Cytogenetic Techniques in Mutagenicity Testing

by WARREN W. NICHOLS[1])
Institute for Medical Research, Camden, New Jersey

Abstract

Both in vivo and in vitro methodologies have uses as cytogenetic test systems for mutagenicity testing. The in vivo methods are certainly the choice as the primary test system, except perhaps in the case of viruses and some biologicals. When an in vitro method is used anaphase offers some advantages as greater speed and ease of observation, the detection of defects produced in G2 and mitosis, and the ability to distinguish between gaps and breaks, while it sacrifices the morphologic detail and ability to localize defects to specific chromosomes found in metaphase.

In recommended procedures for mutagenicity testing cytogenetic methods have figured quite prominently. There are several reasons for this in addition to the importance of detecting chromosome mutations. The first of these is that it is currently the only system that is directly applicable to humans. Second, they permit visualization of the entire genome in the light microscope; and third, while there may be basic differences in the mechanism of production, there has been a high correlation between the induction of chromosomal abnormalities and the induction of gene mutation produced by irradiation and chemicals. This means that cytogenetic methods can be used as an indicator system for gene mutations, as well as a direct system for chromosomal mutations. Finally, if virus vaccines and biologicals are to be tested for mutagenicity the species specificity exhibited by many viral agents frequently requires a human test system. As mentioned, cytogenetic methods lend themselves well to human testing, both in vivo and in vitro.

Recently an ad hoc committee of the Environmental Mutagen Society and the Institute for Medical Research (1972) made recommendations for chromosome methodologies that could be used as a provisional starting point in mutation testing. In these recommendations, in vivo assay systems were suggested as the primary test system of the cytogenetic methods. It was also recommended that an in vitro test system be considered for use as an ancillary method. Reasons for the recommendation of this ancillary method were that the concentration and exposure time of cells to a compound under study can be controlled accurately; higher doses can be used in vitro when this is desired; it supplies correlative data between the in vivo and in vitro systems, the latter having been most commonly used for detection of cytogenetic effects of drugs and chemicals in the past; and it permits the use of anaphase, which is both more rapid and permits the detection of defects produced in G2 and mitosis that are very difficult to detect in mammalian and especially human studies on metaphase preparations. Finally, as previously mentioned, if virus vaccines and biologicals are to be tested, in vitro methods frequently offer the only practical test system.

In this presentation I would like to examine briefly when in vitro materials should be selected for cytogenetic preparations. After this there will be a brief description of the types of cytogenetic abnormalities that may be detected in metaphase and anaphase preparations. When cytogenetic preparations are made from cells cultured in vitro, the time that the cells are selected for making the preparations is of great importance. If the cultures are viewed in the inverted microscope it is possible to see the numbers of metaphase and anaphase plates that are present (Fig. 1). If

[1]) Head Department of Cytogenetics, Institute for Medical Research, Camden, N.J., USA.

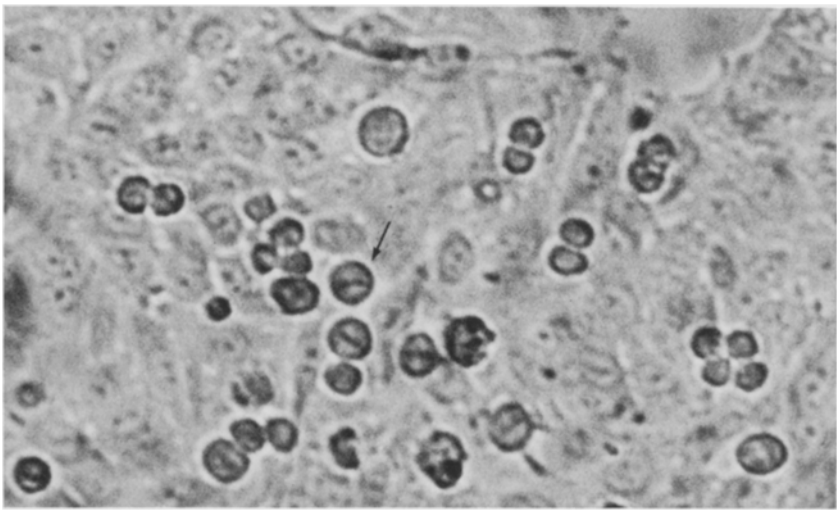


Figure 1
Unstained sheet of cells growing in culture viewed through inverted microscope. Many rounded-up mitotic figures are visible. In some the metaphase plate can be seen (arrow).

3 to 4 cells in mitosis are seen in each field, one can be confident that there is an excellent chance of having good material to examine. If there are few of these figures observed, however, the chances of a good final preparation are very remote.

Assuming that a good preparation now has been obtained, we will examine some of the types of defects that can be recognized. For details of all of the various types of defects that can be seen, the interested reader is referred to several review articles on the subject (SAX [8], LEA [5], EVANS [2], SPARROW [9], KIHLMAN [4], NICHOLS [6]). In metaphase, defects of the open type can be seen as well as chromosomal rearrangements. Each of these can be of either the chromatid or chromosome type. Chromatid lesions involve only one of the two chromatids of the chromosome, while both are involved in a chromosomal defect. Which of these occurs depends on the period of the cell cycle when the defect is introduced. If the damage occurs before that chromosome has replicated its DNA, then the defect is replicated along with the rest of the chromosome, and a chromosomal type abnormality is found. If the defect is produced after the chromosome has replicated its DNA, then it is more likely that one of the two chromatids will be involved, without necessarily involving the other, and a chromatid lesion results. Open type chromatid and chromosome lesions are seen in Figure 2, while rearrangements are seen in Figure 3. Whether the open type defect or the rearrangement results after chromosomal damage is determined by whether or not healing can take place. Healing of broken chromosomes is dependent on intact cellular DNA and/or protein synthesis. If these synthetic processes are operative, chromosomal rearrangements are the usual type of chromosome defect observed. If these are not operative healing cannot take place and open chromosome defects are seen. It has sometimes been stated that chromosomal rearrangements are more significant than the open type because there is a greater possibility of them persisting. However, any agent that is capable of producing open chromosomal abnormalities is also capable of producing chromosomal rearrangements if the

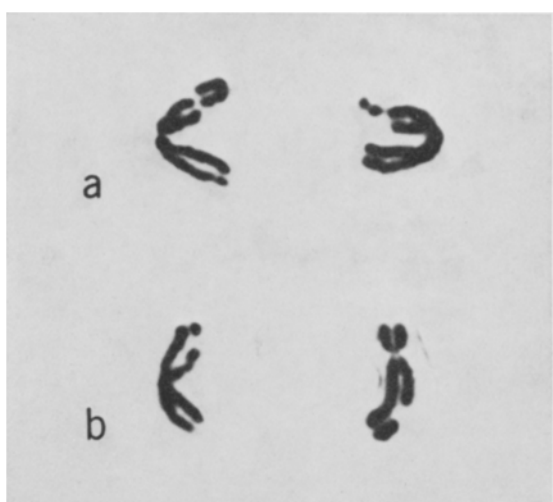


Figure 2
Open type chromosome lesions: (a) chromosome breaks, (b) chromatid breaks.

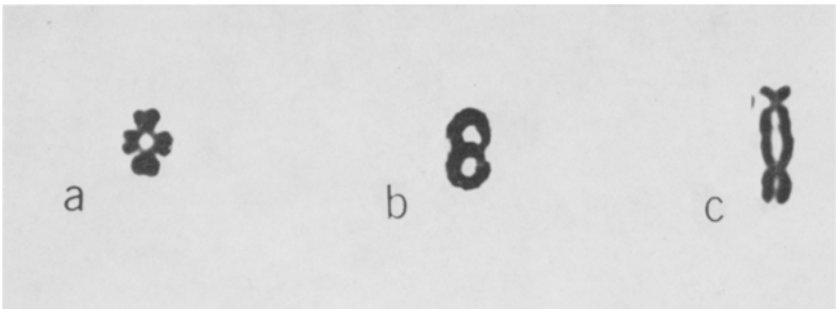


Figure 3
Chromosomal rearrangements resulting from breakage and healing:
(a) interchromatid exchange resulting in quadriradial,
(b) interlocking rings,
(c) interchromosome exchange resulting in dicentric. (From NICHOLS [6], in: *Drug Induced Diseases*, vol. 4, Excerpta Medica.)

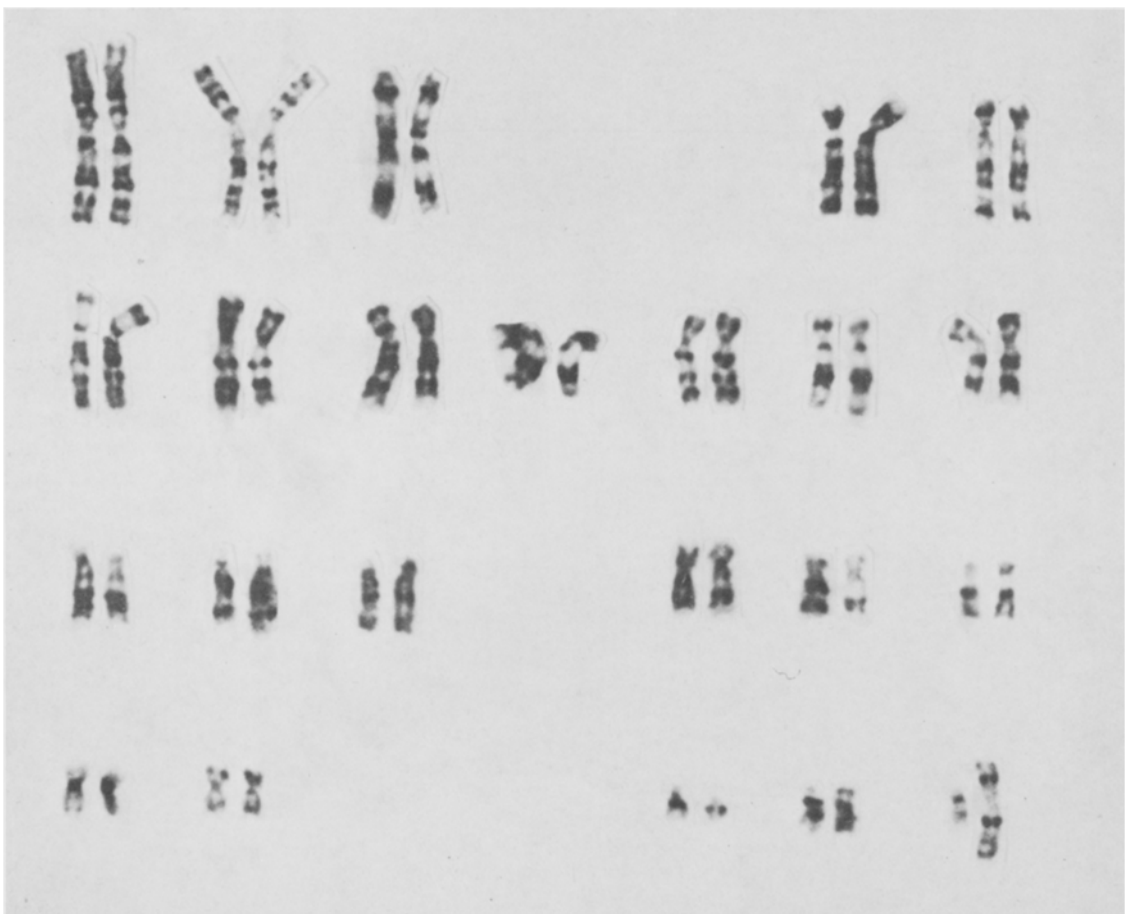
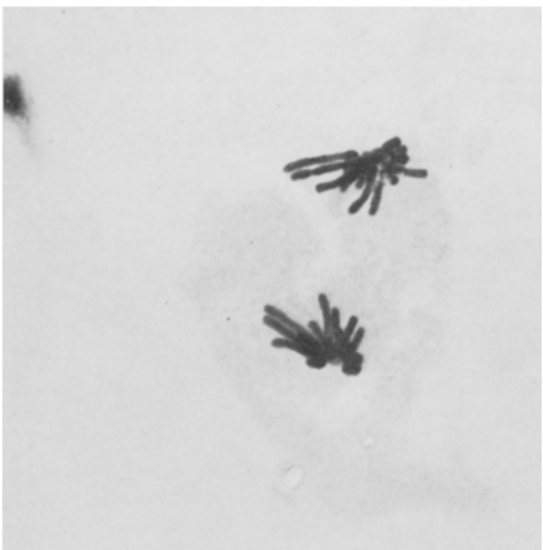


Figure 4
Human karyotype after banding by the trypsin method and Giemsa staining. Each homologous chromosome pair and many chromosomal segments can be identified.

experimental conditions and observations are appropriately altered. In addition to this, if the chromosome lesions are being used as an indicator system for gene mutations, either type defect would seem to be as significant.

These are the type defects that can be easily recognized in a metaphase plate. Reciprocal translocations and other less obvious defects frequently require that the metaphase plate be karyotyped in order to show differences between



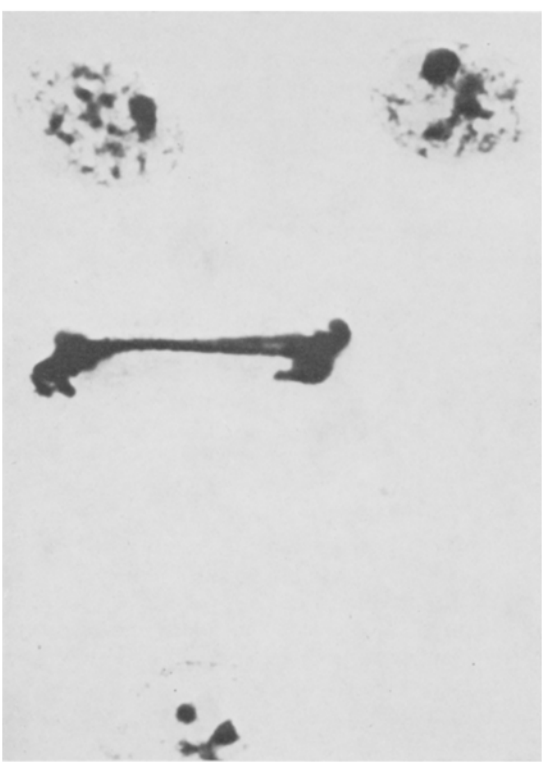


Figure 5
(a) Normal anaphase with chromosome groups at opposite ends of cell, (b) acentric chromosome fragments at equator of the cell after the main chromosome groups have migrated to opposite ends of the cell, (c) chromosome bridge stretched across cell as the result of rearrangement, resulting in dicentric chromosome.

the members of a homologous pair of chromosomes for detection. If the reciprocal translocation involves approximately equal lengths of two chromosomes, then the conventional karyotype is inadequate and the new banding techniques must be utilized. A karyotype after one of these techniques is illustrated in Figure 4. It can be seen that each pair of chromosomes can be differentiated by these techniques and many chromosomal segments can be differentiated, so that resolution of smaller abnormalities is much greater.

It is important to point out however, that in monitoring and test systems whenever these subtle abnormalities are induced by an agent, the more obvious defects should also be detected, so that banding techniques are not ordinarily required in a mutagenicity screening test.

If we now turn our attention to anaphase, we can see a normal anaphase in Figure 5a. It is readily apparent that the chromosomal morphology is not nearly as good in anaphase as in metaphase. If it is important to localize defects to specific chromosomes or chromosomal segments, anaphase is not useful.

When observations are made for chromosomal abnormalities in anaphase preparations, in simplistic terms we look for chromosomal material between the two main chromosome groups that are arranged at the polar areas of the cell. This can be seen either as acentric fragments

(fragments that are the counterpart of open chromosome breaks and have no means of attaching to the spindle mechanism), or as chromosome bridges, when rearrangements result in configurations with more than one centromere (Fig. 5b, c). Since in anaphase the entire cells is viewed as a single unit rather than the necessity of looking at each individual chromosome as is the case in metaphase, this method is considerably faster and easier. As mentioned earlier, an examination of anaphase also permits the detection of defects produced in the G 2 and mitosis phases of the cell cycle. This type of defect is much more difficult to recognize in metaphase preparations. Defects formed in G2 and mitosis have a typical appearance in anaphase, termed 'side-arm bridges' or 'pseudochiasmata'. Figure 6 shows one of these defects in plant material that demonstrates their classic appearance particularly well. The term 'side-arm bridge' is derived from the appearance of the attenuated chromatid stretching across the anaphase figure, that involves the chromatids to the point where the exchange has taken place. Distal to this point of exchange, the two chromatids are out to the side, as side-arms. Pseudochiasmata comes from the resemblance of these figures to chiasma found in meiosis. Side-arm bridges can be seen in metaphase, but here they are much more difficult to distinguish. A group of chromosomes exhibiting the appearances found with side-arm bridges in metaphase are illustrated in Figure 7. Those in the upper row are the result of exchanges between chromatids of the same chromosome (intra-chromosome exchange). This results in the two chromatids coming into close proximity at some point along their length. On the bottom row the appearance of side-arm bridges resulting from chromatid exchanges between more than one chromosome (interchromosome exchange) are illustrated. Here a puckering of a chromatid towards a similar site in another chromatid is frequently all that is seen, and sometimes a small amount of material can be seen passing between these two points. Anyone familiar with metaphase chromosome preparations will immediately recognize that it is extremely difficult to distinguish between positional artifacts and side-arm bridges in metaphase. In anaphase the bridge appearance eliminates this difficulty.

Figure 6
Side-arm bridge as viewed in anaphase. Note attenuated bridge with chromatid segments distal to exchange projecting to side. (From KIHLMAN [3], Hereditas *65*).

Another advantage offered by anaphase preparations is the ability to distinguish between chromosome gaps and true chromosome breaks when this is necessary. As the name implies, a chromosome gap is an achromatic or destained area in a chromosome or chromatid, in which some or all of the chromosomal material still persists between the proximal and distal fragments. This is distinguished from a true break in which there is a true discontinuity with no bridging material between the proximal and distal fragments. In metaphase it is frequently difficult to distinguish between an open chromosome break in which the distal fragment has not been displaced from the proximal fragment and a chromosome gap. In anaphase when a true break has been produced the distal portion of the chromosome or chromatid remains at the equator of the cell as an acentric fragment, while a gap can either not be distinguished or is seen as an attached or lagging fragment in which the fragment is aligned with the main chromosomal group but lagging slightly behind the other members.

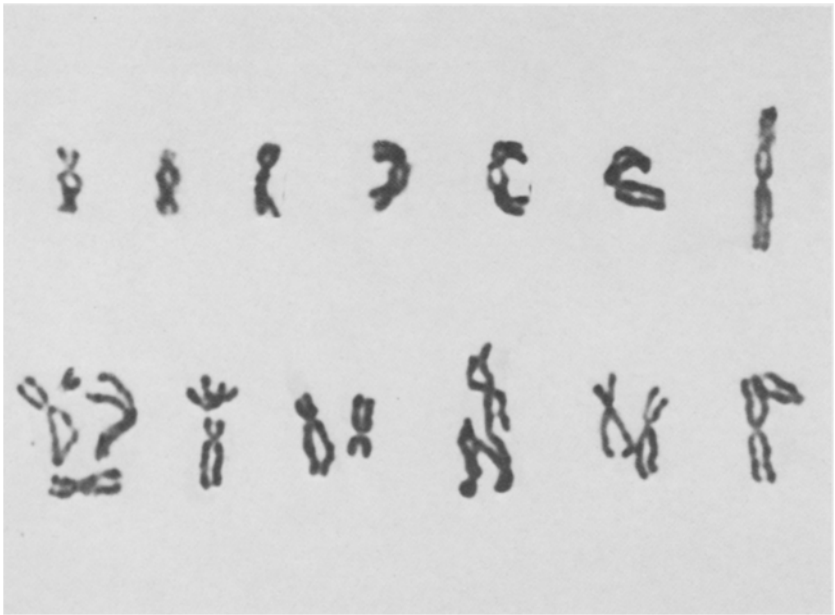


Figure 7
Side-arm bridge (subchromatid exchanges) as viewed in metaphase. Upper row is appearance when subchromatid exchange is between two chromatids of the same chromosome. Lower row is appearance when exchange is between chromatids of different chromosomes.

Until recently it was only possible to carry out the examination of anaphase material in vitro. This was because of the combination of the relatively short duration of anaphase in the mitotic cycle, and the considerably lower mitotic rates that are obtained in vivo when compared to in vitro material. This is overcome in metaphase preparations by utilizing mitotic inhibitors to collect cells at metaphase. It was felt that this was not possible for preparation of anaphase because an intact spindle is necessary for chromosomal migration to the polar areas of the cell. However, work in Dr. Legator's laboratory (PALMER et al. [7]) has demonstrated that the incidence of anaphase could be considerably increased by a colcemid arrest followed by release. This is carried out by injecting the animal with colcemid 2 hours before sacrifice. At the end of this time the bone marrow cells are obtained, washed, and allowed to incubate in complete medium for 2 hours. This incubation permits the spindle fibers to reform, and anaphase to progress in the metaphase cells that have been arrested. It is important in the technique to use colcemid and not colchicine. Cells treated with colchicine do not usually recover within the same mitotic cycle. They usually form restitution nuclei and go back into metaphase, and are therefore not available for anaphase studies. This method offers great advantages over previous attempts at in vivo anaphase. However, the quality of the preparations is still far below that achieved in in vitro materials, and results obtained with these procedures must be subjected to critical evaluation to determine if the period of colcemid arrest influences the detection of abnormalities. Dr. Robert Miller in our laboratory has obtained preliminary data that confirm that whenever metaphase abnormalities are seen in bone marrow cells after treatment with Mitomycin C, anaphase abnormalities are also found using these procedures. It would appear however, that the method of choice for an in vivo cytogenetic examination will probably not be an anaphase procedure. The micronucleus method on polychromatophilic red cells described in this symposium by Dr. Werner Schmid offers many advantages for an in vivo cytogenetic method.

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