DNA Strand Breaks Induced in Human T-Lymphocytes by the Combination of Deoxyadenosine and Deoxycoformycin

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ABSTRACT

There is a progressive loss of human T-lymphocyte viability upon incubation with deoxycoformycin, an adenosine deaminase inhibitor, and low concentrations of deoxyadenosine (drug concentration that reduced cell count at 48 hr after initiation to 50% of value for untreated control culture, <1 µM). The loss of viability was evidenced by vital staining with fluorescein diacetate and by changes in forward single light scatter measured by flow cytometry. This loss of lymphocyte viability is detectable 18 to 20 hr after the addition of deoxyadenosine and is earlier than has been reported by other investigators using trypan blue as the vital stain. Alkaline elution studies show that the incubation of T-lymphocytes with the combinations of deoxycoformycin and deoxyadenosine gives rise to DNA single-strand breaks. These DNA strand breaks are dose and time dependent and are readily detected 4 hr after the addition of deoxyadenosine. These DNA lesions are not observed with deoxycoformycin or deoxyadenosine alone. Incubations of T-lymphocytes with deoxycoformycin and deoxyadenosine (1 and 5 µM) for 7 hr result in DNA strand breaks with a frequency of 145 and 280 rad equivalents, respectively. Preliminary studies indicate that the ability of lymphocytes to repair this damage is dependent upon deoxyadenosine concentration and exposure time. The relationship of these DNA lesions to loss of lymphocyte viability in the presence of deoxycoformycin and deoxyadenosine remains to be established.

INTRODUCTION

The in vivo inhibition of adenosine deaminase with inhibitors such as 2'-deoxycoformycin or the genetic deficiency of this enzyme results in the development of lymphopenia or a severe immunodeficiency state, respectively (7, 18). Deoxycoformycin produces toxicity by potentiating the toxicity of substrates of adenosine deaminase which normally are rendered nontoxic through deamination. When whole animals are treated with deoxycoformycin, the adenosine deaminase substrate that is most important for toxicity is believed to be deoxyadenosine (4).

The mechanism of deoxyadenosine toxicity is still unresolved, but inhibition of ribonucleotide reductase by nucleotides formed from deoxyadenosine and inhibition of S-adenosylhomocysteine hydrolase by unmetabolized deoxyadenosine have been proposed as important effects (6, 9). The significance of the inhibition of replicative DNA synthesis as a mechanism of deoxyadenosine toxicity has been much easier to rationalize in the studies of cultured human lymphoblastoid cell lines than it has been in the case of mature peripheral blood lymphocytes. The initial report by Kefford and Fox (12), followed by a similar study by Carson et al. (3) on the lysis of mature lymphocytes by deoxyadenosine, will probably prove to be significant contributions to our understanding of the clinical effects of adenosine deaminase inhibitors.

This latter work together with the recent observations that mature circulating lymphocytes appear to have significant levels of DNA strand breaks or alkaline sensitive sites prompted the current study to determine whether deoxycoformycin and deoxyadenosine may cause additional DNA lesions which may contribute to the observed toxicity (11).

MATERIALS AND METHODS

Human lymphocytes were isolated from blood, which had been diluted 1:1 with RPMI 1640 medium, by centrifugation over a layer of 60% Percoll (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). Prior to this centrifugation, the blood had been treated with carbonyl iron so that monocytes would not be present in the mononuclear fraction (1). T-lymphocytes were subsequently prepared by rosetting with neuraminidase-treated sheep erythrocytes (22). The incubation medium used throughout these studies was RPMI 1640 with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY).

Lymphocyte viability was monitored by staining with either trypan blue or fluorescein diacetate (17). In the latter case, cells were stained with fluorescein diacetate (5 µg/ml) for 15 to 30 min, and the proportion of viable fluorescent cells was determined by fluorescence microscopy or by flow cytometry using a Coulter Electronics Epics V system. In the flow cytometric analysis, excitation was at 488 nm, and the emission between 530 and 590 nm was measured using the standard 3 log amplifiers on the Epics V system.

The alkaline elution studies were carried out basically as described by Kohn et al. (15, 19) with modifications related to the fact that DNA elution had to be monitored by a fluorescence procedure because the DNA of mature lymphocytes could not be prelabeled with radioactively labeled thymidine. In this study, DNA elution was followed by staining with Hoechst 33258 dye (21). In these studies, it was necessary to use sodium EDTA (pH 12.2) instead of tetrapropylammonium hydroxide for DNA elution because of the high fluorescent blanks generated by this latter reagent. The DNA from approximately 2 × 10⁶ lymphocytes was eluted from the filters at a flow rate of 1.5 ml/hr.

The 2.5-cm polyvinyl chloride filters (2-µm pore size) were obtained from the Millipore Corp., deoxyadenosine was from Sigma Chemical Co., and 2'-deoxycoformycin was from Drug Research and Development, National Cancer Institute, Bethesda, MD.

RESULTS

Viability studies of T-lymphocytes treated with 2'-deoxycoformycin (1 µg/ml) and deoxyadenosine gave essentially the same results as those reported by Kefford and Fox (12), in that with continuous deoxyadenosine exposure the drug concentration that reduced cell count at 48 hr after initiation to 50% of value for untreated control culture for the loss of viability of mature T-lymphocytes was less than 1 µM. Deoxycoformycin was added...
at least 2 hr prior to the addition of deoxyadenosine. In an experiment in which T-lymphocytes were incubated with 2'-deoxycoformycin and 5 μM deoxyadenosine, analysis by high-performance liquid chromatography indicated that greater than 90% of the deoxyadenosine remained after 24 hr. However, we have observed that the use of fluorescein diacetate as a vital stain indicated earlier and progressively faster loss of lymphocyte viability than did trypan blue. With flow cytometric analysis in which at least 30,000 cells were counted, fluorescein diacetate staining indicated loss of viability starting 18 to 20 hr after the addition of the deoxyadenosine, whereas loss of viability by trypan blue staining was not evident for 24 to 36 hr. Lymphocytes appeared normal by conventional Wright's staining for approximately 36 hr after the addition of deoxyadenosine. Kefford and Fox (13) have reported a 10-fold difference in deoxyadenosine toxicity between T-lymphocytes and lymphocytes from CLL patients using trypan blue as a vital stain. In a limited study of only 2 CLL patients to date, we have not observed as large a difference in deoxyadenosine toxicity when fluorescein diacetate is used as the vital stain. This is not entirely unexpected, inasmuch as it is well recognized that the assessment of viability using vital stains must be interpreted cautiously. Chart 1 shows a typical fluorescein diacetate viability study using flow cytometry. Twenty hr after the addition of deoxyadenosine (1 μM), the viability of the T-lymphocytes had dropped to 83% (control was 97%). The reduction in viability over the next 24 hr was progressively faster, with only 44 and 11% of the lymphocytes being viable at 40 and 48 hr, respectively. Deoxycoformycin-treated control cultures maintained a 92% viability at 48 hr. Flow cytometric analysis of the forward angle light scattering (10–20°) by lymphocytes also indicated changes in viability of deoxyadenosine-treated lymphocytes at times comparable to that of the fluorescein diacetate staining, but these changes were difficult to quantitate. The nonviable lymphocytes scatter less light in the forward direction than do viable lymphocytes. This is apparent as a shoulder on the normal light scatter profile and is therefore not readily quantitated.

In the presence of 2'-deoxycoformycin (1 μg/ml), deoxyadenosine induces either DNA single-strand breaks or alkaline sensitive sites in mature human T-lymphocytes, as evidenced by alkaline elution studies (Chart 2). DNA strand breaks in the deoxyadenosine-treated cultures result in an increased rate of DNA elution from the polyvinyl chloride filters as compared to the cultures treated with deoxycoformycin alone. These DNA strand breaks or alkaline sensitive sites are dose and time dependent and are easily detected with the existing methodology as early as 4 hr after the addition of deoxyadenosine. 2'-Deoxycoformycin alone has no effect on viability, as evidenced by fluorescein diacetate staining and does not induce the DNA strand breaks. In the absence of deoxycoformycin, these low concentrations of deoxyadenosine are rapidly deaminated by lymphocytes, and there is no loss of viability or of DNA strand lesions produced. The alkaline elution profiles were essentially superimposable for either control untreated lymphocytes, for deoxycoformycin-treated lymphocytes (1 μg/ml), or for lymphocytes treated with deoxyadenosine alone, at concentrations to at least 10 μM. As shown in Table 1, the frequency of DNA single-strand breaks induced by a given concentration of deoxy-

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3 The abbreviation used is: CLL, chronic lymphocytic leukemia.
background level of DNA strand breaks. The elution rate of DNA from peripheral blood lymphocytes, as compared to that of DNA from RPMI 6410 cells, which are a human lymphoblastoid cell line, would indicate a frequency of DNA strand breaks in peripheral blood lymphocytes of approximately 50 rad equivalents. The frequencies of DNA strand breaks shown in Table 1 are therefore over and above this background level as observed in the untreated T-lymphocyte controls.

**DISCUSSION**

The results presented show that the combination of deoxyadenosine and deoxycoformycin causes significant levels of DNA single-strand breaks in mature human T-lymphocytes. This biochemical effect is easily detected as early as 4 hr after the addition of deoxyadenosine. The appearance of these DNA lesions precedes the maximal buildup of cellular dATP levels (13), and also precedes the loss of lymphocyte viability as evidenced by fluorescein diacetate staining and light scattering measurements by at least 16 hr. To date, the only other described biochemical effect of deoxyadenosine that occurs as early as these DNA lesions is the inhibition of the S-adenosylhomocysteine hydrolase (3, 14). These deoxyadenosine-induced DNA lesions are dose dependent and increase with time to an apparent plateau value which is dose dependent.

It is not currently understood how these DNA lesions arise or how they may be related to loss of viability and cell lysis. However, the observed level of DNA strand breaks is sufficient to result in lymphocyte death if one assumes that the data on the effects of irradiation on lymphocytes are comparable (20). It is conceivable that these lesions may arise from either an alteration in the level of DNA methylation due to the inactivation of the S-adenosylhomocysteine hydrolase or the inhibition of a DNA repair process by elevated dATP levels. There are several human diseases in which it has been clearly shown that defective DNA repair mechanisms may lead to immunologically deficient states (8). Whatever their origin, it appears that these DNA strand breaks are not repaired as long as deoxyadenosine is present.

We are currently investigating the mechanism of DNA repair in these deoxyadenosine-treated lymphocytes after the removal of deoxyadenosine. It has been shown by Kefford and Fox (13) that the loss of lymphocyte viability is related to both the deoxyadenosine dosage and exposure time. Preliminary studies have shown that the DNA lesions induced by a 7-hr treatment with 5 μM deoxyadenosine are almost completely repaired after a 16-hr incubation in deoxyadenosine-free medium. Such an exposure does not result in loss of lymphocyte viability, as evidenced by vital staining.

Kefford and Fox (13) have reported that T-cells are an order of magnitude more sensitive to deoxyadenosine than are non-T-cells but stated that their non-T-cell preparations contained 30 to 40% monocytes. However, it must be remembered that even after removal of monocytes, these non-T-cell fractions isolated from different individuals are comprised of varying proportions of B-cells and null cells, so that eventually it will be necessary to carry out the deoxyadenosine toxicity studies on purified normal B-cell preparations. As yet, we have not examined the effect of deoxyadenosine on human B-cells due to the number of cells required and the isolation procedures involved. The apparent controversy in the literature as to whether CLL lymphocytes are closer to pre-B-cells or mature B-cells in their degree of differentiation, together with the described biochemical and biological abnormalities of these cells, would suggest that these readily available B-lymphocytes may not be a good model for studies of deoxyadenosine toxicity in normal B-cells (10, 16).

With the alkaline elution procedure, we have observed an increased background frequency of DNA single-strand breaks in normal human T-lymphocytes as compared to the RPMI 6410 human lymphoblastoid cell line of approximately 50 rad equivalents. In the initial description of DNA strand breaks in mature lymphocytes, Johnstone and Williams (11) used the method of nucleosid sedimentation but did not give any information on the frequency of DNA strand breaks in terms of rad equivalents. While the biological significance of this low level of DNA breaks in human lymphocytes remains to be established, it is possible that they are related to some deficiency in DNA repair or to some cellular differentiation process, as has been proposed by Farzanek et al. (5). With respect to the question of lymphocyte capacity for DNA repair, it has been shown that circulating lymphocytes are extremely susceptible to radiation damage, whereas phytohemagglutinin-stimulated lymphocytes are considerably more resistant (20).

The relationship of the described deoxyadenosine-induced DNA lesions in T-lymphocytes to cell lysis will require additional studies on the repair and specificity of the DNA damage in the different lymphocyte subsets. It is our opinion that the understanding of the clinical effects of deoxycoformycin will most likely come from studies on the biochemical basis of the deoxyadenosine-induced lysis of peripheral blood lymphocytes, as opposed to studies on cultured lymphoblastoid cell lines.

**REFERENCES**


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