Induction by alkylating agents of sister chromatid exchanges and chromatid breaks in Fanconi’s anemia

(5-bromodeoxyuridine/33258 Hoechst fluorescence/mitomycin C/ethylmethane sulfonate/DNA repair)

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ABSTRACT Sister chromatid exchanges, which may reflect chromosome repair in response to certain types of DNA damage, provide a means of investigating the increased chromosome fragility characteristic of Fanconi’s anemia. By a recently developed technique using 33258 Hoechst and 5-bromodeoxyuridine, it was observed that the baseline frequency of sister chromatid exchanges in phytohemagglutinin-stimulated lymphocytes from four males with Fanconi’s anemia differed little from that of normal lymphocytes. However, addition of the bifunctional alkylating agent mitomycin C (0.01 or 0.03 μg/ml) to the Fanconi’s anemia cells during culture induces less than half of the increase in exchanges found in identically treated normal lymphocytes. This reduced increment in exchanges is accompanied by a partial suppression of mitosis and a marked increase in chromatid breaks and rearrangements. Many of these events occur at sites of incomplete chromatid interchange. The increase in sister chromatid exchanges induced in Fanconi’s anemia lymphocytes by the monofunctional alkylating agent ethylmethane sulfonate (0.25 mg/ml) was slightly less than that in normal cells. Lymphocytes from two sets of parents of the patients with Fanconi’s anemia exhibited a normal response to alkylating agents, while dermal fibroblasts from two different patients with Fanconi’s anemia reacted to mitomycin C with an increase in chromatid breaks, but a nearly normal increment of sister chromatid exchanges. The results suggest that chromatosomal breaks and rearrangements in Fanconi’s anemia lymphocytes may result from a defect in a form of repair of DNA damage.

Fanconi’s anemia is a hereditary disease characterized by pancytopenia, congenital malformations, and patches of increased skin pigmentation (1). Chromosomes from affected individuals exhibit structural lability (2, 3), and there is a strong predisposition for the development of neoplasia (4, 5). Because of the high frequency of chromosomal abnormalities, predominantly breaks, it has been suggested that an error in DNA repair exists in Fanconi’s anemia (5).

Bifunctional alkylating agents such as mitomycin C (6), as well as γ-rays (7), are unusually effective in inducing breaks in chromosomes of peripheral lymphocytes from individuals with Fanconi’s anemia. Similarly, mitomycin C has been reported to be highly lethal to fibroblasts cultured from patients with Fanconi’s anemia (8). Monofunctional alkylating agents exhibit less selective toxicity to Fanconi’s anemia cells than do their bifunctional counterparts (6, 8). One recent study has suggested that Fanconi’s anemia fibroblasts may also have a reduced ability to excise thymine dimers (9).

The observation that alkylating agents are able to induce large numbers of sister chromatid exchanges in chromosomes from normal cells (10–12) provides a new approach for investigating Fanconi’s anemia. Since sister chromatid exchanges involve the interchange of DNA duplexes between chromatids followed by ligation, they may constitute a repair response to chromosome damage. Such an analysis is facilitated by the recent development of fluorescent (13–15) and related techniques (16–18) which have largely supplanted autoradiography (19) for the detection of sister chromatid exchanges. In the present report, fluorescence microscopy is used to study sister chromatid exchanges in Fanconi’s anemia cells and determine the influence of alkylating agents on the frequency of these exchanges.

MATERIALS AND METHODS

Sister chromatid exchanges in human lymphocyte chromosomes were detected by fluorescence microscopy after staining with the dye 33258 Hoechst (11, 13, 19–21). This dye was kindly supplied by Dr. H. Loewe, Hoechst AG, Frankfurt, A.M., Germany.

Lymphocytes were grown for 3 days in medium containing 10−5 M BrdUrd, 6 × 10−6 M Urd, and 4 × 10−7 M FdUrd. The cells were protected from light during this period. Because of the ability of BrdUrd to inhibit the enzyme ribonucleotide reductase (22), dCyd was added to most cultures. Although dCyd does not counter the lethal effect of BrdUrd on HeLa cells or human fibroblasts (22), addition of 10−4 M dCyd to human lymphocyte cultures increased the proportion of second division metaphases observed after 3 days of growth. Fibroblasts from individuals with Fanconi’s anemia were obtained from the Human Genetic Cell Repository, Camden, N.J. (repository nos. GM 391 and GM 449), and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 25% fetal bovine serum to which 10−4 M dCyd and 2.5 × 10−5 M BrdUrd were added. The alkylating agents mitomycin C and ethylmethane sulfonate (EMS) were both purchased from Sigma Chemical Co.

RESULTS

If chromosomes from cells that had replicated twice in medium containing BrdUrd are stained with 33258 Hoechst, sister chromatids exhibit unequal fluorescence. Sister chromatid exchanges are apparent as sharp reciprocal changes in dye fluorescence along these chromosomes (13, 18, 20). In agreement with a previous report (23), there is little difference in the baseline frequency of sister chromatid exchanges in normal and Fanconi’s anemia lymphocytes (Table 1). The increase in the frequency of sister chromatid exchanges per chromosome with metaphase chromosome length mirrors that found with normal cells (Fig. 1).

Normal lymphocytes exposed to 0.03 μg/ml of mitomycin C for the third day of culture exhibit an average of about 29 sister chromatid exchanges per cell (Figs. 2 and 3; Table 1), compared with a baseline frequency of less than 16. When lymphocytes from patients with Fanconi’s anemia are exposed to the same concentration of mitomycin C, an average

Abbreviation: EMS, ethylmethane sulfonate.
Table 1. Sister chromatid exchanges in cultured lymphocytes: Effects of mitomycin C and EMS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fanconi's anemia patientsa (I-IV)</th>
<th>Parents of patientsb (III, IV (M, F))</th>
<th>Normals (V-VIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noneb</td>
<td>(0.12-0.17)</td>
<td>(0.00)</td>
<td>(0.00-0.02)</td>
</tr>
<tr>
<td>BrdUrd</td>
<td>8.6-9.5</td>
<td>6.3-9.2</td>
<td>7.9-11.2</td>
</tr>
<tr>
<td>BrdUrd + dCyd</td>
<td>10.0-10.5</td>
<td>13.4-17.8</td>
<td>13.9-16.2</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>11.8-14.2</td>
<td>18.3-25.8</td>
<td>20.7-23.1</td>
</tr>
<tr>
<td>(10 mg/ml) + dCyd</td>
<td>(0.22-0.69)</td>
<td>(0.00-0.04)</td>
<td>(0.01-0.04)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>13.8-17.1</td>
<td>25.5-30.8</td>
<td>27.3-30.3</td>
</tr>
<tr>
<td>(30 mg/ml) + dCyd</td>
<td>(0.72-2.20)</td>
<td>(0.00-0.02)</td>
<td>(0.03-0.04)</td>
</tr>
<tr>
<td>EMS3,d</td>
<td>22.9-29.5</td>
<td>32.9-39.7</td>
<td>30.4-39.4</td>
</tr>
<tr>
<td>(250 μg/ml) + dCyd</td>
<td>(0.11-0.24)</td>
<td>(0.02-0.11)</td>
<td>(0.00-0.03)</td>
</tr>
</tbody>
</table>

a Sister chromatid exchanges were detected by 33258 Hoechst fluorescence after growth of lymphocytes in medium containing 10⁻⁶ M BrdUrd, 6 × 10⁻⁶ M Urd, and 4 × 10⁻⁹ M FdUrd. The top entry in each box represents the range of the average number of exchanges per cell. An average of 86 cells (range 19-257) was examined for each individual and set of conditions (total for table = 6113 cells). Second division metaphases were photographed at random, and all suitable photographs were scored. The range of the average number of morphological aberrations per cell (mostly chromatid breaks) is given in parentheses. The alkylating agents mitomycin C and EMS were present during the third and final day of culture. dCyd (10⁻⁴ M), when added, was present throughout the time of culture.

b In cells not grown in the presence of BrdUrd, sister chromatids could not be differentiated by 33258 Hoechst fluorescence and sister chromatid exchanges were not detected.

c In other cultures, alkylating agents were present during all 3 days of growth. For these cultures, the average number of exchanges after treatment with 0.01 μg/ml of mitomycin C was 18.4 (107 cells) in Fanconi's anemia and 28.1 (193 cells) in normal subjects, while with 0.25 mg/ml of EMS, the average number of exchanges was 29.0 (75 cells) in Fanconi's anemia and 42.7 (118 cells) in controls.

d In cultures not containing dCyd, the average number of exchanges in the presence of 0.03 μg/ml of mitomycin C was 15.6 (57 cells) in Fanconi's anemia and 21.6 (161 cells) in controls, while in the presence of 0.25 mg/ml of EMS, the average number of exchanges was 21.6 (161 cells) in Fanconi's anemia and 30.3 (180 cells) in controls.

e The patients were all males, age 11 (I), 15 (II), brother of (I), 14 (III), and 24 (IV). Medications received were prednisone (10 mg/day, all patients) and methandrostanolone (60 mg/day for I or 2.5 mg/day for II) or oxymetholone (60 mg/day, III). Patient IV had one (deceased) sibling with Fanconi's anemia and five unaffected siblings, while patient III had two unaffected siblings.

f III (M) and III (F) are the mother and father of patient III. Similarly, IV (M) and IV (F) are the parents of patient IV.

g Data on IV (F) were not obtained for this set of conditions.

of only 15 exchanges per cell is found, compared with a baseline frequency of approximately 10. The proportion of second division metaphases in Fanconi's anemia lymphocytes treated with alkylating agents is greatly increased by the addition of dCyd, which also increases the baseline sister chromatid exchange frequency. The numerical increment of exchanges induced by mitomycin C in Fanconi's anemia lymphocytes is only 0.4 of normal when based on differences in the exchange frequency above a baseline with both BrdUrd and dCyd added. This baseline, which varies between the two populations, may itself reflect the ability of cells to respond to a background level of DNA damage. If the increments in exchanges are divided by their respective pretreatment baselines (to obtain a relative increase in exchanges), the response of the Fanconi's anemia lymphocytes is approximately 0.6 of normal.

In contrast to the reduced number of sister chromatid exchanges, the frequency of chromatid breaks, quadriradial figures, and other exchange forms in these Fanconi's anemia lymphocytes is much greater than in identically treated normal cells (Table 1). In this tabulation, achromatic gaps without a marked discontinuity in chromatid structure and/or orientation were not included. Nearly half of the breaks (218 out of 456 observed in 777 cells treated with 0.03 μg/ml of mitomycin C) occurred at sites of incomplete sister chromatid exchange, i.e., sites of sister chromatid interchange with apparent link-up of the exchange products in only one of the two sister chromatids. (e.g., Figs. 2, 4, and 5A). Approximately half of the breaks induced in normal lymphocytes by mitomycin C are similarly located, although the total number scored was considerably less. Intercromosome junctions in exchange figures (Fig. 5B and C) also often involve sister chromatid exchange points.

The average increment in chromosome aberrations in second division Fanconi's anemia lymphocytes after exposure to mitomycin C (Table 1) is less than the associated reduction in sister chromatid exchanges. However, if the total numbers of breaks and exchanges in normal and Fanconi's
anemia cells are divided by their respective pretreatment baselines, the difference is much less. The data may be biased by selection, i.e., only minimally damaged cells reach the second metaphase after addition of BrdUrd, even if mitomycin C is present for only the third and final day of culture. However, the reduction in the average sister chromatid exchange frequency in mitomycin C-treated Fanconi's anemia cells appears due to a shift in the distribution of exchanges (Fig. 3) rather than a reproportioning of a bimodal distribution composed of unperturbed and normally responding cells.

The baseline level of sister chromatid exchanges in two strains of dermal fibroblasts (9.2 ± 3.0, 36 cells, GM 391; 9.2 ± 3.8, 26 cells, GM 449) from patients with Fanconi's anemia does not differ greatly from that in fibroblasts from normal individuals and from individuals with Fanconi's anemia. Cells were grown in medium containing BrdUrd, Urd, FdUrd, and dCyd. Mitomycin C (0.03 μg/ml) was added for the third and final day of culture. Compared to the normal lymphocyte (A) (35 sister chromatid exchanges), the Fanconi's anemia cell (B) exhibits fewer exchanges (21), but more chromatid breaks (7), many occurring at junctions of incomplete sister chromatid exchanges (arrows).

**FIG. 2.** The effect of mitomycin C on chromosomes from lymphocytes of normal individuals and from individuals with Fanconi's anemia. Cells were grown in medium containing BrdUrd, Urd, FdUrd, and dCyd. Mitomycin C (0.03 μg/ml) was added for the third and final day of culture. Compared with the normal lymphocyte (A) (35 sister chromatid exchanges), the Fanconi's anemia cell (B) exhibits fewer exchanges (21), but more chromatid breaks (7), many occurring at junctions of incomplete sister chromatid exchanges (arrows).

**FIG. 3.** The distribution of sister chromatid exchanges in normal (I) and Fanconi's anemia (II) lymphocytes: effects of dCyd, mitomycin C, and EMS. Lymphocytes were cultured for 3 days in medium containing 10⁻⁵ M BrdUrd, 6 × 10⁻⁶ M Urd, and 4 × 10⁻⁷ M FdUrd. In all but (A), 10⁻⁴ M dCyd was also present throughout culture. Mitomycin C was present during the third and final day of culture at final concentrations of 0.01 μg/ml in (C) and 0.03 μg/ml in (D). In (E), EMS was present at a final concentration of 0.25 mg/ml during the third day of culture. In each histogram, the number of cells is plotted against the appropriate number of sister chromatid exchanges per cell. Vertical lines indicate the mean exchange frequency, while the brackets represent the standard deviation of a single observation (several times the standard error of the mean).

**FIG. 4.** Extensive chromosome damage induced by mitomycin C in a Fanconi's anemia lymphocyte. Cell growth and treatment were identical to those of Fig. 2B, while arrows again indicate chromatid breaks at sites of incomplete sister chromatid exchange.
DISCUSSION

The reduced ability of cells from patients with Fanconi's anemia to form additional sister chromatid exchanges after exposure to alkylating agents suggests that a defect related to chromosome repair exists in these cells. The convenience and sensitivity of the present cytophotometric technique for detecting DNA damage and repair is somewhat offset by the bias introduced when observing cells at metaphase of the second cycle after addition of BrdUrd to cultures. Selection effects might be circumvented by the utilization of biochemical methods for detecting DNA interchange in material isolated from interphase cells.

Although the DNA repair systems in human cells have not yet been fully delineated, there is evidence that mechanisms analogous to those present in bacteria (26, 27) may exist. Interest in incisional repair, related to the uvr system of bacteria, has been stimulated by observations that most xeroderma pigmentosum cells are defective in an early step involving the excision of thymine dimers from DNA (28, 29). An alternate repair system, related to recombination, also exists in bacteria (30). Certain cells (recA) defective in this system are deficient in undergoing genetic recombination and are especially sensitive to killing by a variety of agents (31), while possession of this function is necessary for bacteria to respond to DNA crosslinking with an interchange between sister DNA molecules (32). The increase in sister chromatid exchanges in human cells treated with alkylating agents bears at least a superficial resemblance to recombinational repair (20). It is tempting to speculate that this process may somehow be defective in Fanconi's anemia lymphocytes. Whatever its basis, the reduced inducibility of sister chromatid exchanges observed in Fanconi's anemia lymphocytes may ultimately be of diagnostic use.

The possibility that the paucity of induced sister chromatid exchanges may be related to the increase in chromatid breaks in Fanconi's anemia lymphocytes is supported by the observation that many of these breaks occur at sites of incomplete sister chromatid exchange. The fraction of breaks occurring at such sites may relate to the frequency with which repair of mitomycin C damage is accompanied by the interchange of DNA between sister chromatids. Compared with mitomycin C, EMS induces a relatively greater number of sister chromatid exchanges with a lesser increase in chromatid breaks in Fanconi's anemia lymphocytes. This suggests that a step needed for the repair of damage induced by bifunctional, but not monofunctional, alkylating agents may be deficient in this disease. Whereas, more than one type of reaction product might be expected with each agent, the predominant consequences should differ. Thus, monofunctional agents characteristically react with guanine residues, labilizing the DNA to depurination, which in turn is often followed by a polynucleotide chain break (33, 34). A single such series of events occurring after a bifunctional alkylation, e.g., with mitomycin C (34, 35), would still leave a fragment attached to a second polynucleotide chain, to be removed. In analogy with the heterogeneity of the lesion in xeroderma pigmentosum (36), it is possible that errors in any of a group of steps might produce the chromosome aberrations observed in Fanconi's anemia. Examination of the effects of other agents capable of inducing sister chromatid exchanges should assist characterization of DNA repair processes in Fanconi's anemia.

The parents of patients with Fanconi's anemia exhibit a normal sister chromatid exchange response after mitomycin C or EMS treatment. This is consistent with an autosomal recessive inheritance mode which has previously been postu-
lated (1) for this disease. Fanconi’s anemia occurs in both sexes, although in one large study (1), females accounted for only one-third of the cases observed. It is thus possible that there is a sex-dependent effect on the expression of this disease, and the existence of several forms of the disease, with a heterogeneous genetic basis, cannot be excluded.

Abnormal chromosome morphology in Fanconi’s anemia has been observed in peripheral lymphocytes (6, 7), bone marrow cells (37), and cultured fibroblasts (3). The principal observation reported here is that phytohemagglutinin-stimulated lymphocytes from patients with the clinical diagnosis of Fanconi’s anemia exhibit a markedly deficient ability to respond to mitomycin C with an increase in sister chromatid exchanges. They exhibit an abnormally high number of chromatid breaks, many occurring at sites of incomplete sister chromatid exchange. The nearly normal increment of exchanges after mitomycin C treatment of the lines of dermal fibroblasts examined suggests that different tissues may be unequally affected in Fanconi’s anemia. Examination of fibroblasts and lymphocytes from the same individuals would help to test this hypothesis. Alternatively, the results may reflect differences between details of the culture conditions, which in turn exert unequal selective pressures on lymphocytes and fibroblasts. While the results of the present study could account for the characteristic chromosome changes in Fanconi’s anemia lymphocytes, it is not yet clear how they relate to clinical features of the disease.

The defective sister chromatid exchange response in Fanconi’s anemia lymphocytes contrasts sharply with the marked increase in exchanges observed in Bloom’s syndrome (23) in the absence of alkylating agents. Both diseases involve chromosome structural abnormalities and a predisposition for the development of neoplasia. In a third disease, ataxia telangiectasia, which is also characterized by these same two features, the frequency of sister chromatid exchange is normal (23), but specific rearrangements involving chromosome no. 14 have been observed (38). Cytological data differentiating the diseases described should stimulate a search for associated biochemical changes in these and related conditions.

Note Added in Proof. Sister chromatid exchanges have been analyzed in three additional fibroblast lines. In cells from two normal individuals, the baseline sister chromatid exchange frequencies were 7.3 ± 2.0 (18 cells) and 8.1 ± 3.1 (20 cells), while the exchange frequencies after mitomycin C treatment were 38.3 ± 8.5 (32 cells) and 33.8 ± 6.4 (12 cells), respectively. In one line of Fanconi’s anemia fibroblasts (HG 261, American Type Culture Collection) the frequencies were 10.3 ± 5.8 (16 cells), control, and 27.7 ± 7.1 (3 cells), after mitomycin C treatment.

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