Inhibition of Neutrophil Chemotaxis by 4-Hydroxycyclophosphamide

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SUMMARY

4-Hydroxycyclophosphamide, phosphoramid mustard, and nor-nitrogen mustard are compounds considered as candidates for the biologically active, alkylating metabolite of cyclophosphamide. These compounds were tested for inhibition of chemotaxis in neutrophils from normal human donors. Of those tested, only 4-hydroxycyclophosphamide caused a significant (p < 0.01) decrease in the chemotactic index. The chemical precursor of 4-hydroxycyclophosphamide, 4-hydroperoxycyclophosphamide, caused a barely significant (p < 0.05) decrease; 4-ketocyclophosphamide had no effect.

INTRODUCTION

Cyclophosphamide, an alkylating agent clinically used for the treatment of neoplastic diseases, is itself inactive as an alkylating agent (1, 8, 11). Of the several metabolites identified, there is debate as to which metabolite(s) is the active alkylating agent(s). To test possible active metabolites of cyclophosphamide, we used mature neutrophils because they are easily purified (3), are nondividing (2), and their chemotactic activity is measurable (7).

Previous studies with experimental animals have emphasized the effect of cyclophosphamide on immature dividing myeloid cells (6, 12). These studies did not test the functional capacity of circulating mature nondividing myeloid cells.

The studies reported below indicate that, of the cyclophosphamide metabolites tested, only 4-hydroxycyclophosphamide and its precursor, 4-hydroperoxycyclophosphamide, inhibit neutrophil chemotaxis significantly.

MATERIALS AND METHODS

Materials

Cyclophosphamide (Cytoxan) was provided by Mead Johnson Laboratories, Evansville, Ind. The following cyclophosphamide derivatives were provided by R. Struck, Kettering-Meyer Laboratory of the Southern Research Institute, Birmingham, Ala.: 4-ketocyclophosphamide, phosphoramid mustard, nor-nitrogen mustard and 4-hydroperoxycyclophosphamide. DMSO was obtained from Sigma Chemical Co., St. Louis, Mo.

Methods

Preparation of 4-Hydroxycyclophosphamide. Because 4-hydroxycyclophosphamide is unstable, it was supplied as the stable precursor, 4-hydroperoxycyclophosphamide, and then deoxygenated to 4-hydroxycyclophosphamide immediately prior to use. For each experiment, 10 mg of 4-hydroperoxycyclophosphamide were dissolved in 0.5 ml of acetone and allowed to stand for 10 min at 20°C. One drop of triethyl phosphate was added and exposure to 20°C continued for 5 min. Acetone was evaporated in a stream of nitrogen. Excess triethyl phosphate and resulting triethyl phosphate were removed via vacuum pump at room temperature. The resulting 4-hydroxycyclophosphamide was dissolved in DMSO and used in the chemotaxis experiments. Thin-layer chromatography studies (R. Struck, personal communication) demonstrate that this procedure gives an essentially quantitative yield of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide.

Preparation of Neutrophil Suspension. Leukocyte suspensions yielding approximately 85% neutrophils were prepared from heparinized (10 units/ml) venous blood obtained from healthy adult donors by utilizing standard dextran sedimentation and subsequent hypotonic lysis of erythrocytes (3). For chemotaxis studies, neutrophil preparations were more highly purified by use of Hypaque-Ficoll gradients (3). This technique yielded neutrophil cell suspensions containing 98 ± 1% neutrophils without contaminating erythrocytes or platelets. Subsequently, the neutrophils were suspended in the buffers indicated below for specific experiments.

In some experiments, the neutrophil suspensions were incubated for the time period indicated with the test alkylating agent in Gey’s buffer (9). Then the cells were washed with Gey’s buffer and utilized in the test systems described.

Measure of Neutrophil Chemotaxis and Neutrophil Random Migration. Neutrophil chemotaxis was measured by the technique of Gallin et al. (7). In brief, highly purified neutrophil preparations were suspended in chilled modified Hanks’ solution at a concentration of 2.0 x 10⁷ neutrophils/ml. Neutrophil labeling with ⁵¹Cr was accomplished by the addition of 1 μCi of Na₅¹CrO₄ in 0.9% sodium chloride solution (New England Nuclear, Boston, Mass.) per 10⁷
neutrophils. The neutrophil suspension was incubated with agitation in plastic tubes at 37° for 30 min. The neutrophil suspension was washed with modified Hank’s solution, visible cell clumps were removed, and the remaining neutrophils were suspended in Gey’s medium. The cell concentration was adjusted to 2.3 x 10^6 neutrophils/ml.

Modified Boyden chemotactic chambers (NeuroProbe Nucleopore Corp., Pleasanton, Calif.) with a 3-µm micropore membrane filters (Sartorius, West Germany) were used. The chemotactic stimulus was generated by incubation of 2.0 ml of the cell donor’s serum with 1 µg of endotoxin at 37° for 1 hr. (Escherichia coli 0127:B8, Difco Laboratories, Detroit, Mich.).

The 51Cr-labeled neutrophil suspension was added to the upper compartment, and the chemotactic stimulus, to the lower compartment. Chambers were incubated for 3 hr at 37° in 5% carbon dioxide and 100% humidity. Subsequently, the fluid from the upper and lower chambers was aspirated. The lower filters were removed, rinsed in 0.9% NaCl solution, and placed in individual γ counter vials.

To account for variable 51Cr specific activity, the cpm were adjusted to 10,000 cpm/10^6 neutrophils. A chemotactic index was calculated as follows:

\[
\text{Observed lower filter cpm} \times 10,000
\]

\[
\text{cpm/10^6 neutrophils}
\]

The chemotactic index was calculated as the mean of at least 3 duplicate chambers. A random migration index was calculated in the same way using chambers which omitted the chemotactic stimulus. Standard error was used for variance estimates. Student's t test was used to compare means. Neutrophil viability was tested by trypan blue dye exclusion (14).

**RESULTS**

**Neutrophil Chemotaxis in Presence of Cyclophosphamide and Cyclophosphamide Metabolites.** Cyclophosphamide or cyclophosphamide metabolites were added in equal concentrations to both the upper and lower compartments of the chemotactic chambers. The results are shown in Table 1. With the exception of 4-hydroxycyclophosphamide and 4-hydroperoxycyclophosphamide, neither cyclophosphamide nor any of the other metabolites tested demonstrated any effect on neutrophil chemotaxis or random migration, compared with simultaneously determined control values. At 200 nmoles/ml, 4-hydroxycyclophosphamide produced a statistically significant decrease in the chemotactic index (p < 0.01) from 2394 ± 1054 (mean ± 2 S.E.) in control chambers to 313 ± 140 in test chambers. There was no influence on random migration at any concentration of 4-hydroxycyclophosphamide tested. The precursor of 4-hydroxycyclophosphamide, 4-hydroperoxycyclophosphamide, also had an inhibitory effect on chemotaxis that barely (p < 0.05) achieved statistical significance. Cell viability was not impaired by any metabolite tested, as judged by trypan blue dye exclusion.

Control experiments were conducted to ensure that the effect of 4-hydroxycyclophosphamide was not the result of inhibition by the organic solvent DMSO, used to dissolve the metabolite. DMSO, in concentration up to 1.7 volume %, had no effect on neutrophil chemotaxis.

**Neutrophil Chemotaxis after Incubation in 4-Hydroxycyclophosphamide.** Since it was possible that 4-hydroxycyclophosphamide altered the complement chemotactic factor rather than the neutrophils, neutrophils were incubated in Gey’s buffer containing 4-hydroxycyclophosphamide (200 nmoles/ml) for up to 2 hr at 37°. Control cells were incubated in buffer only. Then, the cells were washed 2 times with buffer and placed in chemotactic chambers. There was a significant reduction (p < 0.01) in the chemotactic index from 1515 ± 343 (mean ± 2 S.E.) for cells incubated in buffer to 608 ± 404 for cells incubated in 4-hydroxycyclophosphamide. There was no effect on neutrophil random migration. Neutrophil viability was judged at greater than 96% by the trypan blue exclusion technique.

Because of the possibility that 4-hydroxycyclophosphamide caused the neutrophil 51Cr membrane label to release resulting in falsely low chemotactic indexes. 51Cr-labeled neutrophils were incubated in Gey’s buffer in 4-hydroxycyclophosphamide (200 nmoles/ml) or in buffer alone in a shaking water bath at 37° for up to 5 hr. The cell suspension was sampled every hr and centrifuged at 400 x g, the cell pellet was washed twice in buffer, and the radioactivity of the cell pellet was determined. There was no loss of the neutrophil 51Cr label over the 5-hr period in either the control or 4-hydroxycyclophosphamide-exposed cells. The variability in counting between samples was low (3.0% S.E.).

**DISCUSSION**

The results support a direct inhibitory but sublethal effect of 4-hydroxycyclophosphamide on neutrophils, as manifest by impaired chemotaxis. Control studies exclude the possibility of an effect of the 4-hydroxycyclophosphamide on either the membrane label or the chemotactic factor. Neutrophil viability is supported by exclusion of the vital dye trypan blue, retention of the chromium isotope membrane label, and the absence of membrane leakage of the cytoplasmic enzyme, lactic dehydrogenase, into the media (unpublished observations).

Studies of the metabolism of cyclophosphamide have identified many potentially biologically active metabolites (1, 4, 5, 10, 11, 17, 18, 19). The metabolite 4-hydroxycyclophosphamide has been considered a likely candidate for the responsible cytotoxic agent (18, 19). However, a recent in vivo study in mice questioned the importance of 4-hydroxycyclophosphamide (18). After administration of cyclophosphamide i.p., no detectable 4-hydroxycyclophosphamide was found in the mouse blood by thin-layer chromatography techniques (18). In contrast, high blood levels of phosphoramide mustard and nor-nitrogen mustard were found. On the basis of these results, the alkylating activity of various metabolites and available data on in vitro cytotoxicity, it was concluded that phosphoramidate must was the most likely biologically active alkylating metabolite of cyclophosphamide produced in vivo (18). Other authors have arrived at the same conclusion (5).

Thus, it is of interest that phosphoramidate mustard in high concentration had no effect on neutrophil chemotaxis. The
same was true of nor-nitrogen mustard. The reason for this discrepancy may relate to the nonreplicating nature of the mature neutrophil. In replicating cells alkylating agents are known to combine with cellular DNA and RNA nucleosides, resulting in interference in nucleic acid and protein biosynthesis (8, 10). Even if a lack of such binding explained the resistance of the mature neutrophil to nor-nitrogen mustard and phosphoramid mustard, the nature of 4-hydroxycyclophosphamide neutrophil chemotaxis inhibition is unclear. An extranuclear site of alkyla-tion as recently suggested by the effect of nitrogen mustard (2) and cyclophosphamide on lymphocyte function (20).

Chemotaxis is an energy-dependent neutrophil function (21). A major energy source for the neutrophil is glycolysis via the hexose monophosphate shunt. It is of interest that NAD is an important coenzyme in this pathway, and NAD tissue levels are reported to be reduced in animals treated with cyclophosphamide (10, 15). Recent studies localize NAD to the neutrophil plasma membrane and suggest a role for NAD in initiating the metabolic response of the cell to stimulation (16). Interference with NAD function and other possible mechanisms of inhibition are presently under investigation.

To our knowledge, no other studies on the effect of alkylating agents on in vitro neutrophil chemotaxis have been published. In rats given i.p. cyclophosphamide, no effect on in vivo chemotactic responsiveness to a carrageenan-induced inflammatory reaction was observed (13).

### REFERENCES


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