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EFFECT OF NOVOBIOCIN ON DNA SYNTHESIS AND STRUCTURE IN HUMAN LYMPHOBLASTOID CELLS

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SUMMARY Novobiocin has an inhibitory effect on DNA synthesis in human lymphoblastoid cells similar to that observed with other mammalian cells. A concentration and time-dependent increase in sedimentation of DNA nucleoids, derived from human lymphoblastoid cells, was observed in the presence of novobiocin. No decrease in the degree of negative supercoiling of DNA, determined by sedimentation on sucrose gradients in the presence of ethidium bromide, was obtained when cells were incubated with novobiocin. The latter findings are contrary to results obtained with other mammalian cells. Alteration in sedimentation rate of nucleoids after novobiocin treatment, at concentrations up to 0.25mg/ml, does not correlate with inhibition of DNA synthesis.

Novobiocin, by binding to the B subunit of DNA gyrase in prokaryote cells, interferes with the energy transduction step during the process of negative supercoiling of DNA(1). The inhibitory effect of novobiocin on DNA replication in E. coli cells can thus be explained by its ability to prevent production of supercoils in DNA(2,3).

It has also been reported that novobiocin inhibits DNA synthesis in both intact and permeable Chinese hamster ovary cells(4), synthesis of SV40 DNA in CV-1 cells (5), and synthesis of rat liver mitochondrial DNA(6). Incubation of Chinese hamster cells with novobiocin reduces the sedimentation of nucleoids in neutral sucrose indicating a reduction in the number of supercoils(4). Evidence for a reduction in the amount of supercoiled DNA after novobiocin treatment has also been provided by determining changes in density of closed circular SV40 DNA using CsCl-ethidium bromide density gradient analysis(5). It has been suggested that the ability of novobiocin to inhibit supercoiling of mitochondrial DNA is consistent with the presence of a mitochondrial DNA gyrase(6).

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Evidence outlined above for changes in supercoiling does not by itself prove the existence of a DNA gyrase in mammalian cells. Recent results demonstrate that inhibition of SV40 DNA synthesis by novobiocin can be explained by its inhibitory effect on the enzyme DNA polymerase α(5). Furthermore novobiocin inhibition of DNA synthesis in yeast is not specific for a particular enzyme(7).

I have examined further the effect of novobiocin on supercoiling of DNA in human cells and its relationship to inhibition of DNA synthesis. The results show a time and concentration-dependent increase in nucleoid sedimentation in the presence of novobiocin. No changes in the degree of negative supercoiling is evident and increased sedimentation appears to be due to a structural change other than a change in the amount of supercoiling.

MATERIALS AND METHODS

Chemicals and radioactive nuclides: Novobiocin and bovine serum albumin were purchased from Sigma. Ethidium bromide was obtained from Sigma, GF/C filters (25mm diameter, 0.45μ pore size) were used to retain acid - precipitated DNA. In experiments measuring DNA synthesis GF/C strips (1.5 x 20mm) were used. [^{3}H] thymidine (26 Ci/mmol) was obtained from the Radiochemical Centre, Amersham.

Cell culture: Epstein Barr Virus-transformed lymphoblastoid cells were used in this study. Lymphocytes were transformed at the Queensland Institute of Medical Research as described previously(8). Cells were grown in RPMI 1640 medium (Gibco) supplemented with streptomycin(60μg/ml), penicillin (100 IU/ml) and 10% foetal calf serum, in an atmosphere of 5% CO_2 in air at 37°C.

Measurement of DNA synthesis: Cells(5 x 10^5/ml) were plated out and incubated for one generation, and novobiocin was added at various concentrations ranging from 0-2mg/ml 15 min prior to addition of [^{3}H]thymidine (5μCi/ml). Novobiocin (20mg/ml)was freshly prepared in RPMI 1640 medium for each experiment. The cells were then incubated for a further 2 hr in the presence of drug. In some experiments novobiocin was removed by washing the cells repeatedly, before incubation with [^{3}H]thymidine. At the end of the incubation period cells were aliquoted out into microtitre plates, 10^5 cells/microwell and placed in a -70°C REVCO. On thawing, cells were collected onto GF/C strips using a multi-harvesting device. Washing was carried out with H_2O(5ml/well) followed by 5ml of ice-cold 5%(w/v) trichloroacetic acid(TCA). This was followed by washing strips in ethanol to remove TCA. Samples were dried and subsequently counted using a toluene scintillator in a Beckman LS-250 liquid scintillation counter. Incorporation of [^{3}H] thymidine in acid-precipitable DNA was expressed as dpm/10^6 cells.

Sucrose gradient analysis: The method of Cook and Brazell(9) was used to determine nucleoid sedimentation. Nucleoids, structures resembling nuclei, contain superhelical DNA, nuclear RNA, and depleted in protein, are formed by cell lysis using a non-ionic detergent and high salt conditions(9). DNA in these structures appears to be intact, supercoiled and circular in character(10). Sucrose gradient (10-30% sucrose; 13.4ml; pH 8.0) contained 1.95M NaCl, 0.01 M Tris, and 0.001 M EDTA. In some experiments different concentrations of ethidium bromide were

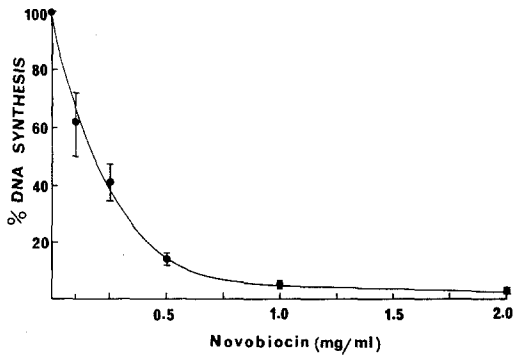


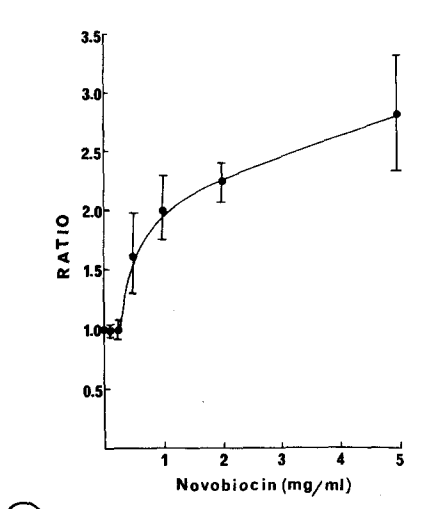
Fig. 1. Inhibition of DNA synthesis in lymphoblastoid cells by novobiocin.

included in the sucrose solutions. Cells(10^6/ml) were incubated at various concentrations of novobiocin for the appropriate time. After incubation cells were harvested by centrifugation at 300g for 10min. The cell pellet was resuspended in Hank's balanced salt solution. In some experiments novobiocin was subsequently removed from cells by repeated washing with Hank's solution. A mixture containing 100µl of cells (2-4 x 10^5) in Hank's balanced salt solution and 3 volumes of lysis solution (2M NaCl, 0.01 M EDTA, 0.5% Triton X-100; pH 8.0) was layered on top of a sucrose gradient - using a wide bore pasteur pipette. Lysis was allowed to proceed for 10 min before centrifugation at either 25,000 rpm for 15-20 min or 30,000 rpm for 25-30 min at 12°C in a Beckman L2-65B ultracentrifuge using an SW41 rotor. The position of the nucleoid peak in the gradient was determined by pumping the gradient through an absorbance monitor at 254nm wavelength. Nucleoids from untreated cells were run in each gradient set and the relative position of nucleoids from treated cells was determined by reference to that of untreated cells.

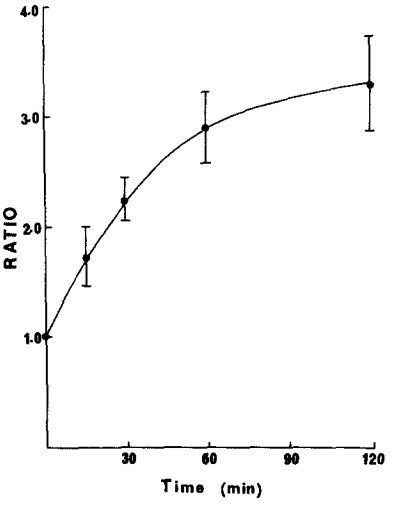
RESULTS

Novobiocin inhibition of DNA synthesis:

Incorporation of [^{3}H]thymidine into DNA in lymphoblastoid cells was inhibited in a dose-dependent manner by novobiocin(Fig.1). Synthesis was reduced to approximately half the control value at 0.1-0.25mg/ml novobiocin. The extent of inhibition is in keeping with results obtained with other mammalian cells. Removal of the drug from the cells by extensive washing followed by an incubation period of 5 hr prior to labelling with [^{3}H]thymidine resulted in a reversal of the inhibitory effect of the drug, on DNA synthesis, at concentrations up to 0.5mg/ml (results not shown). This would suggest that novobiocin does not bind covalently to DNA or other macromolecules in these cells.



(2)



(3)

Fig. 2. Effect of novobiocin concentration on sedimentation of nucleoids from lymphoblastoid cells. Distance sedimented is expressed as a ratio relative to untreated nucleoids. Error bars give standard error of the mean (S.E.M.)

Fig. 3. Effect of incubation time of novobiocin(2mg/ml) on the sedimentation of nucleoids. Error bars represent S.E.M.

Effect of novobiocin on DNA structure:

Lysis of mammalian cells in non-ionic detergent and high salt concentration results in the release of structures resembling nucleoids which contain super-helical DNA. The introduction of single strand breaks into nucleoids reduces the degree of supercoiling and in turn the sedimentation coefficient of these structures on sucrose gradients(9). I have examined the effect of increasing concentration of novobiocin on the supercoiled structure of DNA in lymphoblastoid cells using sucrose gradient analysis. It is evident from the results in Fig. 2 that at concentrations up to 0.25mg/ml no significant change in the sedimentation of nucleoids from these cells relative to untreated cells is observed. However at higher concentrations of novobiocin a dose-dependent increase in sedimentation occurs reaching a value approximately three times that observed with untreated cells at a concentration of 5mg/ml novobiocin.

This increase in sedimentation in the presence of novobiocin is also time-dependent. A linear response with time is observed up to 30 min,in the presence

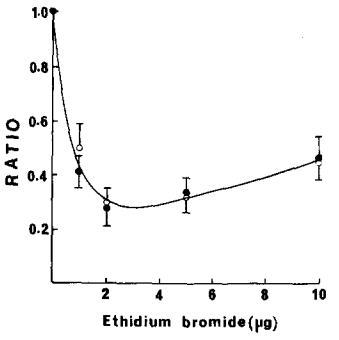


Fig. 4. Effect of ethidium bromide concentration on sedimentation of nucleoids from untreated(○), and novobiocin(2mg/ml) treated cells (●).Error bars represent S.E.M.

of drug, levelling off at longer times(Fig. 3). The results obtained in Fig. 2 and Fig. 3 are consistent with an increase in the degree of supercoiling of DNA in cells treated with novobiocin.

Effect of ethidium bromide:

A characteristic drop in the sedimentation of supercoiled DNA occurs in the presence of low concentrations of ethidium bromide(9,11). This decrease is biphasic and appears to be indicative of an initial relaxation of negatively supercoiled DNA at low concentrations of ethidium bromide followed by the formation of positive supercoils at higher concentrations(12,13). I have used sedimentation of nucleoids in the presence of ethidium bromide to determine if the increased sedimentation rate in the presence of novobiocin is due to an increased degree of supercoiling. The results in Fig.4 describe the sedimentation of nucleoids from untreated and novobiocin treated cells in the presence of increasing concentration of ethidium bromide. A characteristic biphasic pattern is observed in both cases. Maximum relaxation occurs at the same concentration of ethidium (about 2μg/ml) in treated and untreated cells and the extent of relaxation in both cases is the same. Since a minimum in sedimentation occurs in both untreated and novobiocin treated nucleoids at 2μg/ml it seems likely that the same amount of negative supercoiling of DNA occurs in both.

DISCUSSION

The concentration of novobiocin which leads to 50% inhibition of DNA synthesis in human lymphoblastoid cells was found to be similar to that causing the same degree of inhibition in other mammalian cells(4,5). In eukaryote cells several enzyme activities associated with DNA synthesis are inhibited by novobiocin(5,7,14). Data obtained with eukaryote cells would suggest that the mechanism of inhibition of DNA synthesis by novobiocin is more complex than that described in prokaryote cells where only DNA gyrase activity is inhibited(1).

The results obtained in this study add to the complexity of effects observed after novobiocin treatment of eukaryote cells. In the presence of novobiocin an increase in the sedimentation value of DNA nucleoids is obtained(Fig.2). These results do not agree with those obtained using Chinese hamster ovary cells in which novobiocin caused a decrease in nucleoid sedimentation(4). However, it should be pointed out that the decrease in nucleoid sedimentation observed in that study, at a concentration of 1mg/ml novobiocin, is marginal whereas my results show a twofold increase in sedimentation at the same concentration of novobiocin. Furthermore the effect observed in this investigation is not specific to lymphoblastoid cells since MM96, a human melanoma cell line, showed a similar increase in sedimentation rate after incubation with novobiocin(results not shown) Mattern and Painter(4) have demonstrated that a lower concentration of ethidium bromide is required to completely relax superhelical DNA in novobiocin treated cells than in untreated cells. This is consistent with a decrease in the degree of negative supercoiling(12). The DNA of HeLa nucleoids isolated from cells which were incubated with novobiocin was also found to be less negatively supercoiled than that from untreated cells(15). Another study, examining novobiocin inhibition of SV40 DNA replication, shows that SV40 DNA made during a pulse-labelling period in novobiocin treated cells has a greater density in CsCl-ethidium bromide than that made in control cells, indicating less negative supercoiling(5). Contrary to the results in other mammalian cells an increased sedi-

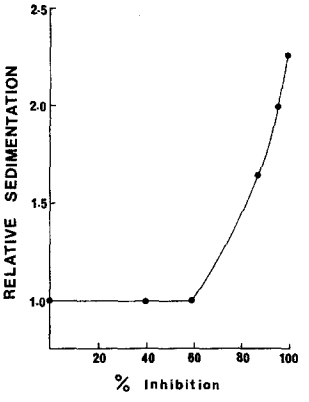


Fig. 5. Relationship between inhibition of DNA synthesis and change in sedimentation of nucleoids with increasing concentration of novobiocin.

mentation of nucleoids is observed in human lymphoblastoid cells in the presence of novobiocin. This increased rate is consistent with an increase in the degree of supercoiling. However, the biphasic sedimentation pattern in the presence of increasing concentration of ethidium bromide demonstrates a similar degree of negative supercoiling in both treated and untreated cells(Fig.4), and indicates that the increased sedimentation of these structures(nucleoids) is due to other changes in chromatin structure. This could be due to an alteration in the amount of protein remaining bound to DNA or to structural alterations due to changes in the affinity of binding of different proteins to DNA in the presence of novobiocin. Alternatively intercalation or some other form of association of novobiocin with DNA might also account for the increased sedimentation. It is possible that association of the drug or drug-induced alteration in protein-nucleic acid association might also account for the density changes and differences in sedimentation in the presence of ethidium bromide reported elsewhere(4,5)

Comparative studies, using coumermycin and novobiocin to inhibit SV40 DNA replication, suggest that the difference in supercoiling observed with novobiocin is due to a secondary effect of the drug(5). It also seems likely that the alteration in sedimentation behaviour of nucleoids by novobiocin, observed in this study, is secondary to inhibition of DNA replication, at least at lower con-

centrations of the drug. This is evident when change in sedimentation with increasing concentration of novobiocin is plotted against degree of inhibition of DNA replication over the same range of novobiocin concentrations(Fig.5). It is clear that no change in sedimentation occurs at concentrations of novobiocin which give up to 60% inhibition of DNA replication. A rapid increase in sedimentation value is obtained as the degree of inhibition of DNA synthesis increases from 60-97%. In summary novobiocin alters significantly the sedimentation value of lymphoblastoid nucleoids and does not decrease the degree of negative supercoiling in these cells.

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REFERENCES

1. Sugino, A., Higgins, N.P., Brown, P.O., Peebles, C.L. and Cozzarelli, N.R. (1978) Proc. Natl. Acad. Sci. U.S. 75, 4838-4842.
2. Gellert, M., O'Dea, M.H., Itoh, T. and Tomizawa, J. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4474-4478.
3. Drlica, K. and Snyder, M. (1978) J. Mol. Biol. 120, 145-154.
4. Mattern, M.R. and Painter, R.B. (1979) Biochim. Biophys. Acta. 563, 306-312.
5. Edenberg, H.J. (1980) Nature 286, 529-531.
6. Castora, F.J. and Simpson, M.V. (1979) J. Biol. Chem. 254, 11193-11195.
7. Nakayama, K. and Sugino, A. (1980) Biochem. Biophys. Res. Commun. 96, 306-312.
8. Moss, D.J. and Pope, J.H. (1972) J. Gen. Virol. 17, 233-236.
9. Cook, P.R. and Brazell, I.A. (1976) Nature 263, 679-682.
10. Cook, P.R., Brazell, I.A. and Jost, E. (1976) J. Cell. Sci. 39, 303-324.
11. Crawford, L.V. and Waring, M.J. (1967) J. Mol. Biol. 25, 23-30.
12. Benyajati, C. and Worcel, A. (1976) Cell 9, 393-407.
13. Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. and Laipis, P. (1965) Proc. Natl. Acad. Sci. U.S. 53, 1104-1111.
14. Hsieh, T. and Brutlag, D. (1980) Cell 21, 115-125.
15. Akrigg, A. and Cook, P.R. (1980) Nucleic Acids Res. 8, 845-854.