Enhancement of *in Vivo* and *in vitro* Murine Immune Responses by the Cyclophosphamide Metabolite Acrolein

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ABSTRACT

Cyclophosphamide (CY)-mediated immunoenhancement has been attributed to the inhibition of suppressor T-cell generation. In order to exert its effects on the immune system, metabolic activation of CY is required. The metabolite of CY responsible for its immunoenhancing properties are not known. Two reactive metabolites of CY which may inhibit suppressor T-cell generation are phosphoramidate mustard and acrolein, compounds known to primarily bind to DNA and sulfhydryl groups, respectively. The objective of this study was to determine whether acrolein and/or phosphoramidate mustard are capable of enhancing the immune response in a manner similar to CY. Administration of 100 μmol/kg of acrolein, i.e., to female C57BL/6 × C3H F1 mice 1 day before antigen exposure (sheep red blood cells) resulted in a 50% increase in the delayed-type hypersensitivity response (foot pad swelling). The Day 4 primary humoral immune response to sheep red blood cells was also increased by 88 and 60% after the administration of 30 and 100 μmol/kg acrolein 1 day before sensitization with sheep red blood cells. Exposure of splenocyte cultures to 3 × 10^{-7} and 10^{-6} M 4-hydroxy-CY or acrolein produced significant increases in the *in vitro* T-dependent antibody-forming cell response. In contrast, the antibody-forming cell response of cultures exposed to 3 × 10^{-8}–10^{-6} M phosphoramidate mustard did not increase above control levels. These results suggest that acrolein is responsible for the CY-induced enhancement of the immune response. Moreover, the enhancement may be produced by the binding of acrolein to sulfhydryl groups of molecules of cells required for the generation of suppressor T-cells.

INTRODUCTION

The antineoplastic drug, cyclophosphamide, is commonly used as an immunosuppressive agent in the treatment of autoimmune diseases and to prevent graft rejection. However, numerous investigators have demonstrated that when administered in low doses before or soon after antigen exposure, CY will also enhance cellular and humoral immune responses (1–7). The immunoenhancing effects of CY have been attributed to a selective inhibition of Ts generation (6–20), a cell population involved in the down regulation of a variety of immune responses, including responses generated against neoplastic cells (21, 22). Subsequent studies demonstrated that this selective effect on Ts generation was due to a greater sensitivity of precursor Ts to CY than effector Ts, precursor and effector cytolytic T-cells, and B-cells (8, 9, 15–20). In addition, antigen-presenting cells involved in the induction of Ts effector cells (Ts3) were found to be sensitive to low doses of CY (23).

Although the immunological mechanisms of the CY-induced immunoenhancement have been extensively investigated, the pharmacological basis for the greater sensitivity of Ts precursors to CY has not been determined. The effects of CY on the immune system, however, are known to be mediated by its metabolites rather than the parent compound (24–26). Under the influence of the cytochrome P-450 monooxygenase system, CY is converted to 4-hydroxy-CY which rearranges to aldophosphamide. Aldophosphamide undergoes β-elimination to form acrolein (2-propanal) and the alkylating compound, PAM (24–26). The binding of PAM to DNA is purported to mediate the antitumor and immunosuppressive effects of CY by inhibiting cell proliferation (27–29). Cells of the immune system that are proliferating at a high rate were observed to be the primary targets of CY when administered at immunosuppressive doses (30, 31). It has been suggested that Ts precursors are proliferating at a greater rate than other cell types of the immune system and therefore are more sensitive to the antiproliferative effects of CY (32). This hypothesis suggests that PAM is responsible for the immunoenhancement produced by CY. On the other hand, since acrolein is known to readily bind to sulfhydryl groups of important cellular macromolecules (25, 29, 33), this reactive aldehyde metabolite should also be considered to mediate the CY inhibition of Ts generation.

The objective of this study was to determine whether the immune response may also be affected by acrolein and/or PAM. This was accomplished by examining the humoral and DTH responses of mice administered acrolein or CY before sensitization with sRBC. In addition, the *in vitro* T-dependent AFC responses of splenocyte cultures exposed to 4-HC, acrolein, or PAM prior to the addition of sRBC were evaluated. 4-HC is known to spontaneously break down in aqueous solutions to 4-hydroxy-CY. Since splenocyte cultures are unable to metabolically activate CY, 4-HC can be used to circumvent the need for a metabolic activation system (e.g., liver S-9 preparation, hepatic enzymes). Several investigators have used 4-HC *in vitro* and have replicated the immunoenhancing actions of CY that were observed *in vivo* (16–20).

MATERIALS AND METHODS

Chemicals. Acrolein (99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Cyclophosphamide was purchased from Sigma Chemical Co. (St. Louis, MO). 4-HC was generously provided by Dr. Michael Colvin (Pharmacology Department, Johns Hopkins Oncology Center, Johns Hopkins University) and PAM was provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

Animals. Female C57BL/6 × C3H F1 mice, hepatitis and Sendai virus free, 8–12 wk of age, were used in this study (Frederick Cancer Research Center, Frederick, MD). Mice were received at 5–7 wk of age, randomized, and quarantined for at least 1 wk prior to use. Plastic cages were used to house the mice with woodchip bedding. Food (Purina Laboratory Chow) and tap water were provided ad libitum. Mice were maintained on a 12-h light-dark cycle at 21–24°C and 40–60% relative humidity.

Delayed-type Hypersensitivity Response. Acrolein was diluted in saline (vehicle) and mice were administered 10, 30, and 100 μmol/kg.
acrolein, i.v. All drugs were administered in a volume of 0.1 ml/10 g body weight. Doses of 200 µmol/kg were found to be lethal, whereas the doses used in this study did not produce any behavioral signs of toxicity. Spleen weight, cellularity, and body weight were not significantly affected. Groups of mice were administered 20 mg/kg CY (72 µmol/kg), i.p. Although the DTH response may be more greatly enhanced with higher doses of CY (100–200 mg/kg) (2, 5, 13), these doses also result in a suppressed humoral immune response (2). A dose of 20 mg/kg had been reported to enhance DTH (5) and AFC (7) responses to sRBC. Mice of all treatment groups were sensitized with sRBC 24 h after drug exposure. The method of sensitization and challenge was similar to that described by LaGrange et al. (3). Mice were sensitized with 10^6 sRBC/mouse i.v. and challenged with 10^6 sRBC in the left hind footpad 6 days after sensitization. Swelling responses were measured with a micrometer 24 h after challenge. The footpad thickness of the right foot was subtracted from the left to obtain the value of footpad swelling. Untreated mice that were challenged but not sensitized had a footpad swelling of 0.2 mm.

Primary IgM Antibody-forming Cell Response. Mice were treated with vehicle, acrolein, or CY as described for the DTH assay. One day after drug exposure, mice were sensitized i.v. with 5 × 10^6 sRBC. An additional group of mice was treated with acrolein (100 µmol/kg, i.v.) or vehicle 1 day after sensitization. Four days after sensitization, mice were sacrificed and the spleens were removed. The number of IgM-producing cells was determined by a modification of the Jerne plaque assay (34). Briefly, spleen cells and sRBC were added to an agar solution. Guinea pig complement was added and the mixture was vortexed and poured on a Petri plate. A glass coverslip was placed on the drop of agar to obtain an even spread of cells. Following a 3-h incubation period at 37°C, cleared areas of hemolysis (plaques) occurring around each AFC were enumerated under magnification. The AFC response was expressed as the number of PFC per 10^8 spleenocytes.

In Vivo T-dependent Antibody-forming Cell Response. Spleen cell suspensions were prepared to 1.1 × 10^7 cells/ml in RPMI 1640 containing 2 mm L-glutamine ( Gibco Laboratories, Grand Island, NY) antibiotic/antimycotic ( Gibco Laboratories, Grand Island, NY; 100 units/ml penicillin and streptomycin, and 0.25 µg/ml fungizone), 2% fetal calf serum (HyClone Laboratories, Inc., Logan, UT), and 50 µM 2-mercaptoethanol. The cell suspensions were added to 24-well cluster plates (0.9 ml/well) and 100 µl of the test compound dissolved in Earle’s balanced salt solution containing 1 mm 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (pH 7.2). Drug solutions were kept at 4°C and prepared 10-20 min before adding them to the cell suspensions. The cultures were incubated in 5% CO2 at 37°C for 60 min while rocking. The wells were centrifuged and washed twice in the wells. The media were used to initially prepare the cell suspensions. The cultures were sensitized with 10^7 sRBC/well and incubated for 5 days (peak day) IgM response in a special gas mixture of 10% CO2, 7% O2, and balanced nitrogen. The number of AFC generated in these cultures was determined by the Jerne plaque assay as described above. Cell viability of the splenocyte cultures was determined by the pronase method (34, 35). Viability measurements by this method were found to be more sensitive than those obtained with conventional trypan blue uptake methodology.

Data Analysis. Each of the in vivo studies presented is representative of at least two studies. The in vitro AFC responses of control cultures were found to vary between experiments. However, the variability of responses of replicate control cultures within an experiment was usually never greater than ± 10% of the mean. Thus, the percentage of control values of replicate cultures of at least 2 experiments (4 replicates/dose group/experiment) was calculated and presented as the percentage of control AFC/10^2 spleen cells. The data were analyzed for homogeneity to determine whether parametric or nonparametric analysis were appropriate. When an analysis of variance of parametric data showed significant differences, control groups were compared to treatment groups by Dunnett’s t test. When nonparametric data showed significant differences, treated and control groups were analyzed by the Wilcoxon rank sum test. When a comparison was made between two groups, Student’s t test was utilized. Groups differing from the control response at the level of P < 0.05 were considered significant.

RESULTS

Effects of Acrolein and CY on the Delayed-type Hypersensitivity Response. The footpad swelling response of mice administered acrolein at doses of 10 and 30 µmol/kg 1 day before sensitization to sRBC did not significantly differ from the response of vehicle-pretreated mice (Fig. 1). However, a dose of 100 µmol/kg produced a 50% increase in footpad swelling. Although a slightly greater swelling response was observed in CY-pretreated mice, the response was not statistically different from the mice treated with 100 µmol/kg acrolein.

Effects of Acrolein and CY on the in Vivo T-dependent AFC Response. Administration of 30 and 100 µmol/kg of acrolein 1 day before sensitization to sRBC increased the PFC response by 88 and 60%, respectively (Fig. 2). A dose of 10 µmol/kg did not produce a significant increase. Cyclophosphamide pretreatment was found to also significantly enhance the number of PFC generated, a response similar to that obtained with 100 µmol/kg acrolein. In contrast, the AFC response of splenocytes of mice administered 100 µmol/kg acrolein 1 day after sensitization with sRBC did not differ from the control response.

Effects of 4-HC, Acrolein, and PAM on the in Vivo T-dependent AFC Response. The AFC response of splenocyte cultures exposed to 3 × 10^{-7} or 10^{-6} M 4-HC was not altered, whereas the addition of 3 × 10^{-7} or 10^{-6} M 4-HC resulted in an AFC response 62 and 65% above control levels, respectively (Fig. 3A). At higher concentrations, 10^{-5} and 3 × 10^{-5} M, the number of AFC/10^2 splenocytes decreased 50 and 99%, respectively. A similarly shaped concentration-response curve was obtained with the exposure of splenocyte cultures to acrolein.
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(Fig. 3B). Significant increases of 20–46% above the control AFC response were observed with exposure to $3 \times 10^{-7}$, $10^{-6}$, and $3 \times 10^{-6}$ M acrolein (Fig. 3B). The number of AFC generated in cultures exposed to $10^{-5}$ and $3 \times 10^{-5}$ M acrolein, however, was suppressed by 50 and 99%, respectively. In contrast to the effects of 4-HC and acrolein, exposure of splenocyte cultures to PAM did not result in an enhanced AFC response. Concentrations of $3 \times 10^{-8}$ to $10^{-4}$ M PAM did not significantly alter the AFC response, while a 99% suppression was observed with the addition of $10^{-3}$ M PAM (Fig. 3C).

Effects of 4-HC, Acrolein, and PAM on Cell Viability of Spleenocyte Cultures. Viabilities of spleenocyte cultures used to examine the effects of 4-HC, acrolein, and PAM on the in vitro AFC response were also determined. At concentrations of metabolites in which AFC responses were observed to decrease by 99%, significant decreases in cell viability were also observed (Fig. 4, A–C). At lower concentrations of 4-HC, acrolein, or PAM, cell viability did not significantly differ from control levels.

DISCUSSION

Since Maguire and Ettore (1) first reported in 1967 that CY pretreatment could enhance the contact sensitization response, numerous investigations were conducted to examine the effect of CY on other immunological responses and to determine the immunological basis for this effect of CY (2–19). Precursors of Ts were determined to be affected and to be more sensitive to CY than other cell types involved in the immune response (8, 9, 15–20). Moreover, Lowy et al. (23) reported that APC are required for the generation of antigen-specific effector Ts (Ts3) (azobenzenearsenate system) and that these cells are CY sensitive. The mechanism for the greater sensitivity of precursor Ts and Ts3-APC in comparison to other cell types, however, has not been elucidated.

In order to mediate its effects on the immune system, it is now well established that CY must first be metabolically activated (24–26). The reactive metabolites of CY, PAM and acrolein, are known to mediate their toxicity by different mechanisms. PAM binds primarily to DNA (27–29), whereas acrolein binds to sulfhydryl groups of proteins and peptides (29, 33). Thus, insight into the mechanisms of CY selectivity on Ts generation may be obtained by determining which metabolite of CY is responsible for its immunoenhancing actions. In this study, the effects of acrolein on the in vivo DTH and primary
IgM AFC response and the in vitro T-dependent AFC response were examined. In addition, the effects of 4-HC, acrolein, and PAM on the in vitro AFC response were examined.

Administration of acrolein 1 day before sensitization was found to enhance the DTH and AFC responses to sRBC to an extent similar to that obtained with CY pretreatment. When acrolein was administered 1 day after drug administration rather than before sensitization, the enhancing effect of acrolein on the primary AFC response was not observed. This timing of drug and antigen exposure is one of the major characteristics of CY-mediated immunoenhancement (2, 10–12). After antigen exposure, Ts precursors differentiate to Ts effector cells (21, 22) and become less sensitive to the effects of CY (15–20). AFC responses of splenocyte cultures were also enhanced with exposure to 4-HC or acrolein at similar concentrations. The concentrations of 4-HC which produced an enhanced AFC response were similar to those used by other investigators to selectively inhibit the generation of Ts (16–20). In contrast to the effect of 4-HC and acrolein, PAM was found to suppress only the AFC response at the highest concentration, a concentration which significantly decreased cell viability. These results suggest that acrolein may mediate the CY-induced immunoenhancement rather than PAM.

Investigators have suggested that precursors to Ts may be proliferating at a greater rate (32) or are less capable of repairing the DNA damage (15), thereby making this cell population more sensitive to CY. This would imply that the DNA alkylating metabolite, PAM, is responsible for the inhibition of Ts induction. This hypothesis is supported by the findings that a variety of antineoplastic drugs are also capable of enhancing the immune response (36). More in-depth studies, however, have demonstrated that Adriamycin (37, 38) and bleomycin (39) mediate their immunoenhancing effects through a mechanism that does not involve Ts. Moreover, results of studies on Ts-precursor proliferation rates in relation to the development of Concanavalin A-induced suppressor activity (antigen-non-specific) indicate that Ts precursors are not a highly proliferating cell type (40). Treatment of cells with mitomycin C, to inhibit DNA synthesis, did not prevent the induction of suppressor cell activity of Concanavalin A-exposed cultures (40). These observations suggest that the antiproliferative actions of CY may not be the mechanism of selectivity on precursor Ts and further support the role of acrolein.

The greatest sensitivity of Ts precursors to CY has also been suggested to be attributed to lower levels of aldehyde dehydrogenase activity (15). CY is metabolized to 4-hydroxy-CY, which rearranges to aldophosphamide and decomposes to PAM and acrolein (24–26). Aldophosphamide may be converted to the inactive carboxyphosphamide by aldehyde dehydrogenase (24, 25). Differences in the susceptibilities of hemopoietic stem cells to CY have been demonstrated to be mediated by differences in aldehyde dehydrogenase activity (41). However, since acrolein was found in this study to mimic the immunoenhancing effects of CY and 4-HC, differences in aldehyde dehydrogenase activity may not be the major factor in determining the relative sensitivities of Ts precursors and/or Ts-APC.

Acrolein is known to mediate its toxic effects on cells by binding to sulfhydryl groups of important cellular molecules (29, 33). Utilizing [3H]CY labeled at the carbon associated with acrolein, Marinello et al. (29) demonstrated that the radioactivity (acrolein) was primarily associated with proteins of the liver. This binding was inhibited with the administration of N-acetylcysteine. In contrast, the binding of [3H]CY labeled on the chloroethyl group was found to be primarily associated with nucleic acids. Thus, inhibition of Ts generation may be mediated by the binding of acrolein to important sulfhydryl groups of molecules in Ts precursors and Ts3-APC. The sensitivity of these cell types to acrolein may be due to the presence of certain sulfhydryl-containing molecules unique and/or critical to the function of these cells (e.g., cell membrane receptors), a lower concentration of intracellular glutathione, or to the inability to regenerate sufficient amounts of glutathione.

In addition to providing evidence for the immunoenhancing effects of acrolein, the in vitro AFC studies demonstrated that higher concentrations (3 × 10^{-5} M) of 4-HC and acrolein suppressed the AFC response without a significant decrease in cell viability. A 33-fold greater concentration of PAM (10^{-3} M) was required to inhibit the AFC response. High concentrations of PAM were also reported to be required to decrease the survival of K562 tumor cells (42) and to inhibit mitogen-induced proliferation of murine splenocytes (43). Differences in potency of PAM and 4-HC in inhibiting tumor cell growth were also reported by Powers and Sladek (44). These results suggest that the immunosuppressive effects of CY may in part be mediated by acrolein and that the antiproliferative effects of PAM may not be the only contributing factor.

**REFERENCES**


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