Time Sequence of Events Leading to Chromosomal Aberration Formation **

Ruth C. Moore¹ and Michael A Bender²

¹Peter MacCallum Cancer Institute
481 Little Lonsdale Street
Melbourne 3000, Australia

²Medical Department
Brookhaven National Laboratory
Upton, New York 11973

²To whom all correspondence should be addressed

** This research supported by U.S. DOE Contract DE-AC02-76CH00016

RECEIVED

MAY 20 1993

OSTI

MASTER

REPRODUCTION OF THIS DOCUMENT IS UNLIMITED *zb*

Abstract

Investigations have been carried out on the influence of the repair polymerases on the yield of different types of chromosomal aberrations. The studies were mainly concerned with the effect of inhibiting the polymerases on the yield of aberrations. The polymerases fill in single-strand regions, and the fact that their inhibition affects the yield of aberrations suggests that single-strand lesions are influential in aberration formation.

The results indicate that:

1. There are two actions of polymerases in clastogenesis. One is in their involvement in a G_2 repair system, in which either of the two chromatids is concerned, and which does not yield aberrations unless the inhibition is still operating when the cells enter mitosis. The second is such that when repair is inhibited, further damage accrues.

2. The second action is affected by inhibiting polymerase repair, but also operates even when the repair enzymes are active.

3. The production of chromosomal exchanges involves a series of reactions, some of which are reversible.

4. The time span over which the reactions occur is much longer than has been envisaged previously.

During the past several years we have examined the yields of different classes of chromosomal aberrations after treatment with clastogenic agents--x rays and others--over a number of different time periods. We have also measured the effects at different times of inhibiting polymerase-dependent DNA repair on the yields of different types of chromosomal aberrations. The inhibiting agent used has been aphidicolin (APC). When we commenced the experiments, it was believed that APC inhibited polymerase α only; later it has been recognized that there are two, or possibly three, similar polymerases in mammalian cells (Weiser et al., 1991). They resemble each other in being sulfhydryl-containing and sulfhydryl-dependent, and are all inhibited by APC, and it seems likely that they are the products of genes of common descent. The polymerase now called α is involved in semi-conservative DNA synthesis, but we have found it is not involved in some aspects of DNA repair which are involved in aberration formation (Bender et al., 1991; Moore and Bender, 1991); the precise functions of those polymerases now called δ and ϵ have not been determined.

We have classified aberration types as follows:

1. Chromosome types, those which occur in cells after irradiation in G_1 and S. They involve both chromatids at the next division. Their frequency gradually declines during S (Moore, 1981), consistent with the reduction in frequency of unreplicated chromosome portions as DNA synthesis proceeds.

The three main aberration types detectable in standard cytogenetic preparations are dicentrics/rings (the asymmetrical moiety of intrachromosomal exchanges, and of interarm chromosomal exchanges), Fragments and sister unions.

The first two types have long been recognized as standard chromosome-type aberrations (Savage, 1974).

Sister unions are not recognized as a classical chromosome-type aberration, but they undoubtedly appear at the next division in cells of the line JU56 after they have been irradiated in G_1 . However, it is only distal sister unions which are easy to recognize. Proximal sister unions are hard to recognize unless they occur some distance from the centromere. In JU56 cells, distal sister unions are extremely obvious, as the line contains only 9 large metacentric chromosomes.

Sister unions also appear in CHO cells at the next division after irradiation in G_1 . Again distal sister unions are easier to recognize than proximal ones. They are relatively easy to identify in CHO Cells, because the line has a modal chromosome number of 22, and only a few acrocentric chromosomes. However, the "acrocentric" chromosomes do not always show a small short arm, but appear to be simply U-shaped, and because of this fact, and because the karyotype varies between cells, as does the number of acrocentric chromosomes, the presence of sister unions is frequently undetected.

It would be difficult, if not impossible, to identify sister unions in cell lines of human or mouse, in the presence of a larger and variable number of acrocentric chromosomes. It is arguable, therefore, that sister unions are common in mammalian cell lines in general after irradiation in G_1 , despite the fact that they have not been reported.

The ratio of the three types, dicentrics: deletions: sister unions, after an x-ray dose of 2.5 Gy to JU56 cells in G_1 is 1:25:2.0 (Moore, 1981).

2. Chromatid type aberrations, which occur following irradiation in S or G₂. There are a number of equivocal types (Moore and Bender, 1993) such as attached sister unions, in which the distal and proximal parts appear to be connected, and terminal triradials, in which an intact chromosome appears to be inserted into a chromatid break. Some of these may be artifacts. Otherwise, the chief types are those which have been recognized for many years (Savage, 1974, and references therein). They are chromatid breaks, chromatid gaps, and exchanges. Intrachromosomal exchanges, in which both exchange points occur close together on one or both sister chromatids, are far more common than would be expected if exchange occurred at random (Moore et al, 1990). Their high frequency may reflect the distribution of interphase chromosomes.

One of the resultant exchange types is a sister union, although it is assumed that the two exchange points do not occur at precisely the same locus.

Although the frequency of chromosome-type aberrations following x-irradiation decreases as cells progress throughout S, the frequency of chromatid-type aberration does not increase (Moore, 1981). The frequency is controlled by factors, as yet unknown, other than the total amount of replicated DNA.

Based on the studies we have performed, a general scheme of the relationship between DNA strand breakage and chromosome aberration formation has been developed. It is illustrated in Figure 1.

It is assumed that the DNA lesion which leads to aberrations of all kinds is a double-strand break. The break may be rejoined (restituted is the term in classical radiobiology). We suggest that, on the molecular level, rejoining is different to exchange. Our reason for believing so is, that we

found that when APC was added to cultures of cells of the permanent line JU56 12-14 hours after irradiation, at a time when cells which were in early S and G_1 at the time of irradiation were passing into mitosis, there was a marked reduction in the number of identifiable exchanges of both the chromosome and chromatid types. It would appear that inhibition at this time brings about the disruption of exchanges previously formed. There was not, however, an increase in the total number of lesions, as would be expected if the inhibitor also disrupted restituted breaks (Moore et al., 1988).

As well as DNA double-strand breaks, which are one of the initial types of damage with x-irradiation, another lesion, which we consider to be a single-strand break, either formed directly or during the repair of base damage, also contributes to the formation of aberrations when repair is inhibited with APC (Bender and Preston, 1982). Temporary exposure of cells of the permanent cell line JU56 in G_1 to APC during and immediately after irradiation, followed by a period of incubation in the absence of the inhibitor, brings about an increase in aberrations (Moore and Bender, 1987). Deletions are increased, although the exact extent of the increase is difficult to gauge, because the yield of aberrations varies with collection time after treatment and APC slows the passage of cells through G_2 (Moore and Randell, 1987), making it difficult to ensure that the same time-cohorts of APC-treated and untreated cells are collected. It is also impossible to distinguish accurately between chromatid breaks and gaps. Exchanges provide a more accurate comparison, as their yield does not alter with time throughout G_2 , and their frequency is Poisson-distributed. The frequency of exchanges increased 100% in one set of experiments where the exposure to APC was for two

hours after irradiation in G_2 (Moore and Bender, 1987), and 50% in another where the exposure was one hour (Moore et al., 1993a).

Exchange frequency is also increased after a temporary exposure to APC following irradiation, of cells of the permanent line JU56 in G_1 , or of human peripheral blood lymphocytes irradiated in G_0 (Bender and Preston, 1981).

Our interpretation of these findings is that, during the period of inhibition of the repair polymerase(s) with APC, single-strand lesions are converted to double-strand breaks. We assume that the conversion involves the action of an endonuclease. We assume that there are two actions competing with each other, one being the repair of the lesion by the polymerase, and the other the conversion of the lesion to a double-strand break. Evidence to be described below suggests that even in the absence of the inhibitor some conversion of single-strand to double-strand breaks occurs.

The next stage in the production of a DNA lesion to a chromosome aberration we consider to be the conversion of the lesion to a form which cannot be rejoined readily by ligation. We consider that the most probable next step is the formation of sticky ends by exonuclease attack. Thereafter, the ends can participate in aberration formation along one of three different paths.

Firstly, the broken ends may come into contact with other sticky ends and chemical hybridization can occur, and that is the preliminary step in the formation of an exchange. This mode of origin of exchanges was first postulated by Taylor (1963). The scheme we propose is almost but not quite the same as that proposed by Taylor. He proposed that following sticky end formation, the remaining single strand regions were filled in by polymerase

action, but according to his proposition, there was some difficulty in disposing of the DNA strand opposite that which re-annealed. It is now known that the polymerase also have exonuclease function (e.g. Weiser et al., 1991), which will bring about digestion of the opposite strand. The proposal of Taylor has already been further elaborated by Bender et al. (1974). The only alternative postulate has been that exchange occurs as a result of recombination between a damaged and an undamaged segment of DNA. Recent experiments by Cornforth (1990) have shown that exchange requires damage to the DNA of both participating chromosome segments before exchange occurs, which favours the hypothesis presented here.

We believe that the initial step in exchange formation does not fully repair the damage to the participating DNA. Experimental support for this view comes from the fact that inhibition of polymerase with APC during G_2 as much as 12 hours after irradiation eliminates most identifiable exchanges (Moore et al., 1988). In the experiments performed, it was necessary to use only low doses of APC, to ensure that equivalent time cohorts were compared; it is possible that at higher doses all the exchanges would be eliminated.

The second route to aberration formation occurs if the broken end folds back upon itself to form a hairpin configuration (Chadwick and Leenhouts, 1978; Bender et al., 1974; Bender, 1980). We assume that the result is that the broken chromosome end is shielded from exonuclease attack, and so the formation of the hairpin can be considered a form of repair. If this route is followed for cells in G_2 , the result at the next division is a chromatid break; after the cells go through a round of replication, the result is sister union. Experimental support comes from the finding that sister unions are

formed if the cells are irradiated in G_1 (Moore, 1981) and at the second division if the cells are irradiated in G_2 (Moore and Bender, 1993).

As we have already discussed, it can be demonstrated that the route leading to sister union is followed in the permanent cell line JU56, where it is easily observed, because the usual karyotype consists of 9 metacentric chromosomes, and the U-shaped configurations of distal sister unions are easily identified.

Sister unions, like other types of exchanges, are not seen if the cells are incubated with APC 12-14 hours after irradiation. It is to be assumed, therefore, that, like other exchanges, the initiating event does not perfectly restore the integrity and continuity of the DNA molecule, the repair of which may be delayed for hours or longer.

Hairpin formation occurs if there is a palindromic sequence, either perfect or imperfect, at the end of a broken DNA molecule, or if such a sequence is revealed after digestion of the broken end by exonuclease. Palindromic sequences may occur in either unique or repetitive DNA sequences; They may also occur close to telomeric sequences, which have been identified in mammals as $(TTAGGG)_n$ sequences (Moyzis et al., 1988). In the latter case, it is possible that the new end of the chromosome may be able to function as a telomere, and that the replicated chromatids may be able to complete separation.

The third possible outcome for the broken molecule is that it does not undergo exchange and remains broken. In cells irradiated in G_2 , this will result in a chromatid break; in cells irradiated in G_1 , it will result in a deleted chromosome and an acentric fragment. In both cases, the broken end will be liable to undergo further digestion.

The general model of aberration formation here proposed has several novel features; firstly, aberration formation is seen as taking place in a number of steps, and to take a long time to complete, even if it appears to be rapid using the split-dose technique, or by premature chromosome condensation; and secondly, it is seen as reversible under some conditions, with exchanges already formed being subject to degradation. We have also found that aberrations can arise de novo between the first and second post-irradiation division after treatment with x-rays (Moore and Bender, 1993). Some lesions do not enter the route which leads to aberration formation until after the first round of replication. Similar reports have been made by others (Weissenborn and Streffer, 1988).

According to this scheme, a deletion may terminate in an unrepaired DNA molecule. Unless the end of the molecule is protected in some way, it is likely to be open to further nuclease attack, resulting in the loss of further DNA, which may be essential, and the loss of which may cause cell death.

We have found that there is a selective loss of cells containing aberrations in general, although not specifically deletions, between the first and the second post-irradiation divisions (Moore and Bender, 1993). The cells were exposed to colcemid at the time of the first division, which prevented cytokinesis, and, therefore, the cause of cell loss could not be loss of fragments or impediment to division due to bridge formation. We suggest that one cause of this death is the loss of DNA from deletions, due to nuclease attack. The loss of exchanges as well as deletions may be partly explained by the fact that cells which contained exchanges at the first division were also more likely to contain deletions; it may also indicate that there is some

unrepaired damage in exchanges which can lead to the degradation of associated DNA.

It has recently been reported that, in CHO cells at the first division after irradiation, there is an excess of aberrations which involve a telomeric sequence (Alvarez and Giaccia, 1993). The authors have interpreted the results to show that telomeric sequences are fragile in some way, but there is an alternative interpretation; telomeric sequences may be resistant to digestion by nucleases, and therefore the process of digestion will stop when it reaches an interstitial telomere. Likewise, a break which occurs close to an interstitial telomere will have an increased survival potential as the telomere will protect the DNA molecule from further attack. Even before the first division, there may be some loss of cells due to digestion of DNA, and some selection of cells with aberrations immune to digestion.

These experiments were carried out on cells of a permanent cell line, not normal cells. The types and copy numbers, and total numbers of active genes are likely to be greatly different in these cells which do not undergo differentiation, but have been selected for their ability to undergo repeated cell cycles in a defined environment. The consequences of loss of DNA sequences are likely to be different and, of course, the types of repair enzymes and nucleases may be different, too.

There are, however, a number of normal cell types which are extremely sensitive to x-ray damage and undergo rapid interphase death. Among the most notable is the lymphocyte. Lymphocytes in G_0 , before stimulation with PHA, have also been used extensively for studies of chromosomal aberrations. Aberrations are measured in that fraction of cells which survive to reach

mitosis. In the case of cell irradiated in G_0 , this means survivors of rapid interphase death.

Such evidence as there is suggests that the ratio of detectable exchanges, dicentrics and rings, to deletions and sister unions, is higher in lymphocytes than it is in permanent cell lines of the same species. For example, after a dose of 3 Gy in G_0 , the frequency of dicentrics is 23%, and of deletions is 50% (Brewen and Preston, 1975) compared to a frequency of dicentrics of 32%, and of deletions and sister unions combined of 125% in JU56 cells after a dose of 2.5 Gy. After 3.0 Gy, the frequency of dicentrics in Chinese Hamster Lymphocytes is 30% and of deletions 39%. In the case of CHO cells, after 2.5 Gy in G_1 , the frequency of dicentrics is 32% and of deletions and sister unions combined, 66%. Furthermore, there do not appear to be sister unions formed in lymphocytes after irradiation in G_0 . We have found they are rare in human peripheral blood lymphocytes; but they were also not detected in lymphocytes of W. Bicolor, the parent species of JU56 (Brewen and Brock, 1968), in which if they had been present, they should have been easily seen.

The evidence suggests that the yield of aberrations, and the types of aberrations found, depends upon the cell type. In the case of lymphocytes, it is possible that the digestion of broken DNA molecules by nucleases is more rapid or efficient than it is in permanent cell lines, so that a higher proportion of deletions are lethal before the first division. It would be of interest to determine the frequency of surviving deletions which terminate in a telomere.

The experimental evidence outlined above indicates that when the repair polymerase(s) is inhibited by APC, there is an increase in the number of

exchanges are found, even after treatment with x-rays or ara-C, both of which, acting alone, produce exchanges.

To sum up, inhibition of the relevant polymerase until the cells enter division produces chromosomal aberrations under conditions where they would not otherwise be formed, and produces an increased number of aberrations when the DNA is modified in fairly minor ways, such as when bromine replaces the 5-OH group on the thymine. It also detects damage produced by agents which themselves produce chromosomal aberrations, such as x-rays, bromine or UV-B, and Ara-C. This damage is only manifested when cells are exposed to the inhibitor until they enter division. Removing the inhibitor before c-metaphases are collected removes the effect for agents such as bromine or UVB, which do not themselves produce aberrations.

Because many of the aberrations are of the isochromatid type, we assume the existence of some repair mechanism which embraces, perhaps physically, the sister chromatids during G_2 , is affected in this case. Furthermore, it appears that inhibition does not itself produce double-strand breaks, since there is no residual chromosome aberrations when the inhibitor is removed. The chromosome damage caused when the inhibitor is left on is most likely produced when the cells enter mitosis, attached to some physical complex which interferes with coiling. The repair system is distinct from that which produces increased DNA double-strand breakage.

Our evidence that single-strand DNA lesions are converted to double-strand breaks even in the absence of inhibition of polymerase-dependent repair by APC is based partly upon the comparative effect of inhibition when the damaging agent is bromine plus UV-B versus x-rays. The ratio of single-strand breaks and/or base damage to double-strand breaks is different for the two

agents, and hence if only double-strand breaks were effective in producing chromosomal damage in the absence of inhibition with APC, and if APC inhibition brought about an increase due to conversion of single-strand breaks or base damage to double-strand breaks, the increase in aberrations should be greater after bromine-UV-B treatment than after x-rays. This would be especially marked in the case of exchanges in G_2 , since their frequency increases with the square of the number of lesions (Moore et al., 1990). In fact, there is little or no difference in the increase brought about by inhibition between the two agents. This suggests that most single-strand breaks or base damage which are converted to double-strand breaks, are converted even in the absence of inhibition by APC, and that inhibition adds only a minor fraction to this component of double-strand breaks.

Further evidence that conversion of single-strand breaks or base damage to double-strand breaks comes from experiments in which the concentration of -SH groups in the growth medium was altered. The results previously described came from experiments in which the cells were grown in α MEM, in the case of JU56 cells, or RPMI, in the case of human lymphocytes. Both these media contain free -SH groups, of cysteine and glutathione, respectively. The polymerases are -SH containing and -SH dependent. It is, therefore, not surprising that their activity varies with -SH concentration (Zeigler, 1985).

As well, the primary effect of damaging agents such as x-rays and bromine -UVB is affected by -SH concentration. Determining the relative influences of -SH concentrations on different stages of clastogenesis is therefore complicated. However, we have shown that JU56 cells must be grown in the presence of cysteine, and cysteine must also be present during x-irradiation, if a temporary exposure to APC is to increase the number of

exchanges. Removing -SH groups has the same effect as adding the inhibitor, and adding the inhibitor in the absence of -SH groups has no additional effect (Moore et al., 1993b).

The cells do not require cysteine for growth, and so DNA repair under the usual conditions of cell metabolism must be quite competent: it is only when additional damage is produced by x-rays or bromine-UVB that the additional level of activity found in the presence of -SH groups reduces the level of aberrations.

It is to be assumed, therefore, that when cells are grown in the absence of thiols, single-strand breaks or base damage, repaired when the repair polymerase is at its most active, are converted to double-strand breaks, although possibly only to a significant extent if the level of initial damage is heavy. It can be assumed that, in the same way, partly stabilized exchanges can be broken down, as they were in our experiments in which the polymerase was inhibited.

To sum up, the formation of chromosomal exchanges apparently occurs in a series of steps, some of which can be reversible. The formation of aberrations in general is not so rapid as has long been thought. Further information on the system can be acquired by further cytogenetic experiments of the type described here, but further information about the function and intracellular distribution of the polymerases would clarify the picture considerably.

Legends: Figures

Figure 1: Postulated routes of DNA damage and repair following treatment with, e.g. x-rays, leading to the formation of chromosomal aberrations. The initial lesions are DNA double-strand breaks, or single-strand breaks, possibly produced during repair of base damage. Polymerases can repair the damage, or endonucleases can convert single-strand regions to double-strand breaks. Exchanges, either between two unrelated DNA segments, or via the formation of a hairpin (which leads to sister union) are completed by polymerase repair. Unrepaired double-strand breaks lead to deletions.

References

- Alvarez L, Giaccia AJ (1993): Behaviour of interstitial telomeres as chromosomal fragile sites. Environ Molec Mutagen (In Press).
- Bender MA (1980): Relationship of DNA lesions and their repair to chromosomal aberration production. In: Generoso WM, Shelby MD, de Serres FJ, DNA Repair and Mutagenesis in Eukaryotes, Plenum NY; 245-265.
- Bender MA, Griggs HG, Bedford JS (1974): Mechanisms of chromosomal aberration formation: III. Chemicals and ionizing radiation. Mutat Res 23:197-212.
- Bender MA, Preston RJ (1981): Role of base damage in aberration formation: interaction of aphidicolin and X-rays. In: Natarajan AT (Ed) Progress in Mutation Research 4:37-46.
- Bender MA, Moore RC (1988) Dose relationships for different effects of aphidicolin in human peripheral blood leucocytes. Mutat Res 198:227-31.
- Bender MA, Moore RC (1990): DNA polymerase alpha does not mediate G_0 - G_1 increase in x-ray-induced exchange aberrations in human peripheral blood lymphocytes. Mutat Res 224:11-114. f
- Bender MA, Moore RC (1991): DNA polymerase delta mediates increase in exchange production by x-radiation in human lymphocytes moving from G_0 to G_1 . Mutat Res 250:319-324.
- Bender MA, Moore RC, Pyatt BE (1991): Role of polymerase alpha and delta in radiation clastogenesis. Mutat Res 262:31-36.

Cornforth MN (1990): Testing the notion of the one-hit exchange. Radiat Res 121:21-27.

Moore RC (1980): Alterations in the frequency of different classes of chromosomal aberrations throughout the first cell cycle following x-irradiation. Radiat Res 81:267-281.

Moore RC Bender MA (1984): Chromosome aberration types in Chinese hamster (CHO) cells x-irradiated in S and G₁. Int J Rad Biol 45:451-458.

Moore RC, Bender MA (1987): The synergistic effect of aphidicolin on the yield of x-ray-induced chromosome aberrations throughout the cell cycle in JU56 cells. Radiat Res 110:385-395.

Moore RC, Randell C (1987): Different effects of 1- β -D-arabinofuranosylcytosine and aphidicolin in S phase cells--chromosome aberrations, cell-cycle delay and cytotoxicity. Mutat Res 178:73-80.

Moore RC, Barber L, Bingham CG (1990): Is it misrepair or lack of repair which kills cells irradiated in G₂? In: Obe G, Natarajan AT, Chromosomal Aberrations: Basic and Applied Aspects, Springer, pp. 42-46.

Moore RC, Bender MA (1993): Chromosomal aberration types in cells at the second division after irradiation in G₂ or G₁. Int J Radiat Biol (In Press).

Moore RC, Bingham CG, Bender MA (1993a): Elucidation of some factors involved in the formation of chromosomal aberrations by inhibiting a repair enzyme. In: Obe G, Natarajan AT, Second International Symposium on Chromosomal Aberrations, Berlin etc., Springer (In Press).

Moore RC, Randell C, Westerman M (1993b): The influence of growth medium on the yield of x-ray-induced chromatid exchanges in the presence or absence of aphidicolin. *Mutat Res* 285:13-18.

Savage JRK (1974): Classification and relationships of induced chromosomal structural changes. *J Med Genet* 12:103-122.

Taylor JH (1963): Radioisotope studies on the structure of the chromosome. In: Wolff S, Radiation-Induced Chromosome Aberrations, Columbia University Press, NY; 123-164.

Trowell OA (1952): Sensitivity of lymphocytes to ionizing radiation. *J Pathol Bacteriol* 64:178-189.

Weiser T, Gassman M, Thommes P, Ferrari E, Hafkemeyer P, Hubscher U (1991): Biochemical and functional comparison of DNA polymerases alpha, delta and epsilon from calf thymus. *J Biol Chem* 266:10420-8.

Weissenborn U, Streffer C (1988): The one-cell mouse embryo: cell cycle dependent radiosensitivity and development of chromosomal anomalies in postradiation cell cycles. *Int J Rad Biol* 54:659-674.

Zeigler DM (1985): Role of reversible oxidation-reduction of enzyme thiol-disulphides in metabolic regulation. Ann Rev Biochem 54:305-329.

RM921101

END

**DATE
FILMED**

7 / 8 / 93