Meta-iodobenzylguanidine ([131 I] MIBG) related thrombocytopenia

Tytgat, G.A.M.

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Chapter 1

Introduction
# Chapter I: Introduction

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NEUROBLASTOMA

I. Introduction

Neuroblastoma is one of the more common solid tumors of childhood. In Europe, the incidence of neuroblastoma is 9.6 per million children per year (1). It displays a remarkable spectrum of clinical behavior; on the one hand there are the highly malignant, therapy resistant tumors with a very bad prognosis and on the other hand it has the highest rate of spontaneous remission described for a malignant neoplasm (2). Such spontaneous remissions occur predominantly in the youngest age group and are characterized by disappearance of the tumor and its metastases or by maturation to a fully differentiated cell type (ganglioneuroma) (3).

I. Pathology

Neuroblastoma was first described in 1864 by a German pathologist Virchow (4). In 1910 the similarity between neuroblastoma tumors and adrenal medulla cells was described by Wright (5). Neuroblastoma is one of the "small, round, blue cell tumors of childhood". The origin of this embryonal tumor is found in the sympathetic nervous system, it is derived from primordial neural crest cells, which ultimately form the sympathetic ganglia, adrenal medulla and other sites. Normally, neuroblasts that form the adrenal medulla, develop into cells that produce and store norepinephrine (chromaffin cells). A neuroblastoma is a highly cellular tumor in which rosettes are visible, which contain a tangle of neurofibrillary material (6). Apart from neuroblastoma there are two variants, ganglioneuroblastoma, a mixed tumor, which also contains mature ganglion cells in addition to undifferentiated neuroblastoma cells, and the benign and fully differentiated ganglioneuroma. It is thought that neuroblastoma develops from immature neuroblasts, ganglioneuroma from more differentiated sympathetic ganglion cells, and phaeochromocytoma from differentiated catecholamines producing chromaffin cells.

I. Biochemistry

Like the postganglionic nerves of the sympathetic nervous system and adrenal medullary cells, neuroblastoma cells have the ability to synthesize, store and release catecholamines (i.e. epinephrine, norepinephrine, and dopamine). The primary metabolite of epinephrine and norepinephrine is 4-hydroxy-3-methoxymandelic acid (HMMA), also known as vanillylmandelic acid (VMA), and the primary metabolite of dopamine is 2-hydroxy-3-methoxyphenylacetic acid (HPMA), that is also known as homovanillic acid (HVA). These metabolites are elevated in most of the patients at diagnosis. Furthermore, the majority of the neuroblastoma tumors contain the norepinephrine transporter (NET). This transport protein is normally expressed in sympathetic nerve endings, where it takes up the norepinephrine, after it has been released in the synaptic junction. This transport system is used when neuroblastoma are visualized or treated by radio-
iodinated meta-iodobenzylguanidine (MIBG). This will be further discussed under MIBG treatment (section II and IV).

I.IV Clinical features

Most patients with neuroblastoma present with symptoms caused directly by the primary tumor or metastases (table 1). The primary tumor is in ± 40% situated in the adrenal medulla and in ± 30% in the abdominal paraganglia or in the sympathetic nervous side chain. Other sites of the primary tumor are the thoracic sympathetic side chain (± 15%), the cervical (± 8%) and in the pelvic sympathetic trunk (± 7%). Only about one third of patients have localized disease at presentation; two thirds have metastases. The most common metastatic sites in patients under 12 months of age are subcutis, liver and bone marrow. In older patients neuroblastoma frequently metastasizes to lymph nodes, bone marrow, orbita, bone and dura.

Table 1 Clinical features of neuroblastoma *

<table>
<thead>
<tr>
<th>Primary tumor</th>
<th>Clinical feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdomen and/ or pelvis</td>
<td>Discomfort; fullness; anorexia and vomiting</td>
</tr>
<tr>
<td></td>
<td>Palpable mass; abdominal pain</td>
</tr>
<tr>
<td></td>
<td>Urinary dysfunction; constipation or fecal incontinence</td>
</tr>
<tr>
<td>Paraspinal (local)</td>
<td>Back pain; cord compression; scoliosis; sphincter dysfunction</td>
</tr>
<tr>
<td>Paraspinal : Thoracic</td>
<td>Dyspnea or dysphagia</td>
</tr>
<tr>
<td></td>
<td>Thoracic inlet obstruction and vena cava superior syndrome</td>
</tr>
<tr>
<td>Paraspinal: Cervical</td>
<td>Horner’s syndrome, palpable tumor</td>
</tr>
<tr>
<td>Metastases</td>
<td>Clinical feature</td>
</tr>
<tr>
<td>Bone</td>
<td>Pain; palpable mass</td>
</tr>
<tr>
<td>Orbita</td>
<td>Uni- or bilateral periorbital ecchymosis and proptosis</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Ranging from asymptomatic to anemia, leukopenia and thrombocytopenia</td>
</tr>
<tr>
<td>Liver</td>
<td>Abdominal distention with or without respiratory problems</td>
</tr>
<tr>
<td></td>
<td>Jaundice</td>
</tr>
<tr>
<td>Skin</td>
<td>Non-tender bluish subcutaneous nodules</td>
</tr>
<tr>
<td>Placental</td>
<td>Fetal: anemia, hydramnion or hydrops fetalis, fetal death.</td>
</tr>
<tr>
<td></td>
<td>Maternal: hypertension, palpitations, signs of (pre-) eclampsia.</td>
</tr>
<tr>
<td>Neurological effects</td>
<td>Paraplegia due to cord compression; ataxia; opsomyoclonus</td>
</tr>
<tr>
<td>Metabolic effects</td>
<td>Hypertension; flushing; irritability; sweating; diarrhea</td>
</tr>
</tbody>
</table>
I.V Clinical staging and risk grouping.

Age is an important factor for the neuroblastoma patients; children of all stages over the age of one year have an inferior prognosis than younger infants. Several staging systems are being used, the oldest is the Evans staging system (8) which still is being used but which is being replaced by the International Neuroblastoma Staging System (INSS) (9). In both systems, the staging of the neuroblastoma is based on findings by physical examination, nuclear imaging (131-I-MIBG, for the INSS), abdominal ultrasound and bone marrow analysis, but the INSS includes surgical intervention to demonstrate metastases, e.g. node positivity. The Shimada system is a histological staging of the neuroblastoma, which includes also the age of the patient, as well as the mitosis-karyorhexis index to include prognostic factors (10).

Biochemical markers that correlate with poor outcome are serum ferritin (11), lactic dehydrogenase (12,13), neuron-specific enolase (14) and the pattern of urinary catecholamine excretion (15,16).

Recent advances in understanding of the biology and genetics of neuroblastoma has led to the insight that specific genetic changes allow tumors to be classified into subsets with distinct clinical behavior. Tumors with a near-triploid chromosome number have been associated with a favorable outcome, although this appears to be useful primarily for patients less than one year of age with advanced stages of disease (17). Amplification of MYCN correlates with advanced stages of disease, rapid tumor progression and poor prognosis (18,19). Gain of chromosome 17q appears also to be associated with a more aggressive subset of neuroblastomas, but it is found in all age and stage groups (20, 21). Gain of 17q is often involved in a novel mechanism leading to allelic loss of chromosome 1p (22), and this loss of heterozygosity for chromosome 1p shows again a strong correlation with poor event-free survival of neuroblastoma patients (23).

So to be able to classify the patients in groups that indicate not only the stage, but also the prognosis and therefore the therapy, the Pediatric Oncology Group (POG) and Children’s Cancer Group (CCG), now together the childrens oncology group (COG), have described three groups based on the INSS stage, patients age, MYCN copy number, DNA index and Shimada histology (24) (table 2). Therapy for neuroblastoma ranges from no therapy at all to the use of a combination of extensive surgery, intensive chemotherapy with bone marrow rescue and sometimes radiotherapy.

I.VI Therapy and prognosis.

Treatment of localized neuroblastoma: the low-risk group (table 2A). This group accounts for 36% of all patients with neuroblastoma. Patients of any age should first be treated with surgery alone. Adjuvant chemotherapy after surgery for infants with localized neuroblastoma is not justified. In the event of incomplete resection, it is becoming increasingly controversial (25, 26, 27) but can be justified in patients with unfavorable biological features. Radiotherapy may be
beneficial in patients whose tumors show MYCN amplification, or in the event of local relapse associated with other unfavorable biological features. The long term event free survival for patients < 1 year is > 90%, and for patients > 1 year it is 75 – 90% (27, 28, 29, 30, 31).

Treatment of unresectable neuroblastoma: intermediate-risk group (table 2B). This group accounts for 10% of all patients. They have a 74% survival rate when given conventional chemotherapy followed by surgery without radiotherapy (24).

Treatment of the high-risk group (table 2C). These patients are the largest group, comprising 54% of all cases, and up to now the long-term survival is maximally 27% despite intensive chemotherapy, surgical resection, and myoablative therapy.

Table 2: Neuroblastoma COG prognostic groups *

Table 2A: low-risk group

<table>
<thead>
<tr>
<th>INSS stage</th>
<th>Patient age(years)</th>
<th>MYCN Status</th>
<th>DNA Index</th>
<th>Shimada histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-21</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>2A/2B</td>
<td>1-21</td>
<td>Non-amplified</td>
<td>NA</td>
<td>Favorable</td>
</tr>
<tr>
<td></td>
<td>1-21</td>
<td>Amplified</td>
<td>NA</td>
<td>Favorable</td>
</tr>
<tr>
<td>4S</td>
<td>&lt;1</td>
<td>Non-amplified</td>
<td>Hyperdiploid</td>
<td>Favorable</td>
</tr>
</tbody>
</table>

Table 2B: intermediate-risk group

<table>
<thead>
<tr>
<th>INSS stage</th>
<th>Patient age(years)</th>
<th>MYCN Status</th>
<th>DNA Index</th>
<th>Shimada histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>&lt;1</td>
<td>Non-amplified</td>
<td>Any</td>
<td>Favorable</td>
</tr>
<tr>
<td></td>
<td>1-21</td>
<td>Non-amplified</td>
<td>NA</td>
<td>Any</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1</td>
<td>Non-amplified</td>
<td>Any</td>
<td>Favorable</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>Non-amplified</td>
<td>Diploid</td>
<td>Favorable</td>
</tr>
<tr>
<td>4S</td>
<td>&lt;1</td>
<td>Non-amplified</td>
<td>Hyperdiploid</td>
<td>Unfavorable</td>
</tr>
</tbody>
</table>

Table 2C: high-risk group

<table>
<thead>
<tr>
<th>INSS stage</th>
<th>Patient age(years)</th>
<th>MYCN Status</th>
<th>DNA Index</th>
<th>Shimada histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A/2B</td>
<td>1-21</td>
<td>Amplified</td>
<td>NA</td>
<td>Unfavorable</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>Amplified</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>3</td>
<td>1-21</td>
<td>Non-amplified</td>
<td>NA</td>
<td>Unfavorable</td>
</tr>
<tr>
<td></td>
<td>1-21</td>
<td>Amplified</td>
<td>NA</td>
<td>Any</td>
</tr>
<tr>
<td></td>
<td>&lt;1</td>
<td>Amplified</td>
<td>Diploid</td>
<td>Any</td>
</tr>
<tr>
<td>4</td>
<td>1-21</td>
<td>Any</td>
<td>NA</td>
<td>Any</td>
</tr>
<tr>
<td>4S</td>
<td>&lt;1</td>
<td>Amplified</td>
<td>Any</td>
<td>Any</td>
</tr>
</tbody>
</table>

NA, not applicable.
* Deduced from Pearson et Philip (24).
Different treatment schedules can be used but treatment schedules were usually divided into three phases: induction therapy, surgery and post surgical treatment. The induction therapy can comprise platinum-based therapy, followed by increasing dose-intensive chemotherapy combinations, myoablative therapy and treatment for minimal residual disease with for example retinoic acid. The different treatment schedules were reviewed by Matthay (32), and Hartmann and Berthold (33). In most of the treatment centers in the Netherlands patients with newly diagnosed irresectable neuroblastoma are being treated with $^{[1^{3}]\text{I}} \text{MIBG}$ (see below section II.II).

II MIBG

II.1a Introduction

Meta-Iodobenzylguanidine (MIBG) is a derivative of the neuron blocking agents bretylium and guanethidine with a structure analogy to norepinephrine (34). It contains an iodinated benzylring with a diamine side-chain, which is positively charged at physiological pH (Fig. 1).

It was developed for imaging the adrenal medulla. When radiolabeled with $^{131}$I or $^{123}$I, MIBG can be used to visualize tumors of neuroadrenergic tissues such as neuroblastoma, pheochromocytoma, paraganglioma, medullary thyroid carcinoma and carcinoids (35,36). MIBG is specifically taken up in neural crest tumors by the norepinephrine transporter (NET), which is the presynaptical re-uptake system of norepinephrine, previously called "uptake-1" (37). Cumulative findings of $^{[1^{3}]\text{I}} \text{MIBG}$ scintigraphy indicate that 91.5% of neuroblastoma concentrate MIBG (38). Higher doses of $^{[1^{3}]\text{I}} \text{MIBG}$ with a high specific activity may be used for the treatment of these tumors. In the Netherlands $^{[1^{3}]\text{I}} \text{MIBG}$ is being used as the first-line treatment for inoperable neuroblastoma, before surgery or chemotherapy, in order to obtain operability (39, 40).

II.1b Biodistribution and pharmacology

After intravenous administration of radioiodinated MIBG, radioactivity is rapidly distributed from the vascular compartment (41, 42), followed by a slow redistribution from the peripheral compartment into the central compartment. Glomerular filtration rate has a major influence on MIBG pharmacokinetics and MIBG is likely to be also excreted by a tubular secretion mechanism (43). During MIBG therapy adequate hydration is therefore propagated. After therapeutic doses
plasma concentrations of MIBG are < 0.1 mM (42, 44). Two components of MIBG uptake have been identified:

1. specific uptake via the NET that is saturable, has a high affinity for MIBG and is sodium-, temperature- or energy-dependent and

2. nonspecific uptake that is nonsaturable, has a low affinity for MIBG and is not sodium-, temperature- or energy-dependent (45).

In the neuroblastoma, MIBG is being taken up from the plasma via the NET, and it is subsequently stored in neurosecretory granules (37). Neuroblastoma contains few neurosecretory granules, and the retention of MIBG in neuroblastoma is thus a constant fraction of the loading level, being a dynamic equilibrium of release and re-uptake (46). The maximal cell loading of MIBG in the neuroblastoma is determined by short incubations at high concentrations, so in clinical practice, short infusions (up to 2 hrs maximum or bolus injections) are propagated. Distribution of radioactivity 24 hr after [131]I-MIBG or [123]I-MIBG administration showed uptake in the salivary glands, spleen, heart and liver and visualization of the urinary bladder. Visualization of the salivary glands, heart and the spleen was due to extensive sympathetic innervation of these organs, whereas the liver was probably depicted because of its volume, vascularity and extraction capacity (47, 48). Lungs, colon and kidneys were less frequently visualized and thyroid was only visualized when pre-treatment with non-radioactive iodide, to completely block the thyroid, had been inadequate or omitted (48).

II.Ic Pre-dosing with unlabeled MIBG.

Unlabeled MIBG used as pre-dose, given prior to administration of [131]I-MIBG changes the biodistribution and led to highly improved selective tumor targeting up to 70% of the subsequent [131]I-MIBG dose in carcinoid patients (36, 50). As a result the relative uptake of [131]I-MIBG is increased in a number of these patients, initially not qualifying for [131]I-MIBG therapy, to levels permitting treatment with the radiopharmaceutical (51, 52). In neuroblastoma patients, and in neuroblastoma xenografted mice this predosing with unlabeled MIBG decreased the uptake of [131]I-MIBG in normal tissues, whereas the tumor uptake was not affected, enhancing the tumor/non-tumor ratio (dr C. Hoefnagel personal communication, 52, 53).

II.II Clinical application
II.IIa The use of [131]I-MIBG.

For therapy MIBG radiolabeled with 131I is used, which decays with a physical half life of 8 days, emitting gamma rays (364 keV) and beta particles (250 – 619 KeV). These gamma rays escape the body and can be used for scintigraphic imaging, and the beta particles deposit their energy locally (maximum range 2.4 mm), i.e. as cross fire in the targeted and neighboring cells. [131]I-MiBG can be given upfront, as first treatment before chemotherapy. Its cross-fire effect
(radiation-induced cell damage caused by radionuclides localized in neighboring cells) is the reason that $^{131}$I-MIBG is being used for large and bulky tumors, as first-line treatment before chemotherapy. In the Netherlands the patients receive 2 to 4 cycles $^{131}$I-MIBG, after which the patients are being operated in order to remove the primary tumor. This is followed by chemotherapy, ablative chemotherapy and autologous bone marrow infusion. Up to now J. de Kraker reported on the use of $^{131}$I-MIBG in 66 patients. Among those, 47 were stage 4, 15 stage 3, three stage 2A and four stage 4S patients according to INSS criteria (39, 40). The results are about the same as with intensive preoperative chemotherapeutic schemes. Long lasting complete responses are still rare after $^{131}$I-MIBG treatment (39, 40, 55), however with few side effects (39, 55). The condition of the patients improved dramatically, as is expressed by relief of pain within 24 to 48 hours, and weight gain of 3 – 11 % (39).

Patients with a recurrent neuroblastoma after previous treatment with chemotherapy and high-dose chemotherapy followed by stem cell rescue, are being treated with $^{131}$I-MIBG. First a group of 36 patients was treated with $^{131}$I-MIBG, and although there were ultimately no survivors, complete remissions were temporarily obtained and prolongation of life was achieved (56). Again the palliative effect of this treatment was considerable; the major toxicity was thrombocytopenia, especially in the patients that had been pre-treated with platinum (56). In order to achieve an increase in life expectancy of this group of patients, hyperbaric oxygen treatment was added to this schema (57). Comparing these two groups of patients, with and without oxygen, a 2-year survival rate of 74 versus 45% was attained, but there are long survivors up to 11 years that received first MIBG (57). In order to increase the effectiveness of the $^{131}$I-MIBG / hyperbaric oxygen therapy, a phase II study was started in 1997, in which the patients are given regular doses of vitamin C. Vitamin C is a substance known to have anti-oxidant properties, which protects cells against oxidative stress. However, under certain conditions vitamin C can also act as a pro-oxidant, e.g. in the presence of Fe$^{+2}$, vitamin C can give rise to the formation of free radicals. Neuroblastoma contains high levels of ferritin (which is an iron binding protein), and addition of vitamin C to neuroblastoma cells causes extensive free radical related damage within the cells (58, 59). The results of the study have to be awaited.

$^{131}$I-MIBG has also been used as part of an intensive consolidation approach after conventional chemotherapy (54, 60). This has led to a prolonged disease-free survival, but after 5 years only one patient out of 13 remained alive (60). In a phase I dose escalation study of $^{131}$I-MIBG with autologous bone marrow support, Matthay and colleagues reported a response rate of 37%, but with two toxic deaths (54).

The relationship between the presence of the NET and the $^{131}$I-MIBG uptake capacity in the neuroblastoma is being studied in a multicenter european study by Dr R. Mairs (Glasgow university) and colleagues using reversed transcriptase polymerase chain reaction (RT-PCR). We have analyzed tumor samples (one or more per patient) for the presence of the NET and the vesicular monoamine
transporter 2 (VMAT 2). The latter indicates the presence of storage granules. In our center, samples from 24 patients have been analyzed (see table 3), and it is clear that the presence of the NET strongly correlates with $[^{131}I]$MIBG uptake in the tumor. None of the patients with a negative MIBG scan showed expression of the NET. One patient with a positive MIBG scan showed no presence of the NET in the tumor sample. If the NET expression had been the golden standard, this patient would not have been treated with MIBG. We therefore advise to rely on a diagnostic $[^{131}I]$MIBG or $[^{123}I]$MIBG scan, prior to the planning of $[^{131}I]$MIBG therapy, and not to rely on the RT-PCR of the NET.

Table 3: Expression of NET and VMAT2 in neuroblastoma tumor biopsies.

<table>
<thead>
<tr>
<th>NET / VMAT2 Expression</th>
<th>MIBG scan positive</th>
<th>MIBG scan negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>++ ++</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>+ / -</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>- / +</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>- / -</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

II.11b The use of $[^{125}I]$MIBG.

The isotope $^{125}$I emits internal conversion and Auger electrons with ultrashort track lengths (< 0.5 mm). The Auger electrons are extremely radiotoxic, provided that they originate within nm distance of target DNA (61). Based on this concept $[^{125}I]$MIBG was given to patients in an attempt to destroy micro-metastases, with negligible radiation exposure of non-targeted neighboring cells (62, 63). In the absence of intracellular concentration, the toxicity of $[^{125}I]$MIBG should be relatively low (64). Sisson hypothesized that 4 mCi of $[^{125}I]$MIBG should cause the same injury as 1 mCi of $[^{131}I]$MIBG (64). The results were not encouraging, and the major toxicity was again hematological toxicity, being prevalent thrombocytopenia. Rutgers et al. demonstrated that $[^{125}I]$MIBG had no therapeutic advantage over $[^{131}I]$MIBG, in mice with a microscopic neuroblastoma (61).

II.111 The use of non-radiolabeled MIBG.

A totally different action mechanism of MIBG is being studied by others (65, 66, 67). The main conclusions of these investigations are being described below.

High doses of non-radiolabeled MIBG appeared to be effective in palliation of the carcinoid syndrome, demonstrating only mild and transient side effects (65). The mechanism of palliation is unknown, but the replacement of serotonin by unlabeled MIBG in the storage granules in the tumor may reduce the sudden release of large amounts of serotonin and the accompanying symptoms. Up till now doses of 135 mg/m² are given intravenously without many side effects (dr. B. Taal personal communication). In carcinoid patients the side effects were stress-related
responses like changes in blood pressure, dizziness and paleness, which disappeared after stopping the infusion (65).

An in vitro study on the cytotoxicity of unlabeled MIBG revealed that it had anti-proliferative effects against a large number of cell lines of various origins. The anti-proliferative effects of MIBG might result from inhibition of the mitochondrial respiratory chain (66), complex I and III (67) as was described by Cornelissen et al. Because of this inhibition, the succinate driven ATP synthesis was inhibited (67), and MIBG caused oxidative stress and up-regulation of anti-oxidant enzymes (68).

Because of the mitochondrial inhibition, the cells are dependent on anaerobic glycolysis for their ATP production. This results in enhanced glucose consumption, increased lactate production and reduced pH. Various strategies to improve the therapeutic index of anti-cancer drugs are based on this phenomenon, as several anti-cancer drugs have higher cytotoxicity at an acidic pH (69, 70).

### III THROMBOCYTOPENIA

The major toxicity of radiolabeled MIBG therapy in patients that have been intensively pre-treated with chemotherapy, with platinum derivates, is hematological, with an often-isolated thrombocytopenia (54, 62, 71, 72, 73).

#### III.I Radiotoxicity of $^{[131]}$I MIBG therapy

After treating 90 patients, all pre-treated with platinum containing chemotherapy regimens, Lashford and colleagues reported that they found that the main toxicity was bone marrow suppression (73). They describe a significant association between prescribed whole-body-absorbed radiation dose and marrow toxicity, and even in this study, the whole-body-absorbed radiation dose measured during therapy was less than the prescribed whole-body-absorbed dose (73). At a whole-body dose of 1.0 Gy, no major toxicity was observed, at 2.0 Gy 31% of the patients developed grade 3 or 4 thrombocytopenia, and at 2.5 Gy 80% (73). Sisson reported that in case of $^{[131]}$I MIBG therapy, the cGy dose of whole-body radiation was predictive of thrombocytopenia (62). However for $^{[125]}$I MIBG treatments, absorbed doses of whole-body radiation had virtually no value in forecasting the changes in circulating platelets, and also blood dosimetry for $^{[125]}$I MIBG had little or no correlation (62). The group of Matthy reported on the use of $^{[131]}$I MIBG in a dose escalation study, defining dose-limiting toxicity without and with autologous bone marrow support (54). In this study it was shown that the level of toxicity was similar to that reported before by Lashford (73), and that increased toxicity at higher whole-body irradiation doses was seen with 15 and 18 mCi/kg. 12 mCi/kg was the maximum tolerable dose without bone marrow
rescue. However nine patients, including three at the 12 mCi/kg level, never recovered platelet independence before death, even though four of these received autologous bone marrow reinfusion (54). In contrast Hoefnagel, using a fixed dose of $^{131}$I-MIBG, hence varying absorbed whole body dose, described therapy-induced thrombocytopenia in 31 of 53 $^{131}$I-MIBG-treated patients (that were also pretreated with chemotherapy) (71). Isolated thrombocytopenia was not clearly correlated with the degree of MIBG-storing tumor deposits within the bone marrow, nor with the whole-body radiation dose (71), although in case of bone marrow invasion a correlation with depression of the whole bone marrow was found (71).

### III.II Other possible causes of thrombocytopenia.

So even though it is known that neutropenia and thrombocytopenia are also common complications of extended-field radiotherapy and of radiolabeled immunoglobulin therapy, the question still arises if the whole-body-absorbed dose is the main cause of the $^{131}$I-MIBG therapy associated toxicity.

Trying to explain and calculate the risk for this thrombocytopenia, it appeared that the absorbed doses to the blood were small and much lower than the whole-body dose (74), because the $^{131}$I-MIBG was cleared rapidly from the circulation (45, 75). Sisson further described that marrow aspirates of three patients contained quantities of radioactivity similar to those in the respective blood specimens obtained at the same time. It was therefore not clear how the bone marrow received so much radiation (74).

For some tumors, radiolabeled immunoglobulin therapy is a cancer treatment that seems more selective than its predecessors (76). In a review, Vriesendorp and coworkers described that the dose-limiting side effects of radiolabeled immunoglobulin therapy are hematological: granulocytopenia and thrombocytopenia (76). The isotopes used are Iodine-131, Indium-111 and Yttrium-90. So of the three bone marrow cell lineages, the thrombocytic lineage is the most sensitive for radionuclide therapy. In contrast to these isotopes, after the use of an Iodine-125 labeled monoclonal antibody in patients with advanced colon cancer, doses up to 350 mCi/m$^2$ did not cause bone marrow toxicity (77). Comparing the use of $^{125}$I-MIBG and $^{131}$I-MIBG, because of the low penetrating capacity of the $^{125}$I derived electrons, and the presumed absence of intracellular concentration of $^{125}$I-MIBG in the hematopoietic cells, the toxicity of $^{125}$I-MIBG should be relatively low (64). Theoretically, 4 mCi of $^{125}$I-MIBG should cause the same injury as 1 mCi of $^{131}$I-MIBG (64). In the case of the iodine-125 labeled monoclonal antibody this hypothesis was correct, but using $^{125}$I-MIBG the major and dose limiting toxicity was again thrombocytopenia, at much lower mCi doses than was expected (62).

The thrombocytopenia, although never extensively described, can last for weeks to months. Matthay described that nine patients never recovered platelet independence, although four of those had received autologous bone marrow reinfusion (54). Most of the patients with recurrent
neuroblastoma had previously been treated with platinum derivates. The radioactive MIBG reactivates the platinum derivates in the kidneys, and gives rise to temporarily impaired renal function, and thus the excretion of the MIBG was slower in these patients, than in patients treated with MIBG upfront. In the patients that received $^{[^{131}]\text{I}}$MIBG without previous chemotherapy, the thrombocytopenia following $^{[^{131}]\text{I}}$MIBG treatment is a lesser problem, but if a patient was treated with ablative chemotherapy followed by autologous bone marrow infusion, the thrombocytopenia can persist for months (personal communication dr. J. de Kraker).

This all suggests that the thrombocytopenia in addition to whole body exposure can be the result from specific $^{[^{131}]\text{I}}$MIBG related toxicity to the precursors of the platelets, the megakaryocytes.

It is known that platelets have the capacity to accumulate MIBG (78, 79). So it is possible that the precursor of the platelets, the megakaryocytes, can selectively bind, or accumulate, $^{[^{131}]\text{I}}$MIBG to a level that would cause injury to these cells, but the quantity would not be apparent in an assay of overall bone marrow radiotoxicity.

Furthermore, a patient was described, in whom a bone scan was associated with abnormal $^{[^{131}]\text{I}}$MIBG uptake in the femoral bone marrow. Instead of an initial diagnosis neuroblastoma, a megakaryocytic leukemia was eventually diagnosed (80).

**IV TRANSPORTERS**

**IV.1 Introduction**

The family of the monoamine transporters is a large family and comprised transporter systems such as the Norepinephrine transporter (NET), Dopamine transporter (DAT) and Serotonin transporter (SERT) (81). These transporters exhibit the pattern of 12 hydrophobic membrane segments, probably transmembrane domains (80) with a predicted topology suggesting NH$_2$ and COOH termini inside the cell and a large glycosylated extracellular loop between transmembrane domains 3 and 4 (81). These transporters are ATP, and temperature dependent, and Na$^+$ and Cl$^-$ coupled (81). In this family the transporters of the NET and SERT are the most closely related (81). In the next chapter, the most important properties of these proteins and the known MIBG uptake capacity will be described.

**IV.11 Norepinephrine transporter**

Pacholczyk first cloned this transporter from the human neuroblastoma cell line SK-N-SH (82). The NET was expressed in COS cells and $^{[^{125}]\text{I}}$MIBG was used to demonstrate the uptake (82). MIBG is very efficiently accumulated by this transporter, in vitro the highest uptake levels were achieved by short time incubations at high dose (40). Because of the lack of many storage granules, the level of retained drug is always a constant fraction of the loading level (47), since these cells retain MIBG by a dynamic equilibrium of release and re-uptake (49). Selective inhibitors of the NET are desipramine and cocaine (82, 83).
Figure 2, deduced from Blakely et al (84). Predicted topological structures of plasma membrane norepinephrine transporters (NETs) and serotonin transporters (SERTs). Both carriers are predicted to span membrane with 12 transmembrane domains. Both NETs and SERTs bear a large hydrophilic loop between transmembrane domains 3 and 4, within which are located the sites for glycosylation. Potential sites for protein phosphorylation are also depicted.

IV.III Serotonin

The human SERT appears to derive from a single genomic locus on chromosome 17q11.2 (84), and has been identified in human brain, human platelets, human placenta, human lung and rat adrenal gland (81). We demonstrated the presence in human megakaryocytes cultured in vitro (See chapter V). Since the cloning, this protein has extensively been investigated. The SERT protein is identical in the human brain and platelets (85), but interestingly these SERT proteins are differently glycosylated (81). N-glycosylation seems important for the physical maturation and surface targeting of the transporter, sugar addition may increase the probability that the transport protein is folded properly and/or is protected from degradation. Differences in SERT inhibitor sensitivities between different species have been described, cloned rat and human SERTs exhibit different sensitivities to tricyclic antidepressants, the human SERT was found to be more sensitive to these compounds (81). The site(s) of species sensitivity for tricyclic antidepressants appear to reside at or near the last transmembrane domain (81). Studies concerning the regulation of the activity of the SERT have shown, that the activity of the SERT is probably intracellularly regulated by second messengers, by altering the phosphorylation of the SERT. For example activation of protein kinase C has been implicated in platelet SERT down-regulation. Studies by Feldman et al. (78) and Guilloteau et al. (79) showed uptake of MIBG (probably via the serotonin transporter) in human platelets. In these studies, however, substrate concentrations were not stated or uptake was measured at markedly different substrate concentrations. Glowniak et al. (86) on the other hand reported that expression of the serotonin
transport protein in normally non-MIBG importing cell lines did not lead to import of MIBG in these cell lines. In this study, no attention was paid to uptake of serotonin in the transformed cell lines. The lack of these positive controls makes Glowniak's study difficult to interpret. Selective inhibitors of the serotonin transporter are the newer generation antidepressants, i.e. the selective serotonin re-uptake inhibitors (SSRIs). Examples of these SSRIs comprise the compounds fluvoxamine, citalopram, fluoxetine and paroxetine (see further chapter IV).

IV. IV Vesicular monoamine transporters

Intracellularly the MIBG storage occurs in a similar way to catecholamine storage. There are two known vesicular monoamine transporters, i.e. VMAT1 and VMAT2 (87), which can use MIBG as substrate (own observations; Personal communication dr M. Rutgers). In the neuroblastoma the uptake of MIBG was by VMAT2 (88), which also uses norepinephrine and serotonin as substrates. The granulas, in which MIBG is stored, have a low intracellular pH. This low pH results from the proton pump activity of an ATPase located in the membrane of the granule. Import of MIBG into the granule is the result of the reserpin (RSP) and tetrabenazine sensitive exchange of intra-granular protons with extra-granular MIBG (89).

IV. V Retention of MIBG in platelets.

The retention of MIBG in platelets was studied, and compared with the retention of serotonin. We assumed that after the uptake of MIBG in the human platelet, it would be stored intracellularly in the storage granules. After incubation of 2 hr for serotonin or 4 hrs for MIBG (both 10^{-8} M), we measured retention of the compounds as a function of time. After 150 minutes the retention of MIBG was still 63 ±10 % (n=3) and that of serotonin 90 ± 13 % (n=4) (appendix). The sympathomimetic drug tyramine, in high concentration, effectively displaced stored MIBG, but much less serotonin. When imipramin (30 μM) was added to 10^{-4} M tyramine, the level of MIBG retention was significantly higher, 45 ± 7% (n= 3) than with tyramine alone, being 21 ± 4% (n=3). This effect was not seen when the retention of serotonin was studied. Tetrabenazin and reserpin are inhibitors of the VMAT2 transporter, and co-incubation of the radioactive compound with these inhibitors did significantly reduce the percentage of both retained compound.

V THROMBOPOIESIS

V.I Introduction

To be able to investigate thrombocytopenia, one has to know about the origin of thrombocytes. For a long time it was not known if a specific growth factor for the megakaryocytes, such as erythropoietin for the erythrocytes, did exist. Only after the cloning of thrombopoietin (TPO),
also called megakaryocyte derived growth and development factor (MGDF), it became clear that indeed such a compound existed. TPO binds to its receptor, the c-mpl receptor, that is expressed on the surface of platelets and bone marrow megakaryocytes.

V.II Thrombopoietin

The TPO locus is localized on chromosome 3q26-27 (90). Numerous in vitro studies have shown that recombinant forms of TPO not only induce proliferation of progenitor megakaryocytes, but also their maturation (91, 92, 93, 94, 95, 96, 97, 98). Furthermore, there are studies that show that the platelets produced from such cultures are morphologically and functionally indistinct from plasma-derived platelets (98).

TPO is primarily produced in the liver and circulates as a hormone. Serum levels of TPO are inversely related with the numbers of circulating platelets and of bone marrow megakaryocytes. Platelets bind TPO with high affinity and internalize and degrade TPO (98). In the literature there are also suggestions that binding of TPO to megakaryocytes may also regulate TPO levels (100).

Two forms of human recombinant thrombopoietin are currently in clinical trials: The full length molecule (rHuTPO) from Genentech and a truncated form of TPO, called recombinant human megakaryocytic growth and differentiation factor conjugated to polyethylene glycol (PEG-rHumGDF) from AMGEN. Unfortunately, when administered to patients, antibodies against PEG-rHumGDF are being formed (101, 102). No such problems have been reported with rHuTPO and development of this preparation still continues. Furthermore there are indications that TPO may play a role in the biology of myeloproliferative disorders (103). So now different groups are trying to use TPO to grow megakaryocytic precursors in vitro, to be able to transplant autologous megakaryocytes, and thus reduce the amount of allogenic thrombocytic transfusions (104).

V.III From megakaryocyte to platelet.

The megakaryocyte is the bone marrow cell that is the precursor of the platelets and is unique in its large size and ploidy.

The hematopoietic system is composed of heterogeneous populations of cells that have been schematically divided into three compartments, i.e., a stem cell, a progenitor cell, and a terminally differentiating cell compartment.

The megakaryocyte progenitor cells are thought to be a heterogeneous population consisting of at least three cell types, each marked by varying degrees of proliferation. The earliest detectable cell in this lineage, the megakaryocyte high proliferative-potential colony-forming cell (MK-HPP-CFC), is followed by the burst-forming unit-megakaryocyte (BFU-Mk), which is more mature but it retains a high proliferative capacity. The most differentiated of the MK progenitor cells is the colony-forming unit CFU-Mk.

The promegakaryoblasts are transitional cells intermediate between the proliferating progenitor
cells and the post-mitotic, mature megakaryocytes. These cells can be recognized by the expression of megakaryocyte/platelet specific markers such as platelet peroxidase, platelet glycoprotein IIb/IIIa, von Willebrand factor etc (105). These cells are restricted in their proliferative potential, being the developmental stage at which megakaryocytes cease to proliferate, but rather continue to increase DNA content (endomitosis).

Morphologically three stages of mature megakaryocytes can be recognized. The megakaryoblast, the promegakaryocyte and the granular or platelet shedding megakaryocyte. The megakaryoblast has a high nucleus to cytoplasm ratio and basophilic cytoplasm, reflecting the large amounts of protein synthesis occurring in these cells. The promegakaryocyte shows both increases in nucleus and cytoplasmatic volume and in the number of platelet specific granules. The most mature, the granular megakaryocyte has a large and lobulated nucleus and abundant and mature cytoplasm. The endpoint of megakaryocyte development is platelet shedding, the release of platelets into the blood stream. Unlike other cells, megakaryocytes continue to synthesize DNA during differentiation, and in the process of endomitosis the lack of cell division results in polyploid cells. The majority of DNA synthesis occurs in the promegakaryoblasts.

Mature megakaryocytes “go to” the sinusoids of the bone marrow and extend pseudopods (pro-platelets) in these sinusoids. Within these pseudopods the platelets are interspaced by a demarcation membrane system, which presumably directs their final packaging and release into the sinusoidal space.

From these pseudopods the platelets are “budded off”, and eventually the entire cytoplasm of the megakaryocyte is broken away. The nucleus undergoes phagocytosis by reticuloendothelial cells, possibly in the lung (106).

V.IV Platelets.

Human platelets are on average 3.6 ± 0.7 mm in diameter and 0.9 ± mM in thickness. The platelet membrane is approximately 7.5 nm in thickness and has a trilaminar unit structure. Overlying the membrane is the surface coat (glycocalyx), a fuzzy, irregular layer ranging from 10 to 50 nm in thickness. This layer is composed of glycoproteins and mucopolysaccharides and contains sialic acids (108).

The platelet contains a canalicular system (surface connecting tubular system), which is a network of vesicles and interconnecting channels that ramify throughout the entire cytoplasm and communicate with the cell surface. That tubular system is found close to storage organelles such as the dense bodies and granules, and is lined with the same membrane and surface coat that covers the external surface of the platelet. Substantial evidence exists to support the theory that substances contained in the platelet organelles are extruded to the exterior trough this canalicular system during the release reaction. The canalicular system also appears to be involved in platelet phagocytosis (106).
VI Megakaryoblastic Leukemia Cells

Before the discovery of thrombopoietin, it was difficult to obtain megakaryocytes, in large quantities needed for uptake studies. To circumvent this methodological problem, continuous cell lines originating from leukemic marrow or peripheral blood have been established. A selection of these cell lines is: a human erythroleukemia cell line (HEL) (107, 108), and the megakaryoblastic cell lines MKPL-1 (106) and CHRF-288-11 (110). These cell lines express a range of megakaryocytic phenotypic properties. In chapter 5 we investigate if these cell lines are a suitable model to study the uptake of serotonin and MiBG in megakaryocytes. Since no uptake of serotonin in these cells was found, we conclude that these cell lines are not a representative model (111).

VII Aim of the Study.

The aim of our study was to clarify the mechanism of \([^{131}I]\text{MiBG}\) related thrombocytopenia. We hypothesized that there was a direct toxic effect of the radiolabeled MiBG on the precursor cells of the thrombocytes, the megakaryocytes. Because it was not possible to obtain large amounts of human megakaryocytes to evaluate the effect of MiBG on these cells, we started the investigation with the daughter cells, the thrombocytes. These cells are easily obtained in large amounts.

First we investigated the possibility that these cells could take up MiBG, and via which mechanism this uptake could precede. Chapter 2 describes that \([^{125}I]\text{MiBG}\) was indeed taken up by the thrombocytes and compares the uptake of \([^{125}I]\text{MiBG}\) with that of serotonin, the natural substrate for the platelets.

Knowing that selective serotonin reuptake inhibitors (SSRIs) can interfere with the uptake, with no effect on the norepinephrine uptake, we further investigated the effect of different SSRIs on MiBG (and serotonin) accumulation in human platelets \textit{in vitro} and in neuroblastoma \textit{in vivo} (chapter 4). In this study, the possibility to selectively inhibit the MiBG accumulation in the human platelets, without reducing the \([^{125}I]\text{MiBG}\) tumor loading \textit{in vivo} is examined.

The hypothesis was that the thrombocytopenia would be the result of a toxic effect of radiolabeled MiBG on the megakaryocytes. Since the culturing of human megakaryocytes \textit{in vitro} was not possible at this time, we sought to find if megakaryocytic cell lines were a suitable model to investigate the uptake and retention of \([^{125}I]\text{MiBG}\) and serotonin in megakaryocytes. In chapter 3 we evaluated the uptake of \([^{125}I]\text{MiBG}\) and serotonin in the megakaryocytic cell lines HEL, MKPL-1 and CHRF-288-11, and studied if they were representative as a model for megakaryocytes.

After the cloning of the thrombopoietin gene, it was then possible to culture human megakaryocytes from human bone marrow progenitors \textit{in vitro} in the presence of recombinant
thrombopoietin. So now the accumulation of serotonin and MIBG by the megakaryocytes could be investigated, and the inhibition of this process by the SSRIs. This is described in chapter 5.

To understand the results of the uptake studies (chapters 2, 3, 4 and 5) the expression profiles of different transporters were studied. In chapter 6 the expression of the cDNA and the protein of the NET, SERT, DAT, and VMAT, in the megakaryocytes, the platelets and the cell lines with the megakaryocytic properties are explored.

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