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Genetics

Different mutations are responsible for the elevated sister-chromatid exchange frequencies characteristic of Bloom's syndrome and hamster EM9 cells

(somatic cell hybridization/complementation/bromodeoxyuridine sensitivity/chromosome instability)

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ABSTRACT **Experimental hybridization of cultured cells was employed to determine whether the strikingly elevated rates of sister-chromatid exchange (SCE) exhibited by Bloom's syndrome (BS) and hamster cell line EM9 have the same or different bases. Seventeen cell lines were developed from polyethylene glycol-treated mixtures of BS and EM9 cells. Cytogenetic analysis proved the hybrid nature of 12 of the lines; 9 of those 12 exhibited low (normal) numbers of SCEs, signifying complementation. The parental BS and EM9 cells, although resembling each other in exhibiting very high SCE frequencies in BrdUrd-containing medium, differ from one another with respect to their proliferative abilities in such medium, the EM9 cells but not the BS cells being exquisitely hypersensitive to BrdUrd. In the low-SCE hybrid lines, hypersensitivity to growth in BrdUrd-containing medium was restored to normal whereas the hypersensitivity was retained by the high-SCE hybrids. It is concluded, first, that the mutations in BS and EM9 cells are different and, second, that both the elevated SCE frequency and the excessive BrdUrd hypersensitivity of EM9 cells are due to the same mutation.**

Three striking phenotypic features are shared by cultured cells from persons with Bloom's syndrome (BS) and Chinese hamster cell line EM9: (*i*) an elevated sister-chromatid exchange (SCE) frequency following growth for one or two cycles of DNA replication in bromodeoxyuridine (BrdUrd)-containing medium (1–3), (*ii*) a markedly elevated number of SCEs following exposure to the mutagen ethyl methanesulfonate (4, 5), and (*iii*) a retarded rate of DNA-chain maturation (4, 6–10). BS is a recessively transmitted growth disorder that predisposes to cancer (11); EM9 is an experimentally induced mutant derivative of Chinese hamster ovary (CHO) cells (1). The similar phenotypic features of cultured BS and EM9 cells suggested the possibility that the molecular defects in the two cell types were the same, in which case EM9 cells might serve as a valuable *in vitro* model for BS. To determine whether the mutation in BS and the mutation in EM9 were, in fact, the same, complementation studies were performed by fusing BS lymphoblastoid cells with EM9 cells. The SCE frequency was found to have been restored to normal in a large proportion of the cell lines derived from BS/EM9 hybrids—i.e., complementation occurred.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human lymphoblastoid cell line (LCL) HG 1270 and hamster cell line EM9 were used in each of the four cell-fusion experiments that were carried out. HG 1270 is an Epstein–Barr virus-transformed B-lymphocyte cell line developed as a clone from a single cell of the peripheral blood of BS patient 15(MaRo) (12). [This line was developed in collaboration with E. E. Henderson (Temple University School of Medicine, Philadelphia, PA). Cell line EM9 was given to us by L. H. Thompson (Lawrence Livermore National Laboratory, Livermore, CA) (1).] Cells from both these lines exhibit strikingly elevated frequencies of SCE after incubation for two cycles of DNA replication in BrdUrd-containing medium (refs. 1 and 13, and Table 1). HG 1270 cells were grown in suspension in RPMI 1640 medium supplemented with 20% heat-inactivated (55°C for 30 min) fetal bovine serum, 2 mM L-glutamine, 100 international units of penicillin per ml, and 100 μg of streptomycin per ml. EM9 cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 μM nonessential amino acids, 100 international units of penicillin per ml, and 100 μg of streptomycin per ml (complete MEM).

Cell-Fusion and Hybrid-Selection Techniques. The cell-fusion method of Yoshida was employed (14). In preparation for each fusion, equal numbers of BS and EM9 cells (1×10^6 of each) were added to a 25-ml flat-bottomed specimen vial (Wheaton Scientific, Millville, NJ) and pelleted by centrifugation ($600 \times g$; 15 min). The cells were resuspended in serum-free MEM and recentrifuged, and the resulting cell pellet was overlaid with 1 ml of serum-free MEM containing 10% dimethyl sulfoxide (Me_2SO, Fisher). The Me_2SO solution was replaced after 5 min with 0.5 ml of serum-free MEM containing 50% (wt/vol) Koch–Light polyethylene glycol (PEG) 1000 (Research Products International, Elk Grove, IL) and 10% Me_2SO. The PEG solution was aspirated after 1 min, and the cell layer was washed gently three times with serum-free MEM. After removal of the final wash, 5 ml of complete MEM supplemented with 0.1% Fungizone (GIBCO) was added, and the cells were incubated for 2 hr at 37°C in 5% CO_2. At the end of the incubation period, the cells were subcultured into five 10-cm petri plates each containing 10 ml of complete MEM with Fungizone. Cells that had adhered to the surface of the vials during incubation were removed by trypsinization (0.05% trypsin/0.02% EDTA).

Twenty-four hr after fusion, unfused (i.e., unattached) BS lymphoblastoid cells remaining in the petri plates were selected against by a vigorous washing of the monolayers with 10 ml of Dulbecco's phosphate-buffered saline. To select against unfused EM9 cells, 5 μg of BrdUrd per ml was added to complete medium supplemented with Fungizone, and the petri plates were incubated in the dark for 96 hr. The medium then was replaced with medium lacking BrdUrd, and the petri

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Abbreviations: BS, Bloom's syndrome; CHO, Chinese hamster ovary; SCE, sister-chromatid exchange; MEM, Eagle's minimum essential medium; Me_2SO, dimethyl sulfoxide; LCL, lymphoblastoid cell line.

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Table 1. Cytogenetic analysis of BS (HG 1270) and hamster EM9 cell lines and of cell lines derived from the fusion of HG 1270 and EM9

Cell lines	SCEs/chromosome		Chromosomes/cell		Human chromosomes/cell
	Mean	Range	Mean	Range	
			Parental		
BS	1.42	0.78–2.15	45.5	44–47	44–47
EM9	4.41	2.85–6.05	20.9	19–23	0
			Hybrid		
Low-SCE lines with human chromosomes					
F20	0.43	0.30–0.64	67.9	57–76	7–15
F24	0.32	0.19–0.61	66.0	59–75	4–11
F25	0.55	0.32–0.85	74.4	61–85	5–12
F32	0.39	0.15–0.75	58.9	51–69	4–9
F33	0.44	0.31–0.57	77.0	68–80	9–19
F35	0.25	0.11–0.38	71.4	48–85	2–15
F37	0.49	0.33–0.66	70.8	57–77	4–10
F38	0.40	0.19–0.57	51.3	46–63	2–5
F39	0.44	0.27–0.63	62.8	60–71	2–7
	0.41	0.11–0.85	66.7	46–85	2–19
High-SCE lines with human chromosomes					
F23	3.43	2.67–4.26	45.3	41–49	2–5
F27	2.96	2.76–3.36	35.4	25–43	1–4
F30	2.82	1.25–3.84	57.7	49–80	1–7
	3.07	1.25–4.26	46.1	25–80	1–7
High-SCE lines lacking human chromosomes					
F22	4.21	3.13–5.42	59.3	43–71	0
F29	4.43	3.57–5.39	60.2	51–66	0
F31	2.89	2.90–3.24	20.6	20–21	0
F34	2.02	1.53–2.56	79.4	66–87	0
F36	2.98	2.29–3.95	34.8	23–49	0
	3.31	1.53–5.42	50.9	20–87	0

plates were reincubated in the dark for 14 days, with changes of medium being made at 4-day intervals.

Colony Isolation and Expansion into Cell Lines. Multiple colonies were present at the end of the 14-day incubation period in each of the petri plates that had been set up from the four separate cell-fusion experiments. One randomly selected colony was removed from each petri plate for expansion into a cell line; a cloning ring was placed around each colony to decrease the chances of cross-contamination by cells from adjacent colonies during the trypsinization procedure. The 20 colonies isolated in this way and the 17 cell lines developed from them were designated F20–F39. Cell lines F21, F26, and F28 failed to thrive and were discarded.

Cytogenetic Analyses. Cells from each of the 17 surviving cell lines were analyzed to determine whether human and/or hamster chromosomes were present and whether the SCE frequency remained elevated like that of the parental cells or had been restored to normal. To accomplish the first objective, Colcemid (6 μg per ml) was added for the final 2.5 hr of incubation, and the cells were harvested by trypsinization, hypotonic treatment (0.075 M KCl for 10 min at 25°C), and fixation in methanol/glacial acetic acid (3:1). Ten metaphase cells from each line were analyzed for the presence of human and hamster chromosomes using the Giemsa-11-staining technique (15). (With this technique, human chromosomes stain pale blue, and hamster chromosomes stain magenta.) For SCE-frequency determinations, cells were cultured in the dark for 29 hr in medium containing 10 μM BrdUrd, and metaphase chromosome preparations were made as described above. Sister chromatids were stained differentially using a previously described procedure (2, 3). Detailed analyses were made of 10 consecutive differentially stained cells from each line.

BrdUrd-Sensitivity Studies. The proliferative response of parental EM9 cells in the presence of BrdUrd (5 μg per ml) was compared with those of three randomly chosen hybrid lines showing low SCE (F24, F32, and F38) and three randomly-chosen hybrid lines showing high SCE (F22, F23, and F36). Duplicate flasks containing 20 ml of complete MEM supplemented with Fungizone were inoculated on day-0 with ≈50,000 viable cells (viable as determined by trypan blue exclusion) from each line. The medium in one flask of each pair was supplemented with 5 μg of BrdUrd per ml; the other flask contained no BrdUrd. The flasks were wrapped in aluminum foil and incubated for 5 days at 37°C in 5% CO_2. On day-5 the number of cells in each flask was determined using a Coulter counter (16). The effect of BrdUrd on cell growth was determined by comparing the number of cells per flask after 5 days growth with and without BrdUrd (Table 2).

RESULTS

Analysis of Giemsa-11-stained chromosomes demonstrated unequivocally that 12 of the 17 cell lines derived from the PEG-treated cultures had arisen from BS/EM9 fusion products (Table 1). Hamster chromosomes varied in number from 25 to 85 per cell, indicating that BS cells originally may have fused either with one or more diploid EM9 cells or with one or more tetraploid EM9 cells. Human chromosomes varied in number from 1 to 19 per cell. Cells from the remaining five lines (F22, F29, F31, F34, and F36) lacked human chromosomes, which is interpreted to mean either that occasional EM9 cells do for unknown reasons survive the 4-day exposure to BrdUrd or that the cell that gave rise to each of the five lines was a BS/EM9 hybrid the progeny of which had lost all human chromosomes after removal of the selective agent.

Both the parental cell lines and the lines developed from the PEG-treated BS and EM9 cell mixtures were examined for (*i*) their SCE frequencies (Table 1) and (*ii*) their abilities to grow in BrdUrd-containing medium (Table 2):

(*i*) The parental BS and EM9 cells exhibited high baseline SCE frequencies (means of 1.42 and 4.41 SCEs per chromo-

Table 2. Comparison of the effect of BrdUrd-containing medium on the proliferative response of cell line EM9 and randomly selected BS/EM9 hybrid lines

Cell lines	Proliferative index*		+BrdUrd/−BrdUrd†
	−BrdUrd	+BrdUrd	
		Parental	
EM9‡	254.1	8.6	0.03
		Hybrid	
Low-SCE lines			
F24	67.9	38.6	0.57
F32	61.7	41.8	0.68
F38	89.9	63.6	0.71
	73.2	48.0	0.65
High-SCE lines			
F22	53.9	9.1	0.17
F23	113.1	10.9	0.10
F36	156.2	12.5	0.08
	107.7	10.8	0.12

*Ratio of the number of cells per flask after 5 days of culture to the number of viable cells in the inoculum.

†Ratio of the proliferative index after 5 days in BrdUrd-containing medium to the proliferative index after 5 days in medium lacking BrdUrd.

‡In previous experiments using EM9, CHO, BS, and normal human cells, the number of cells after 4-day growth in BrdUrd-containing medium was 11%, 47%, 54%, and 52%, respectively, of the number in the absence of BrdUrd (16).

some, respectively) in agreement with previously published reports (1, 4, 13). Among the 12 BS/EM9 lines that had been proven cytogenetically to be hybrids, two types of cells with respect to SCE frequency were detected: 9 of the 12 lines exhibited low numbers of SCEs, with means of 0.25 to 0.55 SCEs per chromosome; the other three lines exhibited high numbers of SCEs, with means of 2.82 to 3.43 SCEs per chromosome (Table 1). Cells from the five BS/EM9 lines that lacked cytogenetically recognizable human chromosomes also had high-SCE frequencies, with means of 2.02 to 4.43 SCEs per chromosome.

(*ii*) Growth of several of the BS/EM9 hybrids in medium supplemented with 5 μg of BrdUrd per ml was compared with that of parental EM9 cells. Three low-SCE and three high-SCE hybrid lines, each chosen at random, were analyzed as indicated in Table 2. Only 3% as many EM9 cells were present after 5-day growth in BrdUrd-containing medium as were present in medium lacking BrdUrd, in agreement with previous reports (16, 17). The three high-SCE hybrid lines resembled the parental EM9 cells with respect to their abilities to proliferate in BrdUrd-containing medium; growth of these hybrid cells in BrdUrd was only 8–17% of their growth in medium lacking BrdUrd. In contrast, the response of the low-SCE hybrids to BrdUrd resembled the responses of CHO, normal human, and parental BS cells to BrdUrd (16, 17); there were 57–71% as many cells after incubation in BrdUrd-containing medium as in BrdUrd-free medium. Thus, those BS/EM9 lines examined that had lost their high-SCE frequency also had lost their hypersensitivity with respect to growth in BrdUrd.

DISCUSSION

The main question addressed was whether EM9 could serve as an *in vitro* model for BS. Shortly before the experiments described here were performed, BS and EM9 cells were discovered to differ from one another in their proliferative responses to BrdUrd. BS cells grow at a slightly reduced rate in BrdUrd-containing medium, as do normal human and CHO cells whereas EM9 cells are extremely sensitive, manifesting extensive chromosome damage (16) and the prompt cessation of proliferation (16, 17). {One report that contradicts these findings had appeared, showing that an LCL derived from a Japanese BS patient was exquisitely sensitive to BrdUrd (18). However, in our laboratory normosensitivity to BrdUrd now has been demonstrated in each of seven high-SCE LCLs derived from BS patients of several ethnic backgrounds, including Japanese [BS patient 93(YoYa) in the Bloom's Syndrome Registry; the line was provided for inclusion in our study by A. Oikawa, Tohoku University, Sendai, Japan], Ashkenazi Jewish, and Western European.} This suggested that the defects in the two cell types might be different, but complementation studies were necessary to confirm this. The experimental production of BS/EM9 hybrid cells with low baseline SCE frequencies, unlike that of either parental cell line, demonstrates unequivocally that the molecular bases for the elevated SCE frequency of BS cells and of EM9 cells are different. Although the results reported here admittedly were derived from the study of a single high-SCE BS LCL (HG 1270) that was developed as a clone, the finding would be expected to extend to BS cells in general because complementation analyses utilizing cells derived from BS patients of diverse ethnic origins have failed to provide evidence for the existence of genetic heterogeneity in BS (19).

BS and EM9 represent two of the six known cell types that feature an elevated baseline SCE frequency when grown in BrdUrd-containing medium. The other four cell types are cell line LA9, a spontaneous mutant derived from the C3H mouse cell line L929 (20, 21), and cell line ES 4, Ac 12, and AC 41, experimentally produced mutants of mouse lymphoma L5178Y cells (22, 23). The mutations in BS and LA9 cells (20, 21) and in ES 4 and AC 12 cells (23) are known to complement one another just as do those of BS and EM9 studied here. Collectively, then, the data indicate not only that the products of multiple genes are involved in SCE formation but also that those several genes have been conserved throughout mammalian evolution. The maintenance of a low (i.e., normal) SCE frequency by cells apparently is a consequence of the coordinated functioning of a number of gene products, any one of which, when defective, can lead to a constitutional elevation of the SCE frequency. Therefore, further studies comparing the several cell types that display the high-SCE phenotype, namely BS, EM9, LA9, ES 4, AC 12, and AC 41 appear warranted. The information derived from the study of such mutations will pertain to the mechanism(s) used by mammalian cells in bringing about exchange between DNA molecules, at least under experimental conditions.

The co-suppression of the elevated SCE frequency and the BrdUrd hypersensitivity in a proportion of the BS/EM9 lines and their co-retention in others (*cf.* Tables 1 and 2) strongly suggest that these two striking phenotypic features of EM9 result from the same biochemical defect. It is probable that the dramatically elevated SCE frequency of EM9 reflects an aberrant response of these cells to proliferation in BrdUrd-containing medium. Supporting this hypothesis are data derived from an analysis of differentially stained endoreduplicated EM9 chromosomes (24) showing that most SCEs are produced during the second cycle of DNA replication when BrdUrd-substituted DNA is used as the template for replication. Similarly, Pinkel and coworkers, using monoclonal antibody to BrdUrd-substituted DNA, reported that the SCE frequency of EM9 cells was reduced significantly when low BrdUrd concentrations were used although it still was elevated above baseline level at the lowest BrdUrd concentration used (25).

Identification of the specific human chromosome that corrects the high SCE frequency of EM9 chromosomes in the BS/EM9 hybrid cells was not attempted in the present experiments, although the material generated would permit such an analysis. However, Siciliano *et al.* (26) recently have shown that chromosome 19 from normal human cells contains the gene that is defective in EM9 cells. The chromosome derived from BS cells that suppresses the elevated SCE frequency of EM9 chromosomes in the hybrids studied here probably is the same chromosome. Identification of the specific hamster chromosome that suppresses the high SCE frequency of BS chromosomes would not have been possible in our experiments because the entire hamster chromosome complement was retained by the hybrids. Fusion of hamster microcell preparations with BS cells should permit identification of the hamster chromosome that contains the wild-type gene for BS.

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