂ production by resting granulocytes and by granulocytes after stimulation with various concentrations of MMF, FMLP, or PMA are shown in Table II. Both MMF and FMLP stimulated oxygen consumption by these cells. Optimal concentrations for MMF and FMLP were 200 μM and 100 nM, respectively. No additive effect of MMF on the FMLP-stimulated oxygen consumption by granulocytes was found (results not shown). In the presence of NaN₃, an inhibitor of mitochondrial respiration, the stimulatory effect of MMF on oxygen consumption was almost completely abolished whereas that of FMLP was hardly affected (Table II), indicating that MMF does not activate the NADPH oxidase complex. In agreement with this notion, MMF did not stimulate granulocytes to produce O₂. This compound did not scavenge O₂.

To find out whether incubation with MMF suppresses the FMLP-stimulated oxygen consumption, granulocytes were exposed to MMF in the presence of NaN₃, or as control to PBS, prior to further stimulation with FMLP.
to stimulation with FMLP. The results showed that the respiratory burst in MMF-incubated cells was significantly lower than in control cells (Table II). Because it has been suggested that an increase in the cAMP concentration inhibits the oxidative metabolism of granulocytes [21,22], we incubated granulocytes with 0.1 mM db-cAMP for 5 min at 37°C before measurement of the FMLP-stimulated O₂⁻ production. The results revealed no effect of MMF on the oxidative activity of db-cAMP-incubated granulocytes (mean ± SEM) (n = 7) than that by control granulocytes.

Effect of MMF on the NADPH Oxidase Activity in the Cell-Free System To elucidate the mechanisms underlying the inhibition of the respiratory burst in MMF-incubated cells, we measured the effect of MMF on the NADPH oxidase activity in the cell-free system. For this purpose MMF, or as control PBS, was added to the mixture of cytosolic and membranous components of the NADPH oxidase complex before the addition of SDS. The results revealed no effect of MMF on the NADPH oxidase activity; the results of the addition of MMF and PBS were 8.4 ± 0.2 µM oxygen/min and 8.2 ± 0.6 µM oxygen/min (means ± SEM; n = 5), respectively.

Effect of MMF on the Binding of FLPEP-FITC to Granulocytes To find out whether the MMF-mediated inhibition of the respiratory burst in granulocytes was due to down-regulation of the FMLP-receptor, the effect of MMF on binding of FLPEP-FITC to these cells was determined. The results revealed no effect of exposure of the cells to MMF for 15 min prior to the incubation with the fluorescent probe (Fig 2). Addition of MMF during the incubation with FLPEP-FITC was without effect as well (results not shown).

Changes in the cAMP Concentration in Granulocytes After Stimulation with MMF and FMLP Because an increase in the cAMP concentration in cells is involved in the negative feedback processes in cells [24], we investigated whether MMF induces a rise in the cAMP concentration in granulocytes. The cAMP concentration in resting granulocytes was 0.72 ± 0.19 µM (mean ± SEM; n = 4). Stimulation of granulocytes with MMF resulted in a transient increase in the cAMP concentration to a maximum value of 1.33 ± 0.14 µM (mean ± SEM; n = 4) 20 seconds after stimulation, which returned to resting values within 1 min after addition of the stimulus. FMLP induced a threefold increase in the cAMP concentration in granulocytes with a maximum value of 2.67 ± 0.60 µM (mean ± SEM; n = 4) 30 seconds after stimulation.

Changes in the [Ca²⁺], in Granulocytes Induced by Various Stimuli The [Ca²⁺], in resting granulocytes amounted to 110 ± 6 nM (mean ± SEM; Table I). Addition of 2 µM to 20 mM MMF to granulocytes induced a dose-dependent increase in the [Ca²⁺]; maximum values were found after stimulation with 200 µM MMF (Fig. 3). The rise in the [Ca²⁺], was already apparent within 5 seconds of the addition of MMF and the [Ca²⁺], gradually returned to resting values within the next 2–3 min. The increase in the [Ca²⁺], after stimulation with MMF is due mainly to the release of Ca²⁺ from intracellular stores. Similar kinetics for changes in the [Ca²⁺], were found for granulocytes stimulated with FMLP and rIL-8 (results not shown). Furthermore, the FMLP-induced increase in the [Ca²⁺], in MMF-incubated and PBS-incubated granulocytes did not differ (results not shown), indicating that MMF did not affect signaling in granulocytes.

To find out whether the effects of methylated fumarate derivatives on granulocytes depend on the presence of methyl groups, the changes in the [Ca²⁺], in granulocytes upon stimulation with DMM, DMM, FA, S-DMM, or R-DMM were assessed. The results revealed that of these compounds only DMM induced an increase in the [Ca²⁺], (Table I).

**DISCUSSION**

The main conclusion that can be drawn from the present results is that the methylated fumarate derivatives MMF and DMM modulate several functional activities of granulocytes. These fumarate derivatives are agonists of granulocytes because they stimulate the polarization and elastase release by granulocytes and enhance the intracellular killing of bacteria by these cells. Some of these effects might be mediated by the rise in the [Ca²⁺], in granulocytes stimulated by these compounds, since Ca²⁺-specific ionophores also stimulate the polarization and elastase release by granulocytes [23–25]. MMF and DMM are not chemotactic for granulocytes or stimulate the respiratory burst in MMF-incubated cells was significantly lower than in control cells (Table II).
late polarization, degranulation, or changes in the [Ca++] in granulocytes. The most convincing evidence for the specificity of the binding site for methylated fumarate derivatives is our observation that DMF, but not its stereoisomer DMM, stimulates granulocytes. Furthermore, we did not observe a change in the [Ca+++] or oxygen consumption by purified monocytes after stimulation with MMF (unpublished observations), indicating that methylated fumarate derivatives react with cells in a selective fashion.

The present results indicate that MMF suppresses the production and release of important mediators of the inflammatory process, such as reactive oxygen intermediates and elastase. In addition to these effects on granulocytes, MMF suppresses the proliferation of human cultured keratinocytes and SVK 14 keratinocyte cell-line cells (unpublished observations). The therapeutic effect of methylated fumarate derivatives in psoriasis can most probably be explained by its anti-inflammatory effects on granulocytes and its antiproliferative effects on keratinocytes.

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REFERENCES


Figures

Figure 3. Effect of various concentrations of MMF on the [Ca++] in granulocytes. For investigation of the effect of MMF on the [Ca++] 4, FURA 2/AM-incubated granulocytes were stimulated with 2 μM to 20 mM MMF and the changes in the [Ca++] monitored. Data, i.e., means ± SEM, represent the peak values (at 10 seconds) after stimulation. n = 3.

‡ Nibbering PH, Pos O, Stevenhagen A, van Furth R: Interleukin-8 enhances the non-oxidative intracellular killing of Mycobacterium fortuitum by human granulocytes (submitted for publication).

ANNOUNCEMENT

The 4th Annual Meeting of the European Hair Research Society will take place in Stockholm on October 1–2, 1993 at the Långholmen Conference Centre. Deadline for abstracts is July 1, 1993. The program of the meeting will tentatively cover the following areas:

Hair proteins—structure and function
Basic research on hair and hair culture models
Immunology and transgenic mice experiments
Hormonal regulation
The pilo-sebaceous unit
Hair diseases
Hair transplantation
Environmental influences on hair and hair care

The Congress Chairman is Associate Prof. Bo Forslind, M.D., Ph.D.
For information on the program, accommodation, etc. write to EHRS Congress Secretariat, Ms. Jenny Bernström, Ms. Margareta Andersson, Ms. Inger Lindbäck, EHRS, Department of Medical Biophysics, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden, Tel, +46 (0) 8 7286790; Fax, +46 (0) 8 32 65 05.