Biochemical Pharmacology, Vol. 40, No. 5, pp. 927–935, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

MECHANISMS OF *IN VITRO* IMMUNOSUPPRESSION BY HEPATOCYTE-GENERATED CYCLOPHOSPHAMIDE METABOLITES AND 4-HYDROPEROXYCYCLOPHOSPHAMIDE

THOMAS T. KAWABATA,* MONICA Y. CHAPMAN, DONG-HYUN KIM,† WAYNE D. STEVENS and MICHAEL P. HOLSAPPLE

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, U.S.A.

(*Received* 17 *May* 1989; *accepted* 10 *January* 1990)

Abstract—Cyclophosphamide (CY) is metabolized to 4-hydroxy-CY which spontaneously breaks down to the reactive intermediates, phosphoramide mustard (PAM) and acrolein. The alkylating property of PAM is thought to mediate the anti-proliferative and cytotoxic actions of CY. Acrolein is known to bind sulfhydryl groups of cellular molecules and may contribute to the action of CY. However, the role of acrolein in the CY-induced immunosuppression remains unclear. The results of studies in which a hepatocyte co-culture system was used suggest that acrolein may play an important role in the cytotoxic action of CY, whereas those investigations using activated derivatives of CY indicate that acrolein is not an important factor. Thus, both approaches of CY exposure were utilized in the present study. Splenocytes of B6C3F1 mice were incubated with syngeneic isolated hepatocytes and CY or with 4-hydroperoxycyclophosphamide (4-HC) (which spontaneously decomposes to 4-hydroxy-CY). The *in vitro* antibody forming cell (AFC) response was found to be suppressed with both methods of exposure to CY metabolites. The addition of DNA to bind extracellular PAM was ineffective in preventing the suppression produced by hepatocyte-activated CY. However, it was also observed that DNA was able to attenuate the PAM-induced suppression. The sulfhydryl compounds 2-mercaptoethanesulfonate (MESNA) (15 μM) or reduced glutathione (GSH) (1 mM) inhibited the suppression of the AFC response of splenocytes incubated with CY and mouse hepatocytes. The suppression produced by 4-HC, however, was not affected by MESNA and only slightly inhibited by GSH. Similarly, the PAM-induced suppression was not affected by MESNA and slightly attenuated with GSH. In contrast, both MESNA and GSH were very effective in abrogating the acrolein-induced suppression, whereas DNA was ineffective. The findings of this study suggest that in the hepatocyte co-culture system, the immunosuppressive actions of CY are mediated by acrolein generated outside of the splenocyte, whereas the 4-HC induced suppression is not mediated by extracellular acrolein. Thus, this difference may explain the contradictory findings of previous studies that used different means of exposing cells to activated CY.

Cyclophosphamide (CY) was developed as an anti-neoplastic agent and later was found to possess immunosuppressive properties. Both humoral and cell-mediated immunity are known to be suppressed with CY treatment, with humoral responses being the most sensitive [1, 2]. It is now well established that the immunosuppression produced by CY is mediated by its metabolites rather than the parent drug [3–7]. Under the influence of the cytochrome P450 monooxygenase system, CY is metabolized to 4-hydroxy-CY. The hydroxylated metabolite nonenzymatically rearranges to aldophosphamide, which breaks down to phosphoramide mustard (PAM) and acrolein. PAM is thought to mediate the immunosuppressive and anti-tumor effects of CY by binding to DNA and inhibiting cell proliferation [3, 5, 8, 9]. On the other hand, acrolein is known to react readily with sulfhydryl groups of important cellular macromolecules [3, 10] and, thereby, may also play a role in inhibiting the immune response.

Although studies to determine the role of acrolein in the immune response have been limited, there are several reports of studies which have examined this question in relation to the cytotoxic and anti-proliferative actions of CY [11–15]. Since many of the target cells (i.e. tumor cells and lymphocytes) are unable to metabolize CY, two approaches have been used to expose cell cultures to activated metabolites of CY. In the first approach, target cells were exposed to activated CY metabolites generated by isolated hepatocytes [11, 15]. The results of these studies suggest that acrolein may play a very important role in the cytotoxic actions of CY. In contrast, the results of studies using compounds such as 4-hydroperoxycyclophosphamide (4-HC) [13] and sulfido [15], derivatives of CY, which spontaneously break down in water to 4-hydroxy-CY, indicate that the cytotoxic actions of CY are mediated by PAM.

The objective of this study was to use both experimental approaches to expose murine splenocytes to activated CY metabolites in order to elucidate the immunosuppressive metabolite. The first approach involved incubating splenocytes with a syngeneic

* To whom requests for reprints should be addressed: Department of Pharmacology and Toxicology, P.O. Box 613, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298-0613.

† Currently in the Department of Biochemistry, Vanderbilt University, Nashville, TN.

murine hepatocyte monolayer in the presence of CY. Following an incubation period, splenocytes were separated from the adherent hepatocyte monolayer, and the *in vitro* T-dependent antibody forming cell (AFC) response was determined. A similar method employing a rat hepatocyte and mouse splenocyte co-culture system has been used successfully to demonstrate suppression of the *in vitro* AFC response by CY [16]. Moreover, a mouse hepatocyte and splenocyte co-culture system has been utilized in examining the *in vitro* immunosuppression produced by dimethylnitrosamine [17]. The second method for CY metabolite exposure involves exposing splenocytes to the 4-hydroxy-CY derivative, 4-HC. Since PAM and acrolein are known to bind primarily DNA [3, 8, 18] and sulfhydryl groups [3, 10], respectively, the addition of DNA, 2-mercaptoethanesulfonate (MESNA, a sulfhydryl containing compound) or reduced glutathione (GSH) to hepatocyte–splenocyte co-cultures may result in the binding of reactive acrolein or 4-HC [19] and attenuate the suppression of *in vitro* AFC response. Addition of DNA to hepatocyte–splenocyte co-cultures has been shown previously to inhibit the suppression of the *in vitro* T-dependent AFC response produced by dimethylnitrosamine [17], a compound known to be metabolized to a DNA alkylating agent [20]. Therefore, trapping agents were used to examine the role of acrolein and PAM in CY-induced suppression of the *in vitro* AFC response.

METHODS

Cyclophosphamide metabolites. Acrolein was obtained from the Aldrich Chemical Co. (Milwaukee, WI). 4-HC was provided by Dr. Michael Colvin (Oncology Division, Johns Hopkins University). PAM was provided by Dr. V. L. Narayanan of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute.

Animals. Female B6C3F1 mice, Sendai and hepatitis virus free, 6–10 weeks of age, were used in these studies. Mice were received at 4–6 weeks of age from the Frederick Cancer Research Center (Frederick, MD) and quarantined for at least 1 week; they were housed four per cage. Food and water were provided *ad lib*. Mice were maintained on a 12-hr light–dark cycle at 21–24° and 40–60% relative humidity.

Hepatocyte–splenocyte co-culture system. The method used to isolate and culture primary mouse hepatocytes has been described previously by Kim *et al.* [17]. Hepatocytes were isolated by a collagenase perfusion and cultured for 24 hr in 6-well cluster plates. This resulted in the formation of a monolayer of adhered hepatocytes that were used for the co-culture experiments. Details of the co-culture system are described in detail by Kim *et al.* [17]. Splenocytes were prepared at a concentration of 3×10^7 cells/mL in RPMI 1640 medium containing antibiotic/antimycotic (GIBCO Laboratories, Grand Island, NY; 100 units/mL penicillin and streptomycin and 0.25 μg/mL fungizone), 2 mM *l*-glutamine (GIBCO Laboratories), 75 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (GIBCO Laboratories) and 0.22% sodium bicarbonate. One milliliter of splenocyte suspension was added to the hepatocyte cultures. Cyclophosphamide (Sigma Chemical Co.), dissolved in RPMI 1640 medium, was added to the cultures to obtain final concentrations of 5 and 10 μM. The final concentrations of DNA, MESNA, and GSH in the respective wells were; 1 mg/mL, 15 μM, and 1 mM respectively. Higher concentrations of the trapping agents were found to suppress the *in vitro* AFC response. Cultures were then incubated for 4 hr in 5% CO_2 at 37°. Splenocytes were separated from the adhered hepatocyte cultures by gentle swirling and were washed subsequently with RPMI 1640. The *in vitro* AFC response of the splenocytes was then determined.

In vivo *T-dependent AFC response.* The method used is similar to that reported by Kim *et al.* [17]. Splenocytes were adjusted to a concentration of 10^7 cells/mL in the same medium described for the co-culture system with the addition of 50 μM 2-mercaptoethanol [2-ME] and 10% fetal calf serum (Hyclone Laboratories, Logan, UT) and 0.5 mL of the suspensions was added per well of 48-well cluster plates. Sheep red blood cells (RBC) (5×10^6 cells/well) were added to each well, and the plates were incubated for 5 days at 37° in a special mixture of 7% O_2, 10% CO_2, and balanced N_2. After 5 days, the number of AFC generated was determined by a modification of the Jerne plaque assay as described by Holsapple *et al.* [21]. The AFC response was calculated as the number of AFC/10^6 recovered spleen cells. Viability of the splenocyte cultures was determined by the pronase method [21]. This method was found to be more sensitive than the trypan blue exclusion method.

Direct addition of CY metabolites. The final concentration of splenocytes used was 10^7 cells/mL of medium, as used in the co-culture studies. Acrolein, PAM, and 4-HC were freshly prepared in Earle's Balanced Salt Solution (GIBCO Laboratories) containing 0.1 M HEPES (pH 7.2), and 100 μL of the drug solution was added to the respective wells. The final concentration of the trapping agents was the same as described for the co-culture studies. The cultures were incubated for 1 hr at 37° in 5% CO_2 while rocking. The plates were centrifuged for 10 min at 1200 rpm, and the supernatant fraction was discarded. Cells were resuspended in 1 mL of medium and centrifuged. The washing procedure was repeated. The cells were resuspended in 1 mL of medium containing 10% fetal calf serum and 50 μM 2-ME. Sheep red blood cells were added (10^7 cells/well) to each well, and the cultures were incubated for 5 days. The AFC response and viability of the splenocytes were determined as described above.

Data analysis. In each experiment, four replicate cultures were prepared for each treatment group. Each experiment was repeated at least twice. To determine if parametric or nonparametric analysis was needed, the data were first analyzed for homogeneity. Dunnett's *t*-test was used to compare the responses of experimental dose groups with a control group of parametric data (data presented in Figs. 1, 2, 4E, 5B, 5D, 5E, 6B, 6D, and 8). The Wilcoxon rank sum test was used to compare treated and

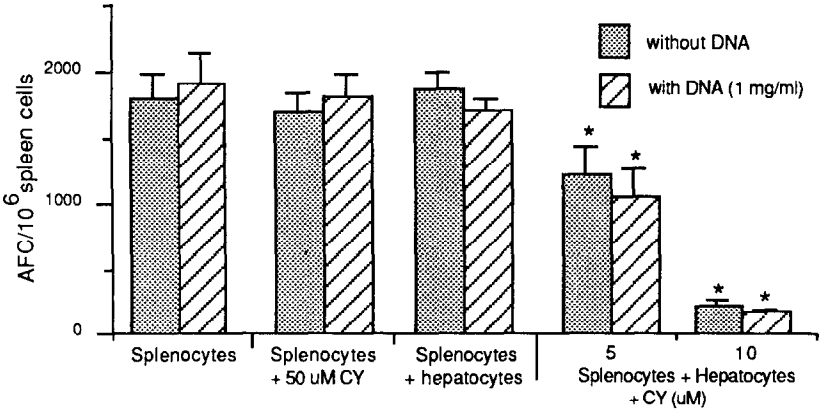

Fig. 1. Effect of DNA on the CY-induced suppression of the *in vitro* AFC response. Splenocytes were incubated with hepatocytes and CY (5 and 10 μM) in the presence (striped bar) or absence (stippled bar) of DNA. As a control, AFC responses of splenocytes only, splenocytes + hepatocytes, and splenocyte + 50 μM CY were also examined with or without the addition of DNA. Each value is the mean (±SE) AFC response from four replicate cultures. Asterisks indicate a significant difference from control responses (splenocytes + hepatocytes) ($P < 0.05$).

control groups of nonparametric data (data presented in Figs. 4A–4D, 4F, 5A, 5C, 5F, 6A, 6C, and 7). Differences between two groups were analyzed by Student's *t*-test (data presented in Fig. 3). A level of $P < 0.05$ was considered to be significant.

RESULTS

CY-induced suppression of the AFC response of splenocytes co-cultured with mouse hepatocytes. Co-incubation of splenocytes with both CY and hepatocytes produced a significant and concentration-dependent decrease in the AFC response (5 and 10 μM) (Fig. 1). At these concentrations of CY, cell viability (measured on day 5) was not affected, although at a higher concentration of CY (50 μM), cell viability was decreased significantly (see Fig. 7A). In all the experiments of this investigation, AFC responses of splenocyte cultures exposed to either CY only or co-cultured with mouse hepatocytes did not differ from responses of cultures with splenocytes only. The addition of exogenous calf thymus DNA to bind and inactivate PAM generated by the hepatocytes was found to be ineffective in reversing the CY-induced suppression (Fig. 1). The AFC response of the control cultures was not affected by the addition of 1 mg/mL DNA (Fig. 1).

In contrast to the effects of exogenous DNA, the addition of 15 μM MESNA was found to attenuate the suppression produced by CY at 5 and 10 μM. A 50% suppression produced by 5 μM CY was abrogated completely with the addition of MESNA (Fig. 2). The AFC response was not affected by MESNA in splenocytes cultures with or without hepatocytes (Fig. 2).

The suppression of the AFC response produced by 10 μM CY in the presence of hepatocytes (7% of control) was attenuated with the addition of 1 mM GSH to splenocyte cultures (74% of control) (Fig. 3). The addition of 1 mM GSH did not alter the AFC response of either splenocytes alone, splenocytes and 10 μM CY, or splenocytes and hepatocytes (Fig. 3).

Effects of 4-HC, acrolein, and PAM on AFC responses and cell viability. Incubation of splenocyte cultures with 4-HC for 1 hr at concentrations of 10 and 30 μM significantly suppressed the AFC response, whereas a significant decrease was not observed with a concentration of 3 μM (Figs. 4A, 5A and 6A). Exposure of splenocytes to acrolein at a concentration of 10 μM resulted in a significant decrease in the AFC response (Figs. 4C, 5C and 6C). At a higher concentration (30 μM), both AFC responses and cell viability were decreased. In contrast to 4-HC and acrolein, 10- to 30-fold greater concentrations of PAM were required to produce a significant decrease in the AFC response. In addition, the concentration–AFC response curve for PAM was much steeper than for 4-HC and acrolein. Concentrations of PAM 300 μM or greater produced a decrease in the AFC response (Figs. 4E, 5E and 6E).

A concentration of 4-HC which produced a 70% suppression of the AFC response did not produce a decrease in cell viability (Fig. 7B). In contrast, a 70% decrease in the AFC response produced by acrolein and PAM was associated with a 30% decrease in cell viability (Fig. 7C and D). At a concentration of 4-HC (30 μM) which produced a 97% decrease in the AFC response, cell viability was decreased by 33% (Fig. 7B). Concentrations of acrolein and PAM which produced a 95 and 99% suppression in the AFC response also resulted in a 65 and 48% decrease in cell viability respectively (Fig. 7C and D).

Effects of DNA, MESNA, and GSH on the suppression of the AFC response produced by 4-HC, acrolein, and PAM. The suppression of the AFC response produced by 4-HC and acrolein was not altered with the addition of calf thymus DNA (Fig. 4, B and D). However, the 80% decrease in the AFC response produced by 350 μM PAM was abrogated with the addition of DNA to a 14% decrease. Suppression of the AFC response produced by PAM was not affected by the addition of 10 and 100 μM

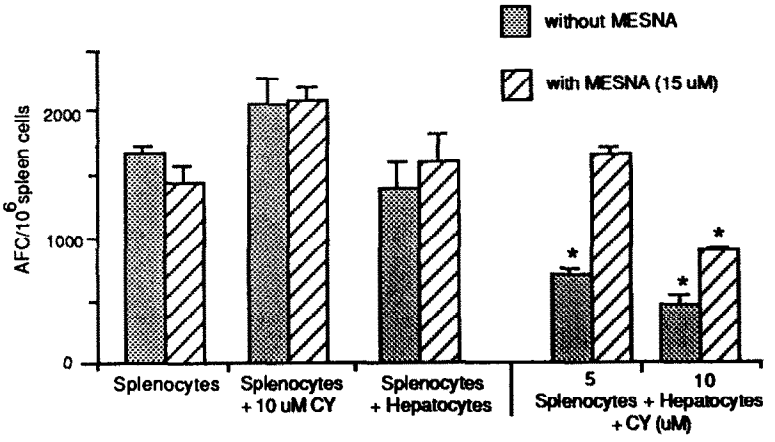

Fig. 2. Effect of MESNA on the CY-induced suppression of the *in vitro* AFC response. Splenocytes were incubated with hepatocytes and CY (5 and 10 μM) in the presence (striped bar) or absence (stippled bar) of 15 μM MESNA. The control cultures were comprised of splenocytes only, splenocytes + hepatocytes, and splenocytes + 10 μM CY. Each value is the mean (±SE) AFC response from four replicate cultures. Asterisks indicate a significant difference from control responses (splenocytes + hepatocytes) ($P < 0.05$).

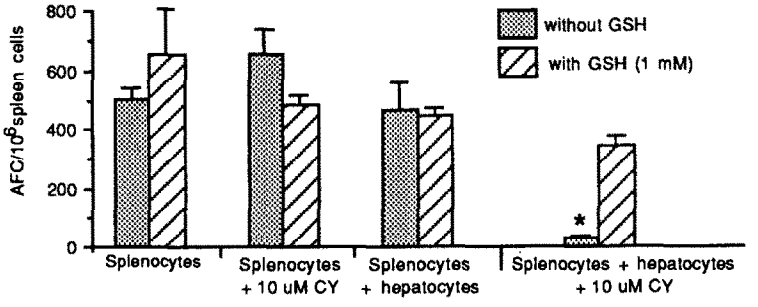

Fig. 3. Effect of GSH on the CY-induced suppression of the *in vitro* AFC response. Splenocytes were incubated with hepatocytes and CY (10 μM) in the presence (striped bar) or absence (stippled bar) of 1 mM GSH. As a control, AFC responses of splenocytes only, splenocytes + hepatocytes, and splenocyte + 10 μM CY were also examined with or without the addition of GSH. Each value is the mean (±SE) AFC response from four replicate cultures. The asterisk indicates a significant difference from control responses (splenocytes + hepatocytes) ($P < 0.05$).

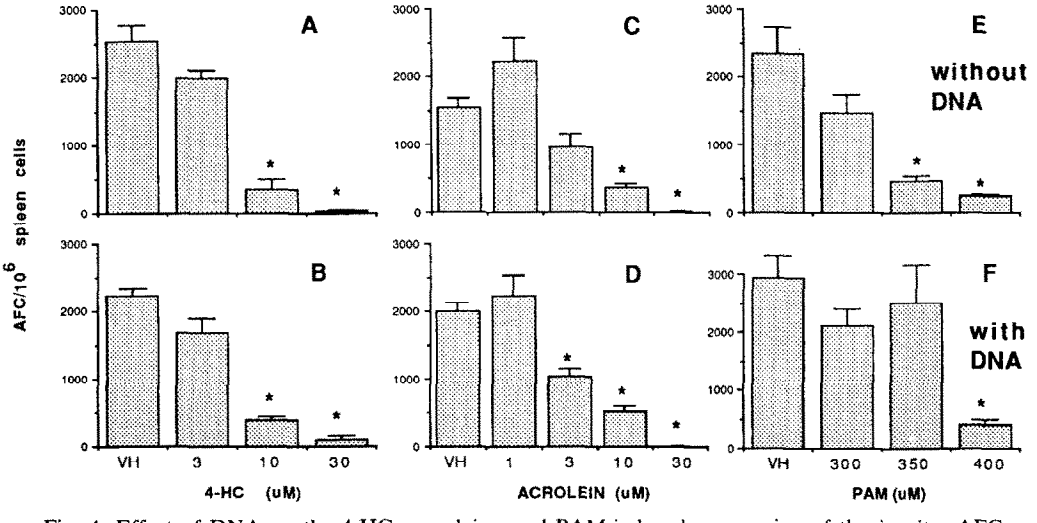

Fig. 4. Effect of DNA on the 4-HC-, acrolein-, and PAM-induced suppression of the *in vitro* AFC response. Splenocytes were exposed to 4-HC (A,B) acrolein (C,D), or PAM (E,F) with (B,D,F) or without (A,C,E) 1 mg/mL calf thymus DNA. Each value is the mean (±SE) AFC response of four replicate cultures. Asterisks indicate a significant difference from AFC responses of vehicle (VH) exposed splenocytes ($P < 0.05$).

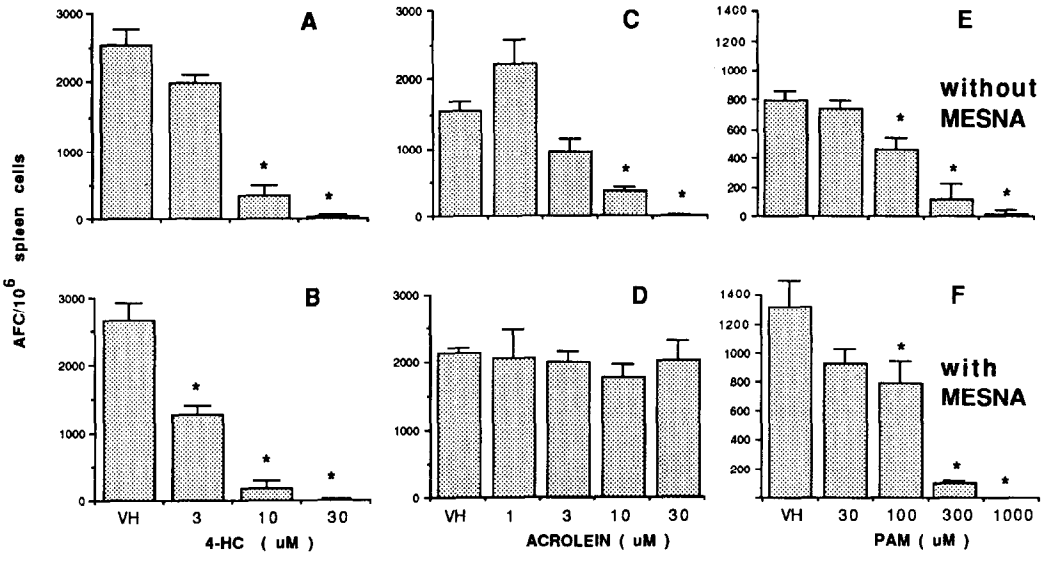

Fig. 5. Effect of MESNA on the 4-HC-, acrolein-, and PAM-induced suppression of the *in vitro* AFC response. Splenocytes were exposed to 4-HC (A,B) acrolein (C,D), or PAM (E,F) with (B,D,F) or without (A,C,E) 15 μM MESNA. Each value is the mean (±SE) AFC response of four replicate cultures. Asterisks indicate a significant difference from AFC responses of vehicle (VH) exposed splenocytes ($P < 0.05$).

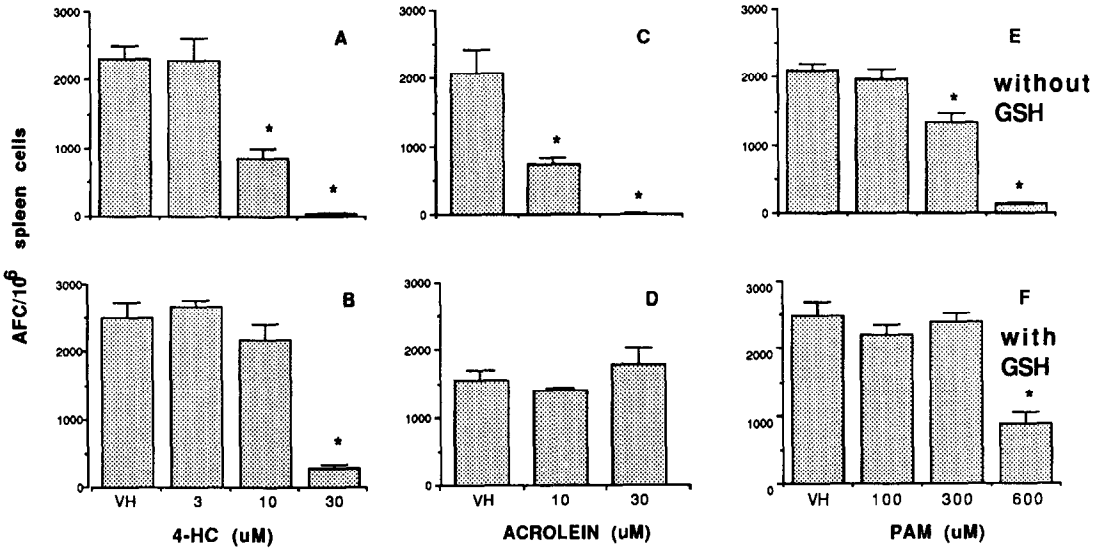

Fig. 6. Effect of GSH on the 4-HC-, acrolein-, and PAM-induced suppression of the *in vitro* AFC response. Splenocytes were exposed to 4-HC (A,B) acrolein (C,D), or PAM (E,F) with (B,D,F) or without (A,C,E) 1 mM GSH. Each value is the mean (±SE) AFC response of four replicate cultures. Asterisks indicate a significant difference from AFC responses of vehicle (VH) exposed splenocytes ($P < 0.05$).

guanosine (Fig. 8B and C). Co-incubation of splenocytes with MESNA did not affect the 4-HC- and PAM-induced suppression of the AFC response (Fig. 5, B and F). The suppression of the AFC response and cell viability produced by 10 and 30 μM acrolein, on the other hand, was reversed completely with the addition of MESNA. The addition of GSH to splenocyte cultures also resulted in the complete abrogation of the acrolein-induced suppression (Fig. 6D). The suppression produced by 10 μM 4-HC and 300 μM PAM was also attenuated with the addition of GSH (Fig. 6B and F); however, the effects of GSH were not as dramatic when observed with acrolein. Exposure to 300 μM PAM produced a statistically

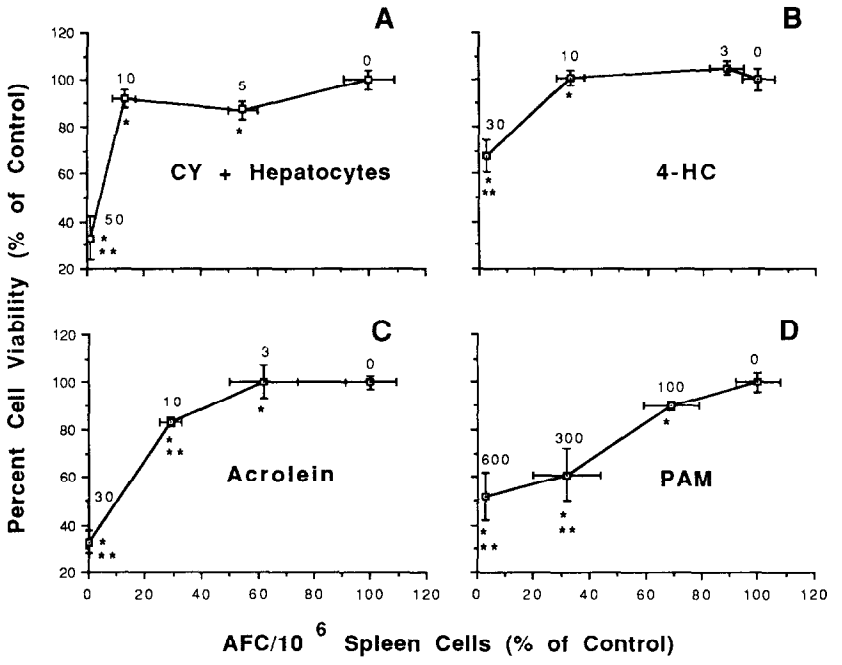

Fig. 7. Relationship between cell viability and AFC response of CY (A), 4-HC (B), acrolein (C), and PAM (D) exposed splenocytes. Each value is the mean percent of control (±SE) cell viability and AFC response of at least two experiments. Percent cell viability of control cultures ranged from 37 to 57%. AFC responses of control cultures ranged from 485 to 2590 AFC/10^6 spleen cells. Three concentrations of CY, 4-HC, acrolein and PAM which produced graded levels in suppression of AFC responses were plotted. The numbers above each data point indicate the micromolar concentration of the corresponding drug. Significant differences from control AFC responses (*) and cell viability (**) are indicated ($P < 0.05$).

significant decrease in cell viability of 18%. With the addition of GSH, cell viability was not affected by this concentration of PAM.

DISCUSSION

Whether PAM and/or acrolein play a major role in the immunosuppressive actions of CY is not clearly understood. There have been several studies to examine the contribution of each metabolite in producing the effects of CY. Many of these studies have concentrated on the anti-proliferative or cytotoxic effects of CY and either used a hepatocyte co-culture system to generate CY metabolites [11, 14] or direct addition of activated derivatives of CY [13, 15]. These activated derivatives spontaneously break down to 4-hydroxy-CY (i.e. 4-HC or 4-alkyl-sulfidocyclophosphamide). The results of studies utilizing these two approaches have been inconsistent with one another in regard to determining the role of acrolein in mediating the actions of CY.

To determine which metabolite of CY produces immunosuppression, splenocytes were exposed to CY metabolites by using a hepatocyte co-culture system or by directly adding 4-HC to splenocyte cultures. To discriminate between suppression produced by either PAM or acrolein, nucleophilic macromolecules were added to cultures to trap extracellular metabolites. DNA was used to trap PAM, and MESNA and GSH were used to bind acrolein. As a control, the effects of these trapping agents on the PAM- and acrolein-induced suppression of the *in vitro* AFC response were also investigated.

Utilizing a mouse hepatocyte and syngeneic mouse splenocyte co-culture method, suppression of the *in vitro* AFC response by CY was attained. This is consistent with a previous report that suppression of the AFC response was produced by CY with a mouse splenocyte and rat hepatocyte co-culture system [17]. At a concentration of 10 μM CY, a 68–98% suppression of the AFC response was observed without a significant decrease in cell viability. The addition of DNA to splenocyte cultures did not alter this suppressive effect, whereas MESNA and GSH were able to attenuate the immunosuppressive effects of CY. In contrast, the suppression of the AFC response produced by dimethylnitrosamine in the presence of hepatocytes was abrogated with the addition of calf thymus DNA [17]. As a control, it was demonstrated that the suppression produced by PAM was attenuated with exogenous DNA. It is also important to note that a 10- to 30-fold greater concentration of PAM was required to produce an equivalent suppression observed with either CY and hepatocytes, 4-HC, or acrolein. Powers and Sladek [22] also found that 4-HC was twenty times more potent than PAM in its ability to kill tumor cells. These findings would suggest that the immunosuppression is not mediated by PAM formed outside of the lymphocyte. In contrast to DNA, guanosine

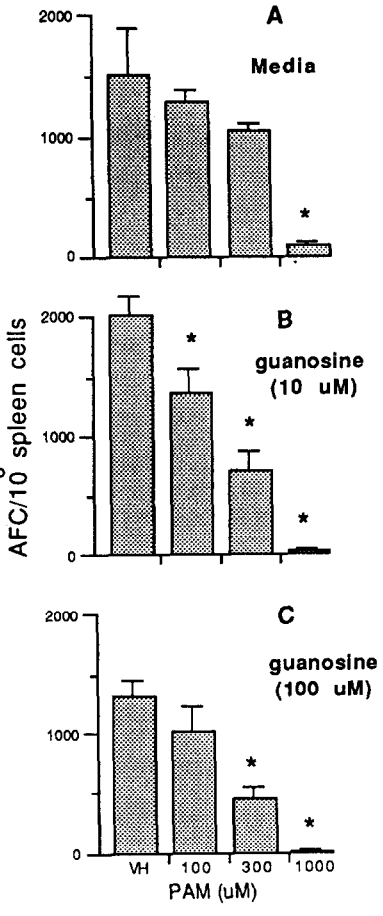

Fig. 8. Effect of guanosine on the PAM-induced suppression of the *in vitro* AFC response. Splenocytes were exposed to vehicle (VH) or PAM and incubated with medium or guanosine [10 μM (B) and 100 μM (C)]. Each value is the mean (±SE) AFC response of four replicate cultures. Asterisks indicate a significant difference from AFC responses of vehicle (VH) exposed splenocytes ($P < 0.05$).

did not affect the suppression produced by PAM. This was unexpected since PAM is known to bind to nucleotides of DNA [3, 8, 18].

The addition of MESNA was very effective in reversing the suppression of the AFC response produced with acrolein. Thus, the finding that MESNA is able to attenuate the suppression produced by hepatocyte-generated CY metabolites suggests that the decreased response was mediated, in part, by acrolein. Another possible explanation may involve the binding of MESNA to the hepatocyte-generated 4-hydroxy-CY. As demonstrated by Hohorst *et al.* [19, 23], sulfhydryl groups of compounds may bind to the hydroxyl group in the 4 position, stabilize 4-hydroxy-CY, and thereby decrease the amount of PAM and acrolein formed. This is supported by the findings that the administration of cysteine or MESNA results in a decrease in the efficacy of 4-sulfidoethylthio-CY derivative (activated) in preventing leukopenia and its antitumor actions [24]. However, the results of this study demonstrate that MESNA is unable to reverse the 4-HC-induced suppression. Furthermore, the results of other investigators suggest that suppressive actions of CY are not attenuated by MESNA. Al-Safi and Maddocks [25] reported that MESNA does not alter the 4-HC-induced suppression of the *in vitro* mixed lymphocyte response. MESNA was also ineffective in altering the mutagenicity of various CY metabolites [26]. Moreover, *in vivo* MESNA treatment to prevent bladder epithelial damage (toxicity mediated by acrolein [27]) has not been found to alter the antitumor effects of CY [28–30]. Thus, contrary to the findings by Hohorst *et al.* [19] and Wagner *et al.* [24], it appears that the sulfhydryl-containing MESNA does not readily bind 4-hydroxy-CY. In light of these findings and those which indicate that cellular uptake of MESNA is limited [31, 32], MESNA may be able to attenuate the CY-induced suppression of *in vitro* AFC responses by binding hepatic-generated extracellular acrolein rather than extracellular 4-hydroxy-CY or intracellular acrolein. In addition, the lack of an effect of MESNA on the 4-HC-induced suppression of the AFC response suggests that the action of 4-HC is not mediated by acrolein formed extracellularly.

Glutathione, like MESNA, was also able to attenuate completely the actions of acrolein. However, in contrast to the actions of MESNA, GSH was found to attenuate slightly the suppressive actions of 4-HC and PAM. The effect of GSH on 4-HC may be attributed to the binding of the sulfhydryl group of GSH to extracellular 4-hydroxy-CY, thereby decreasing its actions. The action of GSH on PAM may be related to the findings that the cytotoxic and anti-proliferative actions of PAM on tumor cell lines were inversely correlated with intracellular levels of GSH and glutathione *S*-transferase activity [33, 34]. The differences between the actions of GSH and MESNA may be attributed to the greater amount of GSH (1 mM) that could be used in the assay in comparison to the concentration of MESNA (15 μM). In addition, the relative reactivities of 4-HC and PAM for sulfhydryl groups may vary between different compounds. Since these results demonstrate that GSH is able to reverse only slightly the actions of 4-HC and PAM, it appears that sulfhydryl containing compounds do not readily bind and inactivate 4-hydroxy-CY and PAM as previously described by others [19, 33, 34].

The finding that GSH is able to abrogate the CY-induced immunosuppression (with a hepatocyte-coculture system) suggests a role of extracellular acrolein. Similar conclusions were obtained by Ohno and Ormstad [11] in which the cytotoxicity of hepatocyte-activated CY produced in isolated kidney cells was found to be attenuated with exogenous GSH. Crook *et al.* [14] detected single-strand DNA breaks in K562 human leukemia cells exposed to either CY or acrolein in the presence of isolated hepatocytes. Exposure to PAM did not produce this type of DNA damage. In contrast, exposure of L1210 cells to sulfido derivatives of CY (spontaneously hydrolyzes to 4-hydroxy-CY) or PAM produces DNA cross-links, while acrolein only produces single-strand breaks [15].

Concentrations of CY, 4-HC, PAM, and acrolein

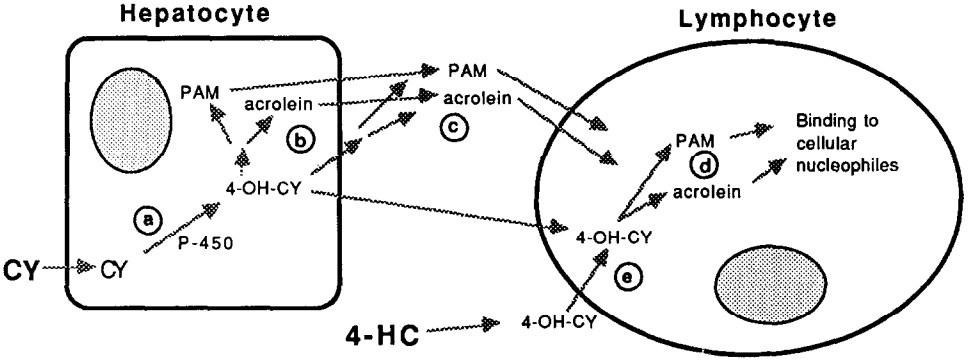

Fig. 9. Model of possible mechanisms of CY-induced immunosuppression by hepatocyte-activated CY metabolites and 4-hydroperoxy-CY. Each of the important steps is indicated by the circled letters (a–e). 4-Hydroxy-CY (4-OH-CY) is produced either by a cytochrome P450-mediated reaction within the hepatocyte (a) or by the spontaneous decomposition of 4-HC in aqueous conditions (e). 4-Hydroxy-CY rearranges to aldophosphamide and spontaneously decomposes to acrolein and PAM intracellularly (b and d) or extracellularly (c). These reactive intermediates may bind cellular nucleophiles and thereby mediate their immunosuppressive actions.

which produced greater than 95% suppression of the AFC response also significantly decreased cell viability. However, an 87% suppression in the AFC response with CY and a 67% decrease with 4-HC did not result in an alteration in cell viability. In contrast, the acrolein- and PAM-induced suppression in the AFC response (70%) was associated with a significant suppression in cell viability. This suggests that the cytotoxic actions of PAM and acrolein may mediate, in part, the suppression of the AFC response. Similar effects on the AFC response and cell viability were also reported previously [35]. The differences between the actions on cell viability with directly added and hepatocyte-generated acrolein and PAM may be attributed to the sudden exposure of the splenocytes to these metabolites above threshold levels, whereas a slower rate of metabolite exposure is obtained with the hepatocyte co-culture system.

Based on the results of this study and others, the mechanism of immunosuppression produced by CY with a hepatocyte co-culture system may be different from that produced with the direct addition of 4-HC. This explains the contradictory findings reported by others. It is hypothesized that 4-hydroxy-CY generated within hepatocytes (Fig. 9a) may break down to PAM and acrolein within the hepatocyte or exit hepatocytes (Fig. 9b). Acrolein and PAM may exit hepatocytes (Fig. 9c), bind to surface or intracellular macromolecules of neighboring lymphocytes, and result in a decreased AFC response (Fig. 9d). Exogenous MESNA or GSH binds to acrolein and abrogates the suppression. This appears to be the major mechanism of immunosuppression by CY *in vitro* with the use of hepatocyte activation. Extracellular 4-hydroxy-CY may enter lymphocytes and break down to PAM and acrolein. This spontaneous reaction may primarily occur intracellulary and thus account for the absence of an effect of MESNA on the 4-HC-induced immunosuppression (Fig. 9e). To determine if acrolein formed within lymphocytes from 4-hydroxy-CY is capable of suppressing the AFC response, agents which are able to increase intracellular levels of GSH in lymphocytes will be examined in future studies (i.e. ethyl-ester of GSH).

Acknowledgements—This work was supported in part by USPHS Grant A125044 and a National Institute of Environmental Health Sciences Contract (ES55094).

REFERENCES

1. Stockman GD, Heim LR, South MA and Trentin JJ, Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. *J Immunol* **110**: 277–282, 1973.
2. Turk JL and Poulter LW, Selective depletion of lymphoid tissue by cyclophosphamide. *Clin Exp Immunol* **10**: 285–296, 1972.
3. Gurtoo HL, Dahms R, Hipkens J and Vaught JB, Studies on the binding of [^{3}H-*chloroethyl*]-cyclophosphamide and ^{14}[C-4]-cyclophosphamide to hepatic microsomes and native calf thymus DNA. *Life Sci* **22**: 45–52, 1977.
4. Connors TA, Cox PJ, Farmer PB, Foster AB and Jarman M, Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. *Biochem Pharmacol* **23**: 115–129, 1974.
5. Colvin M, Padgett CA and Fenselau C, A biologically active metabolite of cyclophosphamide. *Cancer Res* **33**: 915–918, 1973.
6. Foley GE, Friedman OM and Drolet BP, Studies on the mechanism of action of cytoxan. Evidence of activation *in vivo* and *in vitro*. *Cancer Res* **21**: 57–63, 1961.
7. Alarcon RA and Meienhofer J, Formation of the cytotoxic aldehyde acrolein during *in vitro* degradation of cyclophosphamide. *Nature New Biol* **233**: 250–252, 1971.
8. Hemminki K, Binding of metabolites of cyclophosphamide to DNA in a rat liver microsomal system and *in vivo* in mice. *Cancer Res* **45**: 4237–4243, 1985.
9. Colvin M, Brundrett RB, Kan M-NN, Jardine I and Fenselau C, Alkylating properties of phosphoramide mustard. *Cancer Res* **36**: 1121–1126, 1976.
10. Alarcon RA, studies on the *in vivo* formation of acrolein: 3-Hydroxypropylmercapturic acid as an index

of cyclophosphamide (NSC-26271) activation. *Cancer Treat Rep* **60**: 327–335, 1976.
11. Ohno Y and Ormstad K, Formation, toxicity and inactivation of acrolein during biotransformation of cyclophosphamide as studied in freshly isolated cells from rat liver and kidney. *Arch Toxicol* **57**: 99–103, 1985.
12. Slott VL and Hales BF, Enhancement of the embryotoxicity of acrolein, but not phosphoramide mustard, by glutathione depletion in rat embryos *in vitro*. *Biochem Pharmacol* **36**: 2019–2025, 1987.
13. Wrabetz E, Peter G and Hohorst HJ, Does acrolein contribute to the cytotoxicity of cyclophosphamide? *J Cancer Res Clin Oncol* **98**: 119–126, 1980.
14. Crook TR, Souhami RL and McLean AEM, Cytotoxicity, DNA cross-linking, and single breaks induced by activated cyclophosphamide and acrolein in human leukemia cells. *Cancer Res* **46**: 5029–5034, 1986.
15. Erickson LC, Ramonas LM, Zaharko DS and Kohn KW, Cytotoxicity and DNA cross-linking activity of 4-sulfidocyclophosphamides in mouse leukemia cells *in vitro*. *Cancer Res* **40**: 4216–4220, 1980.
16. Yang KH, Kim BS, Munson AE and Holsapple MP, Immunosuppression induced by chemicals requiring metabolic activation in mixed cultures of rat hepatocytes and murine splenocytes. *Toxicol Appl Pharmacol* **83**: 420–429, 1986.
17. Kim DH, Yang KH, Johnson KW and Holsapple MP, Suppression of *in vitro* antibody production by dimethylnitrosamine in mixed cultures of mouse primary hepatocytes and mouse splenocytes. *Toxicol Appl Pharmacol* **87**: 32–42, 1987.
18. Mehta JR, Przybylski M and Ludlum DB, Alkylation of guanisone and deoxyguanosine by phosphoramide mustard. *Cancer Res* **40**: 4183–4186, 1980.
19. Hohorst H-J, Draeger U, Peter G and Voelcker G, The problem of oncostatic specificity of cyclophosphamide (NSC-26271): Studies on reactions that control the alkylating and cytotoxic activity. *Cancer Treat Rep* **60**: 309–315, 1976.
20. Pegg AE, Formation and metabolism of alkylated nucleosides: Possible role in carcinogenesis by nitroso compounds and alkylating agents. *Adv Cancer Res* **25**: 195–269, 1977.
21. Holsapple MP, Tucker AN, McNerney PJ and White KL, Jr, Effects of *N*-nitrosodimethylamine on humoral immunity. *J Pharmacol Exp Ther* **229**: 493–500, 1984.
22. Powers JF and Sladek NE, Cytotoxic activity relative to 4-hydroxycyclophosphamide and phosphoramide mustard concentrations in the plasma of cyclophosphamide-treated rats. *Cancer Res* **43**: 1101–1106, 1983.
23. Hohorts H-J, Bielicki L and Voelcker G, The enzymatic basis of cyclophosphamide specificity. *Adv Enzyme Regul* **25**: 99–122, 1985.
24. Wagner T, Zink M and Schweider G, Influence of mesna and cysteine on the systemic toxicity and therapeutic efficacy of activated cyclophosphamide. *J Cancer Res Clin Oncol* **113**: 160–165, 1987.
25. Al-Safi SA and Maddocks JI, Does 2-mercaptoethane sulphonate (mesna) prevent cyclophosphamide and azathioprine induced immunosuppression? *In vitro* studies. *Br J Clin Pharmacol* **21**: 267–270, 1986.
26. Pool BL, Bos RP, Niemeyer U, Theuws JLG and Schmahl D, *In vitro/in vivo* effects of Mesna on the genotoxicity and toxicity of cyclophosphamide—A study aimed at clarifying the mechanism of Mesna's anticarcinogenic activity. *Toxicol Lett* **41**: 49–56, 1988.
27. Cos PJ, Cyclophosphamide cystitis—Identification of acrolein as the causative agent. *Biochem Pharmacol* **28**: 2045–2049, 1979.
28. Levy L and Harris R, Effect of *N*-acetylcysteine on some aspects of cyclophosphamide-induced toxicity and immunosuppression. *Biochem Pharmacol* **26**: 1015–1020, 1977.
29. Gurtoo HL, Marinello AJ, Berrigan MJ, Bansal SK, Paul B, Pavelic ZP and Struck RF, Effect of thiols on toxicity and carcinostatic activity of cyclophosphamide. *Semin Oncol* **10**: 35–45, 1983.
30. Levy L and Vredevoe DL, The effect of *N*-acetylcysteine on cyclophosphamide immunoregulation and antitumor activity. *Semin Oncol* **10**: 7–16, 1983.
31. Ormstad K, Orrenius S, Lastbom T, Uehara N, Pohl J, Stekar J and Brock N, Pharmacokinetics and metabolism of sodium 2-mercaptoethanesulfonate in the rat. *Cancer Res* **43**: 333–338, 1983.
32. Shaw IC, Graham MI and Jones MS, The fate of [^{14}C]-Mesna in the rat. *Arzneimittelforschung* **36**: 487–489, 1986.
33. McGown AT and Fox BW, A proposed mechanism of resistance to cyclophosphamide and phosphoramide mustard in a Yoshida cell line *in vitro*. *Cancer Chemother Pharmacol* **17**: 223–226, 1986.
34. Wang AL and Tew KD, Increased glutathione-*S*-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat Rep* **69**: 677–682, 1985.
35. Kawabata TT and White KL Jr, Enhancement of *in vivo* and *in vitro* murine immune responses by the cyclophosphamide metabolite acrolein. *Cancer Res* **48**: 41–45, 1988.