Changes in Chromosomes of Bone Marrow after Intravenous Injections of 7,12-Dimethylbenz(a)anthracene and Related Compounds*

E. Douglas Rees,† S. K. Majumdar, and Amy Shuck

DEPARTMENTS OF MEDICINE AND PHARMACOLOGY,
UNIVERSITY OF KENTUCKY MEDICAL CENTER, LEXINGTON

Communicated by Charles Huggins, May 11, 1970

Abstract. Multiple intravenous injections of an emulsion containing 7,12-dimethylbenz(a)anthracene (DMBA) or 7,8,12-trimethylbenz(a)anthracene (TMBA) induce a high incidence of leukemia in rats. Twenty-four hours after a single injection, about half of the metaphase cells in the marrow have chromosomes with breaks. Although breaks were inflicted on chromosomes of various sizes and morphology, these aberrations were nonrandom in that members of the nos. 1 and 2 chromosome pairs were involved to an extent greater than expected on the basis of their size and number. Distinctive karyotypic abnormalities involving the no. 2 chromosome were observed in half of the leukemic rats, whereas these abnormalities were not observed in nonleukemic, DMBA-treated rats. Benzo(a)pyrene and benzo(e)pyrene, polycyclic aromatic hydrocarbons which did not induce leukemia, produced fewer breaks of the no. 2 (and other) chromosomes than did DMBA or TMBA.

The experimental leukemias of rats are of special interest, since spontaneous leukemia rarely occurs in rats but can be induced by radiation and by chemicals—particularly the polycyclic aromatic hydrocarbon carcinogens 3-methylcholanganthrene, 7,12-dimethylbenz(a)anthracene (DMBA), and 7,8,12-trimethylbenz(a)anthracene (TMBA). Despite considerable interest in the chromosomes of leukemic cells and in chromosomal theories of oncogenesis, only scanty data1–3 exist concerning the chromosomal changes occurring in marrow cells shortly after administration of these chemical carcinogens. Multiple doses of DMBA given intravenously under conditions defined by Huggins and Sugiyama4 result in a high incidence of leukemia in Long-Evans rats, and chromosomes from marrow cells can be readily prepared for cytologic study. Also, the morphology of rat chromosomes is such that many of them can be individually identified. Thus the experimental system of intravenous DMBA and rat chromosomes permits study of chromosome changes which occur in normal cells soon after administration of the chemical as well as later study of the chromosomes of cells from animals made leukemic by the chemical. In the present experiments rats were injected with single or multiple intravenous doses of DMBA and related compounds, and the chromosomes of normal and malignant cells were studied.

Methods and Materials. Animals and treatments: Long-Evans rats were ob-
tained as weanlings from Rockland Farms; most of the animals were males. A series of intravenous emulsions\textsuperscript{6} of DMBA (5 mg/ml emulsion) was given beginning at 25–33 days of age; the initial dosage was 40 mg/kg. Subsequent injections were given in a dose of 35 mg/kg with an interval of 10 days between injections. No more than three injections were made in this series of animals, and no more than 5 mg of DMBA were administered, regardless of rat weight. Chromosome preparations were made as early as 2 hr after the first injection and as long as 50 days after the last. Some of the animals (21) which received three injections were not sacrificed but were left to develop leukemia. Two groups of controls were prepared: one group received intravenous injections of emulsion which did not contain DMBA, while another group was untreated.

A few Sprague-Dawley rats, including one that developed leukemia, were studied but this strain is highly susceptible to the toxic effects of DMBA. Female rats of both strains were studied; DMBA produced chromosome abnormalities of comparable type and incidence in both sexes, but we preferred to study leukemia males since fewer mammary carcinomas were induced. These carcinomas become large, ulcerate, and necessitate premature termination of the experiment if they are not excised from the host.

Chromosomes: Animals were decapitated a half hour after intraperitoneal injection of 1 ml of freshly prepared 0.4% colchicine, and chromosomes were prepared from marrow cells in the tibia and femur using hypotonic citrate and the fixing procedure of Moorhead et al.\textsuperscript{6} Chromosomes were stained with Giemsa.

The criteria and nomenclature of Levan et al.\textsuperscript{2} for designating chromosome morphology have been followed: chromosomes with centromere in the terminal, subterminal, submedian, and median regions are called t, st, sm, and m chromosomes, respectively. The no. 1 chromosomes and no. 3 chromosomes (when upper arms are present) are designated st chromosomes, whereas nos. 11–13 members are sm chromosomes. The chromosome numbering system of Hungerford and Nowell\textsuperscript{4} is used. However, in constructing karyotypes it is sometimes necessary for practical reasons (i.e., the X chromosome could not always be unambiguously distinguished, and one or both of the members of the no. 3 chromosomes sometimes have absent upper arms) to include the sex chromosome and one or both members of the no. 3 chromosome pair in the large group of t chromosomes. There is a difference between the karyotypes of cells from the Sprague-Dawley and the Long-Evans rats.\textsuperscript{5} In Long-Evans rats (from several commercial sources) the no. 3 pair of chromosomes is usually heteromorphic,\textsuperscript{4} one member is a st and the other is a t chromosome.

Results. Initial changes: After the rats had been injected with DMBA, the most striking and distinctive observation was the presence of chromatid and (to a lesser extent) isochromatid breaks on the chromosomes of the marrow cells (Fig. 1). The appearance of these lesions was quite rapid. At 2 hr, 5% of the cells had one or more chromosomes containing a break, and at 4, 6, and 24 hr the incidence was 25, 45, and 43%, respectively. Thus, maximum incidence was reached within 6–24 hr. Isochromatid breaks constituted 25% of the total breaks. The incidence of these aberrations increased with DMBA dose as has also been shown by Kurita et al.\textsuperscript{1} Breaks were observed only rarely (less than 1%) in untreated rats of either the Long-Evans or Sprague-Dawley strains. Control rats receiving intravenous emulsion without DMBA had a 3% or less incidence of breaks in marrow cells. In the case of gaps, it was not unusual to find these narrow, sharp, achromatic bands on the chromatids of some chromosomes from some untreated rats. Breaks were visually distinguished from gaps by the displacement (greater separation or malalignment) of the two chromatid pieces and by unevenness of the edges of the breaks. (The interpretation of gaps has been a matter of controversy which is well discussed by Revell,)\textsuperscript{10}
Evans,11 and Kihiman;12 a concise description of present theories of chromosomal aberrations is also found in Rieger et al.13 After the DMBA injection, the incidence of gaps was about the same as for breaks; however, even untreated control animals may have a substantial incidence of gaps. Fragments, occasional dicentrics, and an increased incidence of gaps accompanied the breaks; however, interchanges of chromatids between chromosomes, such as observed by Kato14,15 in hamster cells exposed to DMBA, were seen only rarely—no more than four times in over 1000 cells from our intravenously DMBA-treated rats. The percentage of cells having at least one chromosome with one or more breaks was plotted versus time after injection. After the first dose 2–3 days were required for the incidence of these lesions to decay to half the initial 24-hr value. By the tenth day the incidence had approached the level of the controls. Subsequent injections on day 10 and on day 20 again increased the incidence of breaks. After the third injection many animals showed a persistence of these aberrations.

The effects of other polycyclic aromatic hydrocarbons (given in the same dose as the DMBA) on marrow cell chromosomes were also studied 24 hr after intravenous injection. TMBA, an extremely potent leukemogen, produced breaks in 55% of metaphase cells; whereas, benzo(a)pyrene and benzo(e)pyrene produced breaks in 21 and 11%, respectively. (In the rat, benzo(a)pyrene is not nearly so carcinogenic as DMBA or TMBA, and BeP may be noncarcinogenic; neither benzo(a)pyrene nor benzo(e)pyrene has produced leukemia in our rats.) The location of breaks on individual chromosomes were recorded for each compound, and the total number of breaks observed on a given type of chromosome was divided by the total number of chromosomes of that type analyzed. Figure 2 depicts these data and, in addition, delineates the greater damage of TMBA and DMBA to the nos. 1 and 2 chromosomes. Of course, chromosomes in the M (nos. 11–20) group and T (X, nos. 4–10) group could not be individually identified and, thus, the individual susceptibility to damage could not be determined.

**Leukemic stemlines:** It was important to ascertain whether or not intra-
venous DMBA would produce leukemia in our rats, since this would establish that the procedure was leukemogenic in our hands and would enable study of the karyotypes of cells from leukemic rats. Leukemia did occur, in accordance with the findings of Huggins and Sugiyama,4 Sugiyama et al.,16,17 and Kurita et al.18 Of 21 animals treated with three injections of DMBA and left for up to 7 months, 10 developed leukemia and 11 were sacrificed (during the same interval as the leukemic animals) because of ulcerating cancers of the ear duct or mammary gland. Skin cancers were quite common, especially on Long-Evans rats. Leukemia appeared within 2–7 months and was recognized first by a substantial drop in body weight. Using the classification of Huggins and Sugiyama,4 six of the leukemias were of the hepatic type, three of the lymphatic type, and one was chloroleukemia. Two rats (one with chloroleukemia and the other with hepatic type) died before chromosomes could be prepared, but autopsies were done and tissue was obtained for histologic examination. In several rats, infiltrates of leukemic cells were noted in the adrenal cortex and to a lesser extent in the kidney. Results of chromosome studies on eight leukemic rats are summarized in Table 1. Chromosomes in the marrow cells (other tissues were not examined) of rat A were remarkable in that all the cells had 42 chromosomes, one of which was a large, metacentric marker chromosome. This marker was identical in appearance to the fused no. 2 chromosome found by Sugiyama et al.16,17 and Kurita et al.18,19 Each arm was the same length as that of the single no. 2 chromosome present in all of the metaphase cells. Marrow cells (other tissues not studied) from rat B uniformly had three no. 2 chromosomes in a total of 41–44 chromosomes. In cells with 44 chromosomes there was an extra m as well as a third no. 2 chromosome. The cells (from marrow, liver, and spleen) of rat C were predominantly hypotetraploid; no. 2 trisomy was predominantly present in addition to a lesser but substantial no. 2 tetrasomy. Rat F had greatly enlarged retroperitoneal lymph nodes. Most of the cells had 40–44 chromosomes including two nonidentical marker chromosomes of submetacentric morphology; one marker was somewhat longer and the other somewhat shorter than the single no. 1 chromosome present. The markers were noted in cells from liver, spleen, and lymph nodes as well as marrow. The karyotypes of the other four leukemic animals studied did not have marker chromosomes (except for one sm chromosome in a single cell) or no. 2 trisomy, though other types of karyologic deviations

![Fig. 2.—Chromosomal break incidence 24 hr after polycyclic aromatic hydrocarbon injection. T is the X, 4–10 group of chromosomes and M is the 14–20 group.](image-url)
## Table 1. Analysis of cells from leukemic animals: Comparison with other groups.

<table>
<thead>
<tr>
<th>Rat, leukemia (no. cells)</th>
<th>&lt;39</th>
<th>39</th>
<th>40</th>
<th>41</th>
<th>42</th>
<th>43</th>
<th>44</th>
<th>&gt;44</th>
<th>&lt;80</th>
<th>&gt;80</th>
<th>81</th>
<th>82</th>
<th>84</th>
<th>&gt;84</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Hepatic (50)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>(B) Hepatic (70)</td>
<td>7</td>
<td>14</td>
<td>10</td>
<td>7</td>
<td>59</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C) Hepatic (52)</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>19</td>
<td>19</td>
<td>34</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(D) Hepatic (31)</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td>94</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E) Hepatic (17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>(F) Lymphatic (62)</td>
<td>3</td>
<td>3</td>
<td>8</td>
<td>13</td>
<td>29</td>
<td>23</td>
<td>18</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(G) Lymphatic (49)</td>
<td>2</td>
<td>8</td>
<td>12</td>
<td>76</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(H) Lymphatic (37)</td>
<td>8</td>
<td>3</td>
<td>11</td>
<td>5</td>
<td>73</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated controls (252)</td>
<td>1.2</td>
<td>0.8</td>
<td>2</td>
<td>7</td>
<td>89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intravenous emulsion controls (324)</td>
<td>0.3</td>
<td>0.3</td>
<td>4.6</td>
<td>8.6</td>
<td>85</td>
<td>0.9</td>
<td>0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonleukemic intravenous DMBA (152)</td>
<td>2.0</td>
<td>1.3</td>
<td>4.6</td>
<td>87.5</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-9 days after intravenous DMBA (748)</td>
<td>1.7</td>
<td>0.9</td>
<td>3.5</td>
<td>8.4</td>
<td>80.5</td>
<td>3.1</td>
<td>0.3</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments:
- One fused no. 2 chromosome in 100% of diploid cells; two in tetraploid cells.
- No. 2 trisomy in 73% cells.
- No. 2 trisomy in 52% and no. 2 tetrasomy in 33% of cells.
- One cell had no. 2 trisomy.
- Chromosomes were “fuzzy” and less contracted.
- Submetacentric markers and a single no. 1 chromosome in 89% cells.
- Variability in size and satellites of no. 11 and no. 12 chromosomes.
- A few cells had no. 2 trisomy.
- Cells from 14 animals.
- Cells from 16 animals.
- Cells from 10 animals (one submetacentric marker noted).
- Cells from 40 animals.
were not uncommon. Although no breaks were seen in chromosomes from leukemic rat B, the incidence of breaks in the other leukemic animals ranged from 6 to 17%.

**Control groups:** In addition to the leukemic animals and the animals studied within 1 day to 2 weeks after intravenous injection of DMBA, three other groups of animals (Table 1) were studied for comparison: (a) 14 untreated animals, (b) 16 animals receiving intravenous lipid emulsion without DMBA, and (c) 11 that received DMBA intravenously but were not leukemic. The animals in the latter group were sacrificed 2–7 months after their first DMBA dose, during the time that their leukemic partners were studied. Table 1 also indicates the chromosome number distribution for 40 of the rats which were sacrificed within 2 weeks after the last intravenous dose of DMBA. Of the 10 (one animal was not studied) intravenously injected DMBA treated nonleukemic rats, six had cells possessing chromosomes with breaks in an incidence of 5–15%.

**Discussion.** Chromosomal aberrations have been commonly observed in cells from neoplasms, and there has been much discussion as to whether the aberrations are a cause or a consequence of neoplastic disease. For a causal relation to exist it would seem necessary, though not sufficient, that aberrations occur prior to the onset of overt disease. As demonstrated above, DMBA administered intravenously to rats produced considerable damage, particularly breaks, in marrow cell chromosomes in a matter of hours, and leukemia developed later over a period of weeks. Moreover, the damage was nonrandom, the largest st and t chromosomes were especially vulnerable to break formation. After the first and second injection, cells with breaks had an apparent half-life of 2–3 days in the marrow. Whether these cells left the marrow not to return, or were not viable, or for other reasons did not reach metaphase in the marrow again is not known. However, after the third DMBA injection, cells with aberrant chromosomes were persistently present in the marrow. In this regard, it is of interest that 4–6 injections were used by Huggins and Sugiyama and Sugiyama et al. to induce leukemia in high incidence in their rats. A single dose is rarely sufficient to cause leukemia. The question arises as to the relationship between DMBA and the chromosome damage. The simplest postulate is that the damage is directly caused by the chemical; at present, there seems no need to postulate an intermediate, such as a virus.

In view of the special vulnerability of the nos. 1 and 2 chromosomes in marrow cells to breakage by DMBA and 7,8,12-TMBA, the stemline karyotypes of rats made leukemic by these and other agents command attention. Initial chromosome studies by Nowell et al., Dowd et al., and Moloney et al. on rat leukemias induced by 3-methylcholanthrene (intragastric) and/or radiation disclosed stemlines with predominantly normal karyotypes, but the transplantable ILK leukemia of Fichidzian and Pogosianz and Pogosianz et al. had a modal stemline possessing what appears to be three no. 2 chromosomes. More recently, cells from the transplantable IRC 741 rat leukemia (initially induced by the substituted polycyclic aromatic hydrocarbon, acetylaminofluorene) were found by Yakaitis and Rubini to possess consistently three large, nonidentical, sub-metacentric marker chromosomes, and cells from the related R3149 transplant-
able leukemia consistently had a single large submetaacentric marker.\textsuperscript{27} However, the relation of these markers to normal chromosomes is not apparent from the published data. Our results above for DMBA-induced leukemia are in accord with those of Sugiyama \textit{et al.}\textsuperscript{16,17} and Kurita \textit{et al.}\textsuperscript{18,19} who observed no. 2 trisomy (designated C-2 trisomy by the authors) in modal or nonmodal cells of 30–50\% of their leukemic animals. An additional small \textit{m} chromosome was present in several of their rats and was considered to constitute A-6 trisomy. The biologic behavior of leukemias associated with no. 2 trisomy differed from the behavior of those associated with a normal karyotype, and the addition of the A-6 trisomy to the C-1 trisomy was stated to modify the characteristics of the latter.\textsuperscript{17} Two compounds related to DMBA, TMBA, and 6,8,12-TMBA also induce leukemia,\textsuperscript{28} and cytogenetic studies have been performed on cells of these leukemias by Sugiyama and Brillantes\textsuperscript{29} with results comparable to those for DMBA leukemias. In all, there is substantial evidence from several laboratories that some rat leukemias are associated, though not necessarily in a simple way, with certain types of chromosomal abnormalities.

Probably most chromosomal breaks eventually are restituted, lethal, or lead to a nondividing cell. It seems probable, as suggested by Nichols,\textsuperscript{30} that more subtle chromosomal lesions (e.g., point mutations) occur along with the breaks. In order to form a neoplastic cell, a subtle lesion would have to be present in the appropriate position(s) on a specific chromosome(s). Since our data indicate that breaks are unduly frequent on the nos. 1 and 2 chromosomes (possibly because of a greater "affinity" of these chromosomes for DMBA and TMBA, or a greater intrinsic vulnerability \textit{per se} of these chromosomes to damage by these compounds), subtle lesions presumably would also be more common on these chromosomes. However, our concept also requires that the cell must not be rendered inviable or nonproliferative by additional damage elsewhere. These are demanding requirements; even though the nos. 1 and 2 chromosomes are preferentially involved with breaks, they are not uniquely involved. In fact, our findings indicate rather widespread damage to other chromosomes and the extent of nonechromosomal damage to cells has not been determined. Also, a "leukemicized" cell must not succumb to host defenses, if leukemia is to occur. Such considerations suggest that the proper combination of cell damage and favorable environment must be a rather rare event; a view which is in accord with the observation that, despite many cells in the marrow and a high incidence of damage to these cells following DMBA or TMBA, in a substantial proportion of leukemic hosts a distinctive abnormality is found in a high proportion of metaphase cells.

\* Supported by an AMA-ERF grant and by contract 12-14-100-9524(73) from the Agricultural Research Service, U.S. Department of Agriculture, Eastern Utilization Research and Development Division. The expert assistance of Mr. John Lowry and Mrs. Helen Lipscomb is acknowledged. Emulsion of TMBA was a gift from Dr. Charles Huggins and DMBA emulsion was provided by Dr. Paul Shurr of the Upjohn Co.

\dag Requests for reprints may be addressed to Dr. E. D. Rees, Departments of Medicine and Pharmacology, University of Kentucky Medical Center, Lexington, Ky. 40506.


7 Levan, A., K. Fredga, and A. A. Sandberg, Hereditas, 52, 201 (1964).
9 Long-Evans rats obtained from Dr. Charles Huggins, Ben May Laboratory, University of Chicago, have a karyotype comparable to that of the Sprague-Dawley, i.e., both members of the no. 3 pair are generally st as previously noted by Sugiyama et al.16