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# Searching for Genetic Effects of Toxic Chemicals: Chromosomal Aberration and Sister Chromatid Exchange

Patricia Monteleone

Department of Pediatrics, St. Louis University School of Medicine, 1465 S. Grand Blvd., St. Louis, MO 63104

There have been many allegations that dioxin may not only cause malignant tumors but also may play some role in sterility in those patients exposed and may produce spontaneous abortions, birth defects, chromosome aberrations and other illnesses. These allegations are being investigated in large control studies but, in spite of the voluminous data on the biological effects of phenoxy-type pesticides and the associated chlorinated dioxins, there is really very little substantive cytogenetic evidence for the many claims that have been made against these compounds in man. Indeed, we have a lot of work to do, cytogenetically, to see if they do have any effect and, if so, what it means for the patient and for the patient's off-spring.

We know that data from some experimental animals reported over the last several years indicate that TCDD is a toxic material. We know, for example, in reports by Nubert (1972) and Courtney (1976), that TCDD was found to be teratogenic in mice at a dosage lower than that required by any other compound in any species. It seems to be astonishingly specific, especially in the production of cleft palate and kidney abnormalities in those mice of a special type. Although dioxin has been found to be fetotoxic in some other animals -- rats, sheep, monkeys, rabbits, and hamsters -- it has not been found to be teratogenic in any very good study in any species except the mouse.

It is very important for us to note that this chemical, as well as several others, does induce genetic changes by the Ames test, which is one of our best mutagenic tests. It really has not been well-tested, however, in terms of large populations of humans having their chromosomes examined in any particular fashion. This has been sporadically studied and a couple of studies now are getting underway to examine things as fertility, sperm number and so forth. Lamb, in 1981, analyzed fertility in patients exposed to dioxin and analyzed sperm number, motility and morphology in mice exposed to various mixtures of dioxin in their diet for several weeks (Lamb et al. 1981). Bone marrow sister chromatid exchange studies, which I will describe in a few moments, also were evaluated in that group.

Green and Moreland, as far back as 1975, started to report an increased number of chromosome aberrations in the bone marrow of male rats that were exposed (Green and Moreland 1975). Whether or not this is applicable to humans is another question, and certainly whether or not it is statistically significant in that study has been questioned. One of the most extensive cytogenetic studies that will be important to examine is the final results of the Seveso, Italy experience.

How can we monitor dioxin effects cytogenetically? What would we do if we wanted to see if a patient exposed to dioxin had some type of cytogenetic effect, some type of effect on their chromosomes? A chromosomal analysis is absolutely necessary to determine several things -- first, to assess whether or not alterations in the chromosomes are non-random and whether they have any specificity; and, second, which chromosome aberrations occur most frequently. The controlled analysis of chromosomes in the stage of metaphase in peripheral blood lymphocytes at 48 hours of culture would be the most effective way to evaluate cytogenetic or chromosome alterations in individuals who were exposed to dioxin. It is also important that a control be included with the patient because of the variability in the results of the tests. The determination of the presence of acquired alterations in lymphocyte chromosomes is important because these may reflect the alterations that have occurred in the haploid cells of the individual and, therefore, would be important in providing some kind of evidence for teratogenicity or for chromosome abnormalities in any off-spring of the individuals exposed. It is also important because of the other alterations that have occured with chromosomes in cancer cells. We know that there are relationships of certain non-random, acquired chromosome markers that are found in several specific types of human leukemias and malignant tumors. If one can find a non-random marker, one can begin to study its association with disease and interpret its relationship to the specific patient. It is even feasible that one can conceivably predict the type of tumor or leukemia that the patient exposed to a chemical such as dioxin would contract at a later date. Cytogenetic effects can, therefore, be used as a biological dosimeter of exposure. While there are not enough data currently available to know whether or not this is even predictive of any particular kind of cancer, it is known that with exposure of humans to several other known carcinogens the cytogenetic studies were positive.

Chromosome Aberration: Technique. The frequency of chromosome aberrations can be analyzed in cells from any tissue. Lymphocytes are used primarily because they are easy to work with. They are very practical and they have a very low spontaneous rate of aberrations. Not many breaks or gaps are seen normally in a patient's lymphocyte chromosomes. We do have controls for aberrations; each laboratory has its own controls that are periodically rechecked. The method used to study the chromosomes is to incubate the lymphocytes into a McCoy's spinner modified media for 48 hours, harvest the cells and then count the number of breaks, aberrations, and so forth, to see

how many there are per cell. One will get the most accurate level of aberrations if the cells are taken out of culture at 48 hours instead of the usual 72 hours because many cells that will have an alteration will not make it through to the second division. If the cells are taken out at 48 hours, one will be able to examine them right after the first division.

To briefly review the technique: The lymphocytes from approximately 10 drops of whole blood are incubated for 48 hours with a mitotic stimulant. Most laboratories use phytohemagluttinin, which is a substance made from an extract of kidney beans and it accomplishes two purposes. It stimulates the cells to undergo mitosis at a much more rapid rate than they normally would and it agglutinates the red cells allowing only the lymphocytes to grow and proliferate in the tube. Following this procedure, approximately two hours prior to taking the cells out of culture, one adds some type of an arresting solution, colchimide or velban. The lymphocytes should be arrested at early metaphase in order to have good metaphase plates to look at. Following this procedure the cells literally swell, allowing the membrane to burst and the chromosomes to spill out onto a slide. The chromosomes are then fixed with any one of the simple fixatives that laboratories use, usually methyl alcohol and acetic acid, and the slides are then stained.

Looking for aberrations due to a chemical is done in a completely different way than looking for a congenital abnormality in the chromosomes of a patient. One primarily wants to use unbanded cells, that is cells stained with geimsa applied in such a way that the whole chromosome is solidly stained throughout. One hundred cells are then scored at the microscope for chromosomal aberrations. This is done with a Leitz Ortholux scope with a 35 mm camera attached. To karyotype the chromosomes and see if there is something duplicated or deleted, one would have to de-stain the slide and put on the giemsa stain again for banding to look for small translocations or inversions or to see if there is aneuploidy. The cells are then enlarged, printed and karyotyped. If one wanted to karyotype the chromosomes, it would be necessary to do that to only a small percentage of banded cells. Some people are suggesting 20%, others are saying as high as 50%; but the important point is that 100 cells have to be examined first, de-stained, and then at least 20% have to be banded to count and karyotype them. When one bands the cells, the stain preferentially goes to particular areas of the chromosome, so that the two members of the pair take the stain in a similar dark-light, alternating pattern so that one can identify which two are the pair. The pair numbers of chromosomes have been arbitrarily assigned, but the two partners need to match in their preferential areas for staining.

Figure 1 shows a geimsa banded cell. If there is a break or a gap we do not have to band the chromosomes as one can pick that up on an unbanded cell. However, if there is a small piece of chromosome that has broken off and attached itself, for example, to another chromo-

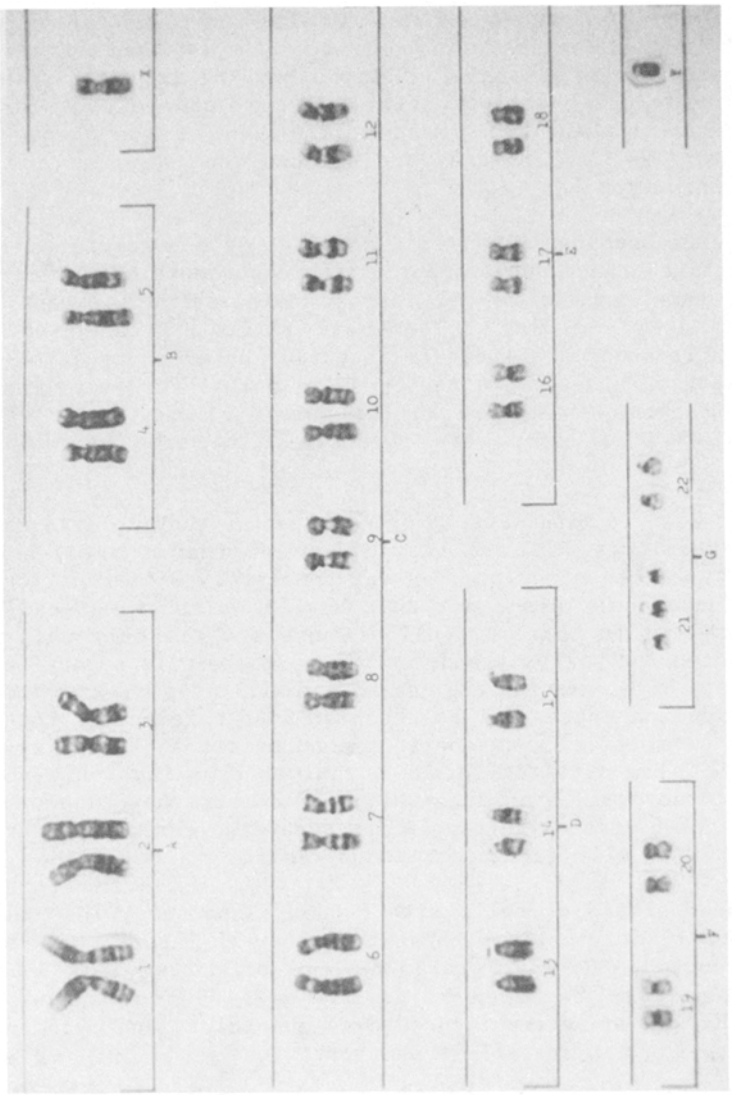


Figure 1. Giemsa banded cell.

some, one probably will not pick that up on an unbanded cell and it is necessary to band that and actually karyotype it. Fluorescent banding using quinacrine is particularly good if we are concerned about the Y chromosome because it intensely fluoresces with this stain. It is also very good if we are questioning some of the satellite ends on the chromosomes that are acrocentric, namely those chromosomes that have a centromere and a long arm but very little short arm. These satellites are often polymorphic and often need to be examined with a quinacome stain. One also can reverse band, that is, everything that bands darkly can be made to band lightly by applying another kind of stain. Reverse banding is used if one is looking for terminal deletions, that is, tiny ends of the chromosomes that have broken off. Most of the ends of the chromosomes light up intensely, and if they are deleted, no intensity will be found with this type of banding.

Another type of banding is called C banding, or centromere banding, which can define changes that occur at the centromere of the chromosome. The same chromosome will appear completely different with different kinds of banding. There are about 300 bands at mid-metaphase while there are literally hundreds at prophase, the stage before metaphase. There is a region and a number for every individual band on every chromosome so the number of abnormalities and aberrations that can occur are phenomenal in terms of one band changes.

One needs to keep in mind several principles in studying cytogenetic effects of chemicals. It is very important that the slides are methodically stained since one can get completely different results if that is not done. Also, one must have a metaphase spread that appears to be in an unbroken cell. These are the only ones that should selected for analysis and, at times, there will be artifacts. In the process of harvesting the cells, one will drop the chromosomes onto the slide and there will be three or four chromosomes that are lost and an experienced cytogenetic technologist can recognize very easily which is an artifact versus a real abnormality. Under high power, the chromosomes should be well-defined and they cannot be in an early anaphase state with completely separated chromatids, otherwise one likewise will get an erroneous result.

To avoid the analysis of cells with random chromosome, loss due to technical error, only cells with a minimum of 45 chromosomes really should be counted. However, large numbers of cells with 43 or 44 chromosomes need to be noted as it may be a sign of aneuploidy. Cells must be scored by experienced cytogeneticists and cytogenetic technologists for all unstable type aberrations.

Potential Chromosomal Abnormalities in Patients with Chemical Exposure. Several types of aberrations are important parameters in gauging whether or not a toxin has done some chromosomal damage (Figure 2). A break in a chromosome is a separation of part of the chromosome from its body. A gap is an area between the broken ends

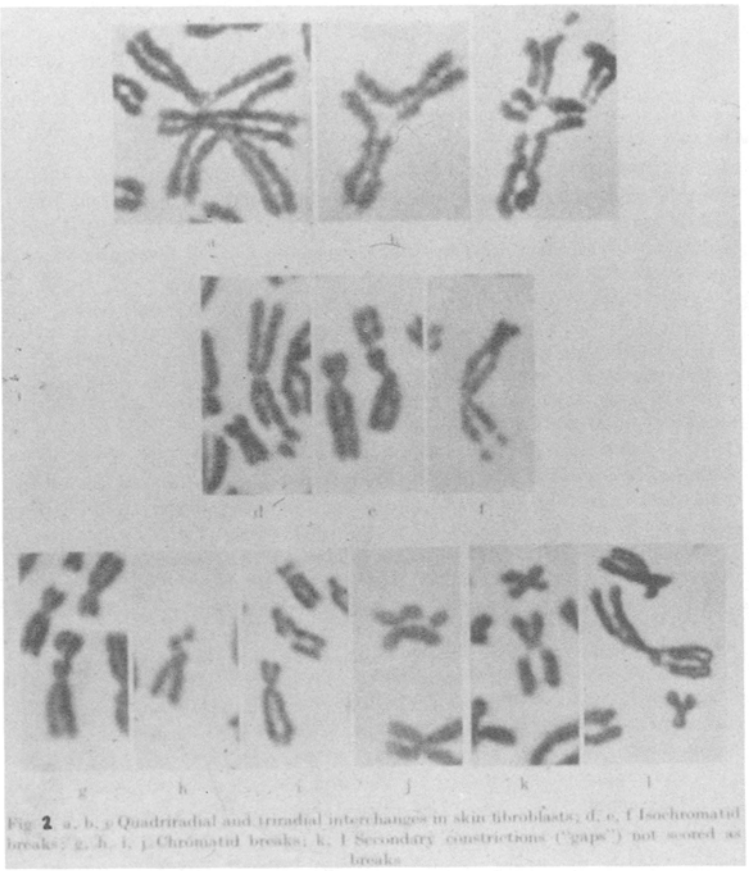


Figure 2. a,b,c: Quadriradial and triradial interchanges and skin fibroblasts; d,e,f: isochromatid breaks; g,h,i,j: chromatid breaks; k,l: secondary constructions ("gaps") not scored as breaks.

of a chromosome with the broken part still remaining in the cell and somewhat attached. Another abnormality to look for in patients who have been exposed to chemicals is dicentric chromosomes, that is chromosomes with two centromeres. If there are pieces of chromo-

somes without a centromere that have just broken off but still remain in the cell, these are called fragments. One wants to look for cells in which there are chromtid breaks and score those separately from chromosome breaks since these are two different kinds of aberrations. A triradius occurs when three chromosomes line up with the ends facing each other in a three-sided figure. One can also see quadriradials with four chromosomes joined in a four-sided figure (Figure 3).

These aberrations do not occur in normal patients. We do not see triradii, quadriradii, dicentric chromosomes, breaks or fragments in more than about one per 10 cells in the normal population. Another unstable chromosome that one looks for in patients who have had an exposure to dioxin or any other chemical is formation of a ring chromosome where a piece of the chromosome has deleted or broken off. The ends are "sticky" and join together after the break to form a ring. Rings are never found in normal patients (Figure 4).

One can assume a partial deletion has occurred if the short arm or the long arm is present in duplicate. These are called isochromosomes and they are not found in normal patients but should be sought in chemically exposed individuals. In the case of exposure to a toxin, the deletion would not be found in every cell but probably would just be found in a few cells and the deletion would appear on a different chromosome in different cells. Figure 5 shows isochromosome formation of an X chromosome meaning that the chromosome has split transversely instead of splitting longitudinally.

Figure 6 shows endoreduplication, meaning every chromosome is duplicated or an exact pattern of itself. It can be seen occasionally in normal patients if they have had exposure to a virus infection or after a vaccination and occasionally we will find one endoreduplicated cell in a normal patient. We will never find more than one in any group of 20 cells. In patients with various types of leukemias and other lymphoproliferative and myeloproliferative disorders, endoreduplication will be found frequently. This is another abnormality to look for in patients exposed to chemicals. Figure 7 shows double minutes which are not whole chromosomes, but small pieces of chromatin. These are seen in patients with various types of malignancies and are not found in normal patients and should be looked for in cells of patients exposed to toxins.

All kinds of factors can influence test results -- the media used and the temperature, for example. If the incubator's temperature varies one degree, that can affect the number of breaks and fragments in the cell, so one has to have very close control when looking at cells from patients exposed to dioxin. It is not necessary to be quite so careful in chromosome analyses for other kinds of things.

Sister Chromatid Exchange. What other tests can we do besides what we call the CA or chromosome aberration test? A second monitoring device is called the sister chromatid exchange test (SCE), and this

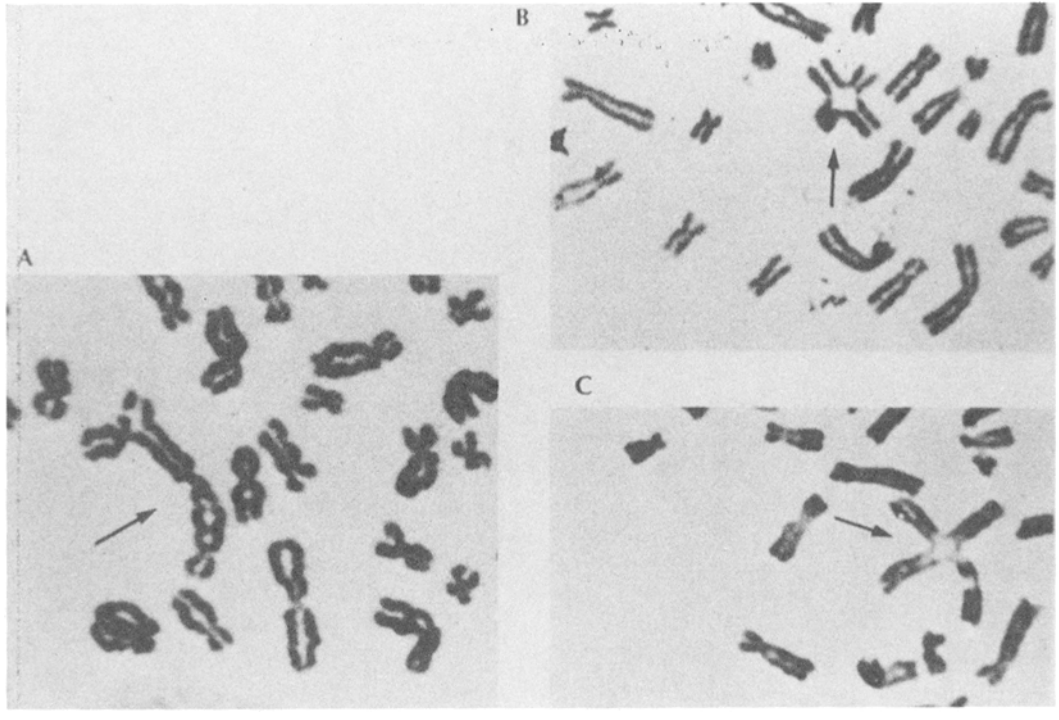



Figure 3. Triradials and quadraradials.

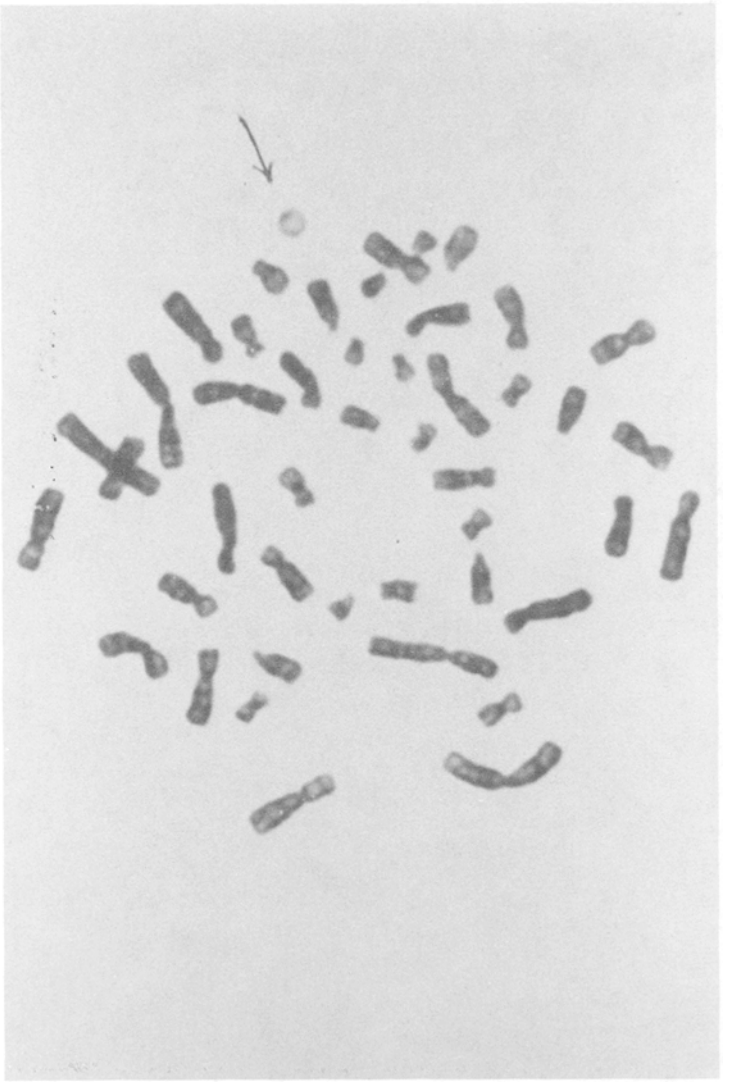


Figure 4. Ring formation.

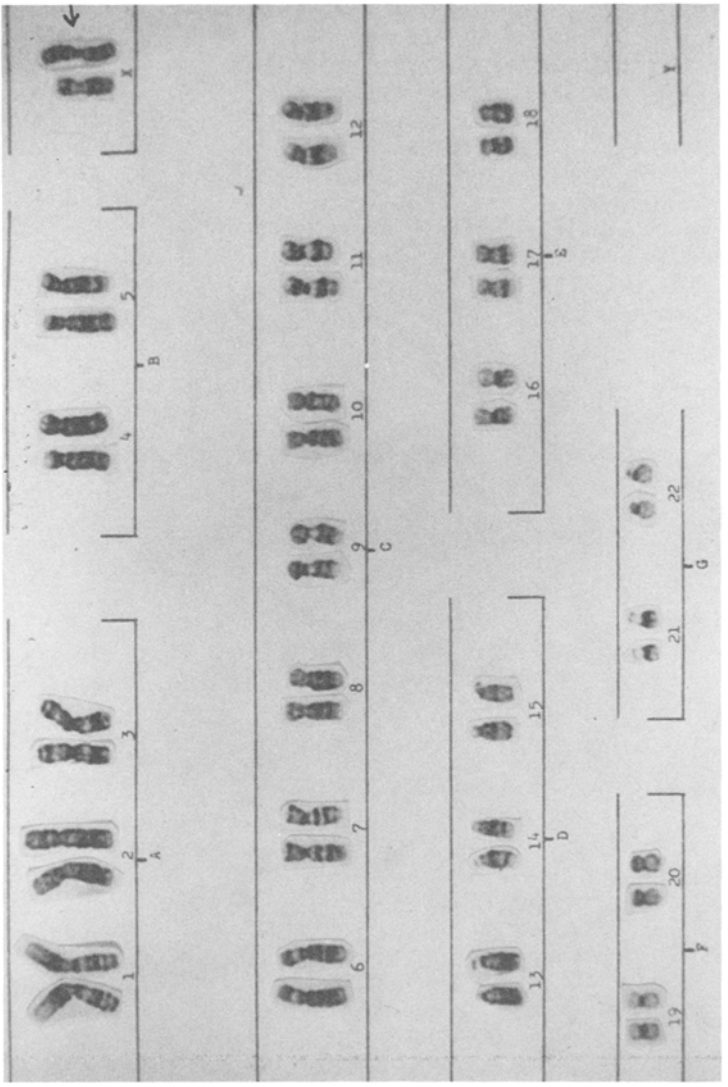


Figure 5. Isochromosome.

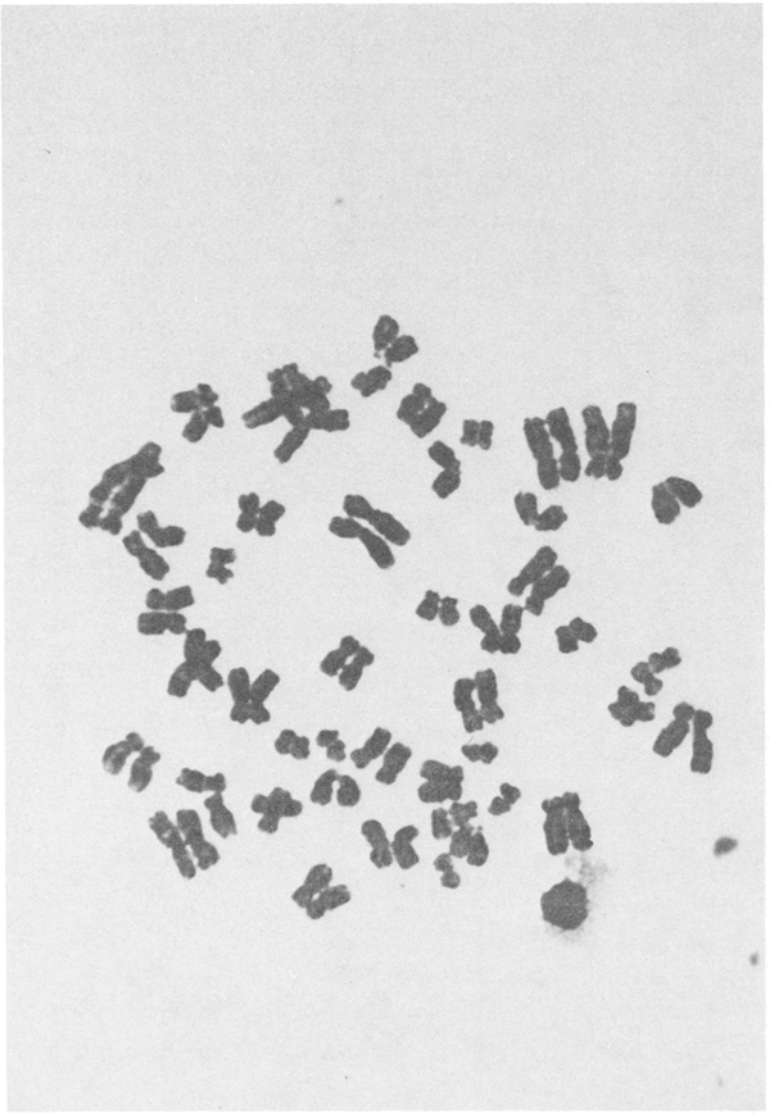


Figure 6. Endoreduplication.

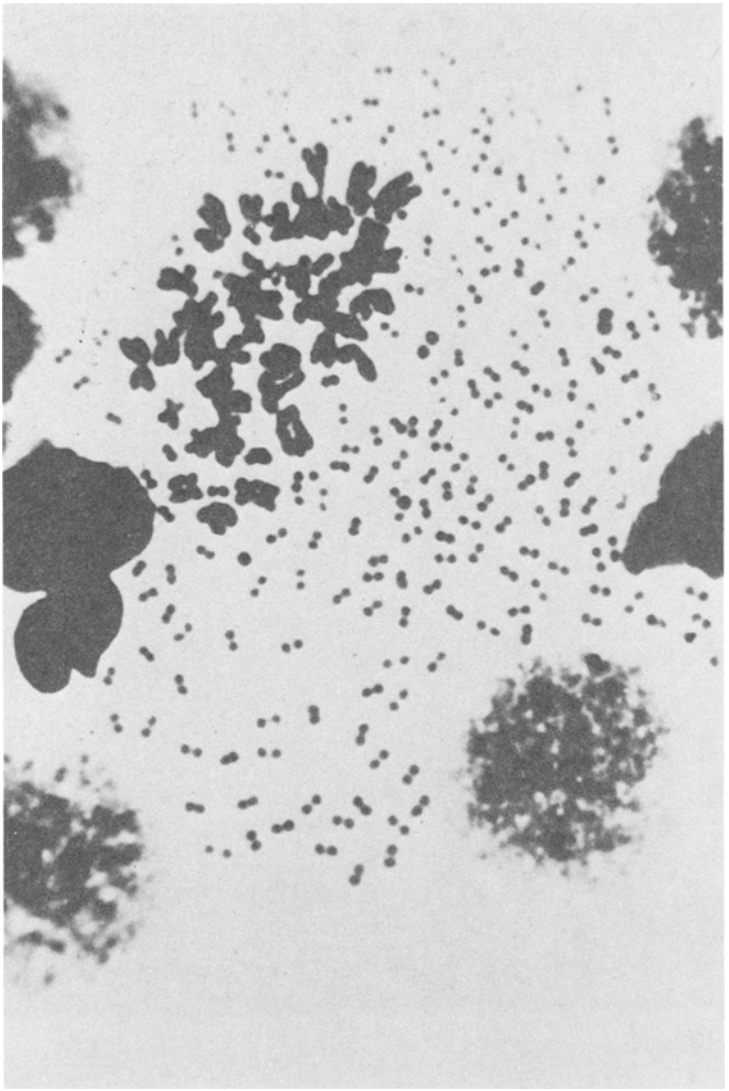
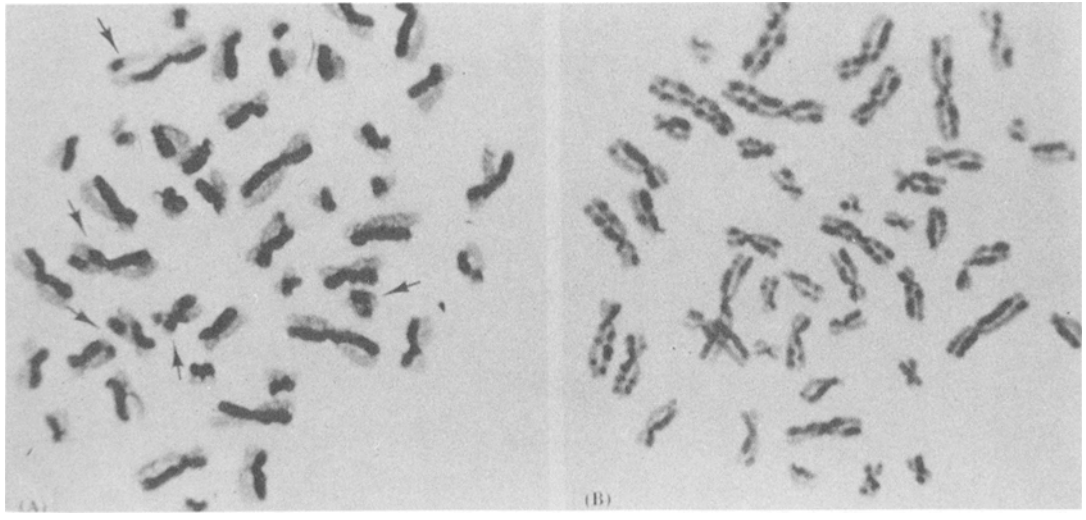


Figure 7. Double minutes.



Figures 8a and 8b. 8a. Normal sister chromatid exchange.
8b. Abnormal sister chromatid exchange.

is being done in a couple of places in the country. What are sister chromatid exchanges? By definition, a sister chromatid exchange is the reciprocal alteration or exchange between sister chromatids of the same chromosome as evidenced by differences in their staining intensity. How is it done? How are sister chromatid exchanges produced? What do they mean?

SCE is a technique that was devised just a few years ago and initially was used in a group of diseases that we call the chromosome fragility diseases, where we knew patients had breaks and fragments of their chromosomes. SCE also was used in patients who had an increased propensity to get cancer as they got older. In the last couple of years, the sister chromatid exchange analysis has been applied to mutagenic and carcinogenic testing as well. It is just now beginning to be used on a large scale basis for toxin studies. Certainly, a lot of work needs to be done in this area because the test results are extremely controversial.

The lymphocytes are exposed to bromodioxyuridine, a substance which is added about 24 hours after initiation of culture. Lymphocytes are used again because they are probably the most practical cell to use. The substance quenches the fluorescence of a Hoechst dye which is placed on the cells a little bit later in culturing time. One then is able to produce, by adding geimsa or acridine orange, differences in chromatid staining intensity. If using fluorescent stains, for example, one is able to fluoresce one chromatid. The other chromatid will be quenched in its fluorescence and will stain very lightly. Then, the pieces of chromatid that have exchanged from one chromatid arm to the other chromatid arm will be very visible. The cells must be exposed to bromodioxyuridine (BrDu) in the culture for the first round of DNA replication, so the cells must be put through more than one cycle. It is probably best to culture these cells for about 76 hours, so they will have gone through three cell cycles.

This analysis takes advantage of the semi-conservative replication of DNA, and allows us to visualize the chromatids in a very nice fashion. Figure 8a shows a sister chromatid exchange analysis in a normal patient demonstrating that one arm is intensely stained with giemsa and the other is very lightly staained. By comparison, Figure 8b shows an abnormal sister chromatid exchange pattern with multiple chromatid displacements.

In a normal control patient, anywhere between three and 20 exchanges is normal. There are a number of variables making test results restrictive. For example, patients who have been smoking cigarettes or are on oral contraceptives will have an increased number of SCEs. Just simple variations in laboratory technique, media, temperature and how long the BrDu is left in the culture are very important factors to consider when trying to decide what is a normal and what is an abnormal SCE test. A number of chemicals have been evaluated showing different SCE responses; some have been positive, some have been negative, some have been correlated with the teratogenic studies and some have not been correlated.

Comparing Tests: Conculsion. Currently there are two tests, the chromosome aberration test and the SCE analysis, both of which can be done to monitor cytogenetic effects of chemicals. There are a number of problems using the SCE test and there are many people who feel that the chromosome aberration test is probably much better. One is limited in the SCE test if it is not an acute exposure; one has to have exposure just a few days prior to doing the test. There are a few people now who are beginning to feel that even an exposure a long time ago may reflect itself in SCE and are beginning to suggest that maybe the SCE test needs to be done even though exposure was several years ago. When to do the SCE and its correlation with human disease is still controversial. The reason the SCE could be important is that there is potentially a use of this test *in utero* to detect teratogenicity of the fetus if the mother has had an exposure. Another area of potential usefulness of both SCE and chromosome aberration tests would be correlation of the results from patients exposed to chemicals with subsequent development of different types of cancer. The relationship of cytogenetic alterations to various malignancies could be very important, simply because of what we know happens to chromosomes in various types of leukemias.

There is a third test which some people feel might have some benefit, namely the micronucleus test; however it certainly is doubtful in its reliability. With this test one can measure the number of breakages and reflect levels of increased aneuploidy, that is changes in total numbers of chromosomes per cell. Some people, primarily those who work in the area of micronuclei testing, think it is a very sensitive index. Others feel it is not reliable at all and, quite truthfully, neglect to use it in most circumstances. Micronuclei are simply small DNA containing bodies. Anytime one has a chromosome aberration at metaphase it will appear as either a break or an exchange, consequently producing fragments of chromosomes that do not have a centromere (acentric fragments). These acentric fragments form what eventually become micronuclei; therefore, finding an increased number of micronuclei indicates chromosome damage.

In summary, the most sensitive test for acute or chronic exposure as well as past exposure is the chromosome aberration test, whereas the most sensitive test for recent exposure would be the SCE test. With more experience, we may find that these tests are useful in the area not only of mutagenicity, but also carcinogenicity. We need long range prospective studies but we also have to be aware of the many ethical conflicts which can arise as a result of such studies. A large group of geneticists demonstrated this awareness when they formed the International Commission for Protection Against Environmental Muta gens and Carcinogens to focus on some of these ethical dilemmas. The whole area of study of effects of toxins on chromo somes may prove to be very important from a public health point of view as well as to the individual and his family.

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