

In pE1A100, the E1a DNA insert is so oriented that a unique Bam HI restriction site occurs upstream of this Sma I site. The pE1A100 was cut with Xma I and treated with Bal31 exonuclease (25). The reaction was stopped at several time points (15, 30, 45, and 60 seconds), and the extent of exonuclease digestion was determined by fine restriction. The 45-second Bal31 digestion time point was selected where only 10 to 20 bp had been removed from the end (5' to the E1a coding sequence) of most of the DNA molecules. The DNA from this Bal31 exonuclease time point was cut with Bam HI, blunt-ended with DNA polymerase (Klenow), and ligated. Ampicillin-resistant transformants were selected and screened for clones in which the Bam HI site was recreated. One of these plasmids, pE1A210, was shown by fine restriction to contain a Bam HI site in which the sixth base of the Bam HI recognition sequence (GGATCC) (G, guanine; A, adenine; T, thymine; C, cytosine) was the first base of the third codon (CAT) of the E1a coding sequence. The entire E1a coding sequence was reconstructed in pE1A210 by inserting the 1748-bp Pvu II fragment from pJN20 (Fig. 1) in the correct orientation at the Pvu II site of pE1A210. This procedure yields pE1A310. The entire E1a coding sequence was moved into the expression vector pAS1 by inserting the 895-bp Bam HI-Hpa I DNA fragment (Fig. 1) from pE1A310 between the Bam HI and Nru I sites on pAS1, to yield pAS1-E1A310. The E1a coding sequence in pAS1-E1A310 is not in-frame with the translation initiation signal supplied by the expression vector. Construction of pAS1-E1A410: The vector pAS1-E1A410 was constructed from pAS1-E1A310 by positioning the E1a coding sequence in-frame with the ATG translation initiation signal supplied by the expression vector. This was accomplished by Bam HI restriction of pAS1-E1A310, followed by treatment with calf alkaline phosphatase, limited digestion with mung bean exonuclease (11) to remove the four-base 5' single-strand overhanging ends, and religation of the vector. The correct construction was identified by fine restriction and by expression of the E1a product (detected by pulse-labeling). pAS1-E1A410 encodes a product that is identical to the authentic product of the E1a 135 mRNA except for the deletion of the second amino acid.

16. T. F. Osborne, R. B. Gaynor, A. J. Berk, *Cell* **29**, 139 (1982).
17. L. T. Feldman and J. R. Nevins, *Mol. Cell. Biol.* **5**, 829 (1983).
18. H. Van Ormondt, J. Maat, C. P. van Beveren, *Gene* **11**, 299 (1980).
19. S. Yee, D. T. Rowe, M. L. Tremblay, M. McDermott, P. E. Branton, *J. Virol.* **46**, 1003 (1983).
20. H. Esche, M. B. Mathews, J. B. Lewis, *J. Mol. Biol.* **142**, 399 (1980); M. Green, W. S. M. Wold, K. H. Brackman, M. A. Cartas, *Virology* **97**, 225 (1979); D. N. Halbert and H. J. Raskas, *ibid.* **116**, 406 (1982); D. N. Halbert, D. J. Spector, H. J. Raskas, *J. Virol.* **31**, 621 (1979); M. L. Harter and J. B. Lewis, *ibid.* **26**, 736 (1978); J. H. Lupker, A. Davis, H. Jochemsen, A. J. Van der Eb, *ibid.* **37**, 524 (1981); J. E. Smart, J. B. Lewis, M. B. Mathews, M. L. Harter, C. W. Anderson, *Virology* **112**, 703 (1981).
21. B. Krippel, B. Ferguson, M. Rosenberg, H. Westphal, in preparation.
22. N. Jones, J. Richter, D. Weeks, L. Smith, *Mol. Cell. Biol.* **3**, 2131 (1983).
23. B. Ferguson and M. Rosenberg, unpublished data.
24. D. Schumperli, B. H. Howard, M. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 257 (1982).
25. R. J. Legerski, J. L. Hodnett, H. B. Gray, Jr., *Nucleic Acids Res.* **5**, 1445 (1978).
26. J. F. Young, U. Desselberger, P. Palese, B. Ferguson, A. R. Shatzman, M. Rosenberg, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6105 (1983).
27. M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976).
28. R. Laskey, A. Mills, J. Gurdon, G. Partington, *Cell* **11**, 345 (1977).
29. All standard molecular cloning techniques were carried out as described in T. Maniatis, E. F. Fritsch, J. Sambrook, Eds., *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982).
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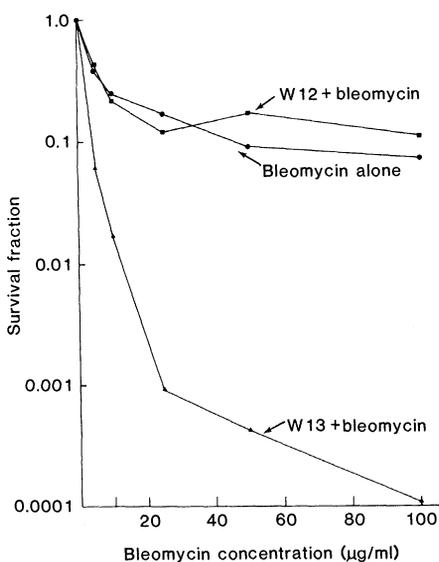
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## Potential of Bleomycin Lethality by Anticalmodulin Drugs: A Role for Calmodulin in DNA Repair

**Abstract.** Treatment of exponentially growing Chinese hamster ovary cells with bleomycin causes a dose-dependent decrease in cell survival due to DNA damage. This lethal effect can be potentiated by the addition of a nonlethal dose of the anticalmodulin drug N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) but not its inactive analog N-(4-aminobutyl)-2-naphthalenesulfonamide (W12). By preventing the repair of damaged DNA, W13 also inhibits recovery from potentially lethal damage induced by bleomycin. These data suggest a role for calmodulin in the DNA repair pathway.

Many intracellular  $\text{Ca}^{2+}$ -dependent events are mediated by the  $\text{Ca}^{2+}$  receptor, calmodulin (1). This protein appears to participate in the regulation of cell proliferation by  $\text{Ca}^{2+}$ , since several anticalmodulin drugs inhibit cell replication (2-6), and calmodulin levels appear to change during the cell cycle (2-4, 7-9). We have shown that calmodulin is selectively increased at the boundary of the  $G_1$  and S phases of the cell cycle in exponentially proliferating Chinese ham-

ster ovary (CHO) cells (2) as well as on release of growth-arrested cells in response to a mitogenic stimulus (3). In both types of proliferative response the entry into and progression through the DNA synthetic (S) phase of the cycle can be specifically and reversibly blocked by the anticalmodulin drug N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) but not by its inactive analog N-(4-aminobutyl)-2-naphthalenesulfonamide (W12). In addition, the antiproliferative effect of interferon is associated with an inhibition in the synthesis of calmodulin that normally occurs at the  $G_1$ -S boundary (10). These data suggest that calmodulin may be important in the commitment to DNA synthesis. We have examined the effect of W13 on the ability of CHO cells to survive after treatment with the DNA-damaging agent bleomycin. Treatment of exponentially growing cells with bleomycin causes a dose-dependent decrease in cell survival. This lethal effect is potentiated by a nonlethal dose of W13 (or another anticalmodulin drug, trifluoperazine) but not by W12. The anticalmodulin drug W13 also prevents recovery from bleomycin-induced potentially lethal damage by preventing the repair of DNA. Taken together, these data suggest a role for calmodulin in the DNA repair pathway and afford a clue to how cell cycle-dependent changes in the concentration of this ubiquitous  $\text{Ca}^{2+}$  receptor may regulate cell proliferation.



**Fig. 1.** Dose-response curve for bleomycin and for simultaneous treatment with W12 or W13. Exponentially growing CHO cells were treated for 1 hour with various concentrations of bleomycin (1 to 100  $\mu\text{g}/\text{ml}$ ) in fresh medium or fresh medium containing W12 or W13 (30  $\mu\text{g}/\text{ml}$ ). The drugs were then removed, and the cultures were washed twice with Puck's solution A. Cells were immediately removed from the dishes by trypsinization, and survival was determined by colony formation as described (2). Cells were immediately plated into replicate culture dishes and propagated in culture for 6 to 8 days to evaluate the ability of the treated cells to form colonies. Colonies were stained with 0.5 percent crystal violet and counted. A cell was considered to have retained reproductive capacity (survival) if it gave rise to a colony of 50 or more cells. The survival index was derived by dividing the number of colonies formed per plate by the total number of single cells initially seeded per plate.

Exponentially growing CHO cells were treated with increasing concentrations of bleomycin for 1 hour in the presence or absence of either W13 or its inactive analog W12 at a dose of 30  $\mu\text{g}/\text{ml}$ . This dose of W12 or W13 has no effect on cell survival over a period of 24 hours (2). The cultures were then washed free of drug, harvested, and evaluated for cell survival by colony formation. Treatment with increasing concentrations of bleomycin alone results in a biphasic decrease in cell survival (Fig. 1). In the acute phase, almost 80 percent of the cells are killed by a dose of 25  $\mu\text{g}/\text{ml}$ . The second phase of the re-

sponse results in only an additional 10 percent increase in cell death at a dose of 100  $\mu\text{g/ml}$ . This refractoriness to higher doses of bleomycin has been attributed to the capacity of cells to repair the damage inflicted by the drug. Simultaneous treatment with the inactive analog W12 has no effect on bleomycin lethality, whereas a marked decrease in cell survival is observed for cells treated with W13 and bleomycin. In the presence of W13, 90 percent of the cells are killed at a bleomycin dose of 5  $\mu\text{g/ml}$ ; and increasing the dose of bleomycin to 25  $\mu\text{g/ml}$  results in a further 99 percent decrease in cell survival. This synergistic effect is maximal at a bleomycin concentration of 100  $\mu\text{g/ml}$ , at which 99.99 percent of the cells are killed in contrast to the 90 percent killed in response to bleomycin alone.

Although these studies show that W13 enhances bleomycin lethality, the possibility existed that the effect of W13 was not mediated by calmodulin. We have therefore evaluated the effect of W13 on growth and bleomycin lethality in bacteria. No effect of W13 on the growth of *Escherichia coli* RRI was observed, even at concentrations up to 125  $\mu\text{g/ml}$  (data not shown). Moreover, when bleomycin was used to induce DNA damage, W13 had no potentiating effect. This strain of *E. coli* has not been shown to contain calmodulin and does not contain a messenger RNA species that cross-hybrid-

izes with eel calmodulin complementary DNA (11) but can repair DNA damage (12); this is suggestive evidence that the effects of W13 noted in the CHO cells were the result of inhibition of calmodulin-mediated activities.

The ultimate effect of bleomycin on cell survival is a balance between the extent of damage inflicted on and the ability of the cells to repair DNA. Therefore, the effect of W13 on the recovery from bleomycin-induced damage was determined by evaluating the ability of CHO cells to recover from bleomycin-induced potentially lethal damage. Recovery from potentially lethal damage is measured by the increase in survival observed when cells are exposed to various incubation or growth conditions after a single exposure to a drug or irradiation treatment. Recovery of CHO cells from potentially lethal damage induced by bleomycin can occur in fresh medium (13). For these experiments, CHO cells in exponential growth were treated for 1 hour with a bleomycin dose of 100  $\mu\text{g/ml}$ , conditions which resulted in 90 percent of the cells being killed when no recovery was allowed (Fig. 1). After this treatment, the bleomycin was removed, and one group of cells was immediately plated for colony formation, no time being allowed for recovery. The other replicate cultures were allowed to recover from the treatment in fresh medium containing W12 (30  $\mu\text{g/ml}$ ) or in fresh medium containing W13 at various concentrations. Bleomycin resulted in less than 10 percent cell survival when no recovery time was allowed (Fig. 2). However, incubation in fresh medium for 30 minutes resulted in an increase in cell survival to more than 70 percent, a value that was maintained for up to 3 hours. Addition of the inactive analog W12 to the fresh medium had no effect on this recovery process. In contrast, addition of W13 to the medium at a concentration of 30  $\mu\text{g/ml}$  not only inhibits the recovery process but also results in a 100-fold increase in the number of cells killed within the first 30 minutes. In addition, only 0.02 percent cell survival is observed for cells exposed to this dose of W13 for 2 hours or longer. Thus, W13 blocks the recovery process in a dose-dependent manner, since greater cell survival is observed as the amount of W13 is decreased until normal recovery is attained at 5  $\mu\text{g/ml}$ . Furthermore, experiments with another commonly used anticancer drug, trifluoperazine, produced results similar to those obtained with W13. At a concentration of trifluoperazine (25  $\mu\text{g/ml}$ ) that had no effect on cell survival, a 100-fold inhibition in recovery from bleomycin-

induced potentially lethal damage was observed (data not shown). The similar effects of two structurally dissimilar anticancer drugs W13 and trifluoperazine (14, 15) support our contention that the effect of W13 is mediated through inactivation of calmodulin.

Since recovery from bleomycin-induced potentially lethal damage is accomplished through a repair mechanism, the most obvious explanation for these results is that calmodulin is required for DNA repair. When calmodulin is neutralized with W13, the cells cannot repair the DNA damage resulting from the bleomycin treatment. To evaluate this possibility more directly, we used the nucleoid technique of Durkacz *et al.* (16) to examine DNA repair. In this procedure, DNA damage and repair is assayed by the loss and restoration of DNA supercoiling in nucleoids. Since the degree of supercoiling affects the buoyant densities of the nucleoids, changes in this measure can be monitored as changes in the relative migrations in neutral sucrose gradients. Thus, DNA damage results in a decrease in migration relative to the control and is expressed as a fraction of the control value. Repair of the DNA reverses this process, resulting in increases in the nucleoid buoyant densities until the relative migrations are ultimately restored to the control values. As

Treatment	Mi- gration ratio
Control	1.00
Bleomycin without recovery	0.61
Bleomycin with recovery in fresh medium	1.00
Bleomycin with recovery in W12	0.97
Bleomycin with recovery in W13	0.59

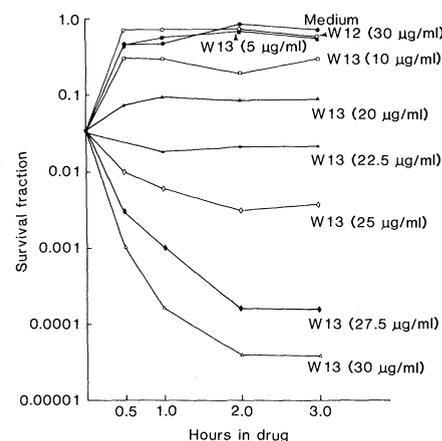


Fig. 2. Effect of W12 and W13 on recovery from bleomycin-induced potentially lethal damage. Exponentially growing CHO cells were treated with bleomycin (100  $\mu\text{g/ml}$ ) for 1 hour. The drug was then removed and the cultures were washed twice with Puck's solution A. One group of cells was immediately processed for colony formation as described in Fig. 1, with no time allowed for recovery. Replicate plates were treated with fresh medium or fresh medium containing either W12 (30  $\mu\text{g/ml}$ ) or W13 in various concentrations. The cultures were then incubated at 37°C and allowed to recover for 0.5 to 3 hours. At appropriate times, replicate plates were removed and prepared for colony formation as described in Fig. 1. Cell survival was determined as described in Fig. 1.

shown in Table 1, control samples are expressed as a relative migration ratio of 1.0. Treatment with bleomycin (100  $\mu\text{g}/\text{ml}$ ) for 1 hour results in damage to the DNA and a decrease in the relative migration of the nucleoids to 0.61. One hour after recovery from bleomycin treatment, the migration ratio has returned to 1.0, consistent with the concept that the DNA damage has been repaired. A similar result is obtained when the inactive analog W12 is added to the recovery medium. In contrast, no repair is observed in cultures to which W13 (30  $\mu\text{g}/\text{ml}$ ) was added during the 1-hour recovery period. These samples have a relative migration ratio of 0.57, which is similar to the samples treated with bleomycin but not allowed to recover. Such data indicate that W13 inhibits DNA repair and that this may be the mechanism by which it increases bleomycin lethality and inhibits recovery from bleomycin-induced potentially lethal damage.

The studies described were predicated on the involvement of calmodulin in cell proliferation and were designed to help elucidate its role in this process. The finding that calmodulin appears to be involved in DNA repair is compatible with its role in the  $G_1$ -S transition, since cells must repair damage to their DNA before successful replication can proceed. If calmodulin participates in DNA repair, then some of the enzymes involved in purine and pyrimidine metabolism, as well as other proteins involved in DNA repair, must be regulated by calmodulin. Identification of these proteins and elucidation of their specific roles in DNA repair are worthy of future investigations.

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#### References and Notes

1. A. R. Means, J. S. Tash, J. G. Chafouleas, *Physiol. Rev.* **62**, 1 (1982).
2. J. G. Chafouleas, W. E. Bolton, H. Hidaka, A. E. Boyd III, A. R. Means, *Cell* **28**, 41 (1982).
3. J. G. Chafouleas, L. Lagace, W. E. Bolton, A. E. Boyd III, A. R. Means, *ibid.* **36**, 73 (1984).
4. K. Okumura-Nuji, T. Kato, R. Tanaka, *Brain Res.* **273**, 17 (1983).
5. H. Hidaka *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4354 (1981).
6. R. A. Hickie, J.-W. Wei, L. M. Blyth, D. Y. Wong, *Can. J. Biochem. Cell Biol.* **61**, 934 (1983).
7. J. P. MacManus, B. M. Braceland, R. H. Rixon, J. F. Whitfield, H. P. Morris, *FEBS Lett.* **133**, 99 (1981).
8. Y. Sasaki and H. Hidaka, *Biochem. Biophys. Res. Commun.* **104**, 451 (1982).
9. A. L. Boyton and J. F. Whitfield, *Adv. Cyclic Nucleotide Res.* **15**, 193 (1983).
10. M. F. Bourgeade, F. Besancon, M. M. Thank, *Biochem. Biophys. Res. Commun.* **111**, 430 (1983).

11. L. Lagace, T. Chandra, S. L. C. Woo, A. R. Means, *J. Biol. Chem.* **258**, 1684 (1983).
12. S. L. Ross, S. Sharma, R. E. Moses, *Mol. Gen. Genet.* **179**, 595 (1980).
13. S. C. Barranco, J. K. Novak, R. M. Humphrey, *Cancer Res.* **35**, 1194 (1975).
14. B. Weiss and R. M. Levin, *Adv. Cyclic Nucleotide Res.* **9**, 285 (1978).
15. T. Tanaka, T. Ohmura, H. Hidaka, *Mol. Pharmacol.* **22**, 403 (1982).

16. B. Durkacz, J. Irwin, S. Shall, *Eur. J. Biochem.* **121**, 65 (1981).
17. We thank Drs. R. Moses, A. Boyd, and D. Medina for their helpful advice. This work was supported by grant BC-326 (to A.R.M.) from the American Cancer Society and by grant RR-05425 (to J.G.C.) from the National Institutes of Health.

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## Autoantibodies to a 64-Kilodalton Islet Cell Protein Precede the Onset of Spontaneous Diabetes in the BB Rat

**Abstract.** *Spontaneous insulin-dependent diabetes mellitus (IDDM) in the BB rat is associated with the presence of antibodies to a 64-kilodalton rat islet cell protein. These protein antibodies appeared in young animals and remained for as long as 8 weeks before the clinical onset of IDDM. Antibodies to a 64-kilodalton human islet cell protein were found to be associated with human IDDM. Detection of the antibodies may therefore be used to predict an early immune reaction against pancreatic B cells.*

Insulin-dependent diabetes mellitus (IDDM) in humans develops after a specific reduction of pancreatic B cells leading to insulin deficiency and inability to control glucose metabolism. The clinical onset is accompanied by a number of immunopathological phenomena (1), in particular the occurrence of autoantibodies that react with antigenic determinants present in the cytoplasmic compartment as well as in the plasma membrane of the B cells (2). Earlier we showed that sera from newly diagnosed IDDM patients, containing islet cell surface antibodies (ICSA's) to B cells, have autoantibodies to a 64-kilodalton (kD) human islet cell protein (3). Evidence suggests that human IDDM has a long prodromal period, and islet cell antibodies have been detected in individuals 2 to 8 years before the clinical presentation of diabetes (4). This premonitory autoimmune response is difficult to study in humans because susceptible individuals are not easily identified. However the BB rat, which spontaneously develops an IDDM analogous to that of humans (5), is amenable for prospective analysis. We have now examined whether rat islet cell proteins are specifically recognized by antibodies in BB rats before and after diagnosis of IDDM.

Serum samples were obtained from two different colonies of BB rats (6). In one colony of outbred animals (BB/Ontario), housed at the Laboratory of Animal Resources in Ottawa, Ontario, rats from two separate lines were studied. Animals from one line had a high incidence of IDDM (60 to 70 percent by 60 to 120 days of age), and animals from the other had a low incidence of IDDM (< 5 percent). The second colony consisted of rats originating from the University of Massachusetts in Worcester that had

been kept in sister-brother matings for 10 to 16 generations; this colony was housed in our laboratory. Animals in one of these lines (BB/Hagedorn) had a high incidence of IDDM (80 to 90 percent by 60 to 120 days of age), while those in another line (the BB w-subline, also from Worcester) in which IDDM did not occur in more than ten generations of brother-sister matings served as controls. ICSA's were determined by assay with  $^{125}\text{I}$ -labeled protein A (7) with a cell line (RIN-5F) developed from a transplantable rat insulinoma (8). Wistar rat islets were labeled with [ $^{35}\text{S}$ ]methionine and lysed with detergent; the lysates were subjected to immunoprecipitation with sera from animals from the two high-incidence groups and their controls as well as from normal Wistar rats (9).

Immunoprecipitation with sera from seven BB/Hagedorn rats obtained 1 to 7 weeks before or at the time of clinical diagnosis of IDDM gave a protein that, by comparison with marker molecules, had a molecular size of 64 kD (Fig. 1, lanes d through j). This protein was not detected in fractions immunoprecipitated with sera from three BB rats of the control BB w-subline (Fig. 1, lanes a, b, and c). The 64-kD component was also specifically detected in rat islet cell proteins (Fig. 2, lanes d, e, and f) and transplantable rat insulinoma cell proteins (Fig. 2, lane g) precipitated with sera from high-incidence BB/Ontario rats, but this component was not detected in proteins precipitated with sera from low-incidence BB/Ontario rats (Fig. 2, lanes a, b, and c) or from outbred normal Wistar rats (Fig. 2, lane h).

Major components of 45 kD (probably actin), 55 kD (probably tubulin), and 73 kD (unknown) were precipitated with both BB sera and control sera (Figs. 1