Cytotoxic Effects of \( m-[^{131}I] \) and \( m-[^{125}I] \) Iodobenzylguanidine on the Human Neuroblastoma Cell Lines SK-N-SH and SK-N-LO

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INTRODUCTION

The catecholamine-analogue compound mIBG\(^4\) has been used in its \( ^{131}I \)- and \( ^{125}I \)-labeled forms for several years in the diagnosis and therapy of pheochromocytoma (1, 2). In 1983 these substances were also successfully introduced for scintigraphic imaging of neuroblastoma (3, 4). Attempts to use m-[\( ^{131}I \)]IIBG in the therapy of neuroblastoma also have been already made in several pediatric clinics, among these being our hospital (5). An up-to-date summary about the experiences gained using mIBG for diagnostic and therapeutic purposes has been presented recently during a meeting held in Rome, Italy (6). Whereas the use of mIBG is well established for the diagnosis of neuroblastoma, its therapeutic significance is not yet fully accepted. Despite some encouraging results that led to dramatic reduction of tumor masses in some children, the clinical outcomes are on the whole still insufficient. Several questions concerning such basic mechanisms of mIBG/neuroblastoma interaction as uptake, storage, toxicity, and pharmacology as well as the better way to schedule application are still unresolved on the subject of controversy.

In order to get more information on the interaction of mIBG with neuroblastoma cells and the cytotoxic effects of radiolabeled mIBG on these cells, we have performed in vitro experiments with two human neuroblastoma cell lines (SK-N-SH and SK-N-LO cells). As we have shown recently, only SK-N-SH cells (but not SK-N-LO cells and most other neuroblastoma cell lines) are able to take up and to store mIBG (7). In this communication, we describe the cytotoxic effects of m-[\( ^{131}I \)]- and m-[\( ^{125}I \)]IIBG on SK-N-SH and SK-N-LO cells as well as on bone marrow stem cells (CFU-c). Our attention was mainly addressed to the following questions: (a) how long can mIBG be stored in the neuroblastoma cells? (b) is unlabeled mIBG cytotoxic with respect to the neuroblastoma cells? (c) does the specific activity of radiolabeled mIBG influence cell killing? (d) are there any differences between the cytotoxicity of m-[\(^{131}I\)]- and m-[\(^{125}I\)]IIBG? and lastly, (e) does radioactively labeled mIBG influence bone marrow cells?

MATERIALS AND METHODS

Chemicals and Cells

The unlabeled mIBG used was a gift from Henning, Ltd., West Berlin. m-[\(^{131}I\)]IIBG (specific activity, 20–30 mCi/mg) and m-[\(^{125}I\)]IIBG (specific activities, 20–30 and 1–5 mCi/mg) were both purchased from Henning, Ltd. and later from Amersham, Braunschweig, West Germany. Catecholamines were determined in cell lysates (5 x 10^6 cells/100 μl 0.1 M perchloric acid) by radioenzymatic assay (8), Amersham. The two human neuroblastoma cell lines SK-N-SH and SK-N-LO were obtained by Drs. J. Fogh and L. Hels, Memorial Sloan-Kettering Cancer Center, New York, NY.

Storage Experiments

For these experiments, cells were grown in 25-cm² flasks (Greiner, Nuertingen, West Germany) as monolayers in Eagle’s minimal essential medium supplemented with 5% fetal calf serum, NaHCO\(_3\) (0.25 g/liter), gentamycin (0.05 g/liter), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (20 mm), and glutamine (0.005 g/liter) in a humidified atmosphere of 5% CO\(_2\) at 37°C. mIBG (final concentration in 5 ml fresh medium, 10–1000 nM, containing m-[\(^{125}I\)]IIBG, specific activity, 1–5 mCi/mg as a tracer) was added to the cell monolayers (about 60% confluency at the beginning of the experiment) for 1 h. The monolayers were washed twice in order to remove mIBG not incorporated by the cells and the incubation continued for 72 h. At 24-h intervals the radioactivity released to the supernatant was determined and the medium was subsequently changed. Finally, the radioactivity was estimated in the cells after lysis with 0.3 M NaOH.

Cytotoxic Effects of mIBG

Monolayer Assays. SK-N-SH and SK-N-LO cells were grown as monolayers in 25-cm² flasks. Before adding the radioactively labeled mIBG (1–200 μCi m-[\(^{131}I\)]IIBG or 100 μCi m-[\(^{125}I\)]IIBG), the cell culture medium was changed and replaced by 5 ml fresh medium. After incubation (1 h, 37°C), the cells were washed twice and once more 3 h later in order to remove most of the radioactivity unspecifically bound to the cells. Subsequently, the cells were transferred into six-well plates (Costar, Ltd., Cambridge, MA). Usually, 2–4 x 10^4 cells were seeded in 4 ml incubation medium/cell/well. After 6 days in culture, the number of viable cells was counted (trypan blue dye exclusion test) and expressed as a percentage of controls (incubation without mIBG). At this time, cells of the control group reached about 95% confluency. In one set of experiments, the cell fraction that survived a first treatment with...
of the controls. The determination of CFU-c was carried out as described above. SK-N-SH cells were incubated with 10 or 100 µCi m-[125I]IBG in 25-cm² flasks as already described. The surviving cell fraction was allowed to grow further in the flasks until a confluent monolayer was formed (within 2–3 weeks). Then, the cells were divided and seeded into two new flasks. The cells in one flask were allowed to grow without any further additives (controls), while the cells in the other flask were treated a second time with the same amount of m-[125I]IBG as the first time. The number of viable cells after this second treatment was determined after 10–12 days in culture.

Soft Agar Colony Assay. Pretreatment of cells with radioactively labeled mIBG was carried out as described above. The soft agar assay was performed according to the method of Hamburger and Salmon (9) with some slight modifications. Briefly, about 50,000 cells suspended in cell culture medium containing 0.3% agarose, 20% fetal calf serum, and 5 µg/liter insulin were transferred into six-well plates over a base layer (cell culture medium containing 20% fetal calf serum and 0.5% agarose). In some experiments, unlabeled or radioactively labeled mIBG was enclosed in the agar test system during the whole culture period (21 days). The number of colonies formed was expressed as a percentage of the controls. The determination of CFU-c was carried out as described (10).

RESULTS

Catecholamine Status of SK-N-SH and SK-N-LO Cells. Most neuroblastoma patients with elevated levels of catecholamines, it is possible to monitor their tumors with m-[123I]IBG. Analogous to this situation, SK-N-SH cells that can take up mIBG also produce catecholamines (dopamine, 18.3 ± 2 (SD) pg/10⁶ cells; noradrenaline, 50.4 ± 12.8 pg/10⁶ cells). By contrast, neither dopamine nor noradrenaline could be detected in SK-N-LO cells.

Storage of mIBG in SK-N-SH and SK-N-LO Cells. In order to correlate the cell killing effects with the amount of radioactively labeled mIBG incorporated into the cells (see below), it was determined how long mIBG could be stored in the neuroblastoma cell lines. In these investigations, SK-N-LO and SK-N-SH cells were incubated with different amounts of unlabeled mIBG (10–1000 nM). Subsequent to uptake, the release of radioactivity was monitored for 72 h, with the medium being changed every 24 h (Fig. 1). About 3–18% of the radioactivity originally present in the incubation medium was found after 72 h in SK-N-SH cells. The less the mIBG concentration used, the lower was the percentage of mIBG still present in the cells after this time, a finding which reflects the fact that the lower the mIBG concentration is, the higher is the portion that is specifically bound in SK-N-SH cells. The nonlinear course of mIBG-release in the semilogarithmic graph may be explained by the fact that cells continue to proliferate during the 72-h test procedure. New cells could therefore incorporate mIBG released previously from the cells already present in the beginning of the experiment. By contrast, no radioactivity could be found in SK-N-LO cells even 2 h after the end of the incubation (data shown in Ref. 7). The reason that SK-N-SH cells can store mIBG for a long time period should probably be sought in the presence of dense core particles in this cell line. Dense core particles have not been detected in SK-N-LO cells (data not shown).

In all further experiments investigating the cytotoxic effects of mIBG, SK-N-SH cells were used as an example of a neuroblastoma cell line that can take up and store mIBG. In addition, SK-N-LO cells were used in parallel as an example of a neuroblastoma cell line that cannot store mIBG in order to distinguish specific and nonspecific effects caused by mIBG.

Cytotoxic Effects of Unlabeled mIBG against SK-N-SH and SK-N-LO Cells. In order to clarify whether the cytotoxic effects generated by treatment with radioactively labeled mIBG are caused by the radioactive part of the molecule or by the substance itself, SK-N-SH and SK-N-LO cells were incubated with different amounts of unlabeled mIBG. As is depicted in Table 1, mIBG was toxic against both cell lines only when concentrations higher than 10 µM permanently present in the test system (soft agar assay) were used. As the experiments with radioactive labeled mIBG were carried out in the concentration range of 30 nM–3 µM, cytotoxic effects are entirely due to radioactivity (see below).

Cytotoxic Effects of m-[123I]IBG against SK-N-SH and SK-N-LO Cells. Investigations were carried out using m-[123I]IBG (specific activity, 20–30 mCi/mg) in the radioactivity range between 1 and 200 µCi. After 1 h incubation time in 5 ml cell culture medium, cells were transferred to six-well plates and grown for 6 days. Fig. 2 shows the concentration-dependent reduction of cell number after this time of growth: under conditions where SK-N-SH cells were clearly affected by the radioactivity, the proliferation of SK-N-LO cells was almost uninhibited. The amount of radioactivity present in SK-N-SH cells after transfer to the six-well plates during a single experiment is shown in Table 2. Combined with the storage data obtained in other experiments depicted in Fig. 1, it can be calculated that arbitrary use of 100 µCi m-[123I]IBG leads to only 5000–10,000 radioactive disintegrations in a single cell occurring during the 1-wk growth period.

SK-N-SH cells surviving the treatment with m-[123I]IBG underwent a drastic change of morphology (Fig. 3): cells with neuroblast-like morphology disappeared and only very large, flattened, tightly substrate-attached cells were still present. They proliferated slowly at first, then after two to four more days, a confluent monolayer was formed (within 2–3 weeks).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>mIBG (mm)</th>
</tr>
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<tbody>
<tr>
<td>SK-N-SH</td>
<td>5 ± 6</td>
</tr>
<tr>
<td>SK-N-LO</td>
<td>0.5 ± 0.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incubation time</th>
<th>CFU-c (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-N-SH</td>
<td>24 h</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>SK-N-LO</td>
<td>48 h</td>
<td>35 ± 6</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>72 h</td>
<td>35 ± 6</td>
</tr>
</tbody>
</table>

Table 1 Cytotoxic effects of unlabeled mIBG on SK-N-SH and SK-N-LO cells

Cells were incubated with the amount of mIBG indicated. In the test system of soft agar colony assay, mIBG was permanently present during the incubation time (21 days). The number of colonies formed is expressed as a percentage of controls (incubation without mIBG).

![Graph showing storage of mIBG (tracer: m-[123I]IBG) in SK-N-SH cells.](Fig. 1. Storage of mIBG (tracer: m-[123I]IBG) in SK-N-SH cells. The amount of mIBG still present in the cells 24, 48, and 72 h after its uptake is expressed as a percentage of the amount of mIBG originally present in the incubation system. A, 10 nM mIBG; B, 100 nM mIBG; C, 1 µM mIBG.)
Fig. 2. Dose-dependent killing of SK-N-SH and SK-N-LO cells after 1 h incubation time with m-[131I]IBG (specific activity, 20–30 mCi/mg). Number of viable cells is expressed as a percentage of controls (incubation without mIBG) after a subsequent growth period of 6 days as monolayer. Mean ± SD (bars) (three experiments).

Table 2 Radioactivity incorporated into SK-N-SH cells after a 1-h incubation
SK-N-SH cells were incubated with the amounts of m-[131I]IBG (specific activity, 20–30 mCi/mg) indicated for 1 h, then washed and 3 h later again and subsequently transferred into six-well plates (cell number seeded per well: 400,000). Radioactivity incorporated into SK-N-SH cells is expressed as dpm/400,000 and as an average of dpm found in a single cell.

<table>
<thead>
<tr>
<th>Incubation with m-[131I]IBG (μCi)</th>
<th>dpm/well</th>
<th>dpm/single cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36,200</td>
<td>0.09</td>
</tr>
<tr>
<td>10</td>
<td>221,000</td>
<td>0.55</td>
</tr>
<tr>
<td>100</td>
<td>1,100,000</td>
<td>2.75</td>
</tr>
<tr>
<td>200</td>
<td>1,200,000</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Fig. 3. SK-N-SH cells grown as monolayer. a, before treatment; b, 6 days after treatment with 100 μCi m-[131I]IBG (specific activity, 20–30 mCi/mg); x 360.

passages they stopped proliferation definitively. The disappearance of neuroblast-like SK-N-SH cells after treatment with m-[131I]IBG resembles the situation obtained after treatment with retinoic acid (11).

Effects of a Second Treatment with m-[131I]IBG on the Surviving SK-N-SH Cell Fraction. Surviving SK-N-SH cells that had previously been treated with 10 and 100 μCi m-[131I]IBG were divided into two flasks. The cells in one flask were allowed to grow further (controls), while the cells in the other flask were treated a second time with the same amount of m-[131I]IBG as the first time. Fig. 4 shows that the amount of cells surviving this second treatment, expressed in relation to controls, is a little higher (but not significantly; P > 0.1, Student’s t test) than after the first application of m-[131I]IBG.

Effects of the Specific Activity of m-[131I]IBG on Cell Killing. The cytoxicity of highly labeled m-[131I]IBG (20–30 mCi/mg) was compared with the cytotoxicity of low-labeled m-[131I]IBG (0.2–0.3 mCi/mg) on SK-N-SH and SK-N-LO cells drawing on the results of the 1-h incubation test system already described. In both cases, the incubation was carried out using 100 μCi (Fig. 5). The amount of radioactivity incorporated into SK-N-SH cells using low-labeled m-[131I]IBG was only about 5.2% of the amount of radioactivity incorporated using highly labeled m-[131I]IBG. Consequently, SK-N-SH cells incubated with low-labeled m-[131I]IBG survived to a much longer extent than did cells treated with highly labeled m-[131I]IBG.

Comparison of the Effects of m-[131I]- and m-[125I]IBG on SK-N-SH and SK-N-LO Cells. SK-N-SH and SK-N-LO cells were both incubated with 100 μCi m-[131I]- and m-[125I]IBG for 1 h. The soft agar assay and the standard monolayer assay were both used as test system and their results compared (Fig. 6). No significant differences could be observed concerning the effects of m-[125I]- and m-[131I]IBG.

Influence of Long-Term Incubation with m-[131I]IBG on SK-N-LO Cells and on CFU-c. The most prominent side effect of therapy with 131I is the cytotoxicity manifested against bone...
DISCUSSION

The therapy of neuroblastoma state IV remains unsatisfactory despite the development of some new approaches, e.g., bone marrow transplantation. Another attempt is the treatment with highly labeled m-[131I]IBG. Although mIBG is taken up by the tumors of more than 80% of catecholamine-positive patients, the therapeutic success in neuroblastoma treatment with m-[131I]IBG still remains poor (5). More information about the interaction of mIBG with neuroblastoma cells is necessary for an improvement of the therapy. We reported here our results obtained with in vitro investigations about the effects of unlabeled and radioactively labeled mIBG on two neuroblastoma cell lines (SK-N-SH and SK-N-LO). SK-N-SH cells were used as an example of a neuroblastoma cell line that can take up and store mIBG and SK-N-LO cells as an example of a neuroblastoma cell line that cannot. The reason for this different behavior is probably the different degree of differentiation found in the two cell lines, especially their catecholamine status. SK-N-SH cells were derived from a vanillylmandelic acid-producing neuroblastoma of a 4-year-old girl (12). Subclones of these cells (S-cells, substrate-adherent cells with epithelial- or fibroblast-like morphology) and N-cells (neuroblast-like cells) that undergo bidirectional morphological and biochemical interconversion in culture (13) could be obtained. In contrast to S-cells, N-cells contain dense-core vesicles and are tyrosine-hydroxylase and dopamine-β-hydroxylase positive; tyrosinase was found in S-cells indicating that melanocytic differentiation could also be possible (14).

Our in vitro investigations demonstrated that, under conditions where SK-N-LO cells can still survive, SK-N-SH cells can be destroyed by radioactively labeled mIBG in a dose-dependent manner. The cytotoxic effect is caused by the radioactive part of the substance and not by mIBG itself, since unlabeled mIBG proved not to be toxic to SK-N-SH cells in the concentration range used in the experiments with radioactively labeled mIBG. Unlabeled mIBG was equally toxic against both SK-N-SH and SK-N-LO cells, but only in much higher concentrations. This cytotoxic effect of unlabeled mIBG on neuroblastoma and other tumor cells was recently investigated in more detail (15). Several items of evidence suggest that the different radiotoxicity of labeled mIBG to SK-N-SH and SK-N-LO cells is caused by its specific storage in SK-N-SH cells and not by unspecific effects occurring during the 1-h incubation time allotted for mIBG uptake. Evidence for this interpretation is provided by the fact that the 1-h incubation time with 100 μCi had only a slight influence on the survival rate of SK-N-LO cells, although this latter cell line was found to be more radiosensitive than SK-N-SH cells. SK-N-LO cells were more effectively destroyed than SK-N-SH cells by treatment with m-[131I]- and m-[125I]IBG when both substances were present in the incubation solution for long time intervals (24 hours). Furthermore, after a 1-h incubation time for each 100 μCi m-[131I]IBG, the survival rate of SK-N-SH cells was heavily dependent on the specific activity of mIBG used, i.e., on the amount of radioactivity incorporated into the cells.

The fact that after a second treatment with m-[131I]IBG a relatively greater part of SK-N-SH cells can survive than after the first treatment is at the first sight surprising, for it might be expected that following in the wake of initial damage a relatively greater part of SK-N-SH cells can survive than after the first treatment. The 1-h incubation time with 100 μCi m-[131I]IBG, a reduction of the colony number to 58 ± 5 and 50 ± 6% (n = 3) (expressed as a percentage of the controls) has been observed.

C. Laubenbacher, H. Glässner, and R. Senekowitsch, personal communication.
population is also less sensitive to a second treatment with m-[\textsuperscript{131}I]MBG.

We further investigated if m-[\textsuperscript{125}I]MBG is more toxic against the two neuroblastoma cell lines than is m-[\textsuperscript{131}I]MBG. Although an exact comparison was not possible because the specific activities of both substances were not necessarily the same (in both cases the activities were only given as 20–30 mCi/mg), it is evident that the effects of both radioactive compounds are almost identical under the in vitro test conditions prevailing. \textsuperscript{131}I emits \(\beta-\) and \(\gamma-\)radiation of high energy, whereas \textsuperscript{125}I decays by electron capture resulting in \(\gamma\) and conversion and Auger electron emissions that are characterized by high linear energy transfer (16) and that cause a highly localized energy deposition (within a radius of 25 nm around the site of decay) (17). Thus, the cytotoxicity of \textsuperscript{125}I strongly depends on its intracellular location: only about 100 or fewer decays are necessary in order to get the dose at which 37\% of the cells survive if \textsuperscript{125}I is incorporated into the DNA (via \textsuperscript{125}Iiododeoxyuridine (18, 19). In contrast, its cytotoxicity outside the cell nucleus is much less pronounced; therefore, more disintegrations are necessary to get the same effects (20, 21).

Due to the biochemical and pharmacological features of mIBG it can be expected that it is not incorporated into the cell nucleus but rather into dense core particles. Therefore, under the test conditions described above using m-[\textsuperscript{125}I]IBG, about 1000–3000 decays were necessary to achieve the 37\% cell survival dose. In summary, apart from practical problems in clinical application because of its long radioactive half-life, the use of m-[\textsuperscript{125}I]IBG does not have any radiobiological advantage compared to m-[\textsuperscript{131}I]IBG.

Since only part of the neuroblastoma cells are obviously able to incorporate and store mIBG, its use in neuroblastoma therapy may be restricted. However, it is not absolutely necessary that all neuroblastoma cells within a tumor must take up mIBG in order to be killed. Because of the long-distance effects of m-[\textsuperscript{131}I]IBG, all that is needed for a sufficient amount of radioactivity to be accumulated within the tumor. It was additionally proposed to use mIBG as an agent for single cell killing in connection with disseminated neuroblastoma. However, the amount of m-[\textsuperscript{131}I]IBG that can be taken up by a single cell, the particular characteristics of \textsuperscript{131}I, and the intracellular localization of mIBG do not seem to be well suited for such a purpose. Moreover, the most important limitation is that not all neuroblastoma cells can take up mIBG.

Finally, care must be taken in using m-[\textsuperscript{131}I]IBG in patients with bone marrow contaminate with neuroblastoma cells because of the high sensitivity of bone marrow stem cells to \textsuperscript{131}I radiation. Indeed, the severely inhibiting effects exercised by m-[\textsuperscript{131}I]IBG on colony formation in cases of high-term cultures in CFU-c can easily give a false impression of the in vitro situation, even in the case of a bone marrow severely contaminate with neuroblastoma cells, because in the in vitro situation (described in “Results”) the radioactivity was permanently present in the test assay. In vivo, a continuous release of mIBG out of the cells occurs. Nevertheless, the formation of colonies in CFU-c was also significantly reduced during a short-term incubation with m-[\textsuperscript{131}I]IBG.

In summary, we suggest, that m-[\textsuperscript{131}I]IBG should be used in neuroblastoma therapy with patients that were previously found to be positive in mIBG scintigraphy but should not be used in cases with severe bone marrow contamination. With regard to its further clinical application, improvements in mIBG-therapy might be possible: e.g., therapy in combination with drugs that allow prolonged storage in neuroblastoma tissues; in combination with differentiation factors in order to raise the number of cells that can store mIBG; or the use of m-[\textsuperscript{131}I]IBG with very high specific activity.

REFERENCES


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