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Characterization and Biological Activities of Humic Substances from Mumie

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Mumie, a semihard black resin formed by long-term humification, is believed to have therapeutic properties. Although mumie has been used in folk medicine since ancient times, there is little information available concerning the physicochemical properties of its constituents and the mechanisms of its therapeutic efficacy. For this study crude mumie was fractionated into fulvic acid (FA), humic acid (HA), humin, hymatomelanic acid, and two low molecular weight fractions (LMW1 and LMW2). The FA fraction was divided into five subfractions, FA1−FA5. The mumie fractions were characterized by IR, UV−vis, and fluorescence spectroscopy. Total carbohydrate content in the fractions was analyzed using the phenol reaction method. The relative content of polar groups and nonpolar hydrocarbon fragments in the mumie fractions correlated well with solubility in an aqueous medium. Biological characterization was performed using only the FA fractions. FA1 and FA2 enhanced the production of reactive oxygen species (ROS) and nitric oxide in murine peritoneal macrophages, as determined with the use of 2′,7′-dichlorofluorescin diacetate and Griess reagent, respectively. The enchancement of ROS and nitric oxide production correlated with the level of total carbohydrates in the fractions. Murine splenic lymphocytes treated with FA1 showed a dose-dependent increase in [^{3}H]thymidine uptake. These findings suggest that FA derived from mumie has immunomodulatory activity.

KEYWORDS: Mumie; humic substances; fulvic acid; reactive oxygen species; nitric oxide; macrophages

INTRODUCTION

Geopolymers and natural biominerals have recently become attractive as food additives and as source materials for the development of drugs. Natural humification products (peat, sapropel, mumie) have been used to develop pharmacologic agents with diverse applications in medical practice (*1*−*3*). Mumie (shilajit) is a semihard black resin formed through long-term humification of *Euphorbia* and *Trifolium* (clover) plants and lichen in mountain regions (*4*). Mumie humus consists of organic matter (60−80%) and mineral matter (20−40%) containing ∼5% trace elements (*4*, *5*).

The people of Tajikistan routinely use mumie in their food. Several biologically active mumie-containing food additives have been patented and are manufactured in that country (*6*, *7*). Mumie is used as a rejuvenator (rasayana) in India, where it has been reported that the active chemical constituents of mumie enhance learning and memory in rats (*8*). Mumie is prescribed for genitourinary diseases, diabetes, digestive disorders, nervous diseases, tuberculosis, chronic bronchitis, asthma, anemia, eczema, bone fractures, and other diseases (*9*, *10*). Although mumie has been used in folk medicine in a number of countries for almost 3000 years, data concerning the structure and physicochemical properties of mumie constituents and mechanisms of therapeutic efficacy are sparse.

For therapeutic applications and as immunostimulating and anabolic food additives, mumie is used in the form of an aqueous extract (processed mumie). The main group of organic substances in the water-soluble fraction of mumie consists of fulvic acids (FA) (*11*). Humic substances of medicinal importance are found abundantly in peat, sapropel, and other humified sources (*1*, *12*). Nevertheless, the biological effects of humic substances can be different, depending on their chemical structure and physicochemical properties. Chemical composition, structure, and functional groups can vary greatly, depending on the origin and age of the humic substance and the conditions of the humification process (humidity, aeration, temperature, mineral microenvironment, etc.) (*13*). The regions of mumie formation are unique biogeocenoses that differ considerably from other regions in terms of the conditions under which the humification process takes place. The physicochemical properties of humic

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substances isolated from soil, peat, sapropel, and aquatic reservoirs are currently under study (*14*−*16*). However, we found only a few publications devoted to the investigation of humic substances from mumie (*11*, *17*). We believe that the systematic approaches used for the characterization of humic substances in general are appropriate for describing the properties of fractions obtained from mumie.

The ability to modulate macrophage and lymphocyte activity plays a pivotal role in determining potential therapeutic effects of medical drugs. The production of nitric oxide and reactive oxygen species (ROS), that is, superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), by activated macrophages is an important cytotoxic and cytostatic mechanism of nonspecific immunity (*18*), and much effort has been directed toward finding natural compounds that influence this production. Macrophages are regulatory cells that are central to cell-mediated and humoral immunity as antigen-presenting, tumoricidal, and microbicidal cells (*19*). Mumie extract has been shown to activate macrophage cell migration in epithelioid granulomas in pulmonary tissue with experimental tuberculosis (*20*).

Aqueous extract of mumie activates phagocytosis and releases cytokines in mouse peritoneal macrophages (*21*). Application of mumie drugs causes proliferation of lymphocytes in the cortical thymus layer and increased migration of these cells into thymus-dependent zones of the lymph nodes and spleen (*20*). FA isolated from soil and water reservoirs stimulate the functional activity of neutrophils and T-lymphocytes (*1*). The effect of FA from mumie on ROS and nitric oxide production by macrophages and lymphocyte proliferation has not been studied.

Thus, the present investigation was undertaken to characterize mumie in terms of fractionation and spectroscopic (IR, UV−vis, and fluorescence) evaluation of its fractions, as well as examination of the biological effects on lymphocyte proliferation and on the production of ROS and nitric oxide by macrophages.

MATERIALS AND METHODS

Reagents. Peroxidase (EC 1.11.1.7) from horseradish, glucose oxidase from *Aspergillus niger*, lipopolysaccharide from *Escherichia coli* O26:B6, 2′,7′-dichlorofluorescin diacetate, concanavalin A from *Canavalia ensiformis* type VI, phorbol-12-myristate-13-acetate, diethylaminoethyl (DEAE)-cellulose, and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO).

Mumie Fractionation and Isolation of Humic Substances. The mumie investigated was a representative sample from a large deposit (>500 kg) of this substance in the mountains of Kazakhstan (Ust'-Kamenogorsk). Mumie from this deposit has been used successfully for medicinal purposes in Russia for many years. The isolation of humic substances from mumie was performed according to the classical method of fractionation based on different solubilities in water at different pH values (*22*). Briefly, 1 kg of mumie was shaken for 16 h at room temperature in 5 L of 0.5 M NaOH. The residue (humin and other insoluble compounds) was separated from the supernatant by centrifugation (3900*g*, 15 min). Then the supernatant was acidified (HCl; pH 1.0), and humic acids (HA) were precipitated. After centrifugation, the FA solution was adjusted to pH 7.0, filtered through a 0.45 μm membrane filter, and fractionated by ethanol precipitation or ion-exchange chromatography. Hymatomelanic acid (HymA) and a dark green viscous matter were extracted with absolute ethanol from HA and humin, respectively. For spectroscopic characterization, an ethanolic extract of crude mumie was also obtained (10 g of raw mumie shaken with 50 mL of ethanol for 24 h at room temperature and filtered through a 0.45 μm membrane filter).

Fractionation of the FA solution into high molecular weight (HMW) and low molecular weight (LMW) fractions by ethanol precipitation was performed as described by Yudina et al. (*23*), with some modification. Briefly, the aqueous solution of FA was mixed with ethanol (final concentration of 66%, v/v) to precipitate the HMW FA. The precipitate was separated out by centrifugation, after which ethanol was added to the supernatant to a final concentration of 85%, and the precipitate again separated out by centrifugation. The two substances obtained by stepwise precipitation with 66 and 85% ethanol were dissolved in distilled water and dialyzed with the use of a 10 kDa cutoff dialysis tube, yielding the FA1 and FA2 fractions, respectively (**Figure 1**). The supernatant from the FA2 fraction was concentrated on a rotary evaporator to ~10% of the original volume, after which an equal volume of ethanol was added. This allowed precipitation to be performed from a significantly concentrated solution. The precipitated grayish-white residue was centrifuged and subjected to further purifying by reprecipitation from a saturated aqueous solution after the addition of an equal volume of ethanol. The result was a white residue [low molecular weight 1 (LMW1) fraction] and a solution. Evaporation of the solution yielded a pellet of bitter black-brown viscous matter (LMW2 fraction).

For fractionation by ion-exchange chromatography, a sample of the FA solution was prepared (**Figure 1**) and then dialyzed with the use of 10 kDa cutoff dialysis tubes, producing the FA_{total} fraction. The FA_{total} fraction was adsorbed to DEAE-cellulose (*24*), and the fractions successively eluted with 0.5 M NaCl, 2 M NaCl, and 0.3 M NaOH were designated FA3, FA4, and FA5, respectively. For subsequent experiments, these fractions were extensively dialyzed against distilled water with the use of 10 kDa cutoff dialysis tubes (**Figure 1**).

Spectroscopic Analysis. UV−vis spectra of liquid mumie fractions (50 $\mu g \cdot mL^{-1}$) were recorded on an Ultrospec 4000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) in a 1 cm quartz cuvette by scanning from 200 to 800 nm. Dry samples were mixed with 800 mg of dry potassium bromide (KBr) and pressed into disks. The infrared spectra (650−4000 cm^{-1}) were recorded with the use of the Specord 71 IR spectrophotometer (Carl Zeiss).

Fluorescence measurements were performed using an F-2000 spectrofluorometer (Hitachi, Tokyo, Japan). Samples of crude mumie, FA fractions (FA_{total} and FA1−5), and LMW fractions (LMW1 and LMW2) were dissolved in distilled water, and the pH was adjusted to 7.0 by the addition of HCl or NaOH solution prior to analysis. HymA and HA were dissolved in 0.1 M NaOH. All samples were evaluated at a concentration of 70 $\mu g \cdot mL^{-1}$. For emission fluorescence analysis, the samples were excited at a wavelength of 254 nm, and the fluorescence intensity was measured from 300 to 500 nm (*25*). Slit width for emission and excitation wavelengths was 10 nm. Synchronous spectra were recorded with the excitation monochromator by scanning from 250 to 600 nm with $\Delta\lambda = 20$ nm (*13*).

Fluorescence spectroscopy can be used to determine the extent of humification by quantifying the shifts in the emission spectrum toward longer wavelengths (*26*−*28*). Previous studies have shown that correction for both primary and secondary fluorescence inner-filtering effects is essential for the accurate representation of fluorescence data and calculation of a humification index (HIX) (*26*). For determination of an HIX value, we used the formula

$$\mathrm{HIX} = \int_{435}^{480} I(\lambda)\, d\lambda \Big/ \int_{300}^{345} I(\lambda)\, d\lambda$$

where $I(\lambda)$ represents the fluorescence intensity spectral curve with excitation at $\lambda_{ex} = 254$ nm. For calculation of HIX values corrected for inner-filtering effects, we performed linear extrapolation on plots of HIX versus transmittance at 254 nm for different concentrations of each fraction. The corrected HIX value corresponds to infinite dilution, that is, to 100% transmittance (*26*). Experimental determination of HIX was performed at fraction concentrations from 20 to 70 $\mu g \cdot mL^{-1}$.

Total Carbohydrate Content. Analysis of total carbohydrate content in crude mumie and mumie fractions was conducted using the phenol reaction method (*29*). Briefly, 0.5 mL of a 50 g/L phenol solution was added to a 0.5 mL sample consisting of 4 mg of the mumie fraction dissolved in 1 mL of distilled water. The sample was mixed, 2.5 mL of concentrated sulfuric acid was added, and the sample was again mixed rapidly. The resulting solution was incubated for 20 min at 27 °C, after which absorbance was measured at 488 nm and compared with that of a glucose standard curve. Each analysis was repeated three

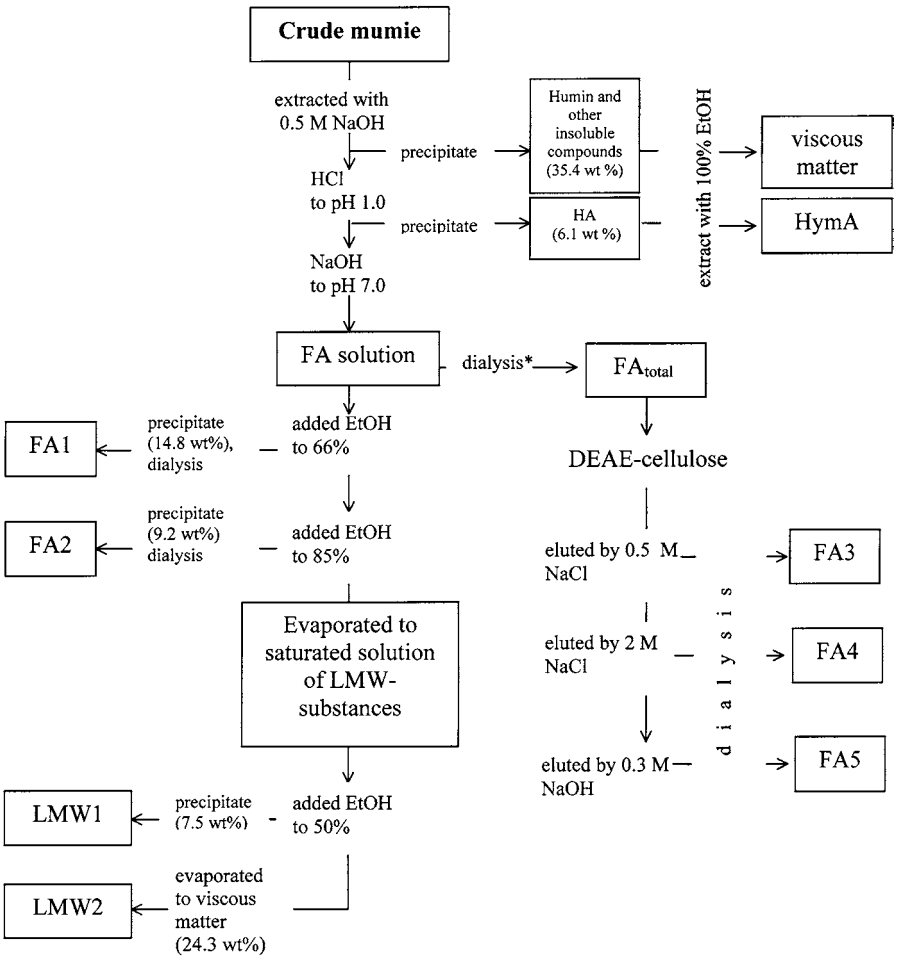

Figure 1. Scheme of mumie fractionation. * Dialysis of sample of FA solution separate from that used in ethanolic precipitation of FA1 and FA2 fractions.

times. The results were calculated as milligrams of carbohydrate per gram (dry weight) of the fraction.

Isolation of Peritoneal Macrophages. Peritoneal macrophages were isolated from 8-week-old male BALB/c mice 4 days after intraperitoneal injection of 1 mL of saline containing 100 μg of concanavalin A (*30*). The peritoneal exudate cells were obtained by lavage with 10 mL of cold RPMI 1640 medium supplemented with 10 units of heparin/mL. The cells were mixed with RPMI 1640 containing 10% fetal calf serum with antibiotics (penicillin and streptomycin) and plated onto 24-well culture plates (10^6 cells/well). After 2 h, nonadherent cells were washed away with prewarmed RPMI medium (without phenol red), and the remaining peritoneal macrophages were incubated with different concentrations of the various mumie fractions.

Assay of ROS Formation. ROS formation was determined by fluorescence using 2′,7′-dichlorofluorescin diacetate (DCFH-DA); this compound, when taken up by cells, is de-esterified to the ionized free acid (DCFH), which then reacts with ROS to form fluorescent 2′,7′-dichlorofluorescin (DCF) (*31*). Briefly, mumie fractions were added to preparations of peritoneal macrophages. The cells were incubated for 90 min or 48 h at 37 °C in 5% CO_2 and then activated with phorbol myristate acetate (PMA) (1 or 5 μM) in the presence of DCFH-DA (3 μM). ROS production was recorded as an increase in well fluorescence (λ_{ex} = 485 nm, λ_{em} = 535 nm) (*32*). Fluorescence was measured at 3−4 min intervals for up to 90 min in a 1420 Wallac Victor 2 multilabel plate reader (Perkin-Elmer, Beaconsfield, U.K.) with temperature controlled at 37 °C.

Determination of Nitric Oxide Production. Peritoneal macrophages were isolated as described above, then challenged with one of the mumie fractions or lipopolysaccharide (LPS, 0.5 μg·mL^{-1}), and incubated at 37 °C in 5% CO_2 for 48 h. At the end of the 48 h culture period, supernatants were removed and assayed for nitric oxide. Nitrite ion (NO_2^-) concentration was used as an indication of nitric oxide production. The amount of NO_2^- in the culture medium was determined according to the colorimetric method using $NaNO_2$ as a standard (*33*). In brief, 100 μL of cell culture supernatant was mixed with an equal volume of Griess reagent [0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid], which was used within 1 h of being mixed. The samples were kept at room temperature for 20 min, after which the absorbance at 540 nm was measured using an Ultrospec 4000 spectrophotometer (Pharmacia Biotech).

Enzymatic System Glucose—Glucose Oxidase and DCFH Generation. To generate DCFH, a 5 mM stock DCFH-DA solution was deacetylated by incubation with 2.5 mM NaOH for 1 h in the dark at room temperature (*34*). Hydrogen peroxide (H_2O_2) was produced enzymatically by glucose oxidase-catalyzed conversion of glucose in the presence of oxygen in aqueous solution. β-D-Glucose was oxidized by glucose oxidase, yielding D-gluconic acid and H_2O_2 using oxygen as the electron acceptor.

Assays were performed in 24-well plates. The reaction was started with the addition of 20 μL of glucose oxidase stock. The final solution contained 5.0 mM glucose, 0.2 unit·mL^{-1} glucose oxidase, 0.015 unit·mL^{-1} horseradish peroxidase, and 30 μM DCFH in 10 mM sodium phosphate buffer (pH 6.5). DCFH oxidation was measured fluorometrically in terms of H_2O_2-mediated oxidation of DCFH to DCF (λ_{ex} = 485 nm, λ_{em} = 535 nm) using the 1420 Wallac Victor 2 multilabel plate reader (Perkin-Elmer) with temperature controlled at 30 °C.

Splenocyte Proliferation. BALB/c splenocytes were isolated and resuspended at a concentration of 5×10^6 cells in 1 mL of RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics, and 0.05 mM β-mercaptoethanol. Samples of mumie fractions dissolved in phosphate-buffered saline with 0.15 mM NaCl, pH 7.2, were added to 96-well plates in triplicate to yield final fraction concentrations of 2, 20, and 100 μg·mL^{-1}; then 0.1 mL of the cell suspension (5×10^5

cells) and 0.1 mL of medium were added. Negative controls consisted of wells without mumie fractions (concentration = 0). Samples containing 5 $\mu g \cdot mL^{-1}$ concanavalin A without mumie fractions served as positive controls. Plates were incubated at 37 °C with 5% CO_2 in a water-jacketed incubator (100% humidity) for 72 h. [^{3}H]Thymidine (1 μCi) was added, and the mixtures were incubated for an additional 18 h. After incubation, the cells were harvested onto a glass fiber mat, washed to remove unincorporated thymidine, and counted by liquid scintillation spectroscopy.

Statistical Analysis. Data from one of three representative experiments are reported. Results are expressed as means ± standard deviations. Statistical comparisons were made using Student's *t* test for paired values.

RESULTS AND DISCUSSION

Mumie Fractionation. In the first step, crude mumie was divided into three main fractions: FA, HA, and humin combined with other components that were not extractable by alkaline solution. Although a milder method of isolation of humic substances from humus materials based on the use of sodium pyrophosphate ($Na_4P_2O_7$) is normally used (*35*), the alkaline extraction provides significantly higher yields of humic substances and is widely used for fractionation of natural organic matter (*13*). Yields of the three main fractions (weight percent) are shown in **Figure 1**.

Ethanol precipitation followed by dialysis was used to divide the FA fraction into two subfractions (FA1 and FA2). On the basis of their solubility in the water/ethanol mixture, these subfractions presumably differed in average molecular weight and in solvation energy. After additional ethanol precipitation with preliminary evaporation, the substances remaining in the 85% ethanol were fractionated into LMW fractions LMW1 and LMW2 as described under Materials and Methods (**Figure 1**).

Ion-exchange chromatography was used to divide the FA_{total} fraction into three subfractions (FA3, FA4, and FA5) that differed in retention time and, thus, in their adsorption, molecular weight, and charge properties. We used DEAE-cellulose as an adsorbent because humic substances are better adsorbed onto this substrate than, for example, onto Amberlite XAD-2 (*24*). The five FA fractions (FA1−5) were powders ranging in color from pale brown to dark brown.

UV—Vis Spectroscopy of Mumie Fractions. The UV−vis spectra of FA_{total}, HA, and LMW2 showed a smooth decline in absorbance with increasing wavelength (**Figure 2A**). Fractions FA1−5 had the same spectral characteristics as FA_{total} (data not shown), which are also common to humic substances from other natural sources (*13*). The similarity is probably the result of the loss of spectral structure as the individual classes of biochemicals present in the plant residue become incorporated into the complex, random structure of mumie humus matter (*26*).

The UV−vis spectrum of the ethanolic precipitate from crude mumie was similar to the previously described spectra of humus samples containing geoporphyrins and chlorins (*36*): the highest extinction was at 402 nm for porphyrin-like pigments and at 665 nm for chlorin-rich sediments (**Figure 2B**). Sedimentary chlorins and porphyrins are diagenetic products of chlorophyll molecules and are considered to be chemical fossils or biomarkers of humus materials. Chlorins are the immediate diagenetic products of chlorophyll, whereas porphyrins result from long-term chlorophyll diagenesis (*36*). On the basis of the assumption that chlorin-like pigments have an absorption maximum near 665 nm and porphyrin-like pigments are characterized by a 410 nm peak, chlorin-rich sediments have been shown to have an E_{410}/E_{665} (porphyrin/chlorin) ratio between 1 and 5, whereas porphyrin-rich sediments have a higher value, between 5 and 10 (*36*). We determined the E_{410}/E_{665} ratio for the ethanolic extract from crude mumie to be ~3.4, enabling us to classify this substance as chlorin-enriched humus material.

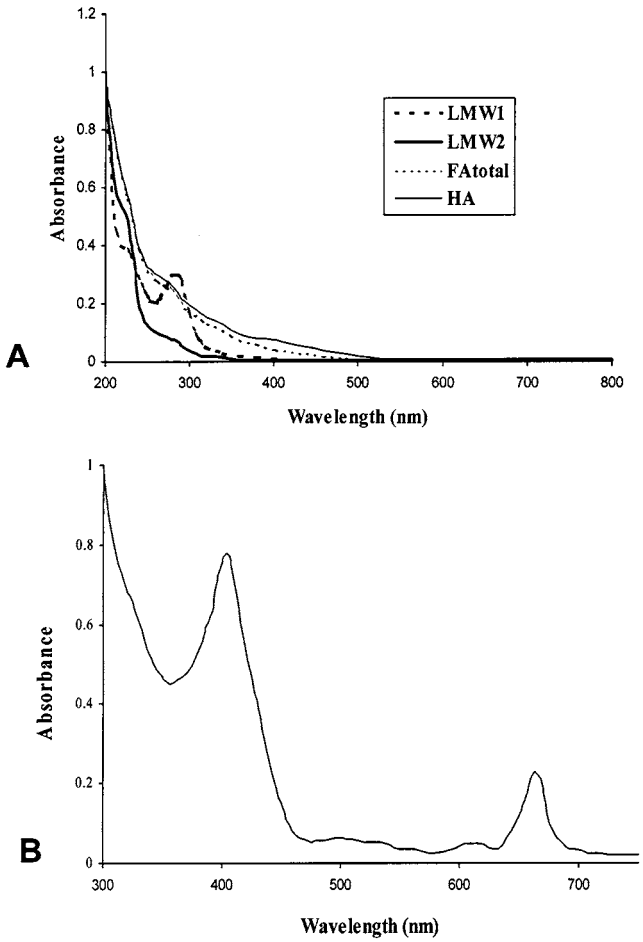

Figure 2. Normalized UV–vis absorption spectra of (A) mumie fractions in water (pH ≈7.0) and (B) ethanolic precipitates from crude mumie in absolute ethanol.

IR Spectroscopy of Mumie Fractions. Unlike pure compounds, which typically have sharp absorption peaks, humic substances from mumie have relatively few, broad IR bands (**Figure 3**), which may result from the overlap of the absorptions of many similar functional groups (*37*). We observed the characteristic absorption bands typical for humic substances in the IR spectra of the FA_{total} and HA fractions. The strong absorption band at 3400 cm^{-1} confirmed the presence of abundant OH groups, as well as amino groups, in these mumie fractions. Absorption maxima present near 2950 cm^{-1} (aliphatic C−H stretching), 1720 cm^{-1} (C═O stretching of COOH and ketonic C═O), 1600−1660 cm^{-1} (aromatic C═C and H bonded C═O), 1420 cm^{-1} (C−H bending of CH_2 or CH_3 groups), and 1240 cm^{-1} (C−O stretching and deformation of COOH) have been reported by others (*5*). In all of our spectra, a broad band was observed in the region of 3300−3350 cm^{-1}, which is related to H-bound OH groups. The presence of a carboxylic functionality was shown by strong absorbance at 1650−1720 cm^{-1}, caused by COOH group vibrations. It should be noted that these bands were located at shorter wavelengths (1720 cm^{-1}) in the samples obtained by extraction with absolute ethanol, corresponding to un-ionized COOH groups.

In the spectra of ethanol precipitates from humin (HymA) and crude mumie, additional bands were observed at 730 and

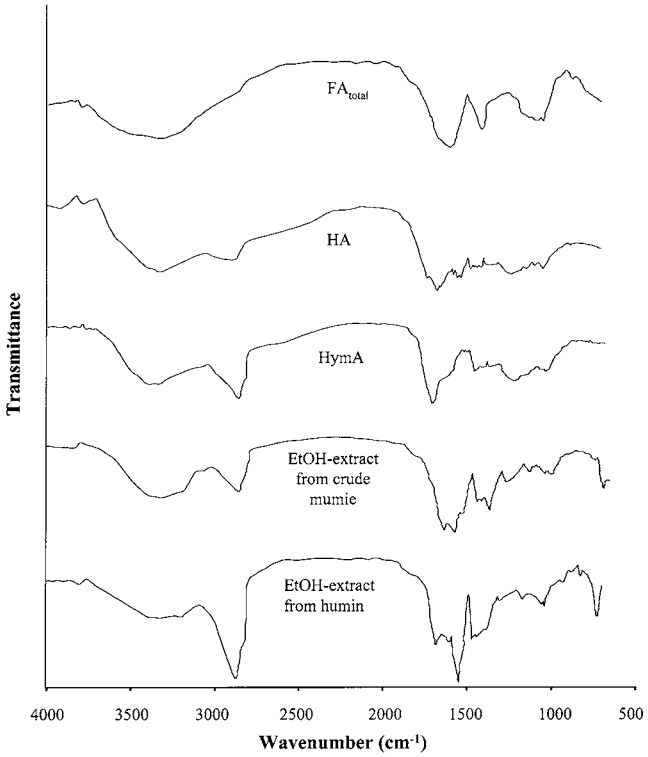

Figure 3. IR spectra of mumie and mumie fractions.

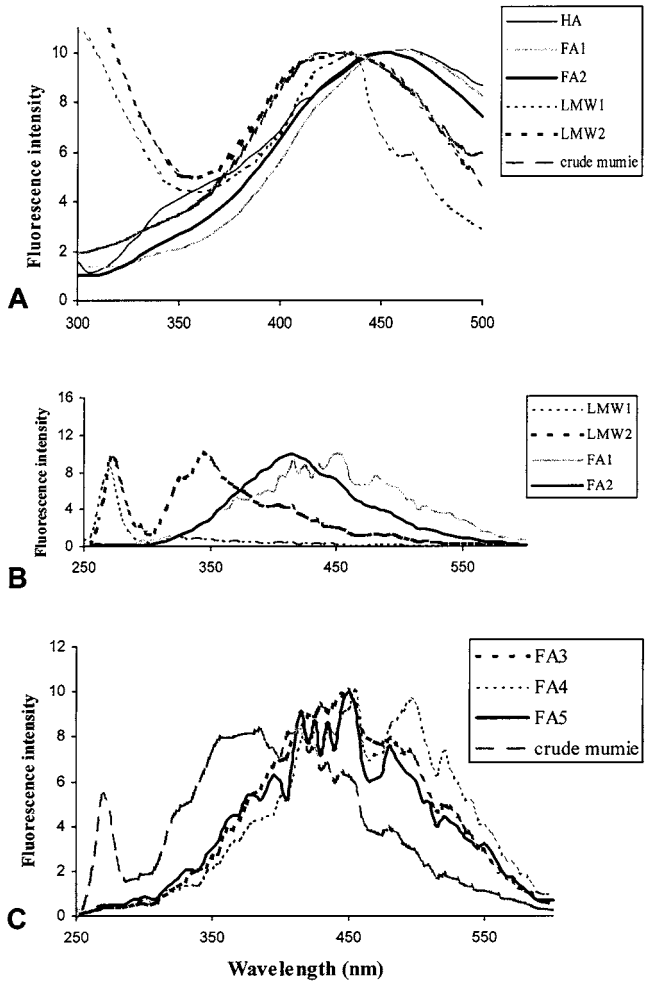

Figure 4. (A) Fluorescence emission spectra of crude mumie and mumie fractions at the excitation wavelength $\lambda_{ex} = 254$ nm; (B, C) synchronous fluorescence spectra of crude mumie, FA fractions, and LMW fractions (LMW1 and LMW2) recorded at $\Delta\lambda = 20$ nm with the excitation monochromator scanning from 250 to 600 nm.

~1570 cm^{-1}, most likely the result of the vibrations of polymethylene chains and/or N−H bonds, respectively. For the spectra of ethanolic extracts of HA, the absorption band in the region of 2950 cm^{-1} was also characteristic, corresponding to the stretching vibrations of C−H bonds in hydrocarbons. The relative intensity of this absorption increased in the order HA < HymA < ethanolic precipitates from crude mumie < ethanolic extract from humin. In the spectrum of FA$_{total}$, increased intensity of the band at 1090 cm^{-1} (corresponding to C−O stretching of polysaccharides) was noted. Also, a relative decrease in peak intensity in the aliphatic region at 2930 cm^{-1} was evident in the IR spectrum of the FA$_{total}$ fraction.

Thus, the ethanolic precipitates from crude mumie and its humin and HA fractions contained molecules with long fragments of hydrocarbon chains. Compounds of relatively low molecular weight identified earlier (*5*, *38*) in mumie may be present in the extracts. According to the literature (*38*), these compounds include long-chain aliphatic hydrocarbons and their derivatives at different levels of oxidation, naphthenes and hydrocymenes, aromatics, phenolics, carboxylic acids and their amino and amino acid conjugates, and N- and S-heterocycles. Other studies have described humic substances as consisting of a skeleton of alkyl/aromatic units cross-linked mainly by oxygen and nitrogen groups, with the major functional groups being carboxylic acid, phenolic and alcoholic hydroxyls, ketone, and quinone groups (*14*−*16*). According to our data, the peaks characteristic of molecules with long hydrocarbon fragments are absent in the FA$_{total}$ spectrum (**Figure 3**).

In the IR spectrum of the ethanolic extract from humin, the absorption band in the region of 2950 cm^{-1} was much more intense than the band of stretching OH vibrations near 3400 cm^{-1}. This indicates fewer polar oxygen-containing groups (OH, COOH) in this sample and is, apparently, also related to a lower solubility in water at pH ≈7 and in an alkaline medium. The IR spectra of the HymA samples also demonstrated a low content of hydroxyl and carboxyl groups and a high content of hydrocarbon fragments. In agreement with this observation, HymA was relatively insoluble in water but was soluble in organic solvents such as ethanol. The peak of low intensity at 2950 cm^{-1} was observed also in the IR spectrum of HA, indicating the presence of hydrocarbon chains; this was not the case for the FA$_{total}$ fraction. Quantitative analysis of oxygen-containing groups in HA and FA$_{total}$ has shown that FA$_{total}$ is enriched with OH, COOH, C═O, and methoxy groups, as compared to HA (*16*).

In general, the relative content of polar groups and nonpolar hydrocarbon fragments in the mumie fractions, determined by IR spectroscopy, strongly correlated with the solubility of these fractions in an aqueous medium. A reduced intensity of the band near 2950 cm^{-1} attributed to C−H bond stretching vibrations, in the order humin > HymA > HA > FA, was accompanied by an increase in solubility in an aqueous medium in the same order; this characteristic served as the basis for mumie fractionation.

Fluorescence Spectroscopy of Mumie Fractions and HIX. All emission fluorescence spectra of HMW fractions (FA fractions, HA) from mumie had the same general shape (**Figure 4A**) and were similar to the spectra of humic substances from other natural sources for which the maximum intensity was observed between 420 and 460 nm (*25*, *39*). The emission spectra for crude mumie and the LMW1 and LMW2 fractions

showed a broad band with the maximum centered at 430 nm; this was a shorter wavelength than was obtained for the HA and FA fractions (FA_{total}, FA1−5; 460 nm). The maximum at $\lambda_{em} \approx 430$ nm for crude mumie can be explained by the high content of LMW fractions (LMW1 and LMW2) in the sample (**Figure 1**).

To improve peak resolution compared with the conventional emission fluorescence technique, we used synchronous fluorescence (*13*, *40*). As shown in **Figure 4B**,**C**, the synchronous spectra of the FA1−5 fractions exhibited spectral line shapes that were somewhat distinct from one another. Significant differences were observed between the spectrum of FA2 and the spectra of FA1 and FA3−5. The FA2 fraction showed a spectrum with a single peak at 415 nm. The FA1 and FA3−5 fractions, however, exhibited spectra with major peaks at 450 and 495 nm, with a number of less-well-defined minor peaks at 370 nm (FA1), 380 nm (FA5), 395 nm (FA1, FA3, and FA5), 415, 425, and 435 nm (FA1, FA4, and FA5), 480 nm (FA1 and FA3−5), and 520 nm (FA1 and FA3−5). In general, the spectrum of FA2 had a shorter wavelength maximum than the FA1 and FA3−5 spectra, suggesting that the FA1 and FA3−5 fractions contained more conjugated aromatic groups (*13*). The synchronous fluorescence spectrum of the LMW2 fraction yielded two main peaks, at 275 and 345 nm, whereas the LMW1 fraction had only one major peak, at 270 nm.

For crude mumie, three maxima were observed: a sharp maximum at 270 nm and two broad maxima around 355−385 and 405−415 nm. The minor peaks between 425 and 520 nm were located at the same wavelengths as the maxima in the spectra of the FA fractions.

The nature of the fluorophores responsible for the fluorescence of HMW humic substances is unknown, although products of oxidative cross-linking of polyunsaturated fatty acids, imine-enamines, and aromatic nuclei associated with lignin-derived structures have been proposed (*40*). A possible reason for the red shift in the emission maxima of FA1 and FA3−5 is a high degree of polymerization of multiple fluorophores that enhances the probability of bimolecular processes such as radiative energy transfer and excimer/exciplex formation (*41*). From the literature it is known that the position of the fluorescence emission maximum depends strongly on the origin of the humic substances and the excitation wavelength; this illustrates the complexity of the fluorophoric structure of the material relative to the level of humification (*40*). The low fluorescence intensities and long wavelengths measured for the major fluorescence peaks of FA and HA fractions from mumie may be ascribed to a high degree of conjugation and the presence of an extended, condensed aromatic π-electron system and other electron-withdrawing functional groups, such as carbonyl and carboxyl groups. The high overall fluorescence and the short wavelengths measured for the main fluorescence peaks of crude mumie and the LMW1 and LMW2 fractions are in agreement with the presence of simple, LMW structural components having considerable molecular heterogeneity and containing electron-donating groups such as hydroxyl and methoxyl (*27*, *40*).

The HIX values for fractions dissolved in water (pH ≈7.0) were ordered as follows: FA3 (11.10) > FA4 (4.26) > FA2 (3.33) > crude mumie (3.13) > FA5 (3.10) > FA1 (2.23) > LMW2 (0.95) > LMW1 (−0.08). The negative value for LMW1 was the result of extrapolation error, which varied between 0.13 and 0.15 ($p < 0.05$) for the fractions investigated. The limits of the variation of HIX values have been shown to exceed somewhat the variation in these values (from 1.23 to 7.65) for samples from soils with different degrees of humification (*13*). The minimum HIX value for the LMW fractions (LMW1 and LMW2) corresponds to an earlier stage of the humification process.

Carbohydrate Content in Mumie Fractions. The highest levels of carbohydrates were observed in the FA1 and FA2 fractions (2.3 and 1.8 mg/g of dry wt, respectively). Carbohydrate content was considerably lower in the FA3, FA4, and FA5 fractions isolated by ion-exchange chromatography (0.8, 0.6, and 0.5 mg/g of dry wt, respectively). It is likely that a large portion of the carbohydrates from FA_{total} was not adsorbed on DEAE-cellulose or eluted subsequently from the column. A low content of carbohydrates was observed in the LMW2 fraction (0.29 mg/g of dry wt), and no carbohydrates were found in the LMW1 fraction. The level of carbohydrates in crude mumie was low (0.4 mg/g of dry wt) because of the high content of low-carbohydrate LMW1 and LMW2 fractions.

Effect of FA Fractions on ROS Production. Our preliminary studies showed that the respiratory burst effect of FA on peritoneal macrophages was observed during long-term (24−48 h) preincubation of cells with FA_{total}. In contrast, short-term (1−2 h) preincubation produced only a priming effect in response to PMA. ROS production by macrophages in response to PMA increased with increased exposure time (0−5 h) (data not shown). A 90 min preincubation followed by removal of FA_{total} from the medium had no significant effect on either spontaneous or PMA-stimulated ROS production by macrophages ($p > 0.05$). The maximum rate of DCFH oxidation occurred in response to PMA in the presence of FA_{total} (**Figure 5A**). The results provide evidence that the presence of FA in the cell medium enhances PMA-induced ROS generation.

To assay the influence of the molecular weight and charge properties of FA on ROS production, cells were pretreated with FA1−5 fractions for 90 min and PMA-stimulated ROS production was recorded during the following 90 min. The FA1 and FA2 fractions produced significantly elevated levels of ROS in macrophages ($p < 0.05$) (**Figure 5B**). Among the FA3−5 fractions, only FA3 enhanced biological activity in the macrophages. The FA4 fraction, characterized by greater charge and adsorption on DEAE-cellulose, showed an inhibitory effect at higher concentrations (70 $\mu g \cdot mL^{-1}$).

Effect of Long-Term Incubation of Macrophages with Mumie Fractions on ROS Production. To investigate the effect of mumie fractions on spontaneous ROS production by macrophages, the cells were incubated for 48 h with different concentrations of the FA_{total}, LMW1, and LMW2 fractions, after which the cell culture medium was replaced with medium containing DCFH-DA and the intensity of fluorescence was recorded for 90 min (**Figure 6**). Measurable activation of ROS production was observed only in cells incubated with FA_{total} at concentrations from 20 to 200 $\mu g \cdot mL^{-1}$ ($p < 0.05$). Cells incubated with the LMW1 fraction at any concentration (2−200 $\mu g \cdot mL^{-1}$) showed no change in ROS production compared with control. Incubation with the LMW2 fraction led to a marked reduction in ROS production, an inhibitory effect that is likely related to the cytotoxic activity of LMW substances in this fraction.

Effect of Mumie Fractions on Nitric Oxide Production. FA1 and FA2 increased nitric oxide synthesis by peritoneal macrophages. The effect was significant at doses >2 $\mu g \cdot mL^{-1}$ for FA1 and at doses >20 $\mu g \cdot mL^{-1}$ for FA2; at a concentration of 200 $\mu g \cdot mL^{-1}$, the activating effect was similar to that of LPS (0.5 $\mu g \cdot mL^{-1}$) for both fractions. The LMW1 and LMW2 fractions did not activate nitric oxide production (**Figure 7**).

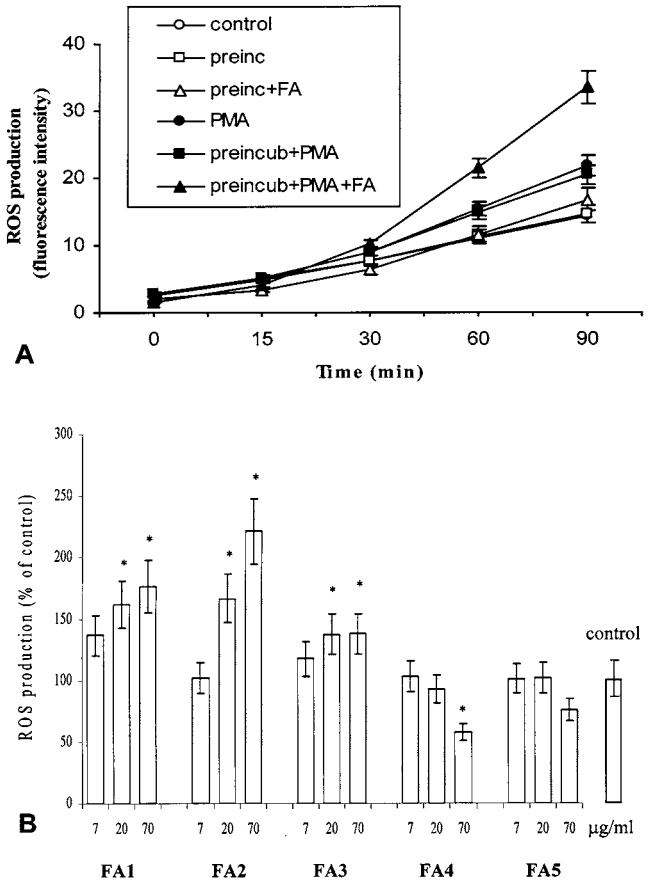

Figure 5. Effect of FA fractions on ROS production by peritoneal macrophages. (A) Macrophages were pretreated or preincubated with FA_{total} (200 $\mu g \cdot mL^{-1}$) for 90 min at 37 °C. The cells were washed and incubated with DCFH (3 mM) for another 90 min with or without FA_{total} (100 $\mu g \cdot mL^{-1}$) with or without PMA (1 μM). Controls consisted of cells without PMA, preincubation, or addition of FA_{total}. (B) Macrophages were preincubated for 90 min at 37 °C with indicated concentrations of FA1–5 fractions. Cells were activated with PMA (5 μM), and production of ROS was measured in the presence of DCFH (3 μM) for 90 min. Controls (100%) consisted of cells not preincubated with FA fractions. * Significant difference from control ($p < 0.05$).

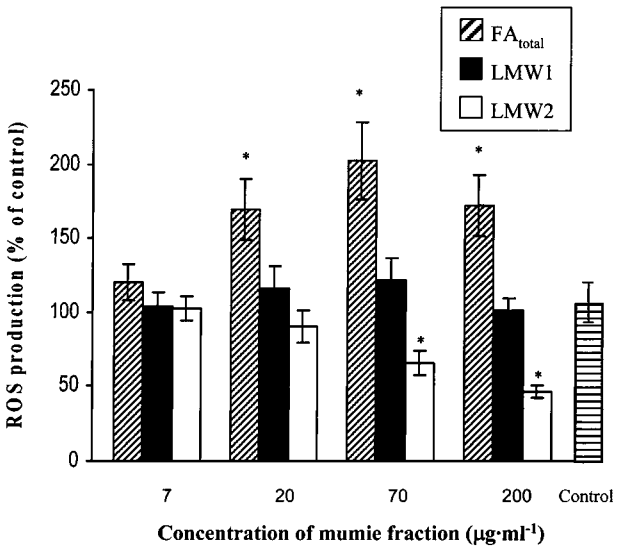

Figure 6. Effect of mumie fractions on ROS production by peritoneal macrophages. Macrophages were incubated with indicated concentrations of mumie fractions for 48 h at 37 °C, and production of ROS was measured in the presence of DCFH for 90 min as described under Materials and Methods. Controls (100%) consisted of cells incubated without mumie fractions. * Significant difference from control ($p < 0.05$).

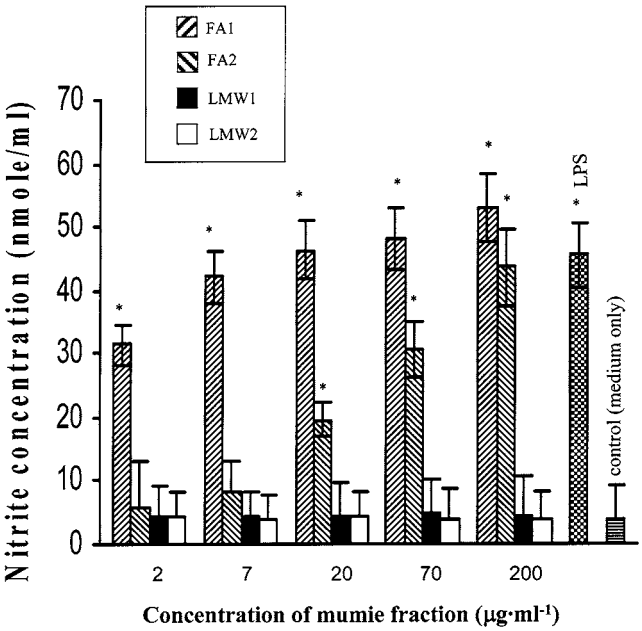

Figure 7. Levels of nitrite synthesis by peritoneal macrophages after 48 h of incubation with mumie fractions. The amount of nitrite in the culture medium was determined with Griess reagent as described under Materials and Methods. Controls consisted of cells incubated without mumie fractions. * Significant difference from control ($p < 0.05$).

Effect of Mumie Fractions on Splenocyte Proliferation. The incorporation of [^{3}H]thymidine in the DNA of lymphocytes exposed to mumie fractions was measured. We found a dose-dependent increase in [^{3}H]thymidine uptake in cells treated with FA1 at doses >20 $\mu g \cdot mL^{-1}$, compared with control ($p < 0.05$; **Figure 8**). The level of [^{3}H]thymidine incorporation produced with FA1 at a concentration of 500 $\mu g \cdot mL^{-1}$ was ~40% of that produced by stimulation with concanavalin A (data not shown). The FA2, LMW1, and LMW2 fractions had no effect on splenocyte proliferation.

Postulated Mechanisms for the Biological Effects of FA and LMW Fractions from Mumie. The mechanism of action of fractions FA1−3 on ROS production in macrophages may be related to their interactions with cellular receptors, ionic channels, and intracellular messengers and the participation of FA in the oxidation−reduction reactions as electron carriers. The role of electron carriers can be conditioned by quinone groups present in FA (*42*, *43*).

To investigate the effect of FA on DCFH oxidation, we used an enzyme system for H_2O_2 generation. The addition of FA_{total} (from 20 to 200 $\mu g \cdot mL^{-1}$) to the H_2O_2-generating enzyme system glucose/glucose oxidase resulted in significantly increased H_2O_2 generation and acceleration of DCFH oxidation after a 15 min incubation ($p < 0.05$) (data not shown). The peroxidases can attack electron-acceptor groups on both FA (*44*) and DCFH (*45*). It is likely that DCFH and the quinone groups in FA (FA-Q) can be electron acceptors for intracellular macrophage peroxidases:

$$\text{DCFH} \xrightarrow{\text{peroxidase}} \text{DCF}^{-\bullet} \quad \text{(a)}$$

$$\text{FA-Q} \xrightarrow{\text{peroxidase}} \text{FA-Q}^{-\bullet} \quad \text{(b)}$$

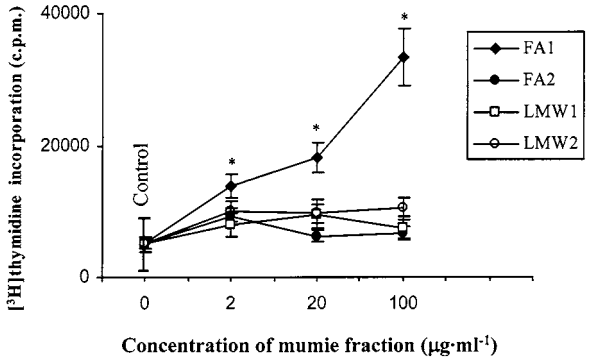

Figure 8. Effect of mumie fractions on [^{3}H]thymidine incorporation in mouse splenocytes. [^{3}H]Thymidine incorporation was determined as described under Materials and Methods. Controls consisted of cells incubated without mumie fractions (concentration = 0). * Significant difference from control ($p < 0.05$).

$DCF^{-\bullet}$ and $FA\text{-}Q^{-\bullet}$ produced in reactions a and b are subsequently air-oxidized with generation of $O_2^{-\bullet}$:

$$DCF^{-\bullet} + O_2 \rightarrow DCFH + O_2^{-\bullet} \quad \text{(c)}$$

$$FA\text{-}Q^{-\bullet} + O_2 \rightarrow FA\text{-}Q + O_2^{-\bullet} \quad \text{(d)}$$

Thus, the mechanism of enhancement of the formation of the fluorescent dye DCF in the presence of FA can be explained by an increase in the rate of ROS generation in concert with spontaneous and enzymatic dismutation of $O_2^{-\bullet}$ formed in reactions c and d. Because FA is a polydisperse mixture of heterogeneous weak polyelectrolytes with strong adsorptive properties (*46*), a significant portion of its molecules can be adsorbed on cell plasma membranes, so ROS induction and formation are likely to be cell surface processes.

FA fractions may stimulate macrophages to produce ROS and nitric oxide by the direct stimulating action of polysaccharides on the cellular receptors. Polysaccharides in mumie are plant-derived products or can be the result of degradation of the mumie humus by microorganisms during the humification process. On the basis of the total carbohydrate content data, it is estimated that 200 $\mu g \cdot mL^{-1}$ samples of FA1 and FA2 contain polysaccharide carbohydrates concentrations of 0.46 and 0.36 $\mu g \cdot mL^{-1}$, respectively.

The stimulatory effect of FA on cells can also be caused by nonspecific binding with cellular membranes. It has been shown that humic substances enhance the permeability of cell membranes to extracellular Ca^{2+} ions, leading to a sustained elevation in cytosolic Ca^{2+} (*47*). It is possible that the molecular mechanism of action of the FA fractions on nitric oxide production by macrophages is related to previously reported data concerning Ca^{2+}-dependent activation of endothelial nitric oxide-synthase by synthetic HA (*48*). The activation of protein kinase C and the increase in intracellular concentrations of Ca^{2+} could mediate the synergistic action of FA and PMA on macrophages (**Figure 5A**). Furthermore, long-term (>24 h) incubation in the presence of enzymes (peroxidases) and H_2O_2 secreted by cells can lead to depolymerization of HMW humic substances to form LMW substances (*44*); these LMW substances are capable of easily penetrating the plasma membranes and may interact with intracellular messengers and/or receptors. For example, at present it is postulated that humic substances can induce PPARγ (peroxisome proliferator-activated receptor γ) and activate the PPRE (peroxisome proliferator response element) reporter gene (*49*).

The inhibitory action of the FA4 fraction on ROS production by peritoneal macrophages may be the result of the charge on this substance and its chelating properties, which perturb the functional activity of the cells. The activation of apoptotic processes by humic substances has been reported (*48*). Furthermore, the carbohydrate level in the FA4 fraction was considerably lower than that of the FA1 and FA2 fractions.

In contrast to the FA fractions, the LMW fractions did not stimulate ROS and nitric oxide production, nor did they enhance [^{3}H]thymidine incorporation into the DNA of lymphocytes. Only the LMW2 fraction has an odor. The smell is caused by the presence of compounds with relatively low molecular weights, such as long-chain aliphatic hydrocarbons and their derivatives at different levels of oxidation, naphthenes and hydrocymenes, aromatics, phenolics, carboxylic acids and their amino and amino acid conjugates, and N- and S-containing heterocyclic compounds (*38*). Most of these substances produce adverse and/or toxic effects (*50*) that could mediate suppression of ROS production by macrophages.

We did not observe specific relationships between the stimulation of macrophages by the FA fractions and the HIX value. It is probable that the biological effect of FA depends on factors that are not clearly reflected by this complex index. In particular, the biological effect could depend on the presence of specific polysaccharide residues in the composition of these HMW organic acids. On the other hand, the effect could be determined by the presence of quinone-hydroquinone moieties that constitute a very small fraction of the functional groups of FA and, apparently, are incapable of measurably affecting the value of such an integral index as HIX.

The results of our studies suggest that the activating effect of mumie on the immune system is caused in part by FA stimulation of macrophages and lymphocytes. Further experiments are necessary to investigate the possibility of FA binding with cellular receptors and molecular mechanisms of sequential intracellular processes of cell activation.

ABBREVIATIONS USED

DEAE, diethylaminoethyl; DCF, 2′,7′-dichlorofluorescin; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; EtOH, ethanol; FA, fulvic acid; FA_{total}, total fulvic acids from mumie after 10 kDa cutoff dialysis; IR, infrared; HA, humic acid; HMW, high molecular weight; HymA, hymatomelanic acid; LMW, low molecular weight; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; Q-FA, quinone functional group of fulvic acid; ROS, reactive oxygen species.

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