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Circulating hydroxy fatty acids in familial Mediterranean fever

(neutrophil aggregation/lipoxygenase/icosanoids/colchicine)

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ABSTRACT **Episodes of fever, serositis, and arthritis in familial Mediterranean fever (FMF) suggested circulating mediators of acute inflammation (e.g., neutrophil activation). The mean serum neutrophil-aggregating activity of 51 FMF patients was 2.5 ± 0.2 cm^2/min, compared to 1.0 ± 0.1 cm^2/min in 20 normal controls ($P < 0.0002$). Lipid extracts of FMF sera retained neutrophil-aggregating activity and had UV absorbance peaks at 269 and 279 nm, indicating the presence of lipids with a conjugated triene structure. Chromatography of extracts yielded peaks that were coeluted with reference dihydroxyicosatetraenoic acids, had UV absorbance peaks at 259, 269, and 279 nm, and possessed neutrophil-aggregating activity. The presence of leukotriene B_4 was excluded by chromatography following methyl-esterification. Monohydroxy compounds identified in FMF extracts by gas chromatography/mass spectrometry included 5-hydroxyicosatetraenoic acid, and 9- and 13-hydroxyoctadecadienoic acids. Hydroxy acids were present in 19 of 31 FMF sera and absent in extracts of sera from 8 patients with active systemic lupus erythematosus, 7 with fever from infection, and 12 normal controls. The finding of circulating mono- and dihydroxy fatty acids in FMF suggests that defects in the formation or elimination of these compounds might play a role in the pathogenesis of FMF.**

Familial Mediterranean fever (FMF) is an autosomal recessive disorder characterized by recurrent self-limited episodes of fever, serositis, arthritis, and, less commonly, dermatitis (1, 2). Histopathologic examination of involved tissues reveals acute inflammation with infiltration by neutrophils (1–3). Synovial effusions are often purulent (4), mimicking septic arthritis. The stimulus to the accumulation and activation of neutrophils is as yet unidentified.

Colchicine reduces the frequency and severity of episodes of FMF (5, 6) by a mechanism that remains unclear, though it has been suggested that it suppresses neutrophil chemotaxis toward an undefined stimulus (6–8). We recently showed that colchicine blocks *in vitro* production by normal human neutrophils of dihydroxyicosatetraenoic acids (di-HETEs), including leukotriene B_4 (LTB_4), a potent lipid chemoattractant and mediator of acute inflammation (9, 10). Therefore, it appeared possible that the inflammatory episodes of FMF were caused by LTB_4 or a related compound; colchicine might act not only by inhibiting neutrophil responsiveness but also by blocking production of the stimulus.

The present study was undertaken to determine whether circulating mediators of acute inflammation might contribute to attacks of FMF. Therefore, we measured the capacity of serum and serum extracts to provoke the aggregation of normal human neutrophils. We report that the sera of patients with FMF possess neutrophil-aggregating activity (NAA); that lipid extracts of FMF sera contain mono- and dihydroxy fatty acids; and that the extracted dihydroxy acids have the capacity to stimulate neutrophils.

MATERIALS AND METHODS

Preparation of Neutrophils. Heparinized (10 units/ml) blood was obtained from healthy donors. Neutrophils were isolated by means of Hypaque/Ficoll gradients (11), followed by sedimentation with dextran and hypotonic lysis of erythrocytes (12). Cells were suspended in a buffered salt solution consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1 mM $MgCl_2$, and 2.6 mM $CaCl_2$ (pH 7.4) (P_iCM) to a final concentration of 3×10^7 cells per ml containing 98 ± 2% neutrophils.

Neutrophil Aggregation. Neutrophil aggregation was studied in a Payton platelet aggregometer as in ref. 13. Cytochalasin B (10 μl; final concentration, 5 μg/ml) and P_iCM (30 μl) were added to the neutrophil suspension (50 μl) in a siliconized cuvette containing a siliconized stirring bar revolving at 900 rpm, and the mixture was allowed to equilibrate at 37°C for 2 min prior to the addition of stimulus. Serum (10 μl) or resuspended lipid extract (10 μl) was added, and the changes in light transmission were recorded. NAA was defined as the area under the light transmission curve during the first minute after the addition of the specimen (14), measured with a Micro-Plan II digital planimeter. With each experiment, fMet-Leu-Phe (final concentration, 0.1 μM) was used as a control stimulus to insure normal responsiveness of donor cells.

Lipid Extraction of Serum. Serum (1 ml) was diluted with water (3 ml), and the pH was titrated to 3.0 by addition of HCl (0.1 M). Chloroform (4 ml) and methanol (2.5 ml) were added, and the mixture was vigorously mixed in a Vortex and then centrifuged at 1600 × *g* at 20°C for 20 min. The chloroform phase was evaporated dry under nitrogen, and the residue was resuspended in methanol (4 ml) and shaken vigorously with diethyl ether (30 ml) and water (15 ml). The aqueous phase was removed, and the extraction was repeated twice. The ether phase was evaporated to dryness, and the residue was resuspended in methanol. UV absorbance scans were obtained with a Beckman model 25 spectrophotometer. For aggregation studies of lipid extracts, an aliquot was evaporated under nitrogen and, after addition of methanol (3 μl), was resuspended in phosphate-buffered saline (50 μl). Aliquots (10 μl) of this suspension were tested for NAA.

High-Performance Liquid Chromatography. Extracts in methanol were analyzed by reverse-phase HPLC (RP-HPLC) with a Beckman Ultrasphere-ODS column (4.6 mm × 25 cm, or 10 mm × 25 cm) with methanol/water/acetic

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Abbreviations: FMF, familial Mediterranean fever; LTB_4, leukotriene B_4; HETE, hydroxyicosatetraenoic acid; di-HETE, dihydroxyicosatetraenoic acid; RP-HPLC, reverse-phase HPLC; NAA, neutrophil aggregating activity.

acid, 75:25:0.01 (vol/vol) as the mobile phase (15). For straight-phase HPLC, samples were methyl-esterified with diazomethane, resuspended in hexane, and analyzed with a Beckman Ultrasphere-Si column (4.6 mm × 25 mm) with hexane/isopropanol/acetic acid, 95:5:0.01 (vol/vol) as the mobile phase (15). Mono- and di-HETE standards were isolated from the supernatants of normal human neutrophils after stimulation with the calcium ionophore A23187 in the presence of arachidonic acid (16).

Gas Chromatography/Mass Spectrometry. Samples were converted to methyl esters with diazomethane in ether at 23°C for 5 min, evaporated under argon, dissolved in pyridine (50 μl), and converted to trimethylsilyl esters (17). The GC/MS assembly (LKB-9000) was equipped with a 1% SE-30 column, and the ionization beam was set at 22.5 electron volts. The Me_3Si derivative of the methyl ester of 13-L-hydroxy-9,11-octadecadienoic acid was prepared as described (18). The mixture of 9-hydroxy-10,12-octadecadienoic acid (75–85%) and 13-hydroxy-9,11-octadecadienoic acid (15–25%) was obtained by incubation of linoleic acid with sheep vesicular gland microsomes (19).

Sera. All samples were allowed to clot at room temperature for 1 hr, centrifuged at 600 × *g* for 15 min, and stored at −20°C until used. Many FMF samples were obtained from patients at Tel-Hashomer Hospital; others were the gift of Keith McAdam at Tufts–New England Medical Center. Systemic lupus erythematosus patients met the revised American Rheumatism Association criteria (20); their sera were selected from patients in the rheumatology clinics or the wards of Bellevue Hospital. Patients with fever from infection were in-patients at Bellevue Hospital when samples were drawn. Normal sera were obtained from healthy laboratory personnel.

RESULTS

NAA of FMF Sera. The aggregation of normal human neutrophils after the addition of serum (10%, vol/vol) was measured. The mean value of 51 FMF sera was 2.5 ± 0.2, compared to a mean of 1.0 ± 0.1 for 20 normal controls ($P < 0.0002$) (Fig. 1).

Whole lipid extracts of FMF sera contained NAA ($n = 3$); extracts of normal serum had no NAA ($n = 2$).

Analysis of Lipid Extracts. UV scans of lipid extracts of FMF sera with high NAA revealed peaks at 269 and 279 nm that are not found in normal serum extracts (Fig. 2 *A* and *B*).

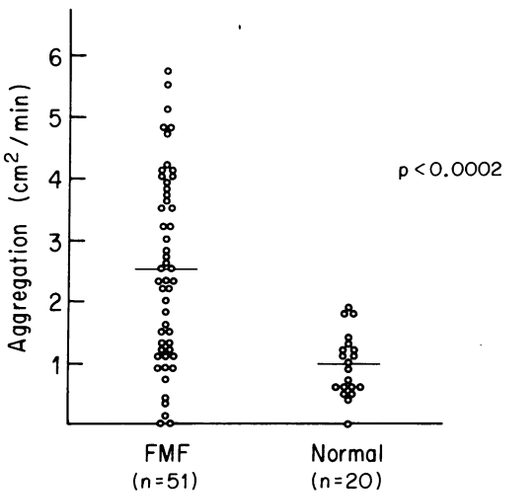

FIG. 1. Aggregation of normal human neutrophils stimulated by sera from patients with FMF. Aggregation was quantitated as described in *Materials and Methods*.

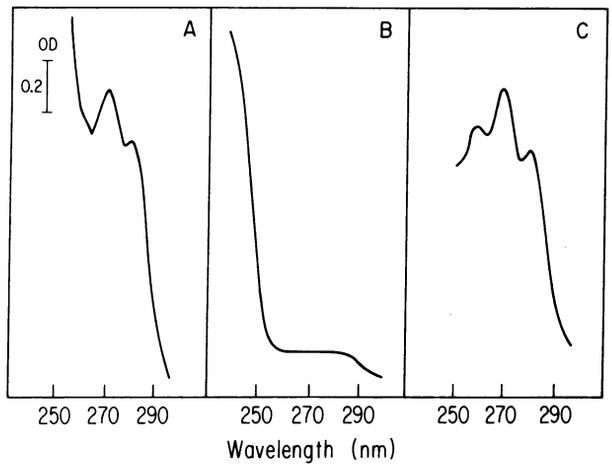

FIG. 2. UV scans of lipid extracts of sera compared to di-HETE spectrum. (*A*) Extract of a FMF patient's serum (1 ml) resuspended in methanol (1 ml). (*B*) Extract of the serum (1 ml) of a healthy volunteer, resuspended in methanol (1 ml). (*C*) Mixture of di-HETEs from ionophore-stimulated normal human neutrophils.

This suggested the presence of fatty acids with conjugated triene structure such as that of the di-HETEs (Fig. 2*C*).

Lipid extracts of FMF and normal serum were analyzed by RP-HPLC and compared to di-HETE standards extracted from the supernatants of ionophore-stimulated neutrophils (Fig. 3). FMF extracts yielded complex peaks (labeled pool A and pool B) that comigrated with the reference di-HETEs. Fractions containing these peaks had NAA and had conjugated triene UV absorbance patterns (Fig. 4).

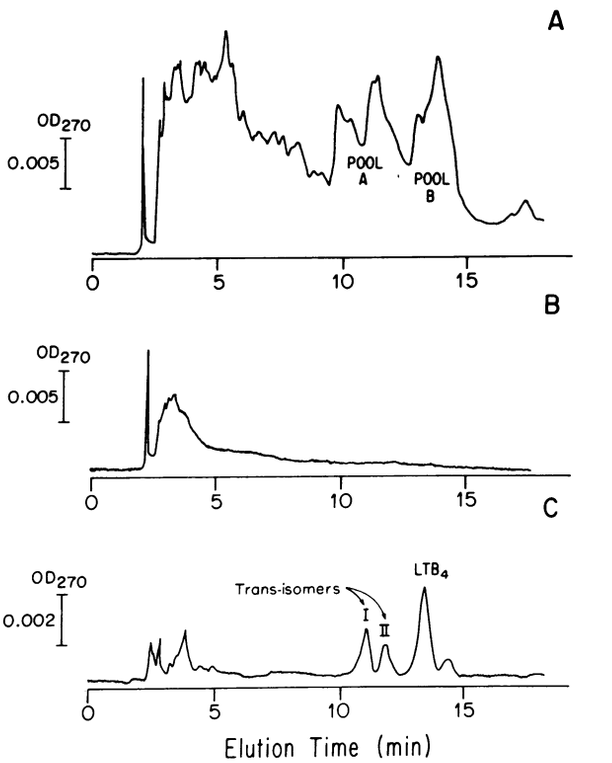

FIG. 3. RP-HPLC analysis of serum extracts compared to di-HETEs (4.6 × 25 cm column; flow rate, 1 ml/min). (*A*) Chromatograph of an extract of FMF serum (500 μl). (*B*) Chromatograph of an extract of normal serum (500 μl). (*C*) Di-HETE standards. *trans* isomer I: (5*S*,12*R*)-5,12-dihydroxy-6,8,10-*trans*-14-*cis*-icosatetraenoic acid; *trans* isomer II; (5*S*,12*S*)-5,12-dihydroxy-6,8,10-*trans*-14-*cis*-icosatetraenoic acid.

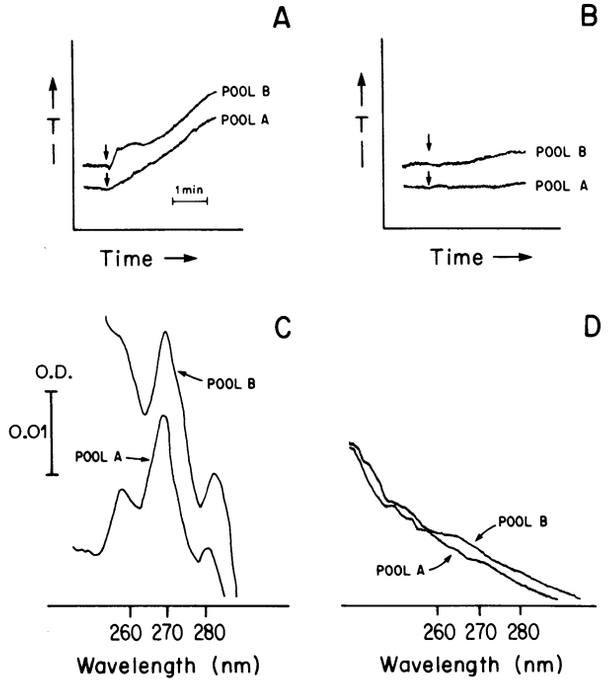

FIG. 4. NAA and UV scans of RP-HPLC fractions of serum extracts. Pool A is elution volume 9–11 ml; pool B is elution volume 12–14 ml (see Fig. 3*A*). (*A* and *B*) Aggregation of normal human neutrophils stimulated by RP-HPLC fractions of FMF and normal serum, respectively. (*C* and *D*) UV scans of RP-HPLC fractions of FMF and normal serum extracts, respectively.

Pools A and B were combined and further resolved by repeated RP-HPLC into seven peaks, none of which precisely comigrated with reference LTB_4 (Fig. 5*A*). These fractions were methyl-esterified with diazomethane and were compared to methyl-esterified di-HETE standards by straight-phase HPLC (data not shown): no peaks comigrated with esterified LTB_4.

In addition to these early-eluted "di-HETE" fractions, RP-HPLC analysis of FMF lipid extracts revealed six less-polar peaks (Fig. 5*B*). Peaks labeled D, E, and F were coeluted with reference 15-, 12-, and 5-HETE, respectively. Peaks C and F, obtained from several sera, were pooled for analysis by GC/MS.

Identification of Monohydroxy Compounds. The UV spectrum of the material that was eluted in peak F of the RP-HPLC showed an absorption band at 235 nm, indicating the presence of a pair of conjugated double bonds (17). GLC analysis of the Me_3Si ether derivative of the methyl ester of this material showed a major peak with an equivalent chain length of $C_{21.5}$. The mass spectrum is given in Fig. 6. Ions were present at *m/e* 406, 391, 375, 316, 305, 255, 216, 215, 203, 190, 155, 150, 143, 136, 105, 80, and 79, in agreement with those published for the Me_3Si ether derivative of the methyl ester of 5-HETE (17). Thus, the identity of the parent compound of this derivative is 5-hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE). However, the stereochemistry of the hydroxyl group at C-5 has not been determined.

The UV spectrum of the material eluted in peak C also showed an absorption band at 235 nm. GC analysis of derivatized material showed a major peak with an equivalent chain length of $C_{19.7}$, suggesting that the material eluted in peak C originated from linoleic acid (18). The mass spectrum of the Me_3Si derivative of the methyl ester of this material is shown in Fig. 7*C*. Ions present included *m/e* 382 (M), 311, 225 (base peak), 186, 143, and 130, indicating a C_{18} ester carrying a Me_3SiO group (18). This suggested that the material eluted in peak C contained monohydroxy acids derived from linoleic acid. For the purpose of comparison, we have included mass spectra of the Me_3Si derivatives of authentic material. The mass spectrum of the Me_3Si derivative of the methyl ester of 100% 13-L-hydroxy-9,11-octadecadienoic acid is given in Fig. 7*A*. Here the base peak is at *m/e* 311, indicating a Me_3SiO group at C-13, with an ion of lower intensity at *m/e* 225 (cf. ref. 18). The mass spectrum of a mixture of Me_3Si derivatives of hydroxyoctadecadienoate methyl esters (approximately, 75–85% 9-hydroxy-10,12-octadecadienoic acid with 15–25% 13-hydroxy-9,11-octadecadienoic acid) is given in Fig. 7*B*. Here, as in Fig. 7*C*, the base peak is at *m/e* 225, with an ion of lower intensity at *m/e* 311. These findings indicate that the peak C material contained a mixture of 9-hydroxy-10,12-octadecadienoic acid plus 13-hydroxy-9,11-octadecadienoic acid.

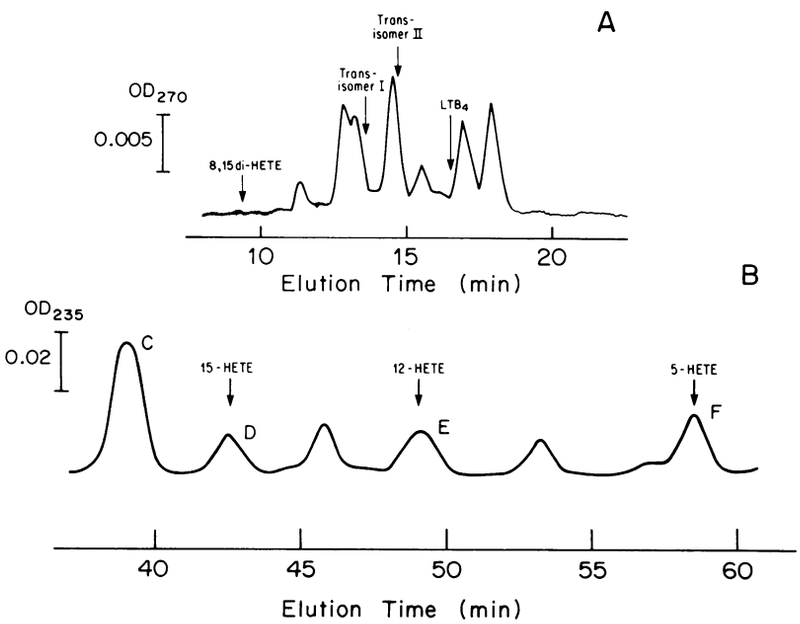

FIG. 5. RP-HPLC analysis of FMF serum extracts (10 mm × 25 cm column; flow rate, 3 ml/min). Elution times of mono- and di-HETE standards are indicated by arrows. (*A*) Early peaks (pools A and B from Fig. 3*A*). (*B*) Late peaks.

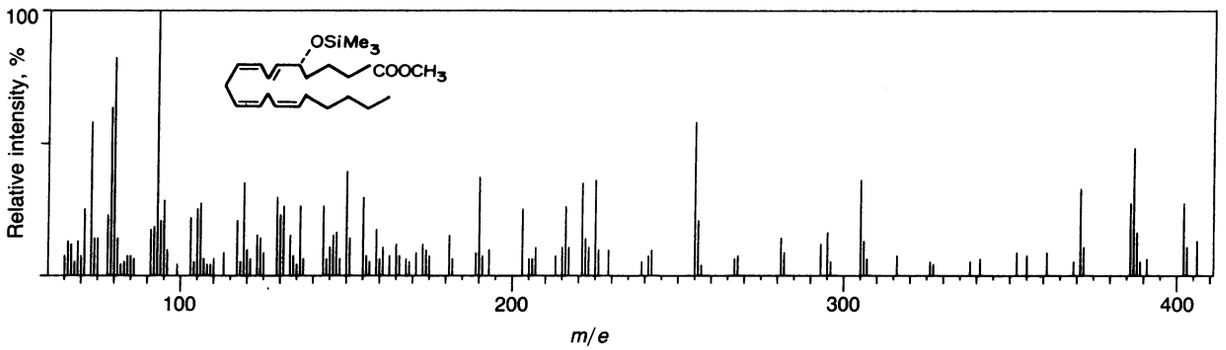

FIG. 6. Mass spectrum of the Me_3Si derivative of the methyl ester of the material eluted as FMF peak F.

Comparison of FMF Sera and Extracts with Controls. Table 1 compares serum NAA and presence of hydroxy fatty acids in patients with FMF, active systemic lupus, fever from bacterial infection, and in normal controls. Of 31 FMF sera that were lipid extracted, 19 contained mono- and dihydroxy fatty acids. The presence of these compounds in FMF sera correlated strongly with NAA: the 19 positive sera had a mean NAA of 3.8 ± 0.3 cm^2/min, whereas the 12 negative sera had a mean NAA of 1.0 ± 0.2 cm^2/min ($P < 0.0001$). As we have previously reported, active lupus patients have elevated serum and plasma NAA (21) in part because of lipid-soluble substances (22). However, extracts of eight active systemic lupus erythematosus sera showed no evidence of circulating hydroxy fatty acids. Sera from patients with fever from infection contained neither elevated NAA nor mono- and dihydroxy acids.

DISCUSSION

FMF is a disease of episodic, acute inflammation and, therefore, attention has focused on the chief effector cell of acute inflammation: the neutrophil. Neutrophil function has been reported to be normal in FMF patients, as measured by pyrogen production, phagocytosis of bacteria and fungi, lysozyme content, and chemotaxis (7, 23). In contrast, increased release of lysozyme by FMF neutrophils has been reported

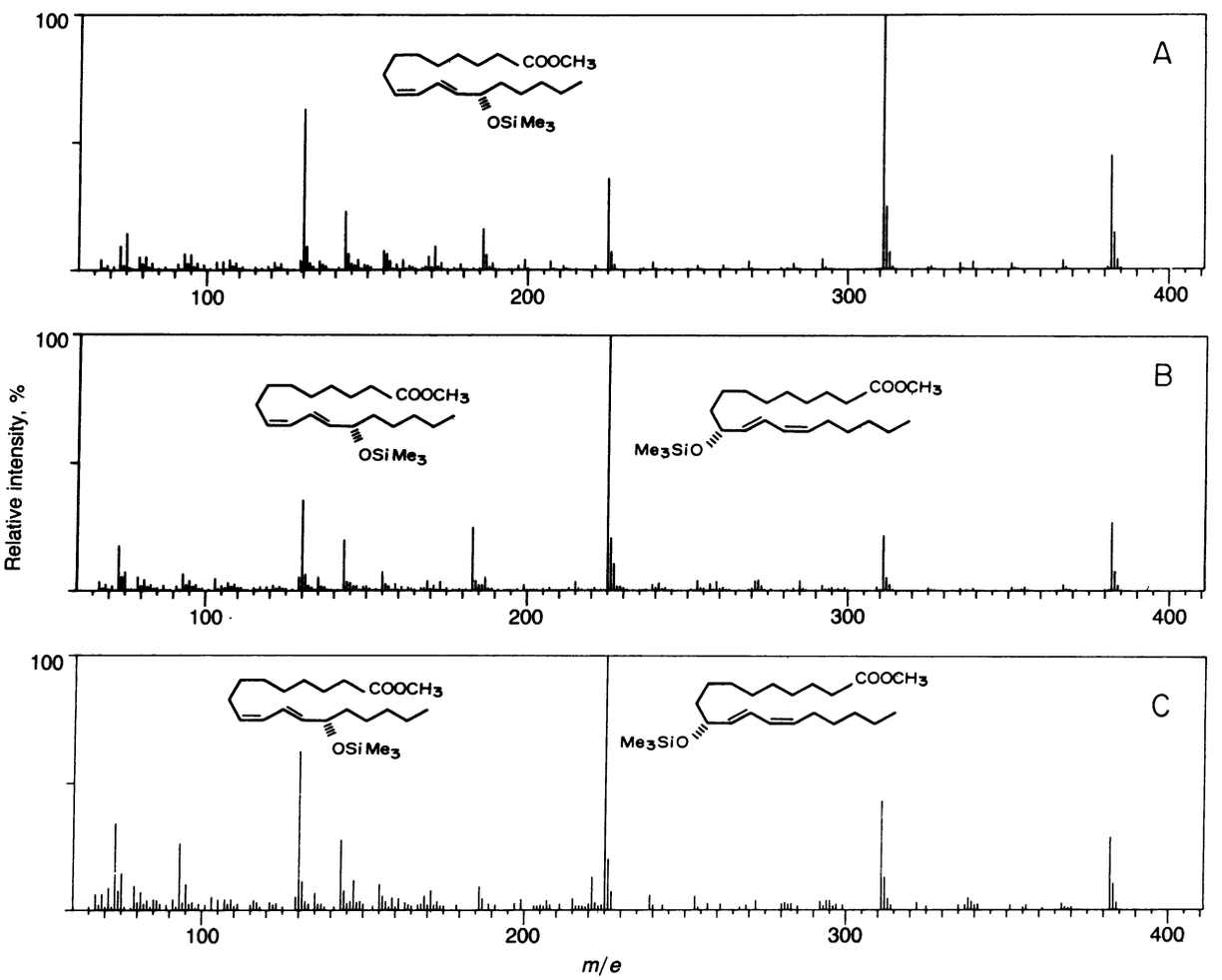

FIG. 7. Mass spectra of the Me_3Si derivatives of the methyl esters of 13-L-hydroxy-9,11-octadecadienoate (*A*), a mixture of 9-hydroxy- and 13-L-hydroxy-9,11-octadecadienoate (*B*), and material from FMF peak C (*C*).

Table 1. NAA and hydroxy acids in human sera

Serum source	NAA, cm^2/min	n	Extracts with hydroxy acids	n
FMF	2.5	51	19	31*
Active SLE	2.6†	26	0	8
Fever, infection	1.2	7	0	7
Normal	1.0	20	0	12

SLE, systemic lupus erythematosus; n, number of patients.
*There was a strong correlation between NAA and the presence of hydroxy acids in the FMF group: FMF sera with hydroxy acids (n = 19) had a mean NAA of 3.8 ± 0.3, whereas FMF sera without hydroxy acids (n = 12) had a mean NAA of 1.0 ± 0.2 ($P < 0.0001$).
†From Abramson *et al.* (21).

at high (≥42°C) temperatures (24), and a recent study suggests that the accumulation and activation of neutrophils in FMF may be caused by a deficiency of an inhibitor of complement component C5a (4).

We present evidence that a majority of patients with FMF have circulating substances that provoke aggregation of normal neutrophils. Analysis of lipid extracts of serum indicates that this activity is at least in part due to lipids that have UV absorbance spectra and HPLC retention times characteristic of di-HETEs. In addition, the majority of FMF sera contain monohydroxy fatty acids, including 9- and 13-hydroxy-9,11-octadecadienoic acids and 5-HETE. 5-HETE is a major product of the 5-lipoxygenase pathway by which leukotrienes are formed in human cells. To our knowledge, none of these compounds has previously been identified in human serum.

We have found NAA and hydroxy acids in the serum of FMF patients during acute flares of their disease as well as inactive periods, but clinical information on many patients who provided samples is incomplete and does not permit a conclusion as to whether there is a correlation between serum levels of NAA or hydroxy acids and disease activity. NAA was not demonstrated in all FMF sera, nor did all contain hydroxy fatty acids. Serial studies might reveal abnormalities in a higher percentage of patients; we have noted fluctuations from normal to abnormal in a few patients from whom serial samples were obtained. Alternatively, the disease may be heterogeneous with regard to these findings.

Our investigation of circulating lipid mediators was in part based on the hypothesis that the effectiveness of colchicine was due to inhibition of a proinflammatory compound such as LTB_4. In fact, we found di-HETEs but not LTB_4, and we found no difference between colchicine-treated and untreated patients. However, the circulation of lipoxygenase products may be a marker of an enzymatic defect leading to the abnormal formation or elimination of oxidized fatty acids. Such a defect could cause the local accumulation of a potent phlogistic substance (e.g., LTB_4) not detected in the serum in response to minor perturbations. Colchicine's effectiveness could then be explained by inhibition of the production of this substance.

Alternatively or additionally, colchicine's action may be at the level of neutrophil responsiveness. Fluctuations in circulating or tissue levels of the lipids that we have extracted (which we have shown to have the capacity to stimulate neutrophils) or the superimposition of a minor exogenous stimulus may lead to the accumulation and activation of neutrophils. Colchicine, in keeping with its known inhibitory effect on neutrophil chemotaxis (25) and degranulation (26), would decrease the intensity of the inflammation by damping the response to the lipid stimuli.

Further characterization of the circulating hydroxy acids, serial studies of serum and body fluids levels of these compounds, and the study of family members of FMF patients may provide further clues to the pathogenesis of this illness.

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