ABSTRACT

Samplings of tumor cells from a patient with Stage IV neuroblastoma were analyzed for chromosome constitution. Chromosome preparations of the tumor cells from a bone marrow sample were compared to preparations of a solid metastatic tumor after growth in the nude mouse host or followed by sample were compared to preparations of a solid metastatic tumor tissue were analyzed for chromosome constitution. Chromosomes 5 and 9 were also involved in structural abnormalities in sublines of the tumor cells. Double minutes were seen in all preparations.

INTRODUCTION

Information on the chromosome constitution of human neuroblastoma ideally is obtained from direct and banded preparations of a primary tumor. It is assumed that the best sampling of the in vivo tumor cell population is thus achieved. Unfortunately, such ideal conditions are not always met, and chromosomes are often studied from metastases, after therapy and/or after the tumor tissue has been grown in cell culture. Tumors may also be analyzed as to how closely these results reflect the original tumor cell composition.

We have had the opportunity to study chromosomes in a case of Stage IV neuroblastoma. These studies enable us to compare results from short-term cultures of bone marrow metastases with those from a solid metastatic tumor transplanted into the nude mouse and/or after many months in culture. A total of 6 different chromosome analyses at various passages of the tumor cells in host animals and in culture showed the presence of consistent marker chromosomes, as well as some variable markers. DMs were seen in all preparations.

CASE SUMMARY

The patient was a 3-year-old male who presented with anemia, recurrent fever, and a right upper quadrant mass. A bone marrow examination revealed clusters of tumor cells which were confirmed as neuroblastoma when the abdominal tumor was biopsied. The patient was treated with cyclophosphamide, vincristine, and Adriamycin plus radiation to the tumor bed. The tumor partially regressed, and 7 months later incomplete surgical removal of the abdominal mass was performed. Shortly thereafter, multiple metastatic lesions to bone, lung, and soft tissues developed. The patient expired 11 months after diagnosis in spite of continued chemotherapy. An autopsy was performed, at which time specimens of tumor tissue were obtained for cytogenetic studies.

MATERIALS AND METHODS

A schedule of chromosome analyses is summarized in Chart 1.

1. Blood lymphocyte cultures were initiated by a standard 72-hr phytohemagglutinin-stimulated culture and harvested after a 1-hr Colcemid treatment. Two cultures were done 5 weeks apart, when the child was undergoing chemotherapy.

2. Skin fibroblast cultures were initiated from a skin biopsy obtained at autopsy. Skin was minced and introduced into 25-cm plastic culture flasks with primary medium [Dulbecco’s modified Eagle’s medium:F-12 medium (1:1), with 20% fetal calf serum and 50 μg gentamicin per ml]. Medium was changed twice weekly. Confluent cultures were trypsinized when growth was predictable. Cells were harvested in log phase after a 4-hr Colcemid (0.1 μg/ml) treatment, by scraping and centrifuging. The cell button was suspended in hypotonic solution of 0.7% sodium citrate and 0.14% KCl for 14 min, followed by several changes of ethanol:acetic acid fixative (3:1).

3. Cells in ascites fluid were concentrated by centrifuging, cultured in primary medium as a monolayer, and harvested in 2 weeks, by the same procedure as for skin fibroblasts.

4. A bone marrow sample was received 30 min after death. A thin smear was stained with Wright-Giemsa to confirm the presence of tumor cells. Several suspension cultures were initiated, grown in primary culture medium (without phytohemagglutinin), and harvested at various times after a 3-hr Colcemid treatment. Good preparations were achieved after 3 days and 2 weeks of culture.

5. All chromosome studies of the neuroblasto tum lines originated from a metastatic tumor of the femur obtained at death. This specimen was minced in culture medium and injected s.c. via trocar into the flank of Nude Mouse I. A tumor palpable after 10 weeks was removed and transplanted to Nude Mouse II. Eight weeks later, the resultant tumor was passaged to a Nude Mouse III. Nude mice were BALB/c background bred from a colony kindly provided by Dr. B. Giovannella.

Cell cultures were initiated from a piece of the tumor from Nude Mouse I and III. This was finely minced and plated in plastic culture flasks using primary medium with 10% fetal bovine serum. Cultures were changed 2 times/week. Cells began to proliferate in 10 weeks and were then subcultured approximately 2 times/month by gentle pipeting. Cultures were treated with Colcemid 4 to 12 hr and harvested as for skin fibroblasts.
Sample 7. Cells grown in culture were injected into Nude Mouse IV. After 10 months in culture at passage 13, cells were pipeted from flasks, centrifuged, and washed twice with Ca^2+ -Mg^2+-free Hanks' solution. Approximately 0.3 × 10^6 cells were resuspended in 0.3 ml Hanks' solution, and Nude Mouse IV was given an s.c. injection of one-half of the suspension on either side of the lateral body wall. In 4 weeks, a 1-cm^3 tumor was removed. A direct preparation of this tumor was made by mincing the tumor, suspending in medium with Colcemid (0.15 µg/ml) for 12 hr, and processing as for skin fibroblasts.

Unbanded, G-, Q-, and C-banding of chromosome preparations were done by conventional methods (8). For each tumor study 27 to 87 metaphases were counted. Chromosomes were scored in the microscope when possible, and 13 to 39 karyotypes were analyzed. Structural abnormalities were identified in banded karyotypes. On the basis of banded karyotypes, structural abnormalities were also scored in unbanded karyotypes and in microscope preparations.

RESULTS

Chromosome analyses of the neuroblastoma cells were done at 6 sampling times, diagramed in Chart 1. Details of chromosome analyses are summarized in Table 1. The 2 analyses from bone marrow metastases are numbered 4A and 4B in Chart 1 and Table 1. The 4 analyses of cell lines from a solid metastatic tumor, after various passages in host animals and in culture, are numbered 5, 6A, 6B, and 7.

In addition to the tumor samples, 2 blood lymphocyte cultures and a skin fibroblast sample from the patient were analyzed and showed a normal male karyotype. Cells from ascites fluid obtained at death and cultured for 2 weeks also had normal chromosomes (Chart 1, Samples 1 to 3).

The bone marrow sample obtained at death showed neuroblastoma cells as the dominant cell type. A portion of this sample was cultured for 3 days and processed for chromosome analysis (Chart 1, Sample 4A). In the 59 second metaphases, chromosome number ranged from 45 to 49 with a majority having 46 or 47 chromosomes. The typical unbanded karyotype showed 47,XY,1p−,+B,+E,+G. There was often a random loss of X, Y, or a C chromosome, accounting for the 46 cells. Giemsa banding showed the deleted chromosome 1 to be del(1)(p31), designated mar 1p−. The identity of the extra B was largely unknown, although the distal region of the q arm appeared to be chromosome 5 material, i.e., 5q15 → qter. This mar 5 was designated t(7q5) in Table 1. A 9p+ chromosome, designated mar 9, replaced a normal 9 in a minority of cells. The extra E chromosome was identified as a probable 17 and the missing G as a 22. It is sometimes difficult to distinguish between +17 and 22q+. However, inspection of a number of banded karyotypes, some with +17 and a normal pair of 22 coexisting, showed convincing +17. A representative karyotype is shown in Fig. 1. Findings were confirmed by Q-banding. Prominent DM were observed in all metaphases varying from 20 to 100 in number (Fig. 2). No homogeneous staining region was identified in banded karyotypes.

The marrow specimen was processed again after 2 weeks in culture (Chart 1, Sample 4B). In 48 metaphases scored, the modal number remained 46 due to a random loss of one chromosome. Mar 1p−, 5, and 9 seen in the 3-day culture were present; the +17 and −22 were more variable. All metaphases had DM.

Although the primary neuroblastoma sample did not grow in the nude mouse or in culture, an autopsy sample of a large metastatic tumor to the femur did grow in Nude Mouse I. This tumor was subsequently passaged in Nude Mouse II and III over a total period of 5 months. Pathology of all nude mouse tumor specimens revealed a small-cell tumor consistent with neuroblastoma from the original patient sample. At the end of this time, a tumor biopsy specimen from Nude Mouse III was initiated in culture. After 4 months, a chromosome preparation was obtained (Chart 1, Sample 5), and 38 metaphases were scored having a modal number of 47. Although banding was

<table>
<thead>
<tr>
<th>Sample Modal No.</th>
<th>Karyotypes</th>
<th>% of cells with markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UB B</td>
<td>1p− t(7q5) 8p+ +17 −22</td>
</tr>
<tr>
<td>1, 2</td>
<td>46 13 3</td>
<td>100 100 24 91 86</td>
</tr>
<tr>
<td>3</td>
<td>46 18 0</td>
<td>100 79 25 38 29</td>
</tr>
<tr>
<td>4A</td>
<td>46 15 8</td>
<td>100 100 38 69 81</td>
</tr>
<tr>
<td>4B</td>
<td>46 13 4</td>
<td>49 61 24 54 95 63</td>
</tr>
<tr>
<td>5</td>
<td>47 13 0</td>
<td>77 23 15 15 77 62</td>
</tr>
<tr>
<td>6A</td>
<td>46 47 28 14</td>
<td>25 75 0 80 60 45</td>
</tr>
<tr>
<td>6B</td>
<td>46 47 13 0</td>
<td>25 75 0 80 60 45</td>
</tr>
<tr>
<td>7</td>
<td>46 16 4</td>
<td>25 75 0 80 60 45</td>
</tr>
</tbody>
</table>

UB, number of unbandied karyotypes; B, number of banded karyotypes.

Second number represents the percentage of cells with a pair of mar 1p−'s.
not possible, all of the unbanded cells clearly showed the mar 1p− and a presumptive mar 5. The majority of these cells had +17 and presumptive −22. DM were seen in most cells although they were often faint.

A sample of neuroblastoma from Nude Mouse I was initiated in culture. A culture of small ovoid human cells, loosely attached to a monolayer of mouse cells (identified by karyotype), grew slowly. Analysis of human cells after 4 months in culture, passage 4 (Chart 1, Sample 6A), showed a range in chromosome number from 44 to 49. About 50% of the cells now had 47 chromosomes (Fig. 3). Some cells in the 47 population had a pair of mar 1p−'s. The majority of cells had +17 and −22. There was a decrease in the number of cells with mar 5 from a majority in previous samples (Samples 4A, 4B, and 5) to about 25% of the cells in Sample 6A. Giemsa banding identified the abnormalities in the karyotypes to be the same as those observed in previous samples. The majority of cells had a mar 9. C-bandning confirmed the identity of the normal and abnormal No. 1 and No. 9 chromosomes.

After 10 months in culture, passage 13 (Chart 1, Sample 6B), metaphases with 46 or 47 chromosomes were predominant. Only unbanded preparations could be analyzed, but it was apparent that most cells had the 1p−, +17, and −22. A few cells had a pair of mar 1p−'s. Only 15% of the cells had the mar 5. DM were still present.

Also at 10 months of culture, the mixed population of mouse and human cells was injected into the Nude Mouse IV, and a tumor appeared in 1 month. A direct preparation of this tumor (Chart 1, Sample 7) was analyzed, and the 54 cells counted had a modal number of 46. Results were similar to other samples except the mar 5 was no longer observed.

Although there was overall evidence of stability of certain marker chromosomes, there was increased heteroploidy seen in all the tumor analyses, compared to cultures of normal cells. In addition to some variability in 1 or 2 mar 1p−'s and in +17 and −22, there was often loss of X and Y and some random loss of chromosomes in all analyses. This accounted for variation in chromosome numbers seen in Table 1.

**DISCUSSION**

Chromosome analyses from direct preparations provide important and often critical information for the cytogeneticist. On the other hand, if one considers the complex cellular composition with subpopulations of differentiating and dividing cells seen in advanced stages of many tumors in vivo, then direct preparations are only a "snapshot" of a final stage in an evolutionary process (10). Cell cultures of the tumors combined with tumorigenicity tests in the nude mouse can be a helpful supplement in sorting out malignant cell lines. Even the study of permanent cell lines after long periods in culture have been informative (3, 4). In such studies, one depends on the stability of the tumor lines in culture as well as in the host animal. Often structural chromosome abnormalities such as marker chromosomes can be useful in following clones of tumor cells (7, 9, 11, 13).

The study of the particular neuroblastoma described here shows that the chromosome constitution of the bone marrow metastasis, as well as preparations derived from a solid tumor metastasis, had consistent chromosome changes including...
1p−, +17, and −22. Furthermore, the solid tumor lines after multiple passages in host animals and in culture retained the characteristic marker chromosomes. Although there is no information on the chromosomes from the primary neuroblastoma, it is reasonable to suppose that the chromosome constitution, at least of the most malignant and therapy-resistant component of the original tumor, was not very different from that of the metastases which were studied (3, 14).

Other marker chromosomes, Nos. 5 and 9, were considered variable, which does not imply that they were unstable. Based on the observation that these markers were present in the original marrow sample, it is probable that they represent different subclones of the original tumor. The 9p+ persisted at some level after many passages. However, there was gradual disappearance of the mar 5 after passage in vitro. Reinjection of the culture into the nude mouse failed to revive this line. The variable occurrence of a pair of mar 1p−′s could have resulted from a tendency for nondisjunction. There was other variable heteroploidy. These exceptions, however, do not contradict the impression of overall stability of the clones and their chromosome markers.

DM have been found in a number of malignant tumors, especially those of neurogenic origin (2, 4, 13). In our study, DM were seen in the majority of cells from all preparations of the neuroblastoma. Their presence lends further support to the conclusion that the cell lines seen in cultures, as well as in the direct preparations, represent the malignant clones of cells.

Other neuroblastomas have been studied as established cell lines. Numerical and structural chromosome abnormalities with a general stability of marker chromosomes have been found (1, 4, 15). Structural changes in chromosomes 1 and 17 are common (4). A partially deleted chromosome 1 has been reported in primary untreated neuroblastoma specimens (5, 6). It is of interest, therefore, that we found a 1p− (at band p31) as one of the consistent chromosome abnormalities. The second consistent marker, +17 as an entire extra chromosome, has been described before in only one neuroblastoma line (4). Abnormalities of 17 are common in myeloproliferative conditions, as well as occasionally in other neoplasia (9, 16). Structural and/or numerical abnormalities of chromosomes 5, 9, and 22 are also considered nonrandom changes in neoplasia (9, 12), but this combination may be rare in neuroblastoma.

The functional significance of marker chromosomes in neuroblastomas and most neoplasia is unknown (9, 12, 13). The fact that some neuroblastomas have apparent normal chromosomes (5) or have very different abnormalities must be kept in mind. The fact that chromosome constitution of other kinds of tumors and tumor lines may be relatively unstable in host animals and in culture must also be kept in mind. Nevertheless, markers, when present as they were in this particular tumor, can serve as useful signs of a tumor cell and clones of a tumor line. Our experience with this tumor suggests that techniques of culture and use of the nude mouse host can be of value in increasing the success rate of karyotyping at least some human tumors without causing artifactual results when the difficult techniques of direct processing fail.

ACKNOWLEDGMENTS

We would like to acknowledge the excellent technical assistance of Geri Weiner.

REFERENCES

Fig. 2. Partial metaphase to show DM from a cell of the 3-day culture of bone marrow sample (Chart 1, Sample 4A).

Fig. 3. Representative Giemsa-banded karyotype of the cultured tumor line (Chart 1, 8A). Arrows, 2 mar 1p−'s, mar 9, +17, and −22.
Chromosome Analysis of a Human Neuroblastoma

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