American Journal of Medical Genetics 35:301–305 (1990)

DNA Polymerase Alpha Defect in the N Syndrome

Kimberly M. Floy, Russell O. Hess, and Lorraine F. Meisner

Wisconsin State Laboratory of Hygiene and Department of Preventive Medicine, University of Wisconsin Medical School (K.M.F., L.F.M.), and Group Health Cooperative (R.O.H.), Madison, Wisconsin

The N syndrome is characterized by mental retardation, malformations, chromosome breakage, and development of T-cell leukemia (Opitz et al.: *Proceedings of the II International Congress IASSMD* pp 115–119, 1971; Hess et al.: *Clinical Genetics* 6:237–246, 1974b, *American Journal of Medical Genetics [supplement]* 3:383–388, 1987). N syndrome fibroblasts were studied to see if the high chromosome breakage rate associated with this apparently X-linked syndrome could be related to a deficiency of DNA polymerase alpha, a product of a gene localized to the X chromosome. Bleomycin, which is known to break double-stranded DNA, produced increased chromosome breakage in normal control, Fanconi anemia, and N syndrome fibroblasts. When aphidicolin was used to inhibit repair mediated by DNA polymerase alpha, both normal control and Fanconi anemia fibroblasts showed significantly more chromosome breakage than was produced by bleomycin alone, but there was no increase in the amount of breakage seen in the N syndrome fibroblasts over that seen with bleomycin alone. This suggests that the N syndrome is due to a mutation affecting the region of the X chromosome on which the gene for DNA polymerase alpha is located, and that the high risk of T-cell leukemia observed in the hemizygote is due to this DNA repair defect.

KEY WORDS: chromosome breakage syndrome, DNA polymerase alpha defect, defective DNA repair, X-linked chromosome instability syndrome, T-cell malignancy, bleomycin-induced chromosome damage, aphidicolin inhibition of DNA repair, N syndrome

INTRODUCTION

The N syndrome is a multiple congenital anomaly-mental retardation syndrome described in 2 brothers [Opitz et al., 1971; Hess et al., 1974b], in which lymphocytes from both propositi and from their unaffected mother showed a greatly increased rate of chromosome breakage. Both brothers died of an acute malignancy clinically resembling T-cell leukemia at ages 5 and 19 years, respectively, while their mother also died of acute leukemia at age 37 [Hess et al., 1987]. A fibroblast culture at the time of autopsy of the 19-year-old brother showed an increased rate of chromosome breakage and rearrangement in all cells.

It is suggested that the observed chromosome instability in this family was the result of a X-linked recessive mutation causing DNA repair deficiency in affected males, predisposing them to the development of a specific lymphoid malignancy. The fact that both brothers died of a malignancy clinically resembling T-cell leukemia, coupled with the observation that childhood T-cell leukemia occurs at least 4 times as frequently in males as in females [Henderson, 1983], suggests an X-linked mutation in the N syndrome males and a somatic mutation involving the same locus in at least some of the nongenetic cases of T-cell leukemia. The occurrence of a high rate of chromosome breakage and leukemia in the normal mother of the propositi [Hess et al., 1987] suggests an increased risk of neoplasia developing in those cells in which the X chromosome containing the mutant allele is not inactivated.

The gene for DNA polymerase alpha, an enzyme known to be involved in DNA replication and repair, has recently been assigned to the X chromosome [Wang et al., 1985]. We hypothesized that the DNA repair deficiency in the N syndrome might be associated with a deficiency of DNA polymerase alpha because of its X-linked inheritance. Therefore, the present investigation was undertaken to evaluate repair of bleomycin-induced chromosome damage in which DNA polymerase alpha was inhibited owing to use of aphidicolin. Bleomycin is an antineoplastic agent isolated from *Streptomyces verticillus*, which is known to cause single- and double-strand breaks in double-stranded DNA [Lazo et al., 1987]. Aphidicolin is an antibiotic that has been shown to inhibit DNA polymerase alpha activity [Ikegami et al., 1978; Hanoka et al., 1979], as well as DNA polymerase delta [Hammond et al., 1987]. Although the exact functional and structural characteris-

Received for publication October 19, 1988; accepted September 8, 1989.

Address reprint requests to Dr. Lorraine F. Meisner, Wisconsin State Laboratory of Hygiene, University of Wisconsin Health Sciences Center, 465 Henry Mall, Madison, WI 53706.

© 1990 Wiley-Liss, Inc.

tics of DNA polymerase alpha and delta are currently under investigation and debate, it is generally accepted that there are functional and even structural relationships between the two polymerases [Hammond et al., 1987]. However, owing to the evidence for an X-linked defect in the N syndrome we focused this study on the role of DNA polymerase alpha. Aphidicolin does not cause G-2 chromatid breakage in human lymphocyte cultures by itself [Hsu et al., 1986]. Lymphocytes exposed to X-rays showed significantly increased chromosome damage when aphidicolin was added to the medium [Bender, 1985], suggesting that aphidicolin increased breakage by inhibition of repair [Hsu et al., 1986].

Several studies have shown that simultaneous treatment of human lymphocytes with bleomycin and aphidicolin during the G-2 phase of the cell cycle results in a synergistic effect on chromosome breakage rates and concluded that DNA polymerase alpha is definitely involved in repairing bleomycin-induced DNA damage [Hsu et al., 1986; Hsu, 1987]. In the present study we used this fact to investigate the activity of DNA polymerase alpha in the N syndrome fibroblasts, since the propositi were deceased and therefore we could not study lymphocytes. It was necessary to establish new dosage levels for these chemicals for use on human fibroblast cultures as opposed to the previous figures for lymphocytes [Hsu et al., 1986], although we expected the synergism of bleomycin and aphidicolin to be comparable to that seen in lymphocytes.

The purpose of the present study was to show that the chromosome instability observed in the N syndrome cells was due to impaired DNA polymerase alpha activity. This was done by comparing breakage in N syndrome cells treated with bleomycin alone to breakage in cells treated with a combination of bleomycin and aphidicolin, using fibroblasts from normal individuals and from a patient with Fanconi anemia (FA) as negative and positive controls. We hypothesized that if DNA polymerase alpha were deficient in N cells it would not matter whether aphidicolin were present or not since DNA repair was already compromised, so the amount of chromosome breakage in the bleomycin-treated N cells should then be the same as that in bleomycin plus aphidicolin-treated N cells.

MATERIALS AND METHODS

This study assessed chromosome damage in cultures grown and passaged according to established techniques maintained using nutrient mixture F-10 (HAM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma, St. Louis), L-glutamine, and Pen-Strep solutions. Control cultures, 25707(3) (i.e., 25707 in the 3rd passage)-male and 25654(7)-female, as well as the FA culture, 25587(7)-female, were established from tissue from recent stillbirths. Control cultures, 7766(6)-male and 6041.1(2)-female, were cells recovered from liquid nitrogen from cultured fibroblasts of children under age 3 years, studied to check for effects of freezing and later passage number on chromosome damage. The N syndrome cells, 1101(6)-male, were also recovered from liquid nitrogen storage. All control cultures were previously found to have a normal chromosome constitution. Chromosome damage was assessed by using bleomycin sulfate (U.S. Biochemical Corp., Cleveland) dissolved in distilled water, 0.5 μg/ml (final culture concentration), and aphidicolin (Sigma, St. Louis) dissolved in 20% ethanol:80% Hanks' balanced salt solution (Gibco, Grand Island, NY), 1.0 μg/ml. Both of these chemicals were frozen at −20°C as stock solutions and defrosted immediately before use. Control cultures received the same amount of ethanol and Hanks' solution (0.25 ml/5 ml culture). Treatment of the normal fibroblast cultures and N syndrome culture was done simultaneously. The treatment of FA cultures was done at a later date; however, all conditions were identical except that the FA cells, which had not been frozen, grew more vigorously so the cultures were heavier at the time of treatment.

Cells were subcultured 2 days before treatment, which consisted of adding bleomycin ± aphidicolin together with 0.25 ml colcemid (final culture conventration 1 μg/ml) for the last 4 hours of culture. Harvest involved a 12 minute hypotonic treatment (0.075 M KCl) followed by fixation with methanol-acetic acid (3:1). Fixed cells were dropped onto wet slides, air dried, and stained with 10% Giemsa (Gurr R66, BDH Chemicals Ltd., Poole, England) without banding.

All preparations were coded for blind microscopic scoring of metaphases using a Zeiss photomicroscope at 1,250×. Metaphase pictures were taken of all scored cells to document chromatid breakage, with the exception that only every tenth untreated cell was photographed unless it contained possible breaks. Chromatid breaks were defined as achromatic regions longer than the width of the chromatid.

RESULTS

The increased chromatid breakage induced in normal human fibroblasts by treatment with bleomycin, aphidicolin, and combination treatments is summarized in Figure 1. Although the untreated control cells showed

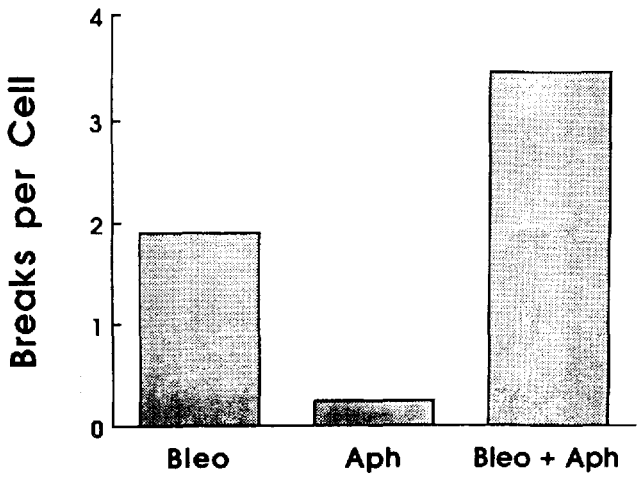

Fig. 1. Histogram showing mean number of chromosome breaks in normal fibroblasts after 4 hour G-2 exposure to 0.5 μg/ml bleomycin and/or 1 μg/ml aphidicolin, based on 100 cells per treatment.

the low background breakage rates expected (0.045 breaks per cell), treatment for 4 hours with bleomycin alone led to a significant increase in breakage. Aphidicolin alone had little effect on chromosome breakage, whereas the use of bleomycin and aphidicolin together resulted in more breakage than would be expected on the basis of an additive model (Fig. 1).

The effects of 4 hour bleomycin treatments with and without aphidicolin are shown in Table I. Because of the increased toxicity observed with the combination treatment, less than 100 acceptable cells were available for scoring chromosome breakage in both the negative control and N syndrome cells; the same was also true in the N syndrome cells treated with bleomycin alone. The untreated N syndrome and FA cells exhibited a higher level of background breakage, as expected. Both control and FA cells exhibited a significant increase in chromatid breakage with bleomycin; this was elevated even further when aphidicolin was used in addition to bleomycin. However, the N syndrome cells that had been treated with bleomycin alone under exactly the same conditions demonstrated a markedly higher breakage rate than control and FA cells treated with bleomycin alone, with no increase resulting from addition of aphidicolin. The data are also summarized in graphic form in Figure 2.

Table II demonstrates the frequency of cells with chromosome damage following each treatment of normal fibroblasts, FA fibroblasts, and N syndrome fibroblasts. The frequency of damaged cells increased in all 3 cell types following treatment with bleomycin. It increased even further when aphidicolin was used together with bleomycin in controls and FA cultures, but not in the N syndrome cells. Also, the percent of cells exhibiting breaks in the N syndrome cultures after bleomycin treatment alone is higher than that seen in control and FA cultures after either bleomycin alone or bleomycin plus aphidicolin.

The increased frequency of breaks per cell is demonstrated in Table III. Treatment with bleomycin not only increased the number of "affected" cells but also increased the amount of damage within "affected" control, FA, and N cells. However, the N syndrome cultures showed neither an increase in "affected" cells nor an increase in the amount of breakage per cell induced by bleomycin when aphidicolin was added. The breakage distribution that was due to bleomycin plus aphidicolin in control and FA cells was equal to that seen in the N syndrome cells treated with bleomycin alone, emphasizing the lack of effect of aphidicolin on the N cells.

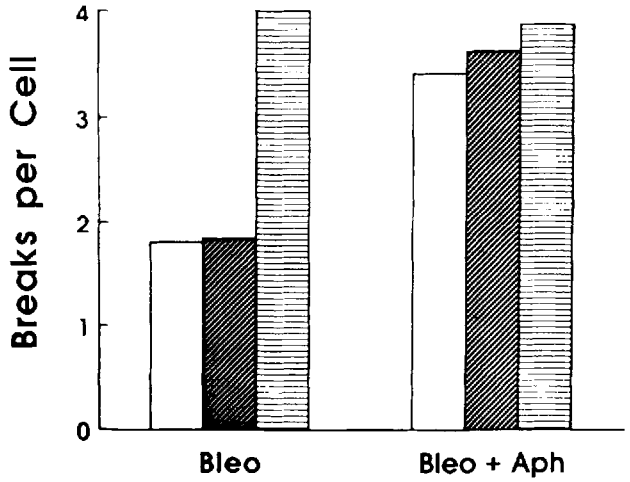

Fig. 2. Histogram showing mean number of chromosome breaks following 4 hour G-2 treatments of normal, FA, and N fibroblasts with 0.5 μg/ml bleomycin alone or with 0.5 μg/ml bleomycin plus 1 μg/ml aphidicolin. Control □, Fanconi anemia ▨, N Syndrome ▤.

DISCUSSION

In the present study we used cultured fibroblasts in an attempt to identify the mechanism of the increased chromosome breakage previously observed in N syndrome lymphocytes and fibroblasts [Hess et al., 1987]. This was necessary because the propositi were deceased, so we had only cryogenically preserved fibroblasts with which to work. Attempting the same dosage of bleomycin previously used in whole blood cultures (30 μg/ml) [Hsu et al., 1986] proved far too toxic for fibroblasts, and the dose had to be modified to 0.5 μg/ml to permit sufficient cell survival for scoring chromosome damage. However, even with such a very low dose of

TABLE I. Total Breaks/Total Cells Scored After 4 Hour Treatment With Bleomycin and Aphidicolin

Culture No.	0 treatment	0.5 μg/ml bleomycin	0.5 μg/ml bleomycin + 1.0 μg/ml aphidicolin
25707 (control)	1/100	151/100	315/91
25654 (control)	8/100	210/100	295/88
1101 ("N" syndrome)	26/100	332/83	298/77
25587 (Fanconi anemia)	17/100	184/100	361/100

TABLE II. Percent of Cells With Chromatid Breaks After 4 Hour Treatment With Bleomycin ± Aphidicolin

Culture No.	0 treatment	0.5 μg/ml bleomycin	0.5 μg/ml bleomycin + 1.0 μg/ml aphidicolin
25707 (control)	1	59	80
25654 (control)	5	64	77
1101 ("N" syndrome)	20	83	74
25587 (Fanconi anemia)	13	67	79

TABLE III. Distribution of Breaks Following 4 Hour Treatment With 0.5 μg/ml Bleomycin ± 1 μg/ml Aphidicolin

Culture No.	Treatment	Total cells	No. of cells showing			
			0	1 break	2 breaks	3+breaks
25707 (control)	0	100	99	1	0	0
	Bleomycin	100	41	24	15	20
	Bleomycin + aphidicolin	91	18	12	16	45
25654 (control)	0	100	95	2	3	0
	Bleomycin	100	36	26	12	26
	Bleomycin + aphidicolin	88	20	16	9	43
1101 ("N" syndrome)	0	100	80	16	3	1
	Bleomycin	83	14	13	16	40
	Bleomycin + aphidicolin	77	20	7	10	40
25587 (Fanconi anemia)	0	100	87	9	4	0
	Bleomycin	100	33	15	17	35
	Bleomycin + aphidicolin	100	21	13	17	49

bleomycin, once aphidicolin was added to the fibroblast cultures to inhibit the repair enzyme DNA polymerase alpha, fewer than 100 scorable cells were found in all except the FA cultures (Table II). The apparently reduced toxicity exhibited in the FA cells may be due to the increased number of cells present in these cultures at the time of treatment, leaving more healthy survivors to be scored after the multiply damaged cells had died. However, the inability to obtain sufficient scorable cells in the N syndrome cultures after the addition of bleomycin alone is consistent with an inherent repair deficiency in these fibroblasts.

Because of the differences in the response of lymphocytes and fibroblasts to bleomycin, among other differences between these two cell lines, before proceeding it was necessary to determine whether the same additive effect reported in lymphocytes with the use of bleomycin plus aphidicolin [Hsu et al., 1986] would also be seen in fibroblasts. When aphidicolin alone was added to control cultures, there was only a small increase in chromosome damage, probably owing to aphidicolin inhibition of repair of spontaneous breakage. However, the combination treatment yielded breakage far in excess of that which would be expected on an additive model (Fig. 1), thus demonstrating the same effects from the use of bleomycin and aphidicolin as was seen in lymphocytes.

The fact that DNA polymerase alpha was evidently capable of repairing bleomycin-induced chromatid breakage in normal fibroblasts permitted testing of the ability of N syndrome fibroblasts to repair bleomycin-induced chromatid breakage with and without aphidicolin. As shown in Tables I–III and in Figure 2, although there was increased spontaneous chromosome breakage in the N cells, with the addition of bleomycin there was a pronounced increase in damage, far in excess of the increase observed in the normal controls and in the FA controls. In fact, as shown in Table I, the amount of bleomycin-induced damage is over twice that seen in control culture 25707 and the FA culture. If DNA polymerase alpha activity is indeed deficient in the N syndrome fibroblasts, we would expect the addition of aphidicolin, a known DNA alpha inhibitor [Ikegami et al., 1987; Hanoka et al., 1979], to have little, if any, effect on the amount of chromosome damage induced by bleomycin. As shown in Table I and Figure 2, this is indeed the case. The small decrease in breaks per cell seen after the addition of aphidicolin to the N cells can be attributed to the lesser number of cells available for scoring owing to increased toxicity.

If bleomycin-induced breakage is repaired by polymerase alpha, which is blocked by aphidicolin, the amount of chromatid breakage seen in normal and FA cultures after treatment with bleomycin and aphidicolin should be comparable to that seen in the N syndrome cells after treatment with bleomycin alone if the N cells already have a reduced DNA polymerase alpha activity level comparable to that produced in the others by the addition of aphidicolin. As shown in Tables I–III, this appears to be the case. The N syndrome cells actually showed more breakage per cell following bleomycin alone than did the others after the addition of aphidicolin (3.4–3.6 breaks per cell vs. 4.0 in the N cells), suggesting that the activity of DNA polymerase alpha normally present in the N syndrome cells is lower than that remaining after inhibition of activity by aphidicolin in cells without this DNA repair defect.

Although both FA and the N syndrome are breakage syndromes, the mechanisms obviously are not at all similar. The chromosome instability associated with the N syndrome is due to defective DNA repair associated with an apparent X-linked mutation, while in FA the genetic lesion is transmitted as an autosomal recessive. FA is also associated with somatic anomalies, as well as the development of pancytopenia in early childhood; if the pancytopenia is not fatal, FA homozygotes almost inevitably develop acute non-lymphocytic leukemia or some other malignancy affecting rapidly dividing cells. Perhaps the chromosome breakage in FA preferentially affects the rapidly dividing cells of the hematopoietic system, resulting in their destruction through genetic damage, so that pancytopenia develops. However, if the pancytopenia is treated successfully, the risk of acute non-lymphocytic leukemia is very high, as the chromosome instability in the bone marrow cells increases the likelihood of a mutation with malignant potential. In contrast, the X-linked N syndrome appears to have as its target cell the long-lived T-lymphocyte, with a mean life span of 5 or more years. Since such long-lived cells, in

the absence of adequate DNA repair of the accumulated genetic damage that they incorporate, could accumulate chromosome translocations with a neoplastic potential, once stimulated to proliferate by infection, a single mutated cell could give rise to a malignant clone culminating in acute T-cell leukemia. By the same token, other DNA breakage syndromes are also associated with risks for specific cancers that are conditioned by their specific genetic lesion (e.g., xeroderma pigmentosum leads to skin cancer because of deficiency of an endonuclease required for repair of UV-induced DNA repair damage).

We conclude that this study demonstrates the first example of a mutation affecting the gene for DNA polymerase alpha, which has been tentatively localized to between bands p21.3 and 22.1 of the X chromosome [Wang et al., 1985]. Since this enzyme is so essential for DNA replication and repair, it might appear that survival would not be possible without its normal function. Clearly the propositi are not normal, but they did survive for 5 and 19 years, respectively, with other repair mechanisms, e.g. DNA polymerase delta, filling in only partially for the defective DNA polymerase alpha gene. Because the alternate pathways of DNA repair could not correct completely for the DNA polymerase alpha deficiency, the propositi ultimately died of leukemia affecting the only cell type that is capable of circulating for years without dividing, while incorporating DNA damage from various mutagenic insults, and that is then forced to proliferate following stimulation by the appropriate antigen. The mother of the affected boys, being heterozygous, did not show the phenotypic effects of malformation and mental retardation associated with this X-linked mutation; but because in half of her lymphocytes the mutant gene was on an active X, she would be expected to incorporate mutations with neoplastic potential in those cells, thus putting her at risk for also developing T-cell leukemia but at an older age than the hemizygous males. Although she died elsewhere with a diagnosis of leukemia, with no autopsy or other information available, we would expect that it was the same type of leukemia as was observed in both of her sons. Similarly, normal males could undergo somatic mutations at the DNA polymerase alpha locus in T lymphocytes, resulting in defective DNA repair leading to preleukemic clones, and thus to a higher rate of T-cell leukemia than females, which has been observed [Henderson, 1983].

ACKNOWLEDGMENTS

We would like to express our gratitude to Dr. Walter N. Strickland of the Enzyme Institute for his help in trying to characterize DNA polymerase alpha. We would also like to thank Dr. Elizabeth Kaveggia who helped to identify the N family and Dr. John Opitz for his continuing encouragement and support.

REFERENCES

Bender MA (1985): Role of DNA polymerase in chromosome aberration production by ionizing radiation. Ann NY Acad Sci 459:245–254.

Hammond RA, Byrnes JJ, Miller MR (1987): Identification of DNA Polymerase delta in CV-1 cells: Studies implicating bone DNA polymerase delta and DNA polymerase alpha in DNA replication. Biochemistry 26:6817–6824.

Hanoka F, Kata H, Ikegami S, Ohashi M, Yamoda M (1979): Aphidicolon does inhibit repair replication in HeLa cells. Biochem Biophys Res Commun 87:575–580.

Henderson ES (1983): Clinical diagnosis in leukemia. In Gunz FW, Henderson ES (eds): "Leukemia." New York: Grune and Stratton, pp 393–425.

Hess RO, Hafez GR, Meisner LF (1987): Updating the N syndrome: Occurrence of lymphoid malignancy and possible association with an increased rate of chromosome breakage. Am J Med Genet [Suppl] 3:383–388.

Hess RO, Kaveggia EG, Opitz JM (1974a): The N-Syndrome: A new multiple anomaly-mental retardation syndrome. Abstract of paper presented at Midwest Society for Pediatric Research 7–8 Nov. 1973, Pittsburgh, PA. J Pediatr 84:920.

Hess RO, Kaveggia EG, Opitz JM (1974b): Studies of malformation syndromes in Man XXVII: The N syndrome. A "new" multiple congenital anomaly-mental retardation syndrome. Clin Genet 6:237–246.

Hsu TC (1987): Genetic predisposition to cancer with special reference to mutagen sensitivity. In Vitro Cell Dev Biol 23:591–603.

Hsu JC, Ramkissoon D, Furlong C (1986): Differential susceptibility to a mutagen among human individuals: Synergistic effect on chromosome damage between bleomycin and aphidicolon. Anticancer Res 6:1171–1176.

Ikegami S, Taguchi T, Ohashi M, Oguro M, Nagano H, Mano Y (1978): Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase alpha. Nature 275:458–460.

Lazo JS, Sebti SM, Filderman AE (1987): Metabolism of bleomycin and bleomycin like compounds. In Pavis G, Prough R (eds): "Metabolism and Action of Anti-Cancer Drugs." New York: Taylor and Francis Ltd., pp 199–210.

Opitz JM, Kaveggia EG, Pallister PD (1971): Studies of genetic malformation syndromes. Proc. II. Int. Congr. IASSMD. Warsaw, Polish Med Publ 115–119.

Wang TS, Pearson BE, Suomalainen HA, Mohandas T, Shapiro LJ, Schroeder J, Korn D (1985): Assignment of the gene for human DNA polymerase alpha to the X chromosome. Proc Natl Acad Sci USA 82:5270–5274.