ANTICANCER RESEARCH *19*: 5355-5360 (1999)

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Effect of Extracellular NADH on Human Tumor Cell Proliferation

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Abstract. *We investigated the antiproliferative effects of extracellular nicotinamide adenine dinucleotide against human malignant CaCo-2 (colon carcinoma), Hep-2 (laringeal carcinoma), MCF-7 (breast carcinoma), CaSki (cervix carcinoma) cell lines, as well as against murine fibrosarcoma and normal human embrional fibroblast (HEF). NADH was very potent in the growth inhibition of murine fibrosarcoma and human Hep-2 cells, regardless of the dose applied. During the observed period (4 or 5 days) only one dose of NADH was sufficient in reducing the growth rate for up to 92%. It had no effect on the growth of other cell lines tested. The identification of DNA-fragmentation and p53 and Ki-67 genes expression suggest that the mechanism of NADH action is different from disregulation of genes considered as checkpoints in cell cycle.*

Nicotinamide adenine dinucleotide in its reduced form (NADH) also known as Coenzyme 1 is important in maintaining growth, differentiation and energy metabolism of cells (1). Previous studies have indicated NADH to be beneficial in the treatment of patients suffering from depression, Alzheimer and Parkinson disease (2-6). In cancer patients, NADH led to better psychophysical condition and improvement of disease. However, the mechanism of NADH action in these patients is unclear. In experimental models NADH was efficient in stimulating DNA repair and synthesis of endogenous cell factors (7). It can also rescue PC12 cells from apoptotic damage (8).

It is well known that tumor cells maintain a high glycolytic rate, very often so great that all of the cell's energy requirements are derived from glycolysis (9). From this fact and the observation that growth factors and oncogene-dependent phosphorylation regulate glycolysis and phosphometabolite pools, it can be assumed that some phosphometabolites, *eg* AMP, NAD, NADH, regulate cell proliferation (10-12). By searching for such metabolic signals it was found that extracellular AMP inhibits DNA synthesis in human breast cancer cell line MCF-7 (13).

Based on these findings the aim of this study was to find out whether NADH can influence tumor cell proliferation rate and to clarify the mechanism of its activity. The approach used was motivated by observations that several anticancer agents trigger in tumor cells programmed cell death (apoptosis) (14-16). During this process, a concomitant regulation of several genes could occur. A question arises whether the same type of mechanism is responsible for the action of NADH.

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Key Words: NADH, tumor, proliferation.

Materials and Methods

Cell lines culturing. Murine fibrosarcoma, several human tumor cell lines (CaCo-2, colon carcinoma; Hep-2, laringeal carcinoma; MCF-7, breast carcinoma; CaSki, cervix carcinoma) and normal human embrional fibroblasts (HEF) were tested for NADH sensitivity *in vitro*. Murine fibrosarcoma, CaCo-2 and Hep-2 cells were grown as monolayers in RPMI-1640 medium while HEF, MCF-7 and CaSki cells were grown in DMEM medium at 37°C in a humidified atmosphere with 5% CO_2. Both media were supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For the purpose of the experiment the cells were plated in sixplicates onto 96-microwell flat bottom plates. The following cell number was plated per well in a volume of 0.2 ml: murine fibrosarcoma - 2×10^3, CaCo-2 cells - 3×10^3, Hep-2, MCF-7 and CaSki cells - 1.8×10^3 and HEF cells - 2.7×10^3. The next day (24 hours later), NADH at different concentrations, 100 μg/ml, 250 μg/ml, 400 μg/ml, 550 μg/ml, 700 μg/ml, and 850 μg/ml, was added to the cells. Control cells (without NADH) were grown under the same conditions.

Cell viability was measured immediately before (day 0) and 24, 48, 72, 96 and 120 hours after addition of NADH, using MTT assay which detects dehydrogenase activity in viable cells. For this purpose the medium was discarded and MTT was added to each well at concentration of 20 μg / 40 μl. After 4 hours of incubation at 37°C the precipitates were dissolved in 160 μl of DMSO. The absorbance was measured on ELISA reader at 570 nm. The cell proliferation was expressed as a percentage of absorbance recorded at certain day in comparison to the absorbance detected on day 0 (expressed as 100%).

Immunocytochemistry. The cells were seeded onto glass slides and 24 hours later the medium was replaced with the fresh one supplemented

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(or not) with 400 μg/ml of NADH. After either 2 or 5 days of incubation the cells were washed with PBS and fixed in methanol with 3% H_2O_2. Applying normal rabbit serum (1:10) for 30 minutes blocked non-specific binding. Primary antibodies p53 (Ab-2, 5 μg/ml, wild and mutant protein; Ab-3, 10 μg/ml, mutant protein, Oncogene Science, USA), Ki-67 (1:10, Dako, Denmark) were allowed to bind overnight at 4°C. Slides were washed three times in PBS. Secondary antibody (rabbit anti-mouse, Dako, Denmark) was applied for one hour at room temperature. Finally, the slides were stained with 0.025% diaminobenzidine tetrahydrochloride (Sigma) containing 4% H_2O_2 for 7 minutes and counterstained with hematoxylin for 30 seconds. The slides were analysed with a light microscope (Olympus). The level of non-specific backgraund staining was established for each measurement using control cells processed in the same way, but without exposure to the primary antibody (17). The concentration of the antigen was assessed by estimating the relative visual intensity of staining, and the results were expressed as weak (+), moderate (++),or strong (+++) staining.

Isolation of apoptotic DNA fragments. Cells were cultured in the medium without or with 400 μg/ml of NADH in 75 cm^2 tissue flasks (Falcon) for 48 and 120 hours. Apoptotic fragments were isolated according to the method described by Herrmann and co-workers (18). Briefly, after harvesting the cell samples were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 seconds with lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5; 10 μl per 10^6 cells, minimum 50 μl). After centrifugation for 5 minutes at 1600 x g the supernatant was collected and the extraction was repeated with the same amount of lysis buffer. The supernatants were brought to 1% SDS and treated for 2 hours with RNAse A (final concentration 5 μg/μl) at 56°C followed by digestion with proteinase K (2.5 μg/μl) for 2 hours at 37°C. After addition of 0.5 vol 10 M ammonium acetate and 2.5 vol 96% ethanol, the DNA was precipitated on 20°C overnight. The DNA was centrifuged (12 000 x g, 30 min) and the pellet was washed with 70 % ethanol, centrifuged again, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA) and separated by electrophoresis (90 V, 400 mA, 45 min) in 1% agarose gels with addition of ethidium bromide.

Results

The influence of NADH on cell proliferation. The influence of NADH on the growth of murine fibrosarcoma and Hep-2 cells is shown in Figure 1. The growth of murine fibrosarcoma was slowed down already after two days regardless of the NADH dose applied. The growth was reduced 180 to 230 % compared to control cell growth (330%) recorded the same day. During the subsequent days the growth of NADH treated cells was kept on the steady-state level (compared to day 2). However, the number of control cells duplicated by each day so the growth inhibition of treated cells was scored as 500% to 650% on day four depending on the dose applied. The results can also be presented in another way. If we consider the growth of control cells (at each particular day) as 100%, then at day four, after the addition of NADH at a concentration as small as 100 μg/ml, the growth of murine fibrosarcoma was inhibited by 72%. The higher concentrations inhibited growth up to 88%.

The growth inhibition of Hep-2 cells was observed already the next day after the addition of NADH regardless of the dose applied. During subsequent days the differences in growth values between control and NADH treated cells were more and more pronounced, due to the control cells

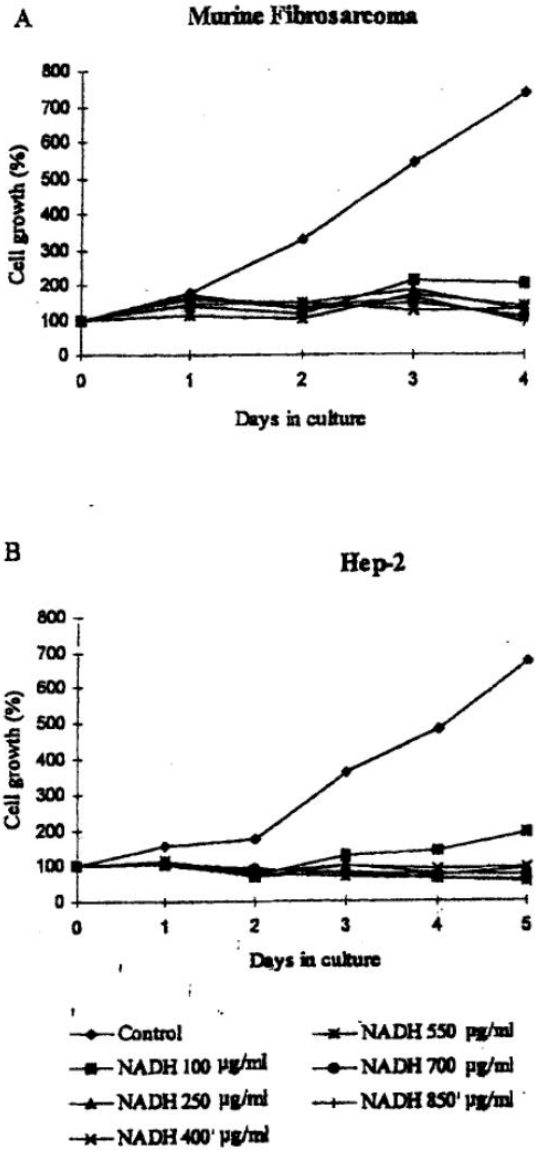

Figure 1. *Proliferation of murine fibrosarcoma (A) and Hep-2 (B) cells grown in the presence of different concentrations of NADH.*

proliferation. However, the growth of NADH treated cells was at the steady-state level. Only the cells treated with the lowest NADH concentration (100 μg/ml) showed a slight proliferation capacity. On day five, the treated cells were inhibited in growth by 480% to 600% in comparison to control cell growth. Again, if we consider the growth of control, untreated cells on day 5 as 100%, the Hep-2 cells treated with NADH at a concentration of 550 μg/ml were inhibited by 92 %.

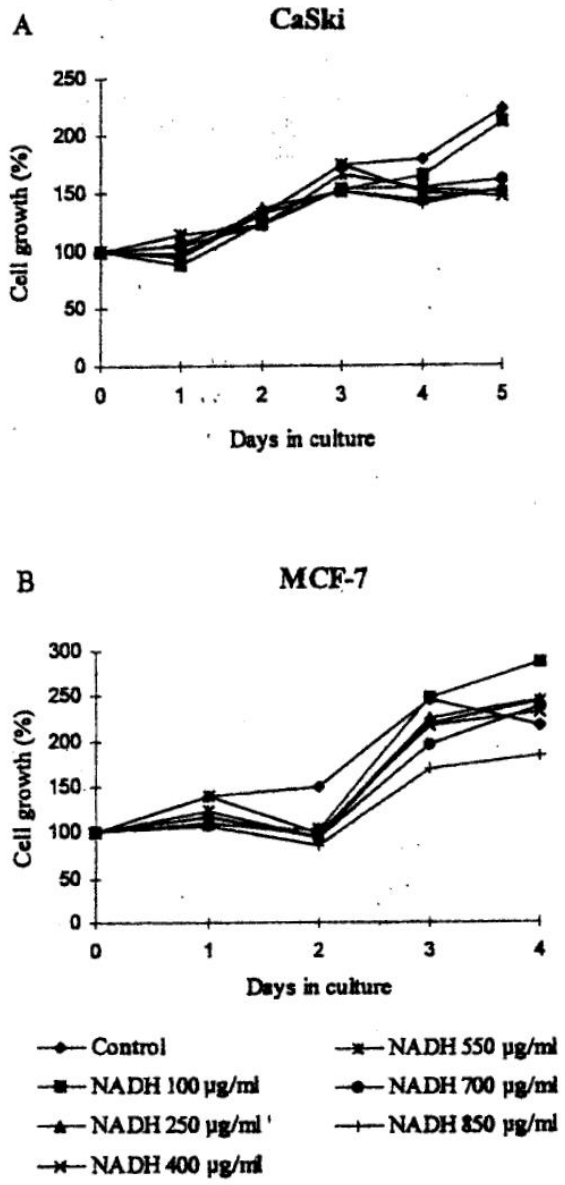

Figure 2. *Proliferation of CaSki (A) and MCF-7 (B) cells grown in the presence of different concentrations of NADH.*

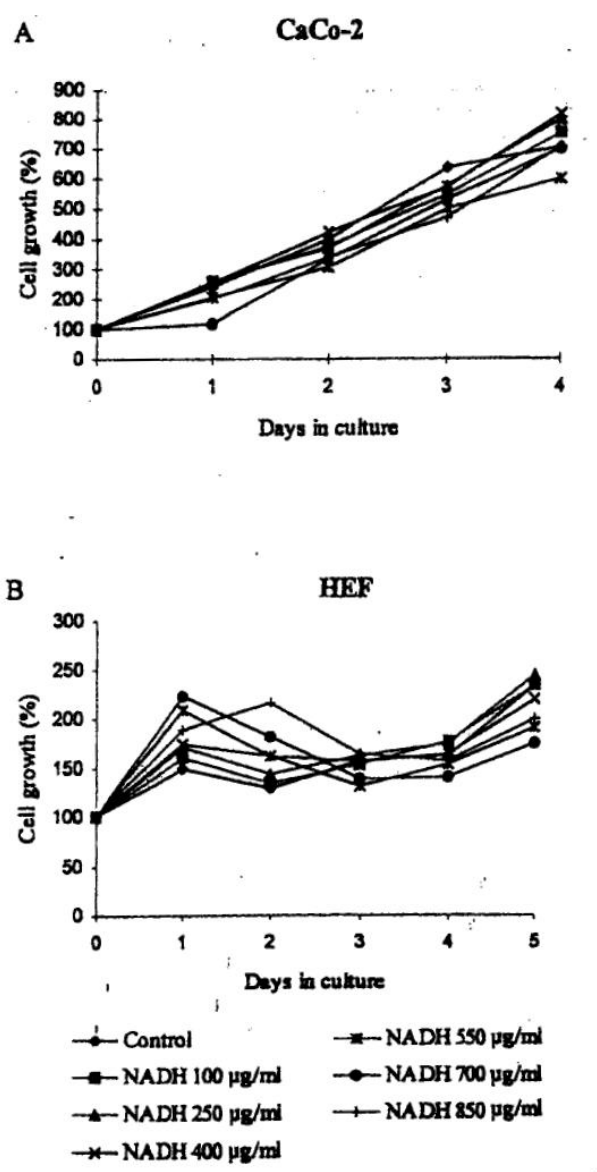

Figure 3. *Proliferation of CaCo-2 (A) and HEF (B) cells grown in the presence of different concentrations of NADH.*

The other cell lines tested did not undergo any significant cytotoxicity in the presence of NADH (Figures 2 and 3). Up to day 4, no growth inhibition of CaSki cells was observed. On day 5, the growth of NADH treated cells was inhibited by 33% (if the growth of control cells is expressed as 100 %) with doses of NADH ranging from 250 µg/ml to 850 µg/ml. The only significant growth inhibition of MCF-7 cells was observed with the highest NADH dose applied. On day 4, NADH treated cells were inhibited in growth by 16%. The experiments conducted on CaCo-2 cells show no growth inhibition of treated cells regardless of the NADH dose tested. Normal human embrional fibroblasts treated with NADH proliferated faster than control cells during first two days of treatment. During days 3 and 4 the growth of both treated and untreated cells was more or less equal, while on day 5, the growth of treated cells was reduced up to 25% (compared to the control cell growth - 100%), however, in a NADH dose independent manner.

The expression of p53 and Ki-67 proteins in NADH treated cells.

Using an immunocytochemical method, the expression of p53 wild and mutant protein as well as Ki-67 protein was examined on all cell lines treated with NADH for 2 and 5 days. In all cell lines the expression of the tested protein was almost the same as in nontreated cells (Table I). The exceptions were detected for wild/mutant p53 protein whose expression was either increased (in Hep-2 cells) or decreased (in CaCo-2 cells) after five days of NADH treatment. The strong increase in Ki-67 protein expression was noted only in MCF-7 cells.

Apoptosis. To investigate whether the cell death induced by NADH was associated with apoptosis, DNA fragmentation in the control and treated cells was examined. The results are shown on Figure 4. Only DNA isolated from treated murine fibrosarcoma and MCF-7 cells showed the presence of DNA fragmentation. In other cell lines (not shown) apoptosis (scored as fragmented DNA) was not visible, even in Hep-2 cells whose proliferation was inhibited by NADH.

Table I. *The expression of p53 and Ki-67 proteins. The intensity of staining was estimated as weak (+), moderate (++) or strong (+++). The localization of staining is indicated in parenthesis (c-cytoplasm; n-nucleus). Ki-67 protein was always found in nucleus.*

	p53 (Ab-2)	p53 (Ab-3)	Ki-67
Murine fibrosarcoma control	++(c+n)	+++(c+n)	+
Murine fibrosarcoma+NADH(2 days)	++(c)	++(c)	++
Murine fibrosarcoma+NADH(5 days)	+++(c+n)	++(c+n)	++
Hep-2 control	+(c)	++(c)	+
Hep-2+NADH(2 days)	-	+(c)	+
Hep-2+NADH(5 days)	+++(c+n)	++(c+n)	+
CaSki control	+(c+n)	-	+
CaSki+NADH(2 days)	-	+(c)	+
CaSki+NADH(5 days)	-	+(c)	+
MCF-7 control	+(c)	+++(c)	+
MCF-7+NADH(2 days)	-	+++(c+n)	+++
MCF-7+NADH(5days)	-	+++(c+n)	+++
CaCo-2 control	+++(n)	+++(n)	+
CaCo-2+NADH(2 days)	+++(n)	+++(n)	+
CaCo-2+NADH(5days)	+(n)	+(n)	+
HEF control	+(n)	+(n)	+
HEF+NADH(2 days)	+(n)	++(n)	+
HEF+NADH(5 days)	+(n)	+(n)	+

Discussion

In this study we described the dual NADH activity on growth of several mouse and human tumor cell lines. While the growth rate of murine fibrosarcoma and human laryngeal carcinoma cell lines was effectively inhibited, neither inhibition nor growth stimulation was observed on other cell lines.

Although some literature evidence exists that high NADH level is essential for cell proliferation, the results are not consistent and they vary between cell clones, transformation state and, expectably, they were different in normal and malignant cell lines (19-22). The observed cell growth inhibition (murine fibrosarcoma and Hep-2) could be explained in several ways. Acid mucopolysaccharides are the component of cell membrane. Their synthesis depends directly on corresponding UDP-galactose 4-epimerase, enzyme whose activity, in turn is inhibited by NADH, as shown by Robinson and coauthors (23). Therefore if NADH is applied in a culture system in excess, one can expect cell growth to be inhibited completely. Another explanation lay in the fact that NADH serves as a substrate in poly-ADP-ribose (poly-adenosine diphospho-ribose) formation by polyADRR polymerase. When active, the enzyme brings ADPR moiety of NAD together, forming homopolymers and liberates nicotinamide concurrently with ADP rybosilation of nuclear proteins, particularly DNA polymerase. The biological function of polyADPR seems to be the regulation of DNA synthesis (19, 24). Namely it was shown that the degree of DNA synthesis inhibition in rat liver nuclei varied directly with the concentration of NAD and inversely with the amount of free nicotinamide (19). If so then the elevated level of NADH is expected to suppress DNA synthesis by stimulating polyADPR formation which, in the form of rybosilated proteins, binds to DNA. On the other hand it has also been shown that the effect of ADPR on DNA synthesis varies from inhibition to stimulation (19-22), which might explain the absence of NADH effect on CaSki, MCF-7, CaCo-2 and HEF cell lines growth rate.

The third possible explanation for tumor cell growth inhibition arose from the observation that reactive oxygen intermediates (ROI) (generally considered as toxic byproducts of normal cell metabolism that are capable of damaging cells), especially small molecules such as nitric oxide are positive mediators of some biological processes. The role of other oxygen derivatives such as radial superoxide (O_2^-) and hydrogen peroxide (H_2O_2) as controllers of cell growth at nonlethal concentrations has been shown as well (25,26). Tumor cells, both *in vivo* and *in vitro*, secrete H_2O_2

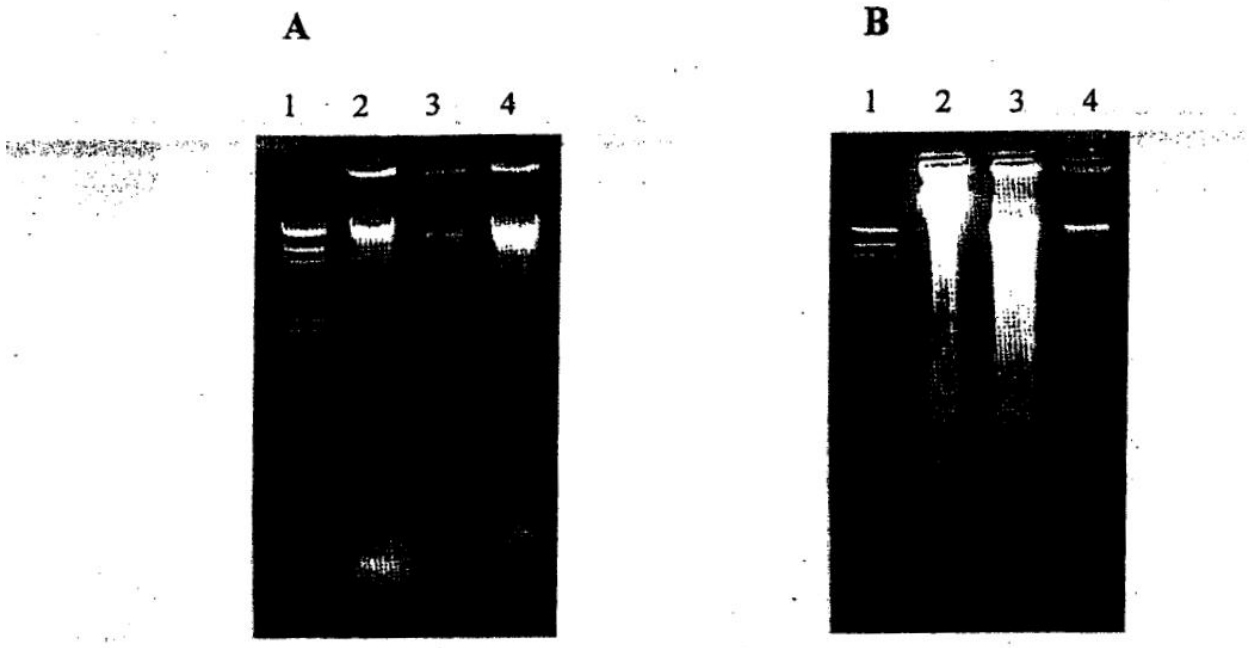

Figure 4. *Agarose gel electrophoresis of DNA isolated from murine fibrosarcoma (A) and MCF-7 cells (B).*
Lanes 1: DNA standard λ/HindIII
Lanes 2: DNA from control, untreated cells
Lanes 3 and 4: DNA from cells treated with NADH (400 μg/ml) for two and for five days, respectively.

which is a necessary factor for its growth (26-28). On the other hand the cell cytosol contains a mixture of reducing agents that protects the cell from damages that might be caused by reactive oxygen intermediates. Among others, NADH has the highest reducing power of any biological material. From this point of view, a high NADH level might reduce H_2O_2 necessary for cell growth, therby blocking cell division.

Taken together, the differences which NADH exerts on the growth of cell lines tested in this study are not easily explained. Could it be that the level of dedifferentiation (malignancy) causes such effects?

When planing the experiments, we expected that the NADH mechanism of action would be expressed as deregulated expression of genes involved in cell cycle regulation and, as a consequence, cell apoptosis. However, apoptosis was observed in only two cell lines, one different from those two where cell growth inhibition was observed. The same was the case for p53 and Ki-67 genes expression.

Taken together, our results indicate that NADH might be useful in reducing the growth of some tumor cell lines, however, by a so far unknown mechanism.

Acknowledgements

This work was supported by grants # 00981103 and # 00981104 from Ministry of Science and Technology Republic of Croatia.

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Received June 7, 1999
Accepted July 8, 1999