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Metaphase Chromosome Anomaly: Association with Drug Resistance and Cell-Specific Products

Abstract. *Large, homogeneously staining chromosome regions which lack the longitudinal differentiation ordinarily revealed by cytogenetic "banding" methods have been found in antifolate-resistant Chinese hamster cells and also in human neuroblastoma cells established in vitro. The drug-resistant cells are characterized by excessive production of the target enzyme, dihydrofolate reductase, while the human neuroblastoma cells have phenotypes of normal neuronal cells. The homogeneously staining region appears to represent a novel metaphase chromosome anomaly which may have functional significance in cells with specialized properties.*

Chromosome staining methods for revealing differential banding patterns along the length of metaphase chromosomes are a useful means of identifying structurally rearranged "marker" chromosomes (*1*). With that objective, we utilized a modified trypsin-Giemsa staining technique (*2*) for investigation of two groups of cell lines having strikingly long marker chromosomes as seen after conventional aceto-orcein staining. The first group comprised a series of amethopterin (methotrexate) and methasquin (a quinazoline antifolate) resistant Chinese hamster sublines (*3*) with very long, seemingly translocated segments on chromosomes 2 and 4 in particular. The second group consisted of continuously cultured human neuroblastoma cells which also possessed long, clearly abnormal marker chromosomes (*4*). Our initial and unanticipated finding was that the long chromosome segments in drug-resistant Chinese hamster cells did not "band," that is, did not manifest the relatively short, sequential regions of differential staining that characterize normal Chinese hamster chromosomes (*5*). We were thus enabled to recognize a similar anomaly occurring in two out of the four neuroblastoma lines which we analyzed. We now present some cytological features of the large, homogeneously staining region (HSR) present on specific chromosomes of antifolate-resistant Chinese hamster cells and on several chromosomes of human neuroblastoma cells.

Chinese hamster cells exposed to high concentrations of either amethopterin or methasquin developed high levels of resistance and cross-resistance to the antifolates

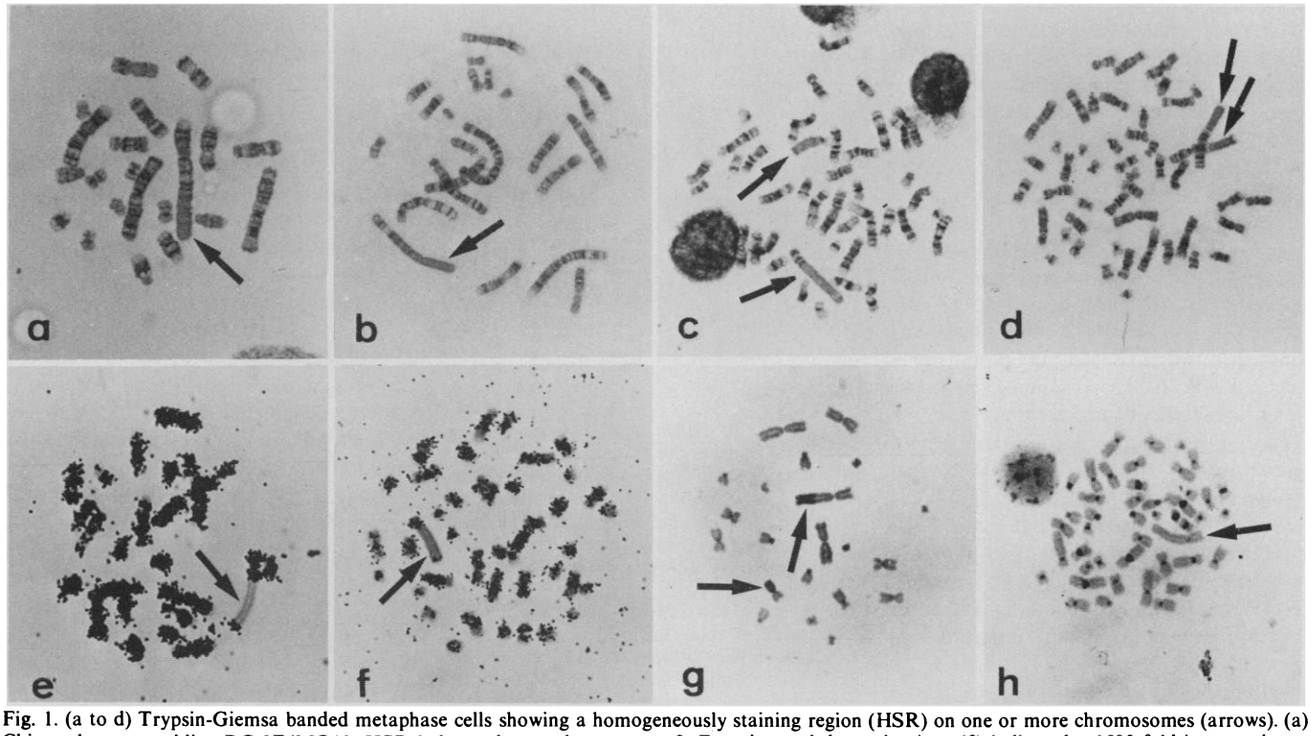

Fig. 1. (a to d) Trypsin-Giemsa banded metaphase cells showing a homogeneously staining region (HSR) on one or more chromosomes (arrows). (a) Chinese hamster subline DC-3F/MQ19. HSR is located on a chromosome 2. Experimental determinations (*3*) indicated a 1583-fold increase in resistance to methasquin and a 151-fold increase in specific activity of dihydrofolate reductase as compared to drug-sensitive parent cells. (b) Subline DC-3F/A3 cell showing unidentified marker chromosome with an HSR. The Chinese hamster line developed a 108,400-fold increase in resistance to amethopterin and a 170-fold increase in dihydrofolate reductase activity after exposure to drug. (c) Human neuroblastoma line SK-N-BE(2). In this cell, HSR's are located on a chromosome 10 and a chromosome 19. (d) A metaphase cell of the IMR-32 neuroblastoma line with overlapping HSR-bearing No. 1 chromosomes. (e) Heavily labeled DC-3F/A3 cell exposed to tritiated thymidine during third quarter of S phase. Except for distal end of arm, there are no silver grains over long segment (arrow) corresponding to HSR. (f) Radioautogram of a metaphase cell of human neuroblastoma line SK-N-BE(2). The No. 10 chromosome (arrow) with an HSR was identified by G-banding prior to application of photographic emulsion. (g) C-banded cell of DC-3F/MQ19 subline. Region corresponding to HSR of chromosome 2 (center arrow) and long arm (arrow) of an X chromosome show positive staining. The entirely heterochromatic X chromosome is absent. (h) C-banded metaphase cell of SK-N-BE(2) neuroblastoma line. Abnormally long chromosome (arrow) is presumptive HSR-bearing chromosome 6. Centromeric regions are strongly C-band positive, while segment or segments corresponding to an HSR in G- or Q-banded cells did not stain by the C-banding technique (× 756).

(*3*). All of the 20 experimentally derived sublines had elevated levels of dihydrofolate reductase activity and, in several of the sublines exhibiting the highest resistance, as much as 2 percent of the total protein was folate-reducing enzyme (*6*). From this large series we chose 13 near-diploid sublines independently selected with either drug for karyotype analysis by the trypsin-Giemsa technique. As previously communicated (*7*), only those seven sublines with enzyme activity increases greater than 100-fold had long "nonbanding" chromosome segments. The HSR's were consistently present on the long arms of either a chromosome 2, a chromosome 4, or, in one instance, on an unidentifiable marker chromosome and comprised 2 to 6 percent of the chromosome complement. Cells from two sublines, DC-3F/MQ19 and DC-3F/A3, are shown (Fig. 1, a and b) and their HSR-bearing chromosomes are represented diagrammatically (Fig. 2, a and b).

Chromosome banding analysis of near-diploid human neuroblastoma cells revealed a similar cytological anomaly. In addition to the cell lines (*8*) established by us from three patients with neuroblastoma, we examined the human IMR-32 line (*9*). Every cell of cell line SK-N-BE(2) analyzed between approximately 8 and 40 weeks of serial cultivation contained at least one chromosome with a long HSR (Fig. 1c) and sometimes a second chromosome as well, with a shorter HSR. Among 35 fully karyotyped cells, HSR's were found on chromosomes 4, 6, 10, 17, and 19 (Fig. 2c). The strikingly long marker chromosome, for example, seen in conventionally stained preparations (*4*) consisted largely of a nonbanding stretch seemingly inserted into the short arm of a chromosome 10. The IMR-32 line consistently possessed two HSR-bearing chromosomes per cell (Fig. 1d) corresponding to the two long, unidentified markers described previously (*9*). Thus, in addition to an apparently normal chromosome 1, cells contained two morphologically identical No. 1 chromosomes with HSR's at a distal site on the short arm (Fig. 2d). Two other established human neuroblastoma lines, SK-N-SH and SK-N-MC, showed no evidence of similar, anomalous chromosome regions.

Homogeneously staining regions of both groups of cells have several attributes in common. First, the regions are of intermediate staining intensity after application of either trypsin-Giemsa or quinacrine fluorescence staining methods. Second, HSR's appear to replicate synchronously, and also rapidly, relative to other autosomal segments of similar size. To determine DNA replication patterns, cells were pulse labeled for 10 minutes with tritiated thymidine (5 μc/ml, specific activity 6.7 c/mmole), rinsed, and reincubated with conditioned medium containing unlabeled thymidine (10 μg/ml), fixed at appropriate intervals, and air-dried on slides. Slides were coated with Kodak liquid emulsion NTB-2 and processed by standard procedures after 2 to 3 weeks' exposure at 4°C. In cells of the amethopterin-resistant Chinese hamster subline, DC-3F/A3, the HSR completed replication before the midpoint of the DNA synthesis period. In fact, in cells that had been exposed to tritiated precursor during the third quarter of the S phase, HSR's were not detectably labeled, while all other chromosomes were heavily labeled (Fig. 1e). Initial attempts to determine the time sequence of chromosomal DNA replication of slowly proliferating SK-N-BE(2) cells were unsuccessful; synchronous replication of the homogeneously staining segments, however, was suggested by radioautographic results (Fig. 1f).

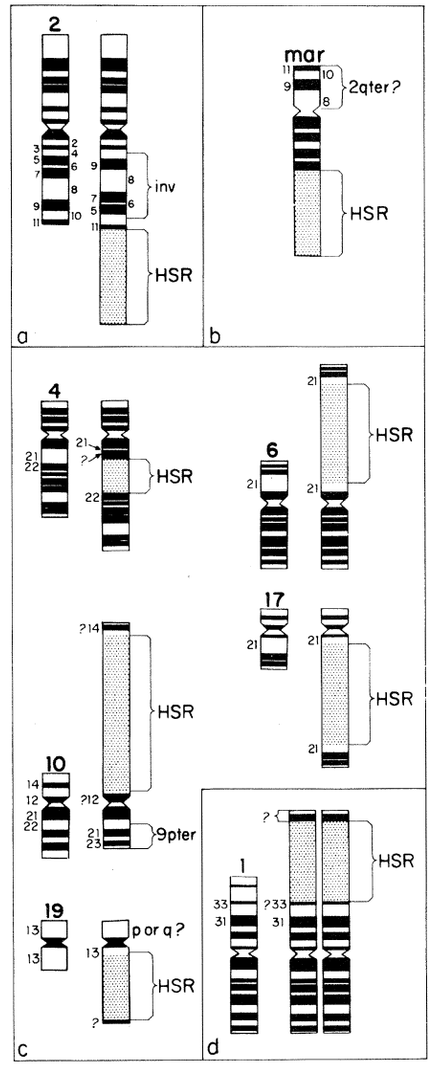

Fig. 2. Diagrammatic representation of the metaphase chromosomes with an HSR and their normal homologs, to show precise location of the region. Recommended nomenclature and numbering systems for chromosome bands in normal human (*16*) and Chinese hamster (*17*) complements are utilized. (a) Chromosome 2 of subline DC-3F/MQ19. In addition to the terminally located HSR, the abnormal No. 2 had a paracentric inversion of a portion of the long arm. (b) Marker chromosome of subline DC-3F/A3. The banding pattern of the short arm resembles that of the distal portion of the long arm of a normal chromosome 2, as diagrammed in (a). (c) The five HSR-bearing chromosomes of SK-N-BE(2). One or two, never more, of these chromosomes were present per cell. In those cells with an HSR on chromosome 10, karyotype analysis revealed a reciprocal translocation between the long arm of the abnormal No. 10 and the short arm of a No. 9 [t(9;10)(p13;q22)]. The so-called short arm of chromosome 10, with an interstitial HSR, does not show an otherwise normal banding pattern. (d) The two apparently identical No. 1 marker chromosomes of IMR-32.

Homogeneously staining regions also differed in several respects from one group of cell lines to the other. Cells were stained for constitutive heterochromatin, that is, C-banded (Fig. 1, g and h), by a modified denaturation-renaturation technique (*10*). While HSR's of the human cells did not C-band (Fig. 1h), the comparable regions on chromosome 2 in DC-3F/MQ19 (Fig. 1g) and on the marker chromosome of DC-3F/A3 sublines stained similarly to the heterochromatic arms of Chinese hamster sex chromosomes. However, unlike the C-banding centromeric heterochromatin of this species, the C-band positive sex chromosomes are deficient in repetitive DNA; the basis for the positive staining reaction is unknown (*11*). Another difference between cell groups is that HSR's in all seven drug-resistant Chinese hamster sublines appear to have a terminal location, while for the six human chromosomes of the two neuroblastoma lines the HSR's are interstitial.

The long homogeneously staining chromosome regions of the human and Chinese hamster cells may represent a hitherto undetected and undescribed phenomenon. However, their presence in two such diverse cell systems suggests that their occurrence is not extremely uncommon. Homogeneously staining regions arose in an experimental series of Chinese hamster sublines under high selection pressure (1 to 50 μg per milliliter of antifolate) and only in those lines developing the highest levels of dihydrofolate reductase activity. Homogeneously staining regions were found in human neuroblastoma cells in vitro, under no obvious selection pressure. These malignant cells display certain specialized properties of normal neurons. For example, IMR-32 cells have high choline acetyltransferase and very low tyrosine hydroxylase activity (*12*), while SK-N-BE(2) cells have dopamine β-hydroxylase activity as do SK-N-SH cells (*13*) which do not manifest the chromosome anomaly. Thus, we found HSR's in two groups of cells

which synthesize special products. Although we have been unable as yet to link a specific cell product to the HSR's of neuroblastoma chromosomes, we are led to speculate from the observed association between the large chromosome regions and markedly elevated levels of dihydrofolate reductase in Chinese hamster cells that the regions are in some way functionally involved with excessive production of one or more proteins specific to the malignant neuronal cells. A first, essential question is whether the HSR's of the drug-resistant cells are transcriptionally active. The finding of increased levels of polysomal poly(A)-containing RNA in three HSR-containing sublines relative to control, drug-sensitive cells (*14*) is consistent with the large amounts of enzyme produced. The origin of the homogeneously staining chromosome region is also speculative. Possibly the region is the somatic mammalian cell chromosomal equivalent of the chromosome "puffs" of lower eukaryotes and/or is the cytological consequence of some process of chromosome amplification or magnification (*15*).

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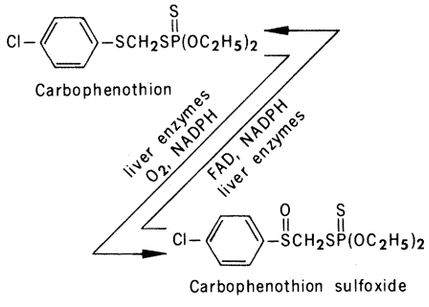
Sulfoxide Reduction in Relation to Organophosphorus Insecticide Detoxification

Abstract. *Carbophenothion sulfoxide, an oxidative metabolite of carbophenothion, is reduced to carbophenothion in the living rat and by an in vitro system containing rat liver enzyme, reduced nicotinamide adenine dinucleotide phosphate, and flavin adenine dinucleotide phosphate. Reduction of sulfoxides, formed metabolically from certain commercial organophosphorus insecticides, may be important in ameliorating the toxicity of these compounds.*

Many important insecticides contain both thionophosphorus and thioether groupings. These compounds undergo metabolic oxidation at both the thionophosphorus and thioether sites, converting them in vivo to potent cholinesterase inhibitors (*1*). This has been assumed to be an irreversible activation process, but we find that the sulfoxide or sulfinyl compound also undergoes reduction, re-forming the thioether or sulfide, under in vitro and in vivo conditions.

Our studies involved the insecticide and acaricide, carbophenothion or Trithion, and living rats and rat liver enzyme systems. These studies were performed with [phenyl-^{14}C]carbophenothion, [phenyl-^{14}C]carbophenothion sulfoxide, and [^{14}C]4-chlorothiophenol. Radiochemical purity of all administered compounds was 97 percent or greater, and [^{14}C]carbophenothion sulfoxide contained less than 0.05 percent carbophenothion.



For the in vitro studies we utilized a system reported by Mazel *et al.* (*2*) to reduce dimethylsulfoxide to dimethylsulfide, in which we replaced the dimethylsulfoxide with carbophenothion sulfoxide. A 20 percent rat liver homogenate was prepared in 0.05*M* tris(hydroxymethyl)aminomethane (tris)-HCl, *p*H 7.2, and centrifuged at 10,000*g*; 9 ml of the supernatant was treated in a Thunburg tube by repeated evacuation and purging with argon. Carbophenothion sulfoxide (1.0 μmole) was added in 0.2 ml of acetone and the evacuation and purge were repeated. Finally, 6.0 μmole of reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 6.0 μmole of flavin adenine dinucleotide (FAD) were added in 1 ml of argon-flushed buffer. After no incubation or incubation for 2 hours at 37°C, the mixtures were extracted five times with 5-ml portions of ethyl acetate, and the combined extracts were analyzed by thin-layer chromatography on silica gel chromatoplates; plates were developed with each of three solvent systems: benzene; benzene + hexane (1 : 1); and 2,2,4-trimethylphentane + chloroform (3 : 2). The following products were found after incubation for 2 hours: unmetabolized carbophenothion sulfoxide, 78 percent; carbophenothion sulfone from oxidation, 1 percent; and carbophenothion from reduction, 12 percent. Two controls, one extracted without incubation and the other incubated for 2 hours but with supernatant boiled for 1 minute to destroy enzyme activity, gave essentially no metabolism: 97 percent carbophenothion sulfoxide; 0.2 percent carbophenothion sulfone, and 0.5 percent carbophenothion. Apparently the reduction is not a result of disproportionation of carbophenothion sulfoxide, as shown by the small amount of carbophenothion sulfone detected.

On oral administration to rats of carbophenothion or carbophenothion sulfoxide at 3 mg per kilogram of body weight, or 4-chlorothiophenol (4-ClTP) at 8 mg/kg, 71 to 80 percent of the radioactive [^{14}C]phenyl label appears in the urine obtained between 0 and 96 hours in each case. Figure 1 shows the identified metabolites, all of which are previously known compounds (*3*). The striking feature of the results is the quantitatively and qualitatively identical pattern of products formed from carbophenothion and carbophenothion sulfoxide. This suggests that one or both of these compounds may convert into the other, resulting ultimately in the same metabolites. Although somewhat similar results are found with 4-ClTP, they differ in several important respects. Both 4-chlorobenzenesulfinic acid (4-ClBSI) and 4-chlorobenzenesulfonic acid (4-ClBSO) are major metabolites of carbophenothion and carbophenothion sulfoxide, but are only minor metabolites of 4-ClTP. Thus, 4-ClBSI arises directly from cleavage of carbophenothion sulfoxide (reaction a in Fig. 1) or indirectly by a different type of cleavage (reaction b) and then oxidation of the 4-chlorobenzenesulfenic acid (4-ClBSE). 4-Chlorothiophenol is equally important as an intermediate in the metabolism of both carbophenothion and carbophenothion sulfoxide, since all metabolites formed through this intermediate, including 4-