Beijing Conference Natural Antioxidants

(14)

BIO-NORMALIZER (NATURAL JAPANESE FOOD SUPPLEMENTATION) AS A MODULATOR OF OXIDATIVE AND IMMUNE STATUS IN AN ORGANISM

J.A.Osato,[1] L.G.Korkina,[2]* E. Mikhal'chik,[2] and I.B.Afanas'ev[2]

[1] Sun-O Intern., 12 Minami-Machi, Bairin, Gifu 500, Japan

[2] Russian Institute of Pediatric Hematology, Leninskii pr. 117, Moscow 117513, Russia

* To whom all correspondence should be directed.

Introduction

Bio-Normalizer (BN) is a natural health food supplementation prepared by yeast fermentation of *Carica papaya* and some other tropical herbs and commercially available in Japan and the Philippines. It has been shown that BN exhibited beneficial therapeutic action in a variety of human pathologies and maintained a high quality of life even in very severe suffered patients. BN is also prescribed to enhance an organism's resistance to infections. However, in contrast to already shown practical advantages of BN application as a perspective pharmacological agent, a mechanism(s) of its biological activity is not fully understandable. We [1] and others [2] have shown that BN is a scavenger of active oxygen species. Therefore, BN may suppress free radical overproduction by the direct interaction with harmful oxygen radicals including hydroxyl radicals. At the same time we found [3] that the BN effects on oxygen radical production by phagocytosing cells are much more complicated. Thus, in some experimental models of aseptic inflammation such as peritoneal dextran-, lung asbestos-, cobalt- and bleomycin-induced inflammatory disorders BN significantly suppressed the inflammatory response including hemotaxis and the release of hydroxyl radicals by inflamed monocytes and macrophages but enhanced macrophage recruitment into the inflammatory *loci* and superoxide production by blood monocytes and tissue macrophages.

These findings suggest that in addition to the direct antioxidant activity of BN, there is probably an even more important regulatory function of BN, which is responsible for its biological activity in various pathologies. In this work we studied the possibility of BN interference with the production of tumor necrosis factor (TNF), which is a potent mediator of cytotoxic activity of macrophages and monocytes [4,5]. On the other hand, the augmentation of phagocyte cytotoxic activity is known to be stimulated by the enhancement of intracellular calcium level [6]. Therefore, it is of utmost interest to consider the effects of BN on the membrane permeability by calcium ions and the production of TNF by phagocytes because the augmentation of both processes by BN will indicate its potential anticancer activity.

Experimental

Chemicals.

BN was produced by Sun-O International Inc., Gifu, Japan, by the fermentation of *Carica papaya* tropical herbal plants. A major component of BN is a mixture of plant and yeast glycopolysaccharides. In addition, BN contains amino acids (tryptophane, leucine, glutamic and aspartic acids, etc.), vitamins B_6 and C, glucose, and the protease/antiprotease system of papain. Under our experimental conditions, the suspension of BN granules was prepared daily in Hanks' balanced salt solution (HBSS). In most experiments BN particles were sedimented by centrifugation after the 2 hours incubation in HBSS, and clear supernatant containing soluble BN fraction (SBN) was used in experiments. SBN contained all the above cited compounds excluding insoluble highly mollecular glycopolysaccharides.

Blood drawing and cell isolation. Venous blood (2 ml) was obtained from healthy subjects after their informed consent. Heparinized blood was carefully layered on dextran-metrizoate mixture (2 ml, 2:5 v/v) and incubated at room temperature for 30 min. Then,

leukocyte-rich top layer was placed on 1 ml Ficoll-Paque (d = 1.119) and centrifuged at 400×g for 30 min. The neutrophil-containing pellet was washed twice in a cold HBSS and finally resuspended in a minimal essential medium (MEM). Cell suspension contained >95% neutrophils as measured by differential cell count using Coulter counter. Neutrophil viability was equal to 90-98% as it was assessed by exclusion of 0.2% trypan blue dye.

Animals and macrophages preparation.

Adult male Wistar rats were injected intraperiloneally with 10 ml HBSS and sacrified 5 min later. The peritoneal lavage fluid was removed, placed on the top of Monoprep solution (d = 1.068), and centrifuged at 400×g for 30 min. The upper portion containing mononuclear cells was washed twice with a cold Ca,Mg free HBSS and resuspended in MEM supplied with 1% heat-inactivated calf serum. Differential cell count was performed microscopically using Giemsa staining: about 80% cells were macrophages and monocytes; the others were lymphocytes, mast cells, and erythrocytes and; > 95% cells excluded trypan blue.

Measurement of intracellular generation of active oxygen species by rat macrophages using fluorescent probe hydroetidine.

Macrophages (3×106 cells) were incubated with 1 mM hydroetidine in 3 ml HBSS at room temperature for 20 min. Then, 10 ng/ml PMA and BN at appropriate concentration was added, and the intensity of fluorescence excited at 473 nm and emitted at 610 nm was measured continuously for 15 min on a Hitachi MPF4 spectrophotometer.

Determination of the rat macrophage permeability by calcium.

The passive permeability of cellular membranes by calicium ions was studied by introduction in the cells of fluorescent calcium indicator Quin 2AM [7]. Rat peritoneal macrophages were incubated with Quin 2AM (2 μM/10^6 cells) and BN at appropiate concentration in Ca-free HBSS at 37^{o}C for 60 min. Then, the cells were twice rinsed and suspended in HBSS. Calcium efflux was started by the addition of 1 mM Ca ions. After measuring the initial rate of calcium incorporation, the cells were lysed with 0.05% Triton

X-100, and the concentration of Quin-bounded Ca was determined. Fluorescence were registered om a MPF-44a Perkin-Elmer spectrofluorimeter.

Measurement of TNF production by human blood monocytes

Human blood monocytes (2.5×10^6 cells/ml) were isolated using the modified Beutler method on a Ficol-Paque gradient (d= 1.077) and incubated with the soluble part of BN at appropriate concentration in the RPMI 1640 medium at 37°C for 1 hour. Then, the cells were cetrifuged, washed and incubated in the same medium supplied with 10% fetal calf serum in the 24-vial planshet for 14 hours. The content of TNF in supernatant was determined using TNF-sensitive murine fibroblast cell line L929. The fibroblasts were placed in the 96 vial planshet, and the supernatant was added to an each vial. The content of nonlysed fibroblasts was measured by light scattering method by staining of the viable cells with crystal violet dye. The TNF concentration in the supernatant was calculated by using the calibration curve obtained with recombinant TNF-α in the concentration range of 16-1000 pg/ml.

Results and Discussion

Present findings suggest that BN affects different pathways of monocyte and macrophage activation stimulating the release of active oxygen species and TNF. Indeed, the incubation of rat peritoneal macrophages with BN resulted in an approximately 115% increase in the intracellular production of active oxygen species measured by hydroetidine fluorescence (Fig.1).

This fact supports our previous findings [1] showed that BN sharply increased macrophage lucigenin-amplified chemiluminescence, which is a specific test of superoxide formation. It may indicate that BN is able to interfere with the activation of phagocyte NADPH oxidase, a major oxygen radical-producing enzyme of phagocytes. This suggestion seems to be supported by the

present study of macrophage permeability by calcium. It is seen (Fig.2) that BN increased calcium efflux into blood neutrophils and rat peritoneal macrophages in a concentration-dependent manner by 1.45-1.6 times. It is well-known that there is a Ca-dependent pathway of NADPH oxidase activation, which probably depends on protein kinase C [8]. Therefore, an increase in calcium efflux by BN appears to be one of main cause of the enhancement of intracellular oxygen radical generation.

Another important effect of BN on phagocyte activity is the stimulation of TNF production by blood monocytes (Fig.3). It is seen that BN increased TNF production by monocytes in a concentration-dependent manner with a maximal TNF level at 1 μg/ml BN. Thus, we can now recognize at least two major mechanisms of BN supposed cytotoxic activity on tumor cells. First one is the stimulation of oxygen radical release by phagocytes

References

1. J.A.Osato, L.G.Korkina, L.A.Santiago, and I.B.Afanas'ev, Effects of Bio-normalizer (a food supplementation) on free radical production by human blood neutrophils, erythrocytes, and rat peritoneal macrophages. (1995) *Nutrition: Intern.J.Appl.Basic Nutr.Sci.* 000-000.

2. L.A.Santiago, J.A.Osato, M.Hiramatsu, R.Edamatsu, and A.Mori, Free radical scavenging action of Bio-catalizer α-ρ no.11 (Bio-normalizer) and its by-product.(1991) *Free Rad. Biol. Med.* 11, 379-383.

3. J.A.Osato, I.B.Afanas'ev, Z.P.Cheremisina, T.B.Suslova, N.E.Abramova, E.V.Mikhalchik, I.B.Deeva, L.A.Santiago, and L.G.Korkina, Bio-normalizer as a modulator of phagocytosis and free radical production by murine inflamed neutrophils and macrophages. (1995) *Phys. Chem. Biol. Med.* 2, 000-000.

4. P.J.Edelson, Monocytes and macrophages: aspects of their cell biology, in: *Cell Biology of Inflammation*, Elsevier, Amsterdam, 470-495 (1980).

5. L.Gagnon, L.G.Filion, C.Dubois, and M.Rola-Pleszczynski, Leukotrienes and macrophage activation: Augmented cytotoxic activity and enhanced interleukin 1, tumor necrosis factor and hydrogen peroxide production. (1989) *Agents Actions* 26, 141-147.

6. J.W.Putney, Stimulus-permeability coupling: role of calcium in the receptor regulation of membrane permeability. (1979) *Pharm.Rev.* 30, 209-245.

7. J.S.Johansson and D.Haynes, Delibirate quin 2 overload as a method for in situ characterization of active calcium extrusion systems and cytoplasmic calcium binding: application of the human platelets. (1988) *J.Memb.Biol.* 104, 107-163.

8. T.Matsumoto, K.Takeshige, and S.Minakami, Spontaneous induction of superoxide release and degranulation of neutrophils in isotopic potassium medium: the role of intracellular calcium. (1986) *J.Biochem.* 99, 1591-1595.