The effect of 6-mercaptopurine on early human placental explants

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BACKGROUND: 6-mercaptopurine (6-MP) is an antineoplastic and immunosuppressive drug. Recently, more women have received this drug during pregnancy. Animal studies have shown that 6-MP has deleterious effects on the fetus, while human data include prematurity, intrauterine growth restriction, low birth weight and malformations that occur especially when the drug is administered in the first trimester of pregnancy. OBJECTIVES: To study the effects of 6-MP on cellular functions of human trophoblast explants. METHODS: Human placental explants (5.5–9 weeks gestational age), that were grown on matrigel, were exposed to medium containing 6-MP for 5 days. Medium alone served as control. Extravillous trophoblast (EVT) cell migration assessment was performed by visual observation. Analysis of proliferating events of the trophoblast cells was assessed by immunohistochemical examination. Apoptosis was analyzed by Tunnel procedure and by anti-caspase 3 staining and hormone level by enzyme-linked immunosorbent assay. RESULTS: 6-MP inhibited migration of EVT cells from the villi to the matrigel with a lower proliferation rate and increased apoptosis of cytotrophoblast cells compared to controls. However, no significant effect of 6-MP on hormone levels was observed. CONCLUSIONS: 6-MP inhibited migration and proliferation of trophoblast cells in first-trimester human placental explant culture.

Key words: 6-mercaptopurine/migration/placenta/pregnancy/proliferation

Introduction

Immunosuppressive and antineoplastic (cytotoxic) agents that are administered during pregnancy may cross the placenta and enter into the fetal circulation, carrying a risk for fetal maldevelopment and malformations (Zemlickis et al., 1992; Pacifici and Nottoli, 1995). Adverse effects of the drugs on the embryo may result from direct damage to the embryo and fetus or from indirect damage through placental dysfunction (Beckman et al., 1990; Kolonin et al., 2002). Most drugs rapidly cross the placenta and pharmacologically significant concentrations equilibrate in maternal and fetal plasma, while other drugs cross the placenta incompletely, and their concentrations are lower in the fetal than in maternal plasma (Pacifici and Nottoli, 1995). Animal and human data suggest that most antineoplastic/immunosuppressive drugs may have deleterious effects on the fetus, including increased incidence of prematurity, intrauterine growth restriction, and low birth weight (Weisz et al., 2001; Prevot et al., 2002). Animal models have also shown a high risk of congenital anomalies, which in human may occur mainly when the drugs are administered during the first trimester of pregnancy (Gibson and Becker, 1968; Thompson et al., 1978).

Human placenta contains cytotrophoblast (CT) stem cells. These cells retain the ability to proliferate, and can differentiate by fusion to form syncytiotrophoblast cells (SCT) that perform gas and nutrient exchange for the developing embryo and fetus and are responsible for hormonal secretion (Malassine and Cronier, 2002). Alternatively, they may give rise to the extravillous trophoblast cells (EVT cells) (Malassine and Cronier, 2002). EVT cells are released from the CT column, progressively migrate and diffusely infiltrate the decidua, and invade maternal uterine blood vessels (Genbacev et al., 1992). Migration, proliferation and apoptotic processes in the trophoblast cells play an important role in determining the structure and function of the placenta in the first trimester of pregnancy (Haanen and Vermes, 1996; Barnea et al., 2000). Migration is performed by EVT cells (Genbacev et al., 1992). Proliferation of trophoblast cells in the villi is observed in the CT cells as well as in the EVT cells before they migrate out of the villi (Malassine and Cronier, 2002). All trophoblast cells may undergo apoptosis (Chan et al., 1999; Levy and Nelson, 2000). Cytotoxic drugs may adversely affect these processes and their regulation and may cause damage to placental structure and function.
6-mercaptopurine (6-MP), an inhibitor of purine metabolism is an antineoplastic and immunosuppressive drug, which is increasingly used in a wide spectrum of immune related disorders (Estlin, 2001; Nielsen et al., 2001). 6-MP may adversely affect the embryo and fetus directly and indirectly (Githens et al., 1965; Schmid, 1984; Norgard et al., 2003). It was reported that 6-MP may cross the human placenta, but that its fetal blood concentration represents 1–2% of its respective maternal blood levels (Saarikoski and Seppala, 1973). Thus, it is reasonable to assume that the adverse effects of 6-MP on the embryo may result from indirect damage to the fetus through placental malfunction (Kolonin et al., 2002). Since nowadays an increasing number of women tend to delay pregnancy until later reproductive age and older age is associated with malignant diseases (Zemlickis et al., 1992), it is expected that the incidence of cancer in pregnancy will rise. Furthermore, the use of immunosuppressive drugs for autoimmune diseases and following organ transplantation is rising as well (Prevot et al., 2002). Cases of women exposed to azathioprine or its metabolite 6-MP during pregnancy are described in the literature and it appears that in the future, women, embryos and fetuses will be exposed more frequently to cytotoxic and/or immunosuppressive agents such as 6-MP during pregnancy (Polifka and Friedman, 2002; Norgard et al., 2003; Tartakover Matalon et al., 2004). We studied the direct effects of 6-MP on trophoblastic cells, by the use of an in vitro system of human placental explants in culture.

Materials and methods

Tissue preparation and culture

Placental tissues (5.5–9 weeks gestational age) retrieved from normal pregnancies terminated legally due to psychosocial causes were used. The use of placental tissues has been approved by the local ethical committee.

The culture techniques were based upon methods developed previously (Yacobi et al., 2002). Briefly, the placental tissue (villi) was dissected from the fetal membranes. Explants of 10 mg wet weight were transferred into Millicell–CM culture dish inserts (Millipore Corporation, Bedford, USA), which had been layered with polymerized Matrigel (BD Biosciences, Bedford, USA) (Figure 1A). Medium (Dulbecco’s modified Eagle’s medium supplemented with l-glutamine (2 mM), sodium pyruvate (1 mM), antibiotics, fetal calf serum (10%); Biological Industries, Beit, Haemek, Israel) was added to the lower well of the culture dish (bottom medium). Cultures were pre-incubated overnight in 5% CO₂, 20% O₂ incubator in the presence of the bottom medium (Figure 1B). No medium was present above the explants. On the following day, medium was added above (top medium) and replaced below the placental explants (Figure 1C). During that time 6-MP (0.1 mM and 0.01 mM, Sigma M7000) was added to the upper medium of the placental culture (these concentrations are similar to those found in the sera, and 10-fold higher). 6-MP was dissolved in 1 M NaOH (50 mg/ml), and diluted with the medium to the appropriate concentration. Medium with NaOH alone served as control. In the first three experiments (out of five experiments) we included an additional control of the same medium without NaOH, to eliminate the possibility of any adverse effect of the 6-MP solvents. Indeed, no differences were observed in the migration and proliferation of EVT cells between explants that were incubated in the presence of medium with or without NaOH (data not shown). Both media from the insert (top, with/without the drug), and from the well (bottom) were changed 24, 72 and 96 h following the beginning of the experiment, and the collected media were stored at −80°C until processing. Villous explants were inspected daily using an inverted phase-contrast microscope for general cellular integrity, cellular migration and outgrowth. Hexaplicates of each treatment for each placenta were performed. Ninety-six hours following the beginning of the experiments, villi with supporting matrigel were dissected out.

Figure 1. (A) Diagram of the well, the insert, the matrigel and the placental explant. (B) Diagram of the well before adding the drug. At that stage cultures were pre-incubated overnight in the presence of the bottom medium only. (C) The experimental period. At this stage medium was added above (top medium) and replaced below the placental explants (bottom medium).
The specimens were fixed in 4% buffered formaldehyde and paraffin embedded for immunohistochemical examination.

**Visual migration assessment of human trophoblast**

The cultured villi were examined daily under a phase contrast microscope in order to follow the migration of trophoblastic cells from the villi to the matrigel. Visual assessment of migration was performed in three ways: (i) by counting the number of explants from which EVT cells migrated out of all explants; (ii) by measuring the percentage of peripheral area from which EVT cells migrated. We simulated the placental explant to a clock, divided into 12 parts. The number of sites from which EVT cells migrated out of 12 possible sites was documented every day, and the percentage of all parts of the circle was calculated. For example: EVT cells that migrated out of one site, as demonstrated in Figure 2 (EVT cells are departing from the placenta in h 6), was considered to have 8.3% of migration out of periphery area (1/12 × 100 = 8.3). In each experiment, six explants were analyzed and averaged for every treatment. Presented data are the mean value of five experiments ± SEM for control and 6-MP 0.01 mM, and of four experiments ± SEM for 6-MP 0.1 mM. And finally; (iii) by counting the number of EVT cells that migrated out from the villi to the matrigel in microscopic sections. EVT cells were counted in three randomly chosen explants for each concentration in every experiment.

**Proliferation in the human trophoblast**

Paraffin-embedded sections were deparaffinized in xylene, dehydrated in ethanol, rinsed in phosphate-buffered saline (PBS) and pretreated with proteinase K. The slides were then immersed in 10 mM (pH 6) sodium citrate buffer and heated in a microwave oven for 15 min at 700 W. Following PBS rinsing, endogenous peroxidase activity was quenched in 1% H₂O₂ (diluted in PBS) and rinsed with PBS. Samples were covered with normal blocker serum (Zymed, San Fransisco, CA), immersed in PBS, and incubated with the primary monoclonal antibody mouse anti-human Ki-67 antigen (mouse IgG1, Zymed, San Francisco, CA) for 60 min at room temperature. The slides were then rinsed and incubated again with the biotinylated second antibody. The specimens were covered with HRP-SA (enhanced horseradish peroxidase-conjugated streptavidin), rinsed with PBS, and covered with AEC–chromogen (biotinylated antibody, HRP-SA and AEC-Zymed laboratories, San Francisco, CA). Sections were counterstained with Mayer's hematoxylin. Proliferation was analyzed in the villous trophoblast cells, excluding SCT. To determine the extent of nonspecific immuno-staining, primary antibody was substituted with mouse immunoglobulin G1 (at the same concentrations as the primary antibodies). Presented data are an average of five experiments ± SEM.

**Apoptotic events in the human trophoblast**

Apoptosis was detected by two assays: Tunnel and anti-caspase 3 analysis.

**Tunnel**

Apoptosis was detected on paraffin sections by ApopTag peroxides labeling of fragmented DNA (KIT S7101, Interjen Company, USA), according to the manufacturer’s instructions. In short, paraffin-embedded sections were deparaffinized, pretreated with proteinase K, rinsed and quenched in 1% H₂O₂. Samples were then incubated with terminal deoxynucleotidyl transferase (TdT), in the presence of nucleotides. (TdT catalyzes a template-independent addition of nucleotide triphosphates to the 3'-OH ends of double- or single-stranded DNA, and forms an oligomer composed of digoxigenin.) The slides were then incubated with stop-wash buffer, incubated with anti-dioxigenin peroxidase, rinsed and stained with AEC–chromogen. Sections were counterstained with Mayer's hematoxylin. Slides that were treated in the same way, but without exposure to TdT, served as negative control.

**Activated caspase 3 analysis**

Activated caspase 3 stained cells were detected on paraffin sections in a similar way to the proliferation assay without proteinase K pretreatment, and by using rabbit anti-caspase 3 polyclonal antibody (Biocare medical, CA) primary antibody instead of anti-Ki67. Purified normal rabbit immunoglobulin (Zymed, San Francisco, CA) served as isotype control.

**Counting the cells**

Quantitative analysis of cell proliferation and apoptosis was done by enumerating the stained cells out of the total trophoblast cells present in the villi. Proliferation and apoptosis analysis were performed on three explants for each treatment in five experiments. They were assessed in the trophoblast cells in the villi, excluding

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**Figure 2.** Measurement of the migration zone. Placental explant was simulated to a clock, divided into 12 parts. The number of sites from which extravillous trophoblast (EVT) cells migrated out of 12 possible sites was documented every day and the percentage out of every part of the circle was calculated. EVT cells that migrated out of one site, as demonstrated in Figure 1 (EVT cells are departing from the placenta in h 6), was considered to have 8.3% of migration out of periphery area (1/12 × 100 = 8.3). (A) Photograph, and (B) an illustration for that photograph.
the non-proliferating cells (i.e. SCT and EVT cells that migrated into the matrigel). Twelve slices from each treatment in every experiment were analyzed (four slices from each of three randomly chosen wells in every treatment). In every slice, cells were counted in three/four villi (thus a minimum of 36 villi were counted). The average number of cells that were counted in each villus during proliferation analysis was $81 \pm 50$, $84 \pm 55$ and $71 \pm 35$ cells for control, 6-MP (0.01 mM) and 6-MP (0.1 mM) treated explants, respectively. The average number of cells that were counted in each villus during apoptosis analysis was $79 \pm 43$, $77 \pm 49$ and $88 \pm 49$ cells for control, 6-MP (0.01 mM) and 6-MP (0.1 mM) treated explants, respectively. Counting was performed at $\times 400$ magnification.

Screening the upper fluid of the human trophoblast for hormones
Twenty-four and 72 h following culture, media were collected from the inserts of control and 6-MP (0.01 mM) treated explants for the analysis of hormone levels. Estradiol (E$_2$), progesterone and HCG were measured by enzyme-linked immunosorbent assay using ICN Pharmaceuticals kits (beta HCG 07B56102, estradiol 07B38102, progesterone 07B70102; ICN Pharmaceuticals, NY), according to the manufacturer’s instructions. Media of three explants were analyzed from each treatment in every experiment. All tests were performed in duplicates.

Statistical analysis
Student’s t-tests were employed in analysis of differences between cohorts. An effect was considered significant when the $P$-value was $\leq 0.05$.

Results

Migration of EVT cells from the villi to the matrigel
Twenty-four hours following the beginning of the incubation period, the first EVT cells started to migrate from the villi into the matrigel in 68% of the control explants. However, significantly fewer explants with migrating EVT cells were observed at that time in the 0.1 mM and 0.01 mM 6-MP treated trophoblasts (27.2%, $P = 0.001$ and 37.9% $P = 0.011$, respectively) (Table I). Significantly more explants with migrating EVT cells were observed after 72 h of 0.1 mM and 0.01 mM 6-MP treatments compared to the 24 h exposure (54.5%, $P = 0.03$ and 58.6%, $P = 0.05$, respectively) (Table I). In contrast, only a small number of additional control explants started to release EVT cells at 72 h compared with 24 h (71.4%, $P = 0.38$) (Table I). We also analyzed the length of the peripheral explant area from which EVT cells migrated. During the first 24 h, EVT cells of 6-MP treated explants migrated from less than a third of the peripheral area compared to EVT cells from control explants ($P = 0.003$ and $P = 0.011$, respectively) (Figure 3). In the next 48 h, EVT cells continued to migrate from other zones of both the 6-MP treated explants and from the control explants. Although the differences between 6-MP treated explants and control explants were reduced by this time, they were still significant ($P < 0.01$ and $P = 0.026$ for 6-MP 0.1 mM and 0.01 mM, respectively) (Figure 3). Counting the number of EVT cells in the matrigel demonstrated that more EVT cells migrated out from the control explants in comparison to the 6-MP treated explants. However, this result was not statistically significant. Two hundred and forty two (242 \pm 106) EVT cells were counted in the matrigel surrounding the control explants, while only 139 \pm 58 and 122 \pm 32 cells were counted in the 6-MP 0.01 mM and 0.1 mM treated explants, respectively.

Proliferation of trophoblast cells in the villous
Proliferating trophoblast cells were analyzed in explants that were fixed at the end of the experiment (96 h following the beginning of the experiment). Forty-six percent of the trophoblast cells proliferated in the control villi compared with only 33% and 37% of the trophoblast cells in the 6-MP 0.1 mM and 0.01 mM treated explants ($P = 0.023$ and $P = 0.007$, respectively) (Figures 4 and 5). This represents a 28% and 19.5% decrease in the proliferation rate in the villi. No staining was observed with the IgG1 (isotype control) as the primary antibody, reflecting the specificity of the anti Ki-67 staining.

Apoptosis of trophoblast cells
Apoptosis of trophoblast cells was analyzed using Tunnel assay and anti-activated caspase 3 staining. Staining was

Table I. Percentage of explants from which extravillous trophoblast cells migrated to the matrigel

<table>
<thead>
<tr>
<th>Time (h) treatment</th>
<th>Control (%)</th>
<th>6-MP 0.01 mM (%)</th>
<th>6-MP 0.1 mM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>67.8</td>
<td>37.9*</td>
<td>27.2*</td>
</tr>
<tr>
<td>24</td>
<td>71.4</td>
<td>58.6**</td>
<td>54.5**</td>
</tr>
<tr>
<td>72</td>
<td>82.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>83.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6-MP, 6-mercaptopurine.
*Significantly different from control at 24 h.
**Significantly different from the same 6-MP treatment at 24 h.
performed in explants that were exposed to 6-MP for 96 h and in the controls at that time point. A small increase in the number of apoptotic events was found in 6-MP treated explants compared to the control with both assays. By Tunnel assay, apoptosis was demonstrated in 2.16% of the CT cells in the control villi compared with 2.7% and 3.2% of the trophoblast cells in the 6-MP 0.01 mM and 0.1 mM treated explants (Figures 6–8). No staining was observed in this procedure without TdT, reflecting the specificity of the antidioxigenin peroxidase. Similar results were obtained when we used anti-caspase 3 antibody. Apoptosis was demonstrated in 1.6% of the CT cells in the control villi compared to 2.7% and 2.9% of the trophoblast cells in the 6-MP 0.01 mM and 0.1 mM treated explants, respectively.

**Hormones**

Hormone levels were analyzed in the media that were collected 24 and 72 h following initiation of experiments.
Beta HCG and E2 levels in the upper fluids of the explants varied between placentae while progesterone level was similar in most of them (Table II). There were no significant differences between the hormone levels in the media of the 6-MP (0.01 mM) treated explants and those of the control explants, at both time points (Table II). A trend towards a higher CG level in the medium of the control compared to 6-MP treated explants was noticed 72 h following incubation.

Discussion

In order to study the effect of 6-MP on the human placenta we used the placental explants culture model, in which the placental explants are located above the matrigel and the EVT cells differentiate and migrate from the villi into the matrigel (Genbacev et al., 1992). Human placental explants contain CT, EVT and SCT cells. Migration, proliferation, apoptosis and hormone production can be measured with this system, which has the advantage of presenting topological and functional villous–extravillous trophoblast inter-relationships.

We found that 6-MP adversely affected trophoblast cells in several ways. 6-MP inhibited migration of EVT cells from the villi to the matrigel. Fewer villi, from fewer explants, released fewer EVT migrating cells in the 6-MP treated explants in comparison with the controls. Moreover, we observed that 6-MP inhibited proliferation of trophoblast cells. The number of apoptotic events increased in the 6-MP treated explants in comparison to control. No significant effect of 6-MP on hormone levels was observed.

The effect of 6-MP on placental explants was similar in both drug concentrations, the one that is similar to the level in the sera (0.01 mM) and the 10-fold higher concentration (0.1 mM).

6-MP is a source of thioguanine nucleotides, which may be incorporated into DNA, yielding abnormal DNA, thus interfering with the function of DNA polymerases, ligases and endonucleases (Platzek et al., 1994; Estlin, 2001). Moreover, 6-MP is catalyzed to inhibitors of enzymes that are important in de novo purine synthesis (Estlin, 2001). Nucleoside metabolism probably plays an important role in the early post-implantation stages of the placenta, since enzymes of the degradation pathways of purine metabolism are enriched at the maternal–fetal interface of mice (Blackburn et al., 1997), and are highly expressed in human maternal-derived decidual cells and in trophoblast cells at that time (Lupidi et al., 1998). Thus, it is not surprising that 6-MP, which affects nucleoside metabolism, will adversely affect the placenta. Inhibition of migration and proliferation, and increased apoptosis of other cell types by 6-MP was already observed in the past. Azathioprine, the mother drug of 6-MP was shown to inhibit leukocyte migration (Szczepanska et al., 1988; Elferink et al., 1997) probably through a cGMP dependent mechanism. This mechanism may have a significant role in EVT cell migration (Cartwright et al., 1999). Inhibition of proliferation by 6-MP was also observed in the past, when 6-MP led to decreased endothelial and T-cells proliferation, by inhibiting the purine/pyrimidine nucleotides in the T-cells and altering endothelial nucleotide balance (Weigel et al., 1999; Quemeneur et al., 2003). Moreover, 6-MP inhibited proliferation of mature B-cells after being triggered with mitogen (Hortelano and Bosca, 1997). In addition to its anti proliferative effect, 6-MP evoked apoptotic cell death in activated cycling T- and activated B-cells (Hortelano and Bosca, 1997; Quemeneur et al., 2003).

In our study, 6-MP’s most vigorous effect was on EVT migration. EVT cells differentiate in vitro by proliferation of cells in the tip of the anchoring villi which later migrate into the surrounding matrix (Genbacev et al., 1993). As a result of cell proliferation and migration, the number of EVT cells in the matrigel increases during the culture period. It is possible that as a consequence of lower proliferative rate, fewer EVT cells with migration potential were formed and therefore fewer migrating cells were observed. However, it is important to emphasize that different regulators control proliferation and migration of EVT cells (Athanassiades et al., 1998; Xu et al., 2002). Thus, it is possible that two different mechanisms of action of 6-MP are responsible for the inhibition of migration and proliferation.

EVT cells play an important role during implantation, when they migrate into the decidua, by remodeling the pregnant endometrium and its vasculature (Malassine and Cronier, 2002). Since 6-MP inhibits EVT cells migration, it is reasonable to assume that this drug may affect the implantation process. Indeed, it was found that azathioprine (Gross et al., 1977) injected daily into pregnant rats has a detrimental effect on the trophoblast of the implanted blastocyst by impeding its penetration, restraining the placental formation with subsequent degeneration of the conceptus (Gross et al., 1977). Moreover, implantation traces of abortion were smaller in pregnant rats that were injected with 6-MP on days 7 and 8 of gestation in comparison with control (Yamada et al., 1985). Thus, our findings suggest a mechanism by which 6-MP may impede implantation and normal placental function not only in animals but also in human.

Interestingly, the highest inhibition of EVT migration occurred in the first 24 h, and was much less vigorous during the rest of the experimental period. It seems that the placenta developed resistance to the effect of 6-MP on migration.

Table II. Hormone levels in the media of 6-MP treated explants and control following 24 and 72 h of incubation

<table>
<thead>
<tr>
<th>Treatment hormone range</th>
<th>6-MP (0.01 mM)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>72 h</td>
</tr>
<tr>
<td>Estradiol (pg/ml/10 mg placenta)</td>
<td>184.9 ± 55.5</td>
<td>136.3 ± 72.9</td>
</tr>
<tr>
<td>Progesterone (ng/ml/10 mg placenta)</td>
<td>26.7 ± 2.9</td>
<td>35.3 ± 1.3</td>
</tr>
<tr>
<td>HCG (mIU/ml/10 mg placenta)</td>
<td>224.6 ± 49.5</td>
<td>296.4 ± 132</td>
</tr>
</tbody>
</table>
Resistance to drugs may result from expression of ATP-dependent efflux pumps (Gottesman et al., 2002) and by other proteins such as heat shock proteins (Vargas-Roig et al., 1998). We are currently studying the mechanisms by which the placental resistance was induced.

No significant differences were observed in the medium hormone levels of 6-MP treated explants in comparison to the medium of the controls. However, although not significant, a higher level of beta HCG was observed in the medium of the control explants 72 h following the beginning of the experiment in comparison with the 6-MP treated explants. The receptor for HCG is found on intermediate trophoblasts (Tao et al., 1995), and HCG increased matrigel invasion of a choriocarcinoma cell line (JEG-3) (Zygmont et al., 1998). Thus, decreased levels of hCG may contribute to the adverse migration of trophoblast cells following 6-MP exposure.

Our results demonstrate that 6-MP adversely affects the placenta. These effects may contribute to failure of the implantation process. The observation that the effect of 6-MP on migration of EVT cells was mainly evident at 24 h after exposure, and substantially decreased with time, suggests that the placenta develops resistance to the drug. This may explain the non-lethal effects of the drug on pregnancy outcome. Further studies will clarify the mechanism of the effect of 6-MP on placental function, and of the placental resistance to the drug.

Acknowledgements

This work was supported by the Tel Aviv University—Toronto Hospital for sick children exchange program and by the Hamer Foundation, The Sackler Faculty of Medicine, Tel-Aviv University, Israel.

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Submitted on May 4, 2004; resubmitted on September 8, 2004; accepted on December 7, 2004.