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ORIGINAL PAPER

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Immunostimulatory effect of natural clinoptilolite as a possible mechanism of its antimetastatic ability

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Abstract *Purpose*: Many biochemical processes are closely related to ion exchange, adsorption, and catalysis. Zeolites reversibly bind small molecules such as oxygen or nitric oxide; they possess size and shape selectivity, the possibility of metalloenzyme mimicry, and immunomodulatory activity. These properties make them interesting for pharmaceutical industry and medicine. *Methods*: The experiments were performed on mice. Different biochemical and molecular methods were used. *Results*: Micronized zeolite (MZ) administered by gastric intubation to mice injected with melanoma cells significantly reduced the number of melanoma metastases. In mice fed MZ for 28 days, concentration of lipid-bound sialic acid (LSA) in serum increased, but lipid peroxidation in liver decreased. The lymphocytes from lymph nodes of these mice provoked a significantly higher alogeneic graft-versus-host (GVH) reaction than cells of control mice. After i.p. application of MZ, the number of peritoneal macrophages, as well as their production of superoxide anion, increased. However, NO generation was totally abolished. At the same time, translocation of p65 (NFκB subunit) to the nucleus of splenic cells was observed. *Conclusion*: Here we report antimetastatic and immunostimulatory effect of MZ and we propose a possible mechanism of its action.

Keywords Micronized zeolite · Clinoptilolite · Oxidative stress · Immunostimulation · T-lymphocyte NFκB

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Introduction

Zeolites are hydrated natural and synthetic microporous crystals with well-defined structures containing AlO_4 and SiO_4 tetrahedra linked through the common oxygen atoms (Breck 1964). Zeolites have properties to act as catalysts, ion-exchangers, adsorbents, and detergent builders (Colella 1999; Garces 1999; Flanigen 1980; Naber et al. 1994; Sersale 1985). Apart from being extensively used in different industrial applications, it is known that silicates and aluminosilicates also possess either positive or negative biological activity. Well-defined structures and catalytic activity make aluminosilicates an attractive model system for protein and enzyme mimetics (Bedioui 1995). Recent results have demonstrated that zeolite was very effective as a glucose adsorbent (Concepcion-Rosabal et al. 1997) as well as a potential adjuvant in anticancer therapy (Pavelic et al. 2001). Zeolites reversibly bind small molecules such as oxygen or nitric oxide, they possess size and shape selectivity, the possibility of metalloenzyme mimicry, and immunomodulatory activity (Ozesmi et al. 1986).

Accumulating evidence has indicated that zeolites play an important role in regulation of the immune system. It was reported that silica, silicates, and aluminosilicates act as non-specific immunostimulators similarly to superantigens (Ueki et al. 1994). Superantigens (SAG) are a class of immunostimulatory and disease-causing proteins of bacterial and viral origin with the ability to activate a relatively large fraction (5–20%) of the T cell population. Activation requires simultaneous interaction of the SAG with the Vβ domain of T cell complex (MHC) class II molecules on the surface of antigen-presenting cells (Ueki et al. 1994). Pro-inflammatory macrophages, that belong to class II MHC antigen-presenting cells, are activated by fibrinogen silicate particulate (Allison et al. 1996; Drumm et al. 1998).

It was shown that exposure of alveolar macrophages to silicate particles leads to activation of mitogen-activated protein kinases (MAPK), protein kinase C, and

stress-activated protein kinases (SAPK) (Lim et al. 1997). Important transcription factors such as AP-1 and NFκB are also activated in lung epithelial cell, and expression of pro-inflammatory cytokines such as IL-1α, IL-6 or TNF-α was enhanced (Simeonova et al. 1997). Modifications of receptor activation kinetics or activity of integrins can be responsible for the observed behavior. Alternatively, particles engulfed by phagocytosis were shown to stimulate production of reactive oxygen species (ROS) that have been found to be important second messengers for signal transduction in general (Martin et al. 1997). Alterations in the redox homeostasis of cells may play an important role in modulating immune functions. For example, transmembrane redox signaling activates NFκB in macrophages and T lymphocytes (Gin-Pease and Whisler 1998; Kaul et al. 1998). Nuclear factor kappa B (NFκB)/Rel proteins are dimeric, sequence-specific transcription factors involved in the activation of an exceptionally large number of genes in response to inflammation, viral, and bacterial infections and other stressful situations requiring rapid reprogramming of gene expression.

Previous results have shown that clinoptilolite treatment of mice and dogs suffering from various tumour types led to improvement of the overall health status, prolonged life-span, and decrease of tumour size (Pavelic et al. 2001). In addition, toxicology studies on mice and rats demonstrated that the same treatment did not have any negative effect (Pavelic et al. 2001). In vitro tissue-culture studies showed that finely ground clinoptilolite inhibited protein kinase B (c-Akt), induced expression of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ tumour suppressor proteins, and decreased cell proliferation in several cancer cell lines. Here we present evidence for antimetastatic activity and immunostimulatory effect of clinoptilolite in vivo. In addition, we propose a possible mechanism of its action.

Materials and methods

Natural clinoptilolite

The fine powder of natural clinoptilolites (MZ: micronized zeolite) from Slovakia was obtained by tribomechanical micronization. Particle-size distribution curves of the MZ were taken by a Mastersize XLB (Malvern) laser light-scattering particle-size analyzer. Tribomechanically treated natural clinoptilolite contained approximately 80 wt % clinoptilolite. The remaining 20% consisted of the silica, montmorollonite, and mordenite zeolite. Chemical composition of clinoptilolite: SiO_2 70.06%, Al_2O_3 12.32%, Fe_2O_3 1.48%, CaO 3.42% MgO 0.96%, TiO_2 0.71%, P_2O_5 0.05%, MnO 0.02%, Na_2O 0.68%, K_2O 2.38%, SO_3 0.17%, and H_2O 7.3%. Humidity at 105 °C was max. 6%, pH 6.9–7.1, specific mass 2.39 g/cm^3, specific area 360–390 m^2/g, NH_4^+ substitution capacity 8,500 mg NH_4^+/kg. Particle size analysis of the clinoptilolite showed that the maximum frequency of particles appeared at 1 μm.

Animals

C57Bl/6 mice were used for the experiment with B16 metastasis. CBA/HZgr and RFM mice were used for oxidative stress parameters and for cellular immune-response measurement. The mice were about 3 months old, weighing 21–26 g. Mice were bred in the Animal facility of the Ruđer Bošković Institute. Food (Domzale, Slovenia) and tap water were given ad libitum. Animals were kept in conventional circumstances: light/dark rhythms 12/12 h, temperature 22 °C, and humidity 55%.

Application of MZ

Since MZ is insoluble, it was administered to the mice either orally by gavage (100 mg/mice per day) or in their diet given as standard food consisting of 12.5% or 25% MZ. Each mouse ate about 4 g food daily, thus consuming 0.5 g or 1 g MZ, respectively. In part of the experiments suspension of MZ was administered intraperitoneally (3 mg/mouse).

Evaluation of antimetastatic effect of MZ

Ten mice (C57Bl/6) were injected i.v. with 7.5×10^4 melanoma B16 cells. For the next 16 days, they were treated daily with MZ (100 mg/ml distilled H_2O per mouse) by gastric intubation. Controls (6 mice) were intubated daily with distilled H_2O. Mice were killed, and lungs were removed and fixed in Bouen. Metastases were counted and statistical analysis was performed by Student's *t*-test.

Isolation of peritoneal macrophages

Peritoneal macrophages were aseptically collected from the peritoneal cavities of mice 24 h after i.p. or 7, 14, 21, and 28 days after per os administration of MZ. Macrophages were collected with Hank's solution (without phenol red; Sigma) and red blood cells were removed by NH_4Cl lysis. The remaining cells were washed three times, resuspended in RPMI 1640 (without phenol red; Sigma) supplemented with antibiotics and 10% fetal calf serum (FCS; Sigma), and adjusted to 2×10^6 cells/ml.

Assay for superoxide anion (O_2^-) release

In macrophages, superoxide release was measured as superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C using a modification of the method of Johnston et al. (Johnston et al. 1978). Samples contained 1 ml of cytochrome C (1 mg/ml) in phenol-free Hank's balanced salt solution and 2×10^6 cells in 100 μl of medium. The specificity of the reaction was tested by the addition of 60 IU SOD per millilitre of the reaction mixture. The reactivity of the cells was tested by the addition of cytochrome C in phenol-free Hank's solution for 30 min at 37 °C. After incubation, the reaction mixture was centrifuged for 5 min at 800×g, and the absorbance of the supernatant was determined spectrophotometrically at 550 nm. The concentration of reduced cytochrome C was calculated using the formula $E_{550nm} = 2.1\times10^4\ M^{-1}\ cm^{-1}$. Experiments were performed in duplicate and the results were expressed as nmol O_2^- $(10^6\ cells)^{-1}$ $(30\ min)^{-1}$.

Measurement of nitrite production

The measurement of nitric oxide (NO) from macrophages was assayed according to Naslund et al. (Naslund et al. 1995). Briefly, cultures of isolated peritoneal macrophages were incubated in plastic 24-well flat-bottom microplates (Falcon, USA) for 48 h at 37 °C and 5% CO_2. Aliquots (800 μl) of each supernatant were placed in tubes and mixed with 800 μl of GRIESS reagent (1% sulfanil amide in 2.5% phosphoric acid and 0.5% naphthylethylenediamine in 2.5% phosphoric acid; 1:1). The resulting colorimetric reaction was measured spectrophotometrically at 540 nm. Nitrite concentration was calculated from a standard curve using sodium nitrite (0–100 μM) as standard.

Measurement of lipid-bound sialic acid (LSA) in serum, total sialic acid (TSA) in spleen, and assay for lipid peroxidation (LPO) in liver

After exsanguination, sera from fed mice were collected and prepared for LSA measurement according to Katopodis et al. (Katopodis et al. 1982). The spleen and liver were removed from i.p. and per os treated mice. Concentration of TSA in the spleen was determined according to Hadzija et al. (Hadzija et al. 1992) and expressed as mg/10^6 spleen cells. Lipid peroxidation (LPO) was estimated according to the presence of thiobarbituric acid-reactive substances (TBARS) in the liver as reported by Ohkawa et al. (Ohkawa et al. 1979). Protein concentration was measured by the method of Lowry, using bovine serum albumin (BSA; Sigma) as standard.

Local alogeneic graft versus host reaction

A modified version of LXGVHR described by Shohat and Trainin (Shohat and Trainin 1980) was used. In our experiment, LAGVHR was done on alogeneic mice instead of rats. For each experiment ten control mice (treated with conventional food) and ten mice in each experimental group were used. CBA mice were fed either 12.5% (0.25 g) or 25% (0.5 g) MZ per day, during a period of 21 or 28 days. Mice were killed by bleeding. The pool of lymphocytes from lymph nodes of 3–5 treated or control mice was prepared, washed two times with Hank's by centrifugation. 2×10^7 living cells were injected intradermally into the shaved abdominal skin of RFM mice (irradiated with 7 Gy, 24 h before), where lymphocytes provoked the GVH reaction and damage of skin. On day 5, the treated mice were injected intravenously with 0.4 ml of 0.5% Evans blue. Five hours later the entire abdominal skin was excised and the blue-stained area was measured with caliper along two opposite diameters. A mean diameter of each spot was shown as a result.

NFκB activation in spleen

Twenty-four hours after i.p. injection of MZ to experimental mice, and Hank's solution to control mice, animals were killed by cervical dislocation. For preparation of cytoplasm and nuclear fractions of spleen, cells were isolated and a crude spleen extract was made. Erythrocytes were removed by ammonium chloride lysis. The nuclear and cytosolic fractionation procedure was a modification of the protocol of Lernbecher et al. (Lernbacher et al. 1993). Cells were washed twice with phosphate-buffered saline without calcium and magnesium and resuspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF). After 60-min lysis on ice, nuclei were spun down, and the supernatant, after additional centrifugation at 17,500×g, was stored as the cytoplasmic fraction. The nuclear pellet was resuspended in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol), vortexed, and incubated on ice for 45 min. Centrifugation at 17,500×g was performed to remove insoluble debris. The supernatant was used as nuclear extract.

Western blot (immunoblot) analysis

Protein concentration in nuclear and cytoplasm fractions was determined by Bradford assay. Equal amounts of nuclear and cytoplasm proteins (20 μg and 80 μg, respectively) were separated by 9% – SDS PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). Levels of loaded proteins were checked by Ponceau S and Commassie blue staining. Membranes were blocked overnight with TBS/2.5% BSA at 4 °C. After that, they were incubated for 90 min with primary antibodies (anti-p50, anti-RelB, and anti-p65), washed in TBS/0.05% Triton X-100, and then incubated for 1 h with appropriate secondary antibody. Following further washes, immunoblots were visualized using enhanced chemiluminescence reagent (POD; Boehringer-Mannheim, Germany). For immunoblots, polyclonal antibodies against p50 and RelB (Santa Cruz, USA) and monoclonal antibody against p65 (Transduction Laboratories, USA) were used. Secondary antibodies were peroxide-conjugated rabbit anti-mouse immunoglobulin (Amersham/Pharmacia, Sweden) and peroxide-conjugated protein A from Kierkegaard and Perry Laboratories.

Statistical analysis

Statistical analysis for all experiments was performed by the Student's *t*-test. A level of $P < 0.05$ was accepted as statistically significant.

Results

Antimetastatic effect of MZ

In the experiment where 15×10^4 melanoma cells were injected into controls and mice treated with MZ, the number of lung metastases was reduced from 36.05 ± 13.09 (in the control group consisting of six mice) to 21.0 ± 4.96 for treated mice (consisting of five animals). This was not statistically significant ($P = 0.056$). On the other hand, if 7.5×10^4 melanoma cells were injected, MZ-treated mice (ten animals) had a reduced number of lung metastasis compared to controls (five animals). While the number of metastasis in the control group was 5.2 ± 1.64, in treated mice the number of metastasis was strongly reduced to 0.7 ± 1.06. In addition, statistical significance was $P < 0.001$.

Influence of MZ on macrophages O_2^- production, lipid peroxidation (LPO) in liver, and lipid-bound sialic acid (LSA) in serum of healthy mice fed with MZ

O_2^- production, TBARS, and LSA concentration were measured in healthy mice fed with MZ (0.5 g or 1 g/day) for 7, 14, 21, and 28 days. The results are shown in Table 1. Concentration of O_2^- (in peritoneal macrophages) started to change slightly 14 days after administration of 1 g of MZ. However, TBARS concentration (in liver) started to change significantly 21 days after administration regardless of MZ concentration. Significant results regarding LSA concentration in serum was obtained with 1 g MZ on day 28.

Effect of MZ on local alogeneic graft versus host reaction

Results of two separately prepared experiments on healthy mice fed MZ 21 and 28 days are shown in Fig. 1. The cells from lymph nodes of mice fed 28 days with MZ (1 g/day) provoked a significantly higher GVH reaction than cells of control group mice. A treatment with a lower dose (0.5 g) of MZ for 21 or 28 days also

Table 1 Influence of MZ on O_2^- production, lipid peroxidation (TBARS), and lipid bound sialic acid (LSA) in healthy mice fed with MZ

	Days	7	14	21	28
O_2^- nmol/2×10^6 macrophages	Control	3.10 ± 0.14	1.50 ± 0.23	0.45 ± 0.07	1.20 ± 0.9
	0.5 g MZ	3.95 ± 0.8	0.68 ± 0.2[a]	2.55 ± 0.21[a]	0.55 ± 0.07[a]
	1 g MZ	3.20 ± 0.56	0.95 ± 0.35	0.57 ± 0.39	1.10 ± 0.14
TBARS nmol/mg liver proteins	Control	0.97 ± 0.11	0.94 ± 0.22	1.89 ± 0.18	1.78 ± 0.09
	0.5 g MZ	0.95 ± 0.11	0.72 ± 0.04	1.20 ± 0.29[a]	1.16 ± 0.08[a]
	1 g MZ	0.94 ± 0.13	0.61 ± 0.09	0.85 ± 0.11[a]	0.85 ± 0.12[a]
LSA nmol/l	Control	0.18 ± 0.07	0.26 ± 0.11	0.29 ± 0.03	0.27 ± 0.03
	0.5 g MZ	0.29 ± 0.13	0.25 ± 0.04	0.42 ± 0.16	0.51 ± 0.17[a]
	1 g MZ	0.28 ± 0.06	0.28 ± 0.08	0.34 ± 0.06	0.49 ± 0.15[a]

[a]Indicates significantly different (Student's *t*-test, $P < 0.05$) result compared to relevant control. The values are Σ ± SD

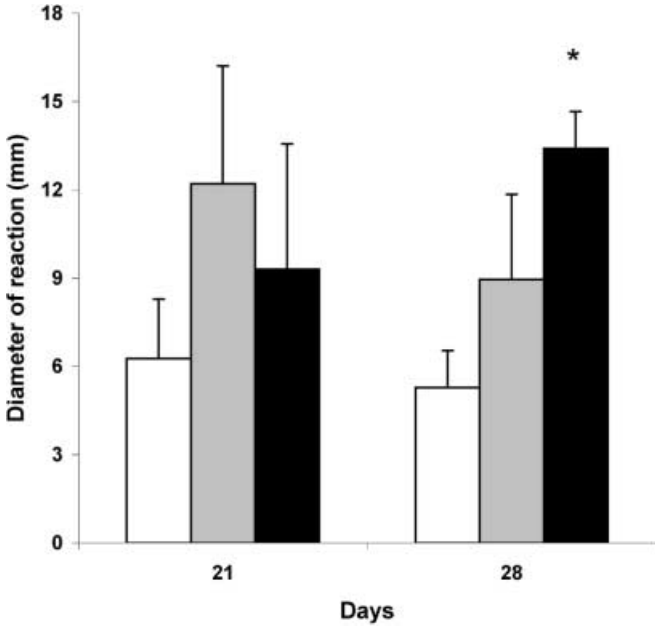

Fig. 1 Effect of MZ on alogeneic graft versus host reaction. The GVH reaction of lymph node cells of control mice (*empty* column), mice fed with 0.5 g (*gray* column), and 1 g (*black* column) MZ were tested

showed the higher reaction than the control group, but it was not significant.

Effect of i.p. administration of MZ on number of peritoneal macrophages, ROIs generation, and oxidative stress (OS) parameters

Intraperitoneal administration of MZ at a dose higher than 3 mg was lethal for mice and a dose of 3 mg was sublethal but proinflammatory. In experiments, a dose of 3 mg was used and the number of macrophages, production of O_2^- and NO, as well as TSA measurement in spleen and TBARS concentration in liver was performed 24 h after intraperitoneal injection of MZ.

MZ provoked accumulation of macrophages in peritoneum. The number of peritoneal macrophages (PM) after treatment was seven times higher than in control mice (Fig. 2a). The concentration of O_2^- was ten

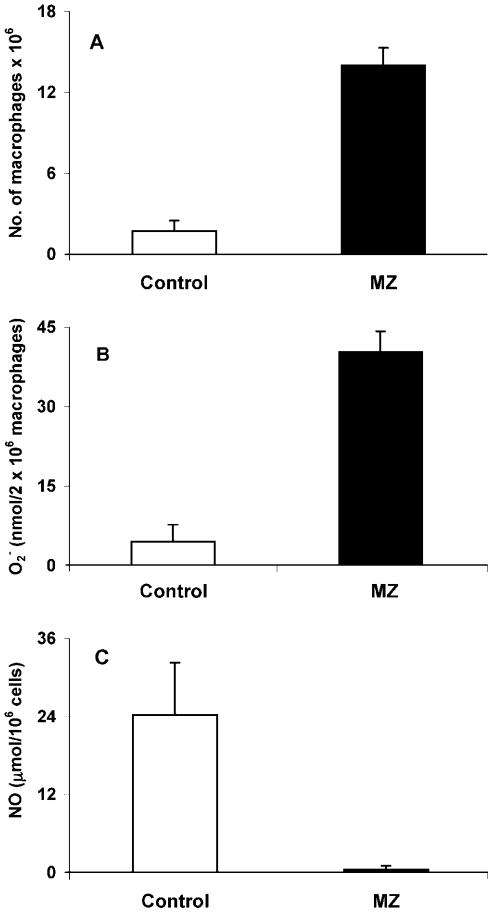

Fig. 2 **a** Number of peritoneal macrophages per mouse, **b** superoxide generation in macrophages, and **c** nitric oxide concentration in peritoneal macrophages ex vivo 24 h after treatment with 3 mg MZ/mouse. There were 18 control and 21 mice treated with MZ in two experiments

times higher in macrophages of treated mice than in controls (Fig. 2b). Since O_2^- release was calculated to 10^6 cells, the increased release was not the result of increased number of macrophages, but represents truly increased activity. Production of NO by peritoneal macrophages isolated from treated mice, and cultivated for another 24 h ex vivo, strongly decreased (Fig. 2c). There was no change in liver TBARS concentrations (expressed in nmol/mg protein) between the control

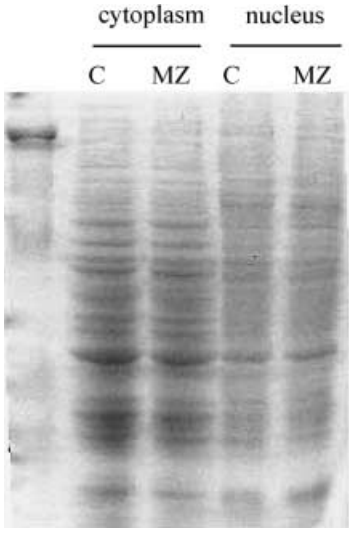

Fig. 3 Amounts of nuclear and cytoplasmic proteins were checked by Coomassie blue staining. *C* indicates splenic proteins of control group, whereas *MZ* indicates splenic proteins of treated animals

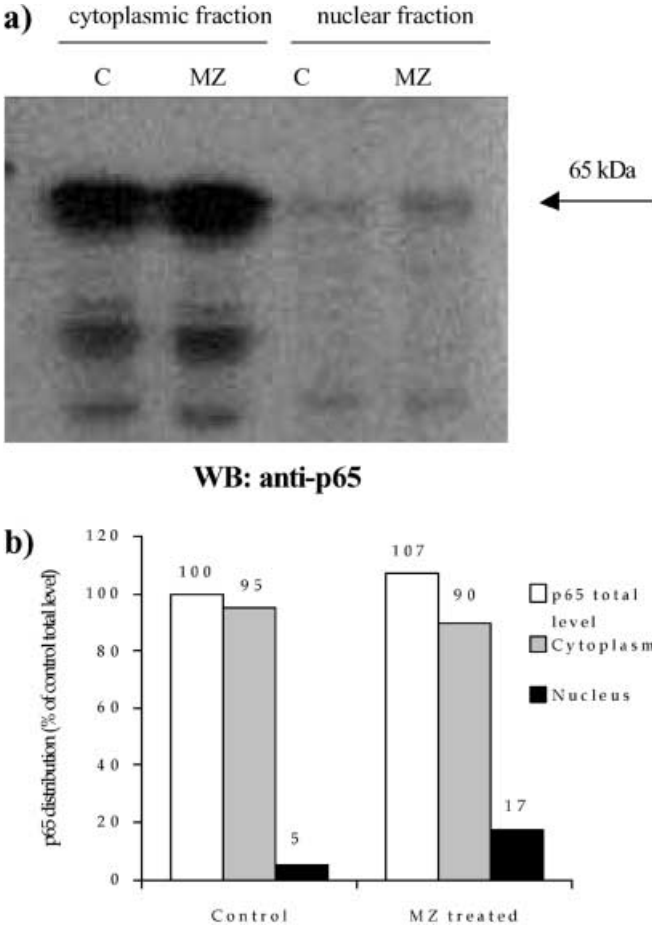

Fig. 4a,b Effect of MZ on level and distribution of p65 subunit of NF-κB. **a** Western blot analysis of p65 in nuclear and cytoplasmic fractions. *C* indicates control group of animals, whereas *MZ* indicates treated animals; **b** densitometric quantification of signal intensities. p65 total level indicates sum of cytoplasmic and nuclear fraction signal intensities in each group (*C* and *MZ*). Results are expressed as percentage of control p65 total level

group (0.907 ± 0.17) and treated (0.886 ± 0.16) group. In addition, TSA concentration (expressed as $\mu g/10^6$ splenocytes) was not changed after treatment with MZ. The value of the control group was 5.25 ± 0.73, and 5.58 ± 0.78 for the treated group.

Effect of MZ on NFκB/Rel proteins

A mass of spleens isolated from animals treated i.p. with 3 mg MZ was significantly higher than that of control animals (120 ± 18 mg for control compared to 175 ± 23.6 mg for MZ-treated mice). The effect of MZ on NFκB/Rel proteins could be seen after preparation of cytoplasm and nuclear fractions, electrophoresis, and Western blot. Equal levels of nuclear and cytoplasm fractions (20 μg and 80 μg, respectively) isolated from spleen of control and MZ-treated mice were loaded on gel, which was confirmed by Commasse blue staining (Fig. 3). MZ treatment increased translocation of p65 subunit into nucleus (Fig. 4) suggesting that the NFκB-containing p65 subunit had been activated. However, MZ treatment did not have any effect on the p50 or RelB subunit concerning translocation into the nucleus (Fig. 5

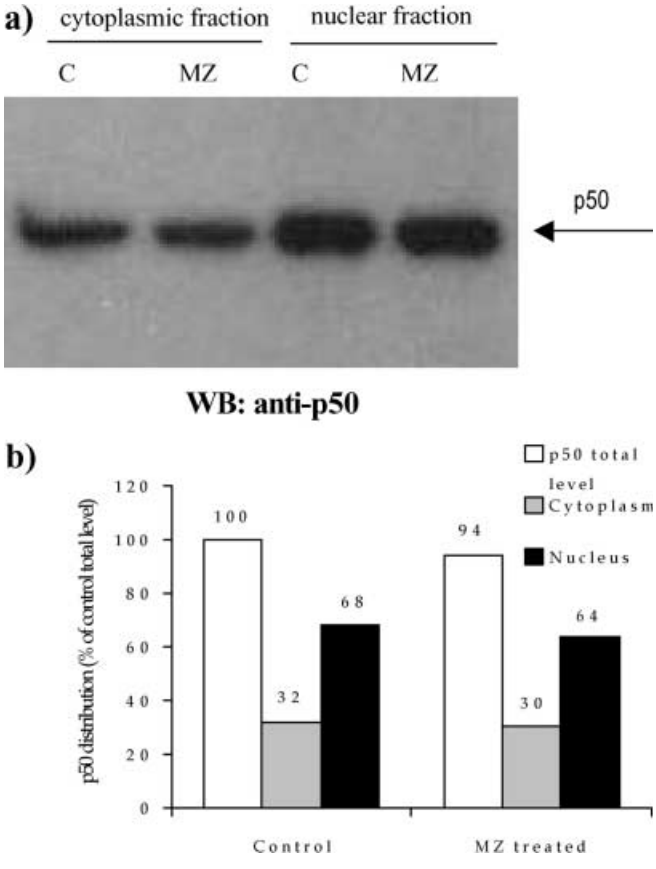

Fig. 5a,b Effect of MZ on level and distribution of p50 subunit of NF-κB. **a** Western blot analysis of p50 in nuclear and cytoplasmic fractions; **b** densitometric quantification of signal intensities. p50 total level indicates sum of signal intensities of cytoplasmic and nuclear fraction in each group (*C* and *MZ*). Results are expressed as percentage of control p50 total level

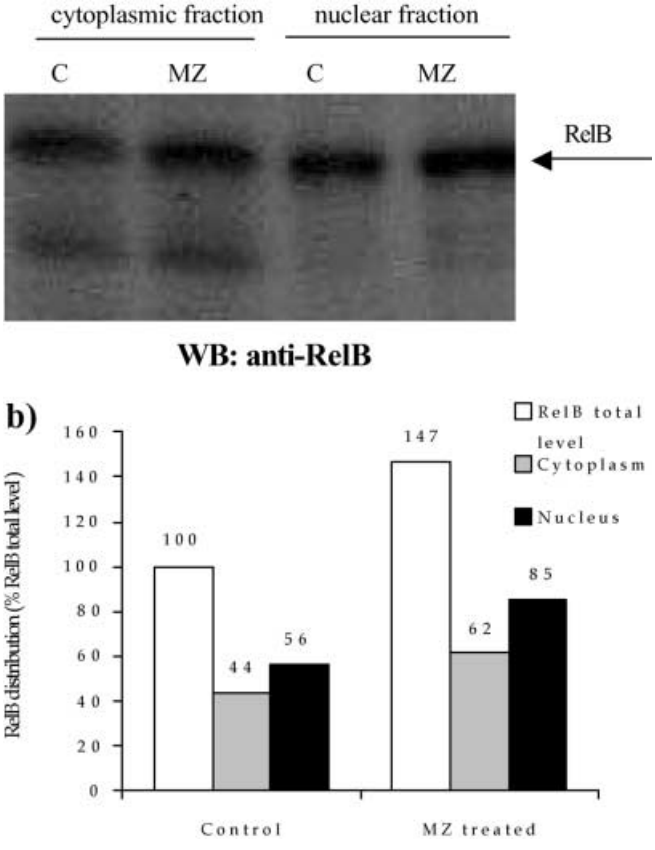

Fig. 6a,b Effect of MZ on level and distribution of RelB subunit of NF-κB. **a** Western blot analysis of RelB in nuclear and cytoplasmic fractions; **b** densitometric quantification of signal intensities. RelB total level indicates sum of signal intensities of cytoplasmic and nuclear fraction in each group (*C* and *MZ*). Results are expressed as percentage of control RelB total level

and 6). However, the total amount of the RelB subunit increased by about 40% and the total amount of p50 slightly decreased compared to controls.

Discussion

Antitumor effect of MZ in vivo

Previous in vitro and in vivo animal studies showed that MZ was a non-toxic compound that acted as an adjuvant in anticancer treatment (Pavelic et al. 2001). The range of effects was diverse, ranging from negative antitumor response, to normalization of biochemical parameters and prolonged life span.

Our previous in vitro experiments (Pavelic et al. 2001) showed 30–50% inhibition of proliferation (by MTT as well as ^{3}H-thymidine test) of several cell lines after incubation with MZ-pre-treated medium (50 mg/ml). We analyzed mitogenic and survival signaling pathways in tumour cells (Pavelic et al. 2001) and the most significant results were observed in the activity of Akt protein that was highly inhibited after incubation of cancer cells with MZ-pre-treated medium. That resulted in growth inhibition and increased apoptosis of cancer cells, but only in the presence of serum in medium.

Since previous in vitro results indicated an indirect effect on tumour cell lines growth and that MZ is not resorbed from gastrointestinal system, the MZ effect in vivo cannot be due to some direct biochemical interaction. The result of the antimetastatic effect of MZ presented here is concomitant with previous results and shows reduction of metastasis in mice. However, since the antimetastatic effect is dependent on the number of tumour cells injected into mice, we assumed that an immunological response is involved in MZ in vivo action. To confirm the possible mechanism of MZ in vivo effect, we used different ways of applying MZ to healthy mice.

Immunostimulatory effect of MZ in vivo

Inoculation of immunostimulators such as BCG or Freund's adjuvant as well as antigens or Silasorb results in a two- to threefold increase of the serum sialic acid level in mice (Sydow et al. 1989). Being a marker for inflammation, sialic acid may have a regulatory role in immunological processes. Inoculation of BCG also provokes an increased formation of macrophages in peritoneum (Sydow et al. 1989). The data presented here demonstrated that application of MZ in food for 28 days increased serum LSA concentration in healthy mice, which is probably associated with inflammatory process, i.e., activation of macrophages. That is confirmed by our data of elevated O_2^- in peritoneal macrophages of MZ-fed mice. We suspect, according to previous results (Sydow et al. 1989), that factors activating and influencing the proliferation or increasing the synthetic capacity of the phagocyte system might cause a change in the serum LSA level. It is also possible that macrophages participate in this process indirectly by releasing TNF-α and interleukin-1 or is connected with an elevation of acute phase proteins.

Although a parenteral route of application is not suitable, to confirm the immunomodulatory effect of MZ we examined the processes that happened after MZ intraperitoneal application. For this purpose, different amounts of MZ were injected in normal, healthy mice. It was shown that effect of MZ was dose dependent. Doses higher than 3 mg/mouse were toxic. However, since lower doses (3 mg/mouse) had a non-toxic, but proinflammatory effect, the immunological parameters were measured. In the acute phase of an inflammatory process, a large number of polymorphonuclear leukocytes (PMNs) migrate from the blood and accumulate in the exudate (Hambleton and Miller 1989). In our experiments, 24 h after MZ administration, a high accumulation of macrophages was found in peritoneum of treated animals. Our results confirmed that macrophages of treated mice were activated since they generated a nine-times higher amount of O_2^- than those of control mice.

Normally, NO reacts with O_2^- in a quite fast reaction, which is completed within less than 1 μs (Huie and Padmaja 1993). Therefore, any NO produced under aerobic conditions is converted rapidly to peroxynitrite anion. Peroxynitrite anion is a strong oxidant with bactericidal activity. At physiological pH it is protonated to form peroxinitrous acid, a relatively long-lived, strong oxidant, which could initiate oxidation of lipids. These reactions could explain the toxic (at higher doses) and inflammatory (at lower doses) effect of MZ administrated in peritoneum. In addition, the shown depletion of NO from peritoneal macrophages might have significantly increased superoxide generation and in this way could have intensified the effect of MZ. Since the concentration of TBARS in liver and TSA in spleen of treated mice remained the same, this is obviously a local reaction.

Phagocytosis per se or reactive oxygen species (ROS) can stimulate macrophages to secrete TNF-α and other cytokines that normally stimulate immunological response (Chaudhri and Clark 1989). One ubiquitous transcription factor of particular importance in immune and inflammatory responses is nuclear factor kappa B (NFκB) (Kopp and Ghosh 1995). Therefore, we wanted to examine the activation of NFκB in splenocytes of MZ-treated mice. Our results showed MZ-induced translocation of p65 to the nucleus of RFM mice spleen cells. This finding suggests that MZ acts as an immunoactivator, activating NFκB, and therefore inducing transcription of genes regulated with NFκB.

A decreased amount of p50 and increased amount of RelB proteins in treated mice compared to control mice could be due to a changed number and/or ratio of B- and T-lymphocytes. The fact that spleens of treated mice were 11% heavier contributes to this assumption. B-lymphocytes have a basal level of p50 homodimer that is not inducible upon stimulation (Liou et al. 1994) and/or serve as regulators of NFκB activity (Kang et al. 1992). A decreased total amount of p50 protein, as well as unchanged translocation into nucleus, could imply that B-lymphocytes were not stimulated by MZ. In addition, RelB/p50 heterodimer is constitutively active in primary lymphoid cells and its presence correlates with constitutive lymphoid-specific transgene expression of genes involved in B- and T-cell development (Lernbecher et al. 1993; Lernbecher et al. 1994). This explains our finding that MZ treatment did not have any (or had only a slight) effect on translocation of RelB subunit into the nucleus of RFM mice spleen cells. These facts also explain a relatively high basal amount of p50 and RelB proteins in nuclei of control splenocytes.

While B-lymphocytes and several other cells exhibit both constitutive and stimulated NFκB activation, only inducible NFκB activity has been described in T-cells or T cell lines (Schreck et al. 1991). However, as complete T-cell activation requires at least two signals provided by the T-cell receptor (TCR) complex and another stimulatory molecule, optimal NFκB activation in the T-cell is also dependent on dual signaling mechanisms (Crabtree and Clipstone 1994). Many agents have been shown to promote activation of NFκB in T-cells including TNF-α (Menon et al. 1995), calcium ionophores (Ginn-Pease and Whisler 1998), and H_2O_2 (Schreck et al. 1991). However, maximal NFκB activation has been observed in response to combinations of stimulants which fulfil the dual signaling requirements of T-cells (Crabtree and Clipstone 1994; Kanno and Siebenlist 1996). ROS have been found to act as second messengers in activation of NFκB/Rel proteins (Schreck et al. 1991) and oxidative stress can modulate the activity of NFκB in T-cells (Ginn-Pease and Whisler 1998). In addition, previous results have shown that NFκB is activated in inflammatory diseases. Therefore, according to all these results, as well as to ours, we concluded that MZ, in our experiments, activated a T-cell immunological (cellular) response that could be involved in an anticancer effect of MZ in vivo.

Possible mechanism of MZ in vivo action

Due to our results, we propose the mechanism of MZ in vivo action. MZ caused local inflammation at the place of application that attracted peritoneal macrophages. Macrophages were activated, which has been shown with increased O_2^- production. We suggest that activated macrophages produced TNF-α that, together with the other stimulants (e.g., other cytokines, ROS or changed calcium concentration), stimulated spleen T-cells. Since products of the genes that are regulated by NFκB also cause its activation, this type of positive regulatory loop may amplify and perpetuate the local inflammatory response. Our hypothesis is that MZ acted the same way after per os administration, affecting intestinal macrophages. The results of experiments with metastases reduction, when a lower number of tumour cells were injected, as well as local alogeneic graft versus host reaction, can partially confirm this hypothesis. Our results are in agreement with the accumulating evidence that zeolites could play an important role in regulation of the immune system as well as with the report that silica, silicates, and aluminosilicates act as non-specific immunomodulators similarly to superantigens.

To additionally confirm the hypothesis, TNF-α in serum and B- and T-lymphocytes should be measured separately, as well as activation of NFκB in macrophages.

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