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DNA Damage Induced by Chloramphenicol and its Nitroso Derivative: Damage in Intact Cells

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We have postulated that the p-NO_2 group of chloramphenicol (CAP) is the structural feature underlying aplastic anemia from this drug. In a series of studies to examine this hypothesis we have demonstrated the toxic nature of the CAP-reduction intermediate nitroso CAP (NO-CAP) and its damaging effect on isolated DNA in vitro. The present study was designed to examine the comparative effects of CAP, NO-CAP, and thiamphenicol (TAP) on the integrity of DNA in intact cells. By using the alkaline elution technique of Kohn, DNA damage in the form of single strand breaks could be readily demonstrated in cultured Raji cells and in PHA-stimulated normal human lymphocytes by small concentrations of NO-CAP (0.05–0.1 mM). A small but reproducible effect was observed from large concentrations of CAP (2 mM). In contrast, TAP, lacking the p-NO_2 group, was without effect.

Key words: nitrosochloramphenicol

INTRODUCTION

On the basis of extensive studies with chloramphenicol (CAP), its analogue thiamphenicol (TAP, in which the paranitro group has been replaced by a methylsulfonyl moiety), and the nitroso derivative of CAP (NO-CAP), we have postulated that aplastic anemia from CAP is mediated by its nitroreduction products [1–7]. According to this hypothesis, reductive intermediates of CAP (nitroso, −NO, and/or hydroxylamine, −NHOH) produced in susceptible individuals cause DNA damage in stem cells ultimately leading to aplastic anemia. In further investigations to examine this hypothesis we have demonstrated that NO-CAP is much more cytotoxic than the parent compound [4] and in small concentrations causes hydrolysis of isolated *E. coli* DNA in vitro [7]. The present study was designed to examine the effect of CAP and NO-CAP on the integrity of DNA in intact cells. By using two separate cell systems, the induction of DNA damage by small concentrations of NO-CAP in intact cells could be readily demonstrated. The possible relevance of this observation to aplastic anemia from CAP and the potential usefulness of the approach used herein to the pretesting of susceptible subjects are discussed.

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MATERIALS AND METHODS

CAP, NO-CAP, TAP, and N-acetylcysteine (NAC) were provided by Zambon SpA, Milan, Italy. Sarkosy NL-30 was from Ciba-Geigy Corp., Greensboro, NC; [Me-^{3}H] thymidine (82 Ci/mmol), [2-^{14}C] thymidine (58 μCi/mmol), and aquasol were obtained from New England Nuclear, Boston, MA; polyvinyl-chloride filter, 25-mm diameter, 2-μm pore size, was from Millipore Corp., Belford, MA; tetrapropylammonium hydroxide was from Eastman Kodak Co., Rochester, NY. All other chemicals were reagent grade.

Cell Preparations

Two cell systems were used:

(1) *Phytohemagglutinin-stimulated lymphocytes:* Blood is collected from normal human subjects in phenol-free heparin and mononuclear cells separated by fractionation on lymphocyte separation medium (LSM Bionetics, Kensington, MD).

(2) *Raji cells*—a rapidly growing human lymphoblastoid cell line (Raji cells, CCL-86, American Type Culture Collection, Rockville, MD)—are maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (DME-FCS). They have a doubling time of 22 hr.

DNA Labeling

Exponentially growing Raji cells (3×10^5 cells/ml) were incubated for 22 hr in DME-FCS containing 5 μCi/ml of ^{5}H-thymidine, a concentration selected to give optimum labeling in these cells. The medium was removed and the cells were suspended in DME-FCS containing 10^{-5} M unlabeled thymidine and incubated an additional 4 hr. Cells were then exposed to the drug for 3 hr followed by washing and alkaline elution.

Isolated normal human lymphocytes were suspended in DME-FCS (2.5×10^6 cells/ml) and incubated with phytohemagglutinin (PHA) for 48 hr; ^{3}H thymidine was then added (0.2 μCi/ml) and incubation continued for an additional 24 hr. Cells were then washed once with medium and incubated in 10^{-5} M unlabeled thymidine for 4 hr. Drug was added for the indicated period, after which cells were washed in cold phosphate-buffered saline and subjected to alkaline elution.

Cell Viability

Cell viability was determined by the Trypan Blue exclusion method. In all experiments cell viability after incubation with drug was over 85% and comparable to control.

Alkaline Elution

In the alkaline elution technique single strand breaks are measured on the basis of an increase in the DNA elution rate [8]. Elution was performed according to the method of Kohn [9], briefly described as follows:

At the end of the drug treatment, about $1.0–2.0 \times 10^6$ cells were loaded onto chilled polyvinyl chloride filters and the cell suspensions were gravity filtered. Then the cells were washed with 5 ml ice-cold phosphate-buffered saline and lysed at room temperature with 5 ml of 0.2% Sarkosyl NL30, 2 M NaCl, 0.04 M Na_2 EDTA, pH = 10, for at least 15 min. Lysis solution was drained by gravity, and the filters

were washed with 3 ml of 0.02 M Na_2 EDTA, pH = 10. The DNA on the filters was eluted with 0.02 M H_4 EDTA solution adjusted to pH = 12.2 with 10% tetrapropylammonium hydroxide. Flow was kept at a constant rate and fractions were collected at 90-min intervals.

RESULTS

DNA Damage Induced by NO-CAP

Figure 1 shows the alkaline elution profile of DNA of PHA-stimulated normal human lymphocytes after treatment of the cells with CAP (2 mM) and NO-CAP (0.07 mM). Treatment of the cells with 0.07 mM NO-CAP for 3 hr resulted in a marked increase in the DNA elution rate as compared to control untreated cells, indicating extensive DNA damage in the form of single strand breaks. In contrast, only a slight increase in the rate of DNA elution was observed in cells treated with the high CAP concentration of 2 mM.

To exclude the possibility that differences in elution rate may be partially due to internal variations between samples, an experiment was carried out in two parts: In one, the cells were labeled with ^{3}H-thymidine and in the other with ^{14}C-thymidine. Prior to elution, equal numbers of ^{14}C- and ^{3}H-labeled cells were mixed, as follows, and each mixture was eluted on the same filter: Control ^{14}C-labeled cells + control ^{3}H-labeled cells; control ^{14}C cells + NO-CAP-treated ^{3}H cells.

As can be seen in Figure 2, ^{3}H and ^{14}C DNA of control cell mixture eluted at a similar rate. In contrast, when ^{3}H-labeled NO-CAP-treated cells were mixed with control ^{14}C cells, ^{3}H DNA separated from the ^{14}C DNA eluting at a very rapid rate while ^{14}C DNA elution remained the same as the control mixture.

Comparative Effect of CAP, NO-CAP, and TAP

Figure 3 compares the rate of elution of DNA of PHA-stimulated normal lymphocytes after treatment of the cells with CAP (2 mM), TAP (2 mM), and NO-CAP (0.1 mM). Here again a marked increase in the rate of elution was observed with NO-CAP, and only a slight increase was noted with the high concentration of 2 mM CAP. In contrast, no effect was observed from TAP.

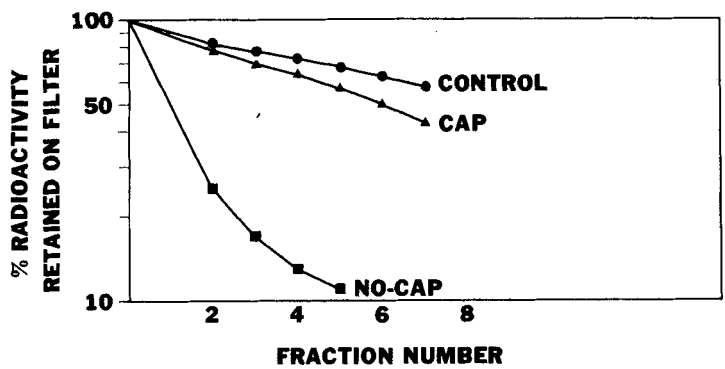

Fig. 1. Alkaline elution profile of DNA of phytohemagglutin-stimulated normal human lymphocytes after treatment of the cells with the nitroso derivative or chloramphenicol (NO-CAP; 0.07 mM) or chloramphenicol (CAP; 2 mM) for 3 hr (see text for methods).

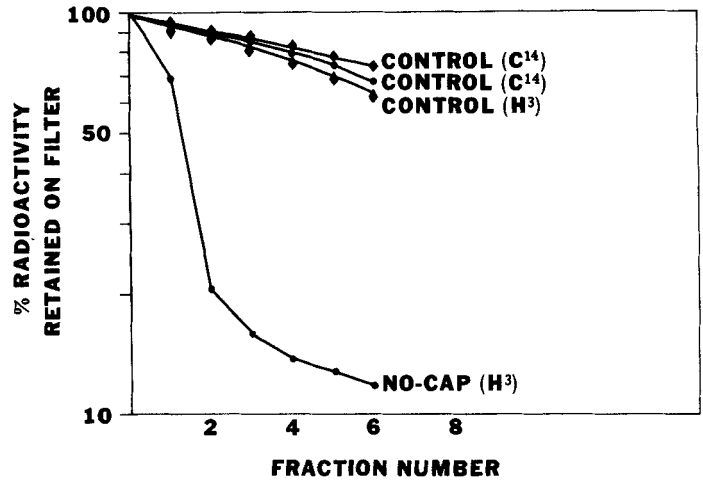

Fig. 2. Alkaline elution profile of DNA of phytohemagglutinin-stimulated normal human lymphocytes after treatment of the cells with 0.1 mM NO-CAP for 3 hr. This experiment was carried out in two parts. In one, cells were labeled with ^{3}H-thymidine and in the other with ^{14}C-thymidine. After treatment with the drug, ^{3}H-labeled treated cells were coeluted with ^{14}C-labeled untreated cells (●) and ^{14}C-labeled untreated cells were coeluted with ^{3}H-labeled untreated cells (♦). Note the rapid elution rate of ^{3}H-labeled DNA of NO-CAP-treated cells compared to ^{14}C-labeled DNA of control cells eluted on the same filter.

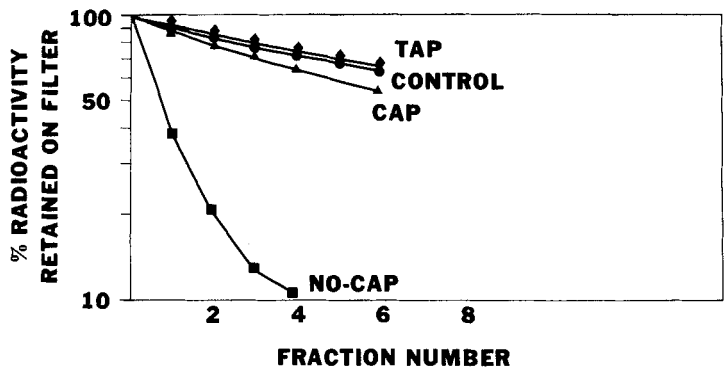

Fig. 3. Comparative effect of CAP (2 mM), thiamphenicol (TAP; 2 mM), and NO-CAP (0.1 mM) on the alkaline elution profile of DNA of phytohemagglutinin-stimulated normal human lymphocytes (see text for methods).

No-CAP-Induced DNA Damage Is Drug Concentration Dependent

Figures 4A and B show the DNA elution profile of phytohemagglutinin-stimulated normal human lymphocytes (A) and of Raji cells (B) after treatment with increasing concentrations of NO-CAP. In each case the rate of DNA elution increases with increasing NO-CAP concentrations.

Neutralization of NO-CAP Effect by Sulfhydryl Compounds

Figure 5 shows the results of an experiment with PHA-stimulated lymphocytes in which NAC was added along with NO-CAP. The NO-CAP effect was completely

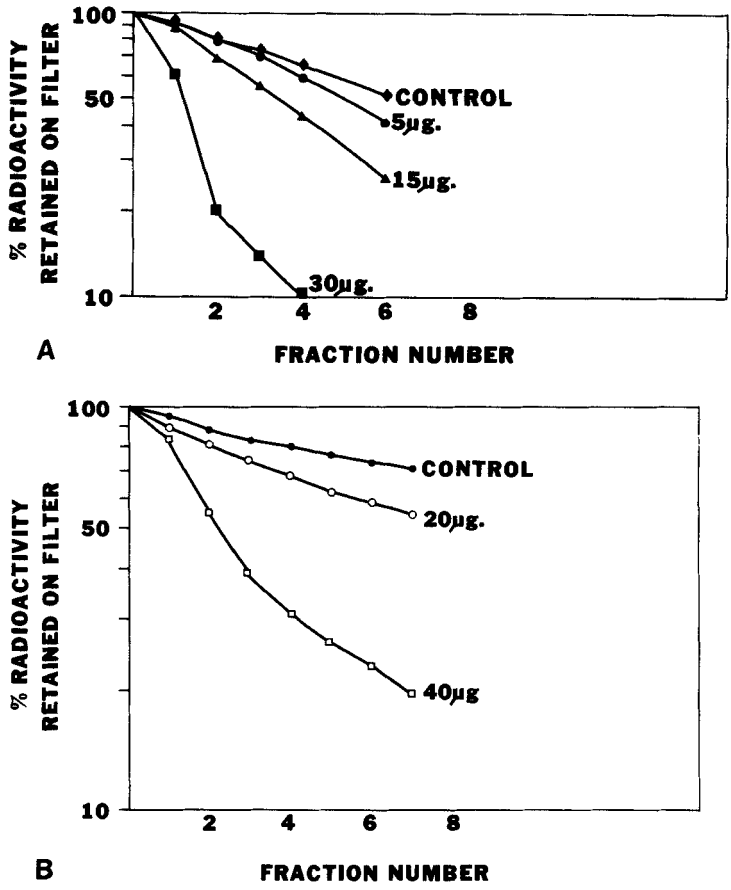

Fig. 4. Alkaline elution profile of DNA of phytohemagglutinin-stimulated normal human lymphocytes (A) and of Raji cells (B) after treatment of the cells with various concentrations of NO-CAP.

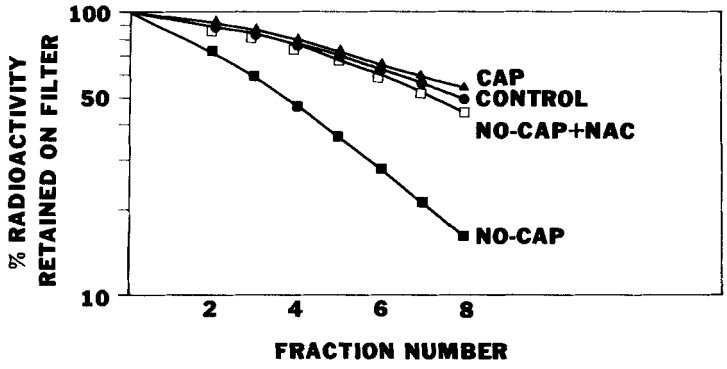

Fig. 5. Neutralization of the NO-CAP effect by N-acetylcysteine (NAC). In this experiment phytohemagglutinin-stimulated ^{3}H-labeled normal human lymphocytes were treated with 0.3 mM CAP, 0.07 mM NO-CAP, or 0.07 mM NO-CAP + 0.2 mM NAC for 3 hr prior to alkaline elution.

neutralized by NAC. This figure also shows the lack of effect of 100 μg/ml CAP (0.3 mM).

DISCUSSION

In previous studies we demonstrated that NO-CAP in small concentrations (0.1 - 0.1 mM) causes the hydrolysis of isolated *E. coli* DNA in vitro [7]. Although CAP in equivalent concentrations had no effect, when used at higher levels (1 mM) it was capable of inducing similar damage to DNA [10]. The results of those studies were consistent with our hypothesis regarding the role of CAP nitroreduction products in CAP-induced aplastic anemia. However, in order to attribute a direct pathophysiologic relevance to NO-CAP-induced DNA damage, one must be able to demonstrate that the drug exerts a similar action in intact cells. The studies described herein were designed with that objective in mind.

It should be emphasized that in our previous studies, as well as the present ones, pure stock solutions of NO-CAP were added to the cultures or reaction mixtures. Chemical or metabolic alterations of the NO-CAP could possibly take place and such products (eg, hydroxylamine) may be the reactive species which are actually involved in the DNA damage. Thus, whenever mentioned, NO-CAP refers to NO-CAP or compounds which can form from it in solution under the experimental conditions employed.

In our attempts to demonstrate DNA damage in intact cells, we explored a number of methods, including sister chromatid exchange and alkaline sucrose gradient sedimentation. We selected the alkaline elution technique because of the high reproducibility in our hands and because of the potential utilization of this technique and its variations in determining the type of DNA damage [8], eg, single strand breaks, double strand breaks, cross linking etc. Both Raji cells and PHA-stimulated normal human lymphocytes proved to be excellent cell systems for our studies. However, most of our experiments were performed with PHA-stimulated lymphocytes because of potential applicability to the testing of human subjects. Since DNA damage can result from cell death and the action of endonucleases, incubation conditions were chosen to avoid cell killing. Thus, viabilities of cells treated for 3 hr with drugs at the concentrations specified were comparable to those of control samples.

Our studies clearly demonstrate the ability of small concentrations of NO-CAP to damage DNA in intact cells. In comparable concentrations, CAP was without effect. However, a slight but reproducible increase in DNA elution rate was observed with the high CAP concentration of 2 mM. This is the same concentration range at which CAP causes hydrolysis of isolated DNA in vitro [10] and inhibits DNA synthesis in mammalian cells [2–10]. TAP, on the other hand, lacking the p-NO_2 group is incapable of inhibiting DNA synthesis [2] or damaging either isolated DNA [10] or intact cell DNA.

The demonstration of DNA damage by NO-CAP in stimulated lymphocytes and Raji cells reported herein is in keeping with our previous observations showing that cells are most susceptible to NO-CAP during proliferation and/or DNA synthesis [11]. Thus, we could also demonstrate DNA damage by NO-CAP in other proliferating cells such as cultured HL-60 cells and normal human bone marrow (not illustrated).

Increased rate of DNA elution at pH 12.1 indicates the presence of single strand breaks [8]. The exact mechanism by which these breaks are produced, however,

cannot be ascertained from these data; ie, direct effect of drug cannot be distinguished from other causes of nicking such as that secondary to DNA repair. The neutralization of NO-CAP by NAC is consistent with previous observations, showing the protection of isolated DNA from NO-CAP by sulfhydryl compounds [7]. Sulfhydryl compounds are known to react directly with aromatic nitroso groups, resulting in their reduction and inactivation [12,13]. This is most likely the mechanism by which NAC neutralized the NO-CAP effect observed in these studies. However, the possibility of an additional action of NAC as a hydroxyl radical scavenger cannot be excluded.

The demonstration of DNA damage in the intact cells induced by small concentrations of NO-CAP lends further support to our hypothesis emphasizing the role of the p-NO_2 group in CAP-induced aplastic anemia [1–7]. The ultimate objective of our studies is to uncover an individual predisposition to CAP-induced aplastic anemia and possibly foretell susceptible subjects. In these subjects, DNA may be more sensitive to damage by CAP or its reduction products. Alternatively, their hemopoietic cells, including lymphocytes, may be capable of metabolizing CAP or some of its derivatives to toxic intermediates ultimately reflected in DNA damage. Accordingly, our observations described herein suggest that peripheral lymphocytes and/or bone marrow cells could potentially be used to screen individuals susceptible to CAP-induced aplastic anemia.

ACKNOWLEDGMENTS

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