EFFECTS OF MITOMYCIN C ON HUMAN CHROMOSOMES

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INTRODUCTION

Mitomycin C (MC) is a chemically reactive antibiotic derived from *Streptomyces caespitosus*. The drug selectively inhibits DNA synthesis (2, 15, 29, 34, 39, 40, 43, 44, 49) and degrades cellular DNA but does not affect the synthesis of RNA or protein (31, 33, 40). MC induces bacteriophage production in lysogenic bacteria (23, 29), increases the rate of genetic recombination among mutant forms of *E. coli* (15, 47, 49), and possesses antitumor activity (15, 47). In tissue culture systems, MC inhibits mitosis, reduces cell viability, and produces nuclear disorganization and giant cells (21, 32).

Several models for the mechanism of MC activity have been suggested. Reich *et al.* proposed that MC acts through the scission of the DNA strands, thereby preventing replication (33). This implies that the template competence of the DNA is destroyed (31). The depolymerization of DNA and the accumulation of acid-soluble fragments implicates the DNA polymerase system as a possible target of MC activity (33, 34). Recently, Iyer and Szybalski suggested that the primary action of MC is the "cross-linking" of the complementary strands of the DNA molecule and that the degradation of DNA may be of a secondary nature (16).

The effects of MC on plant chromosomes have been previously noted. Merz (26) reported that chromatid breaks in the heterochromatic regions of *Vicia faba* root tip chromosomes were induced by MC. Matsuura *et al.* (24, 25) observed no breaks after MC treatment of *Trillium kamschaticum*; however, MC treatment followed by x-ray greatly increased the frequency of chromosome breaks involved in exchanges.

Because MC has a profound effect on DNA synthesis and because another Streptomyces-derived antimicrobial agent (streptonigrin) caused nonrandom breakage of human chromosomes (3, 4), the present study was undertaken. The experiments to be described demonstrate that MC preferentially produces breaks and rearrangements in the paracentric secondary constrictions of chromosomes 1, 9, and 16.

MATERIALS AND METHODS

Mitomycin C was obtained from Bristol Laboratories, Inc., Syracuse, New York. Three-day leukocyte cultures were established from peripheral blood specimens obtained from five apparently healthy, unrelated individuals. Twenty-four hours prior to harvest, MC was added in final concentrations of 0.1, 1.0, and 5.0 μg/ml culture medium in replicate cultures. Cytological preparations were made following a method similar to that of Moorhead *et al.* (27). Coded slides were systematically scanned under low power. Each well spread metaphase encountered was examined under oil and analyzed for chromosome number and morphology. Chromosomes with abnormalities were classified according to the seven groups of the Denver classification (5) and, where possible, identified as individual chromosomes. Both chromatid and isochromatid breaks were scored as single breaks. A chromosomal exchange was scored as a single break in each of the chromosomes involved. The position of each break was measured following a method previously used (4).

RESULTS

General Effects of MC

Treatment with 5.0 μg MC/ml resulted in total cell destruction. At 1.0 μg MC/ml the mitotic rate was significantly lower than normal and numerous chromosome breaks and exchanges were observed, while at a concentration of 0.1 μg MC/ml the chromosome damage was less marked (Table I; last column). At comparable molar concentrations, mitomycin is less potent than streptonigrin (SN): 2.0×10^{-7} M SN for 24 hours resulted in chromosome fragmentation and cell disintegration (3), whereas cultures treated with 1.8×10^{-6} M MC (1.0 μg MC/ml) contained some normal-appearing chromosomes.

Chromosomal Breakage

MC did not break the chromosomes randomly in proportion to their relative lengths (Table I; $P < 0.001$). The largest deviation from randomness was found among the chromosomes of the 6–12 + X group. In this group alone, the deviation was due primarily to the excess of breaks in those chromosomes identified as No. 9 with a

TABLE I

Distribution and Test for Randomness of Chromosome Breaks, Based on Unit Lengths of the Denver Classification

Treatment	Number of breaks by chromosome or group*													
	1	2	3	4-5	6-12+X (except No. 9)	9	13-15	16	17-18	19-20	21-22+Y	Total	Total cells	Mean number of breaks per cell
Control	6	3	4		6	1		1	1			22	626	0.03
0.1 μg MC/ml	58	15	14	11	74	82	12	19	10	13	4	312	416	0.75
1.0 μg MC/ml	45	8	10	17	66	64	16	18	7	10	1	262	114	2.30
Total treated														
Observed	103	23	24	28	140	146	28	37	17	23	5	574	530	1.08
Expected	50.40	47.18	39.55	70.46	181.33	26.58	58.39	17.56	32.26	26.31	23.98	574		

$\chi^2 = 705.16$; df = 10; $P < 0.001$.

* Does not include 42 unidentifiable breaks and 13 acentric fragments.

corresponding deficiency of breaks among the remaining chromosomes of this group (χ^2 = 374.84; P < 0.001).

As noted by other authors (7, 28, 36, 37), human chromosomes Nos. 1, 9, and 16 possess prominent secondary constrictions. These regions appear to be focal points of MC activity (Fig. 1), since 56 per cent, 87 per cent, and 58 per cent of the breaks in these chromosomes, respectively, occurred in these "target areas." Upon removal of these breaks and subsequent testing of the data, a random distribution of breaks was observed among the remainder of the chromatin complement (χ^2 = 9.39; 0.50 > P > 0.25).

Attenuation of Secondary Constrictions

More striking than the chromosome breaks, however, was the exaggerated appearance of the

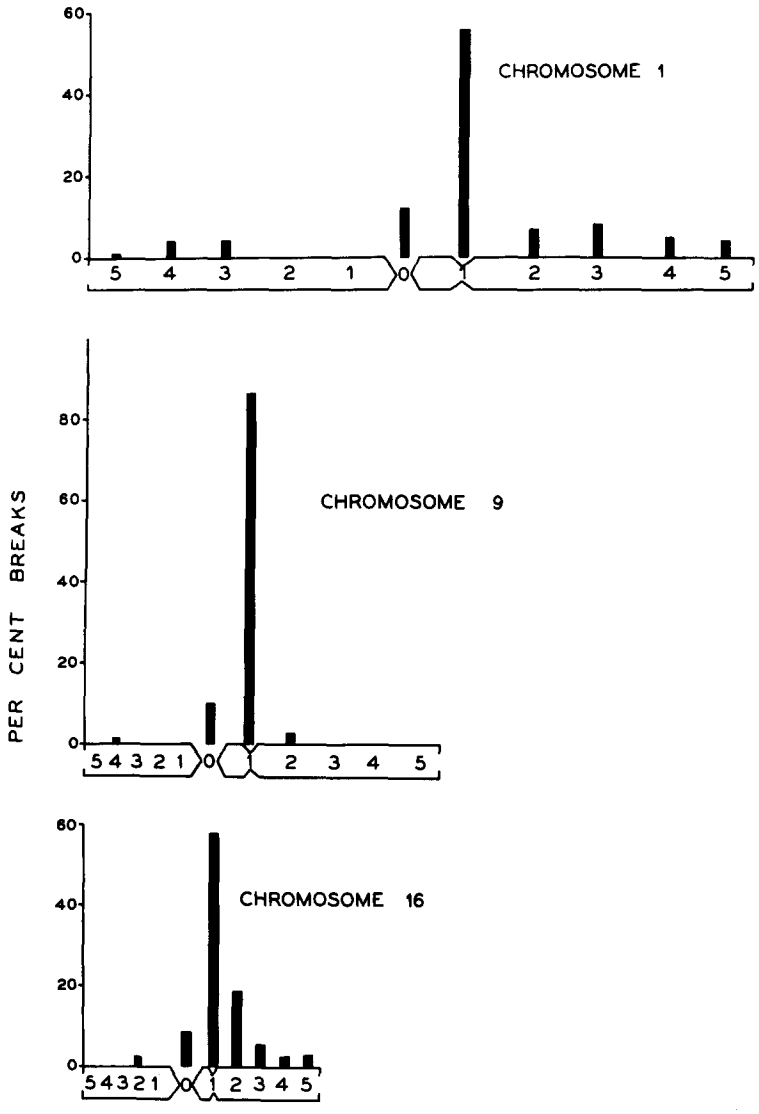

FIGURE 1 The distribution of breaks within chromosomes Nos. 1, 9, and 16. The secondary constriction lies in segment 1 of the long arm in each chromosome.

secondary constriction of No. 9 after MC treatment. A marked distention, attenuation, and uncoiling of this region was observed, resulting in a pale heteropyknotic appearance (Fig. 2). Although this effect was often noted in only one homologue in the treated cells (37.5 per cent), sometimes (11.7 per cent) both members of the No. 9 pair, in male and female cells, were so affected (Fig. 3).

Chromosomal Exchanges

In the MC-treated cells, chromosomal exchanges were observed which resembled the pachytene cross-configurations seen in meiosis after a reciprocal translocation (Fig. 4). The frequency of these exchange configurations increased with the MC concentration (Table III). Among 118

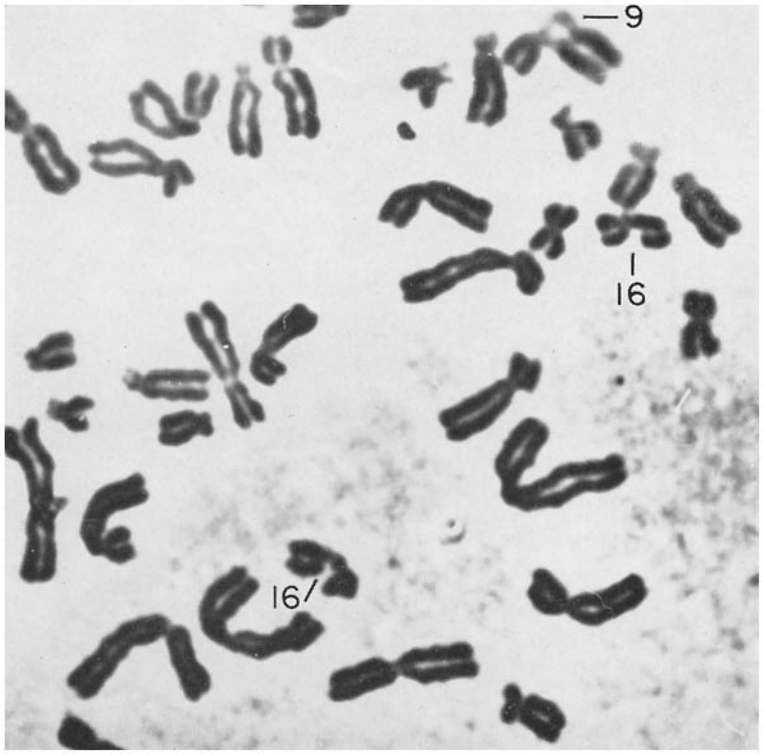

FIGURE 2 Exaggerated secondary constriction in chromosome No. 9; note the attenuated heteropycnotic appearance. Both chromosomes No. 16 show chromatid breaks in the secondary constriction region. × 1500.

A less pronounced increase in frequency of attenuation was also observed at the secondary constrictions of chromosomes Nos. 1 and 16 in the treated cells (Table II). Other chromosomes of the complement in which heterochromatic regions have been described were not apparently strongly affected by MC. Combining the data for attenuation and/or breaks at the secondary constrictions in chromosomes Nos. 1, 9, and 16, it can be seen in Table II that at the 1.0 μg/ml concentration nearly one-half of the No. 9 chromosomes, one-third of the No. 1 chromosomes, and one-eighth of the No. 16 chromosomes were affected.

exchanges analyzed, 60.2 per cent of the figures occurred between homologues or apparent homologues. The probability that any two chromosomes in an exchange figure would be apparent homologues by chance alone (assuming equal probability for each chromosome) is only 15 per cent. Exchanges between non-homologous chromosomes were observed in 29.7 per cent of cases, while 5.1 per cent of the configurations included three or more chromosomes and 5.0 per cent could not be identified. Fifty per cent of the exchange figures included chromosomes possessing a secondary constriction (No. 1 = 17 per cent;

No. 9 = 27 per cent; and No. 16 = 6 per cent). Among these chromosomes the exchange almost invariably occurred at the secondary constriction. Those figures involving apparent homologues suggest that somatic pairing has occurred. A larger sample of these mitomycin-induced exchanges is currently being analyzed in search for cytological evidence of somatic crossing over.

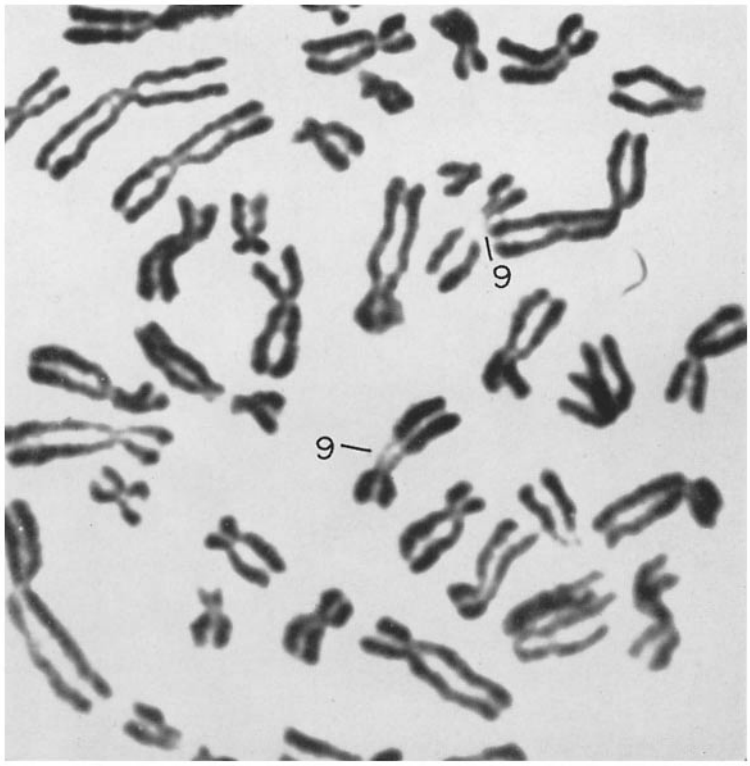

FIGURE 3 Arrows indicate attenuated secondary constrictions in both homologues of chromosome pair No. 9. × 1500.

DISCUSSION

A variety of chemicals has long been known to induce chromosomal damage, and many of these compounds seem to induce effects which are nonrandom, but confined to specific regions of specific chromosomes. For example, chromosomal breaks induced by chemical treatment are frequently localized in the prominent heterochromatic segments: the nucleolar organizing regions, secondary constrictions, and paracentric heterochromatin (see review by Kihlman, 19). Segments of intercalary heterochromatin also exhibit an increased frequency of chromosome breakage (11). More recently, experiments with mammalian cells have revealed the specificity of several chemical agents for preferential breakage at heterochromatic regions (4, 12, 45). Mitomycin also demonstrates such an affinity for certain regions of the chromosomes in human leukocytes; it induces breaks at the three prominent secondary constrictions in chromosomes Nos. 1, 9, and 16. In addition, however, MC exhibits specificities not previously attributed to other radiomimetic agents. This drug causes a marked increase in attenuation and uncoiling of these secondary constrictions (particularly No. 9) and induces exchange figures between homologues suggesting that somatic pairing and crossing-over have taken place.

That MC affected chromosome No. 9 in particular was, indeed, fortuitous, since this is the only member of the 6–12 + X group which can sometimes be identified by a secondary constriction. The appearance of this constriction, by its severe attenuation or breakage, increased the

TABLE II

Distribution of Exaggerated Secondary Constrictions and Breaks in Chromosomes Nos. 1, 9, and 16 Following MC Treatment

Percentages of chromosomes affected are given in parentheses.

Treatment	Chromosome Number									Total cells
	1			9			16			
	*A**	*B*	*Tot.*	*A*	*B*	*Tot.*	*A*	*B*	*Tot.*	
Control	149 (11.9)	6 (0.5)	155 (12.4)	54 (4.3)	1 (0.1)	55 (4.4)	7 (0.6)	1 (0.1)	8 (0.7)	626
0.1 μg MC/ml	209 (25.1)	58 (7.0)	267 (32.1)	264 (31.7)	82 (9.9)	346 (41.6)	29 (3.5)	19 (2.3)	48 (5.7)	416
1.0 μg MC/ml	31 (13.6)	45 (19.7)	76 (33.3)	39 (21.5)	64 (28.1)	103 (45.2)	10 (4.4)	18 (7.9)	28 (12.3)	114

A^* = attenuated secondary constriction; B = breaks in secondary constriction regions.

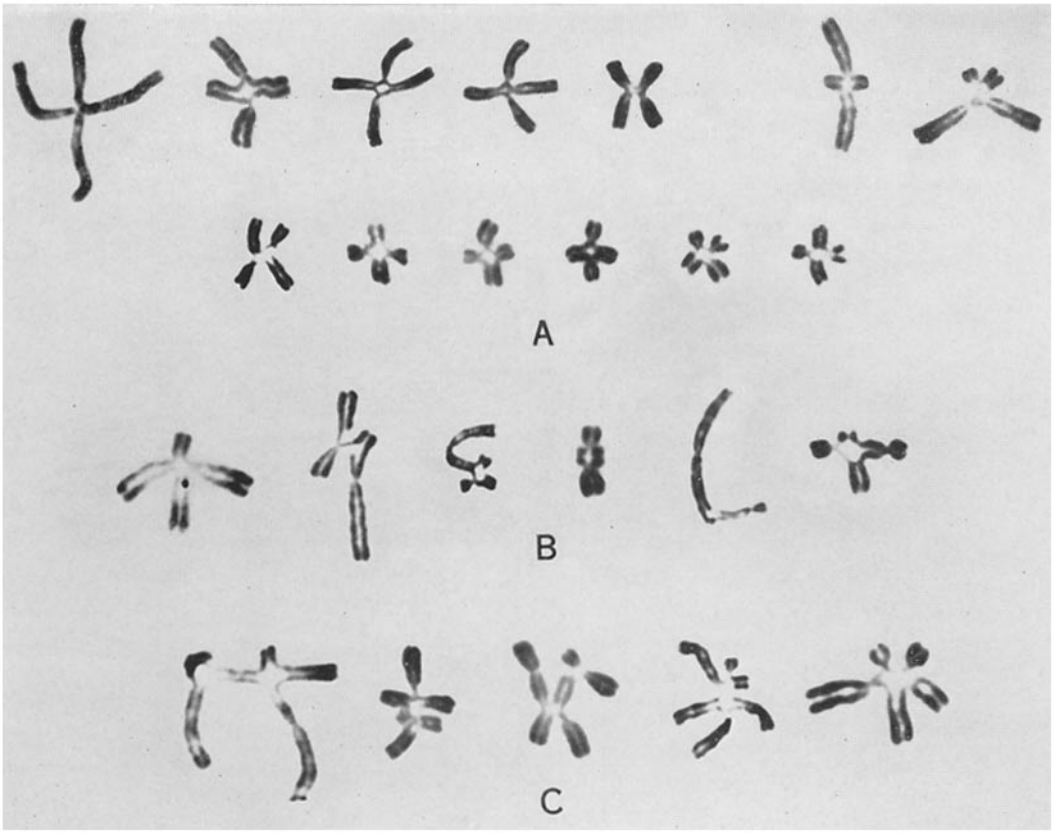

FIGURE 4 Chromosomal exchanges observed as "translocation" configurations. Group *A*, exchanges between apparent pairs of homologues; second row demonstrates exchanges at secondary constrictions in chromosome pair No. 9; Group *B*, exchanges between non-homologous chromosomes; Group *C*, exchanges involving more than two chromosomes. This is a composite plate with variable magnifications.

probability of identifying No. 9 by approximately tenfold after MC treatment. The observation of two such constricted chromosomes in male cells rules out the possibility that the chromosome is the X. MC treatment, thus, is useful in delineating another chromosome in the human karyotype and in the study of anomalies involving the 6–12 + X group.

The effect of MC is, at best, an underestimate of the true activity of the drug. The aberrations which were scored reflect only unrestituted abnormalities. If broken ends rejoin directly or if isolocus breaks of homologous chromosomes are followed by segmental interchange and separation, the chromosomes will appear morphologically normal. Minor structural abnormalities would be undetectable. It may safely be assumed that the healing process is at least as frequent as the observed unhealed breaks, since 95 per cent of the breaks induced by x-ray restitute (22). Thus, the effect and specificity of MC may be many times greater than that observed.

TABLE III

Distribution of Chromosome Exchange Configurations Following MC Treatment

Treatment	Number of cells	Number of "exchanges"	Mean number of "exchanges" per cell
Control	626	0	0
0.1 μg MC/ml	416	42	0.10
1.0 μg MC/ml	114	76	0.67
Total treated cells	530	118	0.22

The data on exchanges suggest that MC induces or potentiates somatic pairing in man. This phenomenon has been described in *Drosophila* (48) and in marsupials (20, 41). Ruddle (35) has demonstrated a close spatial relationship of homologues at metaphase of pig kidney cells. Although evidence suggests that somatic pairing may also occur in man (1, 9, 10, 13, 14, 38), cytological and genetic proof is lacking.

It has been suggested that somatic pairing in occurs. The rosette formation of a group 21–22 chromosome associated with the secondary constrictions of two No. 9 chromosomes (Fig. 5) may be evidence of ectopic pairing maintained by MC. Since all chromosomes are suspected of possessing small blocks of paracentric heterochromatin, they may all be capable of such association, to varying degrees. However, those chromosomes with larger heterochromatic regions would be more likely to exhibit ectopic pairing.

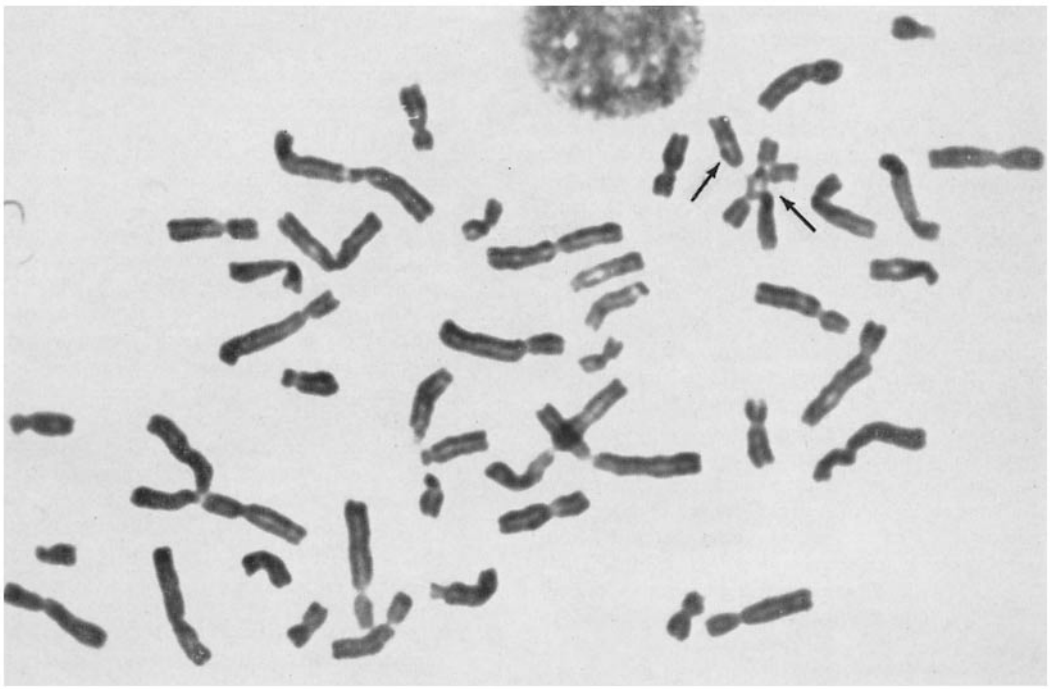

FIGURE 5 A complex rearrangement involving a small acrocentric chromosome (21–22) with the secondary constriction region of two No. 9 chromosomes. One member of the No. 9 pair has an isochromatid break at this region giving rise to a large acentric fragment (arrows). × 1300.

Drosophila may not be limited to homologous regions but may involve heterochromatic segments of non-homologous loci (17, 18, 30, 46). This observation has been termed "ectopic pairing" and was interpreted as a specific property of heterochromatin. A thorough discussion of heterochromatin effects in *Drosophila melanogaster* is rendered by Hannah (11). In man, the association of satellited chromosomes (6) and the association of acrocentric chromosomes with the paracentric region of No. 1 (42), as well as other chromosomes bearing heterochromatic secondary constrictions (8), suggest that ectopic pairing also

Exchange figures are seldom seen in untreated human material (9); therefore, somatic pairing may normally occur only during a specific period in interphase and disappear before metaphase. The role of MC in these associations is not known; however, it is possible that MC may induce such pairing or may act to inhibit the separation of paired homologues.

Somatic pairing, if it can be induced by MC, will provide a mechanism for recombination of genetic material in somatic cells. When suitable cytogenetic markers become available in tissue culture systems, it may be possible, by using

chemical agents such as mitomycin, to make significant strides in human genetics at the experimental level.

SUMMARY

Mitomycin C inhibits mitosis and causes breaks and exchanges in the chromosomes of cultured human leukocytes. The distribution of breaks is nonrandom with a marked excess of breaks in the secondary constriction regions of chromosomes Nos. 1, 9, and 16. When not broken, these regions frequently show extreme attenuation and negative heteropycnosis.

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Addendum: Since this manuscript was submitted, Nowell has published similar effects of mitomycin C on human leukocytes (Nowell, P. C., Mitotic inhibition and chromosome damage by mitomycin in human leukocyte cultures, *Exp. Cell Research*, 1964, **33**, 445). We have recently completed the analysis of a large series of mitomycin-induced chromosomal exchanges and find positive cytological evidence of somatic pairing and crossing over (Shaw, M. W., and Cohen, M. M., Chromosome exchanges in human leukocytes induced by mitomycin C, *Genetics*, in press).

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