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DESCRIPTIVE SUMMARY WITH CONCLUSIONS:

Based on our previous findings that the exposure of mice to vinyl chloride (VC) caused increased lymphocytic transformation in splenic cultures and stimulation indices by phytomitogens, we investigated the possibility that the metabolism of VC played an important role in this phenomenon. Incubation of splenic cultures in an atmosphere with 1000 ppm VC failed to show any effect. A metabolite of VC, thiodiglycolic acid, produced effects similar to VC exposure both during in vivo exposure of mice to this chemical and after in vitro addition to splenic cell cultures. Modification of VC metabolism by ethanol appreciably inhibited the stimulating property. Administration of phenobarbital also reduced the stimulating effect of VC exposure in lymphocyte cultures and this was assumed to be a result of an increased hepatic binding of chloroethylene oxide, a suspected intermediate in VC metabolism. The studies support the view that VC is metabolized to yield products that are responsible for increased mitotic activity of the splenic lymphocytes.

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ROLE OF VINYL CHLORIDE METABOLISM IN THE STIMULATION OF SPLENIC LYMPHOCYTE TRANSFORMATION IN MICE

September 22, 1978

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ABSTRACT

Based on our previous findings that the exposure of mice to vinyl chloride (VC) caused increased lymphocytic transformation in splenic cultures and stimulation indices by phytomitogens, we investigated the possibility that the metabolism of VC played an important role in this phenomenon. Incubation of splenic cultures in an atmosphere with 1000 ppm VC failed to show any effect. A metabolite of VC, thiodiglycolic acid, produced effects similar to VC exposure both during in vivo exposure of mice to this chemical and after in vitro addition to splenic cell cultures. Modification of VC metabolism by ethanol appreciably inhibited the stimulating property. Administration of phenobarbital also reduced the stimulating effect of VC exposure in lymphocyte cultures and this was assumed to be a result of an increased hepatic binding of chloroethylene oxide, a suspected intermediate in VC metabolism. The studies support the view that VC is metabolized to yield products that are responsible for increased mitotic activity of the splenic lymphocytes.

INTRODUCTION

Exposure of mice to vinyl chloride (VC) has been reported to cause stimulation of immune reactivity in splenic lymphocytes as indicated by an enhancement of spontaneous lymphocyte transformation and the increased response of these lymphocytes to the phytomitogens, phytohemagglutinin (PHA) and pokeweed mitogen (PWM) (Sharma et al. 1977 b). This finding supported an earlier report by Ward et al. (1976) showing the presence of immune complexes in workers exposed to vinyl chloride. The effect observed on lymphocyte transformation was not necessarily dose and time related and appeared to be saturable with increasing concentration of and duration of exposure to vinyl chloride. It is hypothesized that vinyl chloride itself is not biologically reactive but is transformed readily to active metabolites, thus it was postulated that the immunologic effects of vinyl chloride are mediated through one or more of its biotransformation products.

There is evidence that vinyl chloride may be metabolized in the body through the epoxide intermediate, chloroethylene epoxide (Hefner et al., 1975; Barbin et al., 1975). Although not shown conclusively, this potent alkylating intermediate has been hypothesized as the possible proximate carcinogen. Since lymphocyte transformation represents increased mitotic activity of these cells and involves increased synthesis of deoxyribonucleic acid (DNA), this effect may be mediated through the reaction of this epoxide with critical DNA receptors. If such is the case, alteration of VC metabolism should be able to alter the lymphocytic transformation property of VC exposure.

In the studies reported herein, the metabolism of VC was altered by the simultaneous administration of phenobarbital and ethanol in mice. In addition, the immunologic effects of two of the metabolites of VC found in the urine of rodents, i.e., thiodiglycolic acid and N-acetyl-S-(2-hydroxyethyl)-cysteine (Watanabe et al., 1976) were also investigated in mice splenic cell cultures.

METHODS

Male, CD-1 mice, obtained from Charles River (Wilmington, Mass.) were used in all studies. The animals were acclimatized with the surroundings for at least 1 week after arrival and were of 5-6 weeks of age (approximately 30 g in weight) at the beginning of vinyl chloride exposures. Vinyl chloride (Matheson Gas Products, Joliet, Ill.) of 99.9% minimum purity was used. The exposures were conducted either in large exposure chambers as described previously (Sharma et al., 1977 b) or in stainless steel inhalation chambers (1 cubic meter). The concentration of vinyl chloride was checked several times a day by gas chromatographic analysis and was maintained at 1000 ppm for 6 hours per day, Monday through Friday. Food and water were withheld during exposures, but were allowed ad lib at all other times.

Splenic Lymphocyte Cultures. After decapitation, the spleens from mice were removed aseptically and stored temporarily in sterilized isotonic saline solution. Immediately thereafter, the spleens were teased out with forceps and lymphocyte suspensions prepared as described previously (Janossy and Greaves, 1971). The cells were finally suspended in culture medium (RPMI-1640, supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 0.03% L-glutamine, and 10% heat inactivated fetal calf serum) and the counts adjusted to 8×10^6 cells/ml after counting the leukocytes. These cells were cultured in microplates (400,000 cells per culture) without any mitogen or with optimal amounts of photohemagglutinin (PHA) and pokeweed mitogen (PWM). The cultures were conducted in triplicate

or quadruplicate for each condition. After incubation of these for 52 hours in humidified air containing 5% CO_2 at 37°C, 0.5 µCi of ^3H-thymidine was added to each culture and incubation continued for an additional 16 hours. The cells were then harvested, washed vigorously with saline, and radioactivity was counted in a liquid scintillation spectrometer. The counts were converted to disintegrations per minute (DPM) using a standard quench curve, averaged for the replicates, and adjusted for 10^6 cells in culture. Stimulation indices for PHA and PWM were determined by dividing the DPM for cultures in the presence of respective mitogen by the culture from the same preparation containing no mitogen. The details of the microculture system have been described elsewhere (Sharma et al., 1977 a). The incorporation of thymidine by the lymphocytes indicated their DNA synthesis, which is indicative of blastogenesis (transformation) of these cells.

When splenic lymphocyte cultures were incubated in the presence of VC, these were held in specially designed humidified glass containers which were well purged with either 5% CO_2 in air or with 1000 ppm VC in 5% CO_2 in air mixture. These containers were kept in a constant temperature incubator at 37°C for the incubation as described above.

Splenic Cultures in the Presence of VC Metabolites. Cultures of lymphocytes prepared from the spleens of normal mice were conducted in the presence of varying amounts of thiodiglycolic acid and N-acetyl-S-(2-hydroxyethyl)-cysteine. These compounds (preparation and purity described elsewhere, Watanabe et al., 1976) were dissolved in culture medium

and aliquots added to the lymphocyte cultures. Spleens from several animals were often pooled for these studies and cultures conducted as mentioned above.

Exposure of Mice to Thiodiglycolic Acid. Groups of mice were exposed to thiodiglycolic acid in drinking water continuously for 14 days. The concentration of thiodiglycolic acid in water was adjusted to provide 0 (control), 5, 50 and 500 mg/kg/day. Spleens were obtained and cultured as described above.

Alteration of VC Metabolism. Groups of mice were injected ip with either saline, phenobarbital sodium (100 mg/kg), or ethanol (3.2 g/kg as 30% solution) 30 minutes prior to VC exposure. The concentration of phenobarbital and ethanol were adjusted so that all animals (including those receiving saline) were given 10 ml solution/kg (0.3 ml/30 g mouse). These were divided into equal subgroups and were exposed either to normal air atmosphere or to 1000 ppm vinyl chloride in 1 meter^3 inhalation chambers for 5 days. Two days after the last exposure, their spleens were removed and cultured as mentioned above.

Statistical. The results are expressed as means and their standard errors. Comparisons were made with the respective control group using a t-test (Steel and Torrie, 1960) and a predetermined value of $p = 0.05$.

RESULTS

The presence of vinyl chloride *in vitro* in the incubating atmosphere of lymphocyte cultures had no stimulating effect on mouse splenic lymphocyte transformation. The results of one such experiment are shown in Table 1. When animals were exposed *in vivo* to 1000 ppm of VC for a period of 8 weeks, the lymphocytes obtained from their spleens had an increased spontaneous transformation when incubated in normal (CO_2:air) atmosphere, as compared to control animals. The presence of 1000 ppm VC in the incubation environment, in fact, slightly decreased the blast formation of lymphocytes enhanced by exposure of mice to 1000 ppm VC for 8 weeks and the values were comparable to those obtained for lymphocytes cultures prepared from mice with no VC exposure. The stimulating indices by PHA and PWM were likewise unaltered.

Experiments similar to those shown in Table 1 were also conducted in the presence of hepatic metabolizing systems. Addition of liver supernatant supplemented with necessary cofactors (required for microsomal oxidation system, Kappus *et al.*, 1976) inhibited the mitotic activity of lymphocytes considerably. Approximately 30% of normal thymidine uptake was seen in the presence of such a metabolizing system and the results were inconclusive (data not shown). The inhibitory effect of VC *in vitro*, as shown in Table 1, for splenic lymphocytes from VC exposed animals, however, was not observed under such conditions.

Since unaltered vinyl chloride did not produce any influence on the mitotic activity of splenic lymphocytes, the effect of two of its available metabolites was also studied upon direct addition of

these to lymphocyte cultures. The results are shown in Table 2. N-acetyl-S-(hydroxyethyl)-cysteine produced no effect on lymphocyte transformation up to the concentrations of 2.5×10^{-4} M in culture media. No effect of this chemical on phytomitogen induced stimulation of transformation was seen. On the other hand, thiodiglycolic acid produced a significant stimulation of splenic lymphocyte transformation at the concentration of 2.5×10^{-4} M. Lower concentrations of this metabolite of vinyl chloride were found ineffective. The mitogen induced stimulation indices were not affected.

The effect of thiodiglycolic acid was studied in mice after in vivo exposures as well. This was the only metabolite that was available in amounts necessary for in vivo exposures. Mice were exposed to 5, 50 and 500 mg/kg/day of this chemical in the drinking water for a period of 14 days after which their splenic cells were cultured as usual. A dose related effect of thiodiglycolic acid on splenic lymphocyte transformation, as illustrated in Figure 1, was observed. Both spontaneous lymphocytic mitosis as well as mitogen induced transformation was higher in treated groups as compared to respective controls but the stimulation indices (calculated as a ratio of transformation with or without the mitogen) were not enhanced significantly.

Culturing of lymphocytes from animals exposed to vinyl chloride and treated simultaneously with chemicals to modify its metabolism provided interesting results. Administration of phenobarbital or ethanol alone to mice increased the transformation rate of their splenic lymphocytes. To

handle this problem, respective controls that were given these chemicals were exposed to room air simultaneously in control chambers. Saline treated controls were also used in both VC exposed and unexposed groups. Since VC exposure increased the lymphocyte transformation in culture, the differences of ^{3}H-thymidine uptake in VC exposed animals and non-exposed animals were calculated for each drug treatment. The results are shown in Table 3. As established previously, both spontaneous and mitogen induced thymidine uptake were considerably increased by exposing animals to vinyl chloride. Administration of 80 mg/kg/day phenobarbital prior to VC exposure reduced this increment in lymphocyte transformation. Administration of 3.2 g/kg of ethanol prior to VC exposure decreased markedly the stimulating effect of VC exposure to an extent that when the cultures were incubated in the presence of PHA and PWM, their transformation rate, as indicated by tritiated thymidine uptake, was lower than those for animals treated with ethanol and not exposed to VC. Thus, the the last two values presented in Table 3 for the ethanol mice are negative.

DISCUSSION

The results presented in this report support the hypothesis that a metabolite of vinyl chloride is responsible for the stimulating effect seen in lymphocyte transformation. Direct exposure of cultures to vinyl chloride, either from normal mice or from VC exposed animals, was unable to produce any effect. In fact, a slight reduction in the transformation rate was noticed when the cultures were conducted in the atmosphere containing 1000 ppm of vinyl chloride. This may be considered a nonspecific effect of altered culture environment. Although the presence of a hepatic metabolizing system did not produce any conclusive results, this may be because no efforts were made to optimize the VC metabolism in such a system. In vitro metabolism of vinyl chloride has been demonstrated (Kappus et al. 1976) but this requires high concentrations of vinyl chloride in the atmosphere and other conditions that are not favorable for maintaining cell culture systems. This fact, together with the finding that liver tissue contains factors inhibitory to cell mitosis (Nadal et al., 1976, Sharma, 1977) necessitated abandoning this approach.

Thiodiglycolic acid, a known metabolite of vinyl chloride (Watanabe et al., 1976) caused effects similar to those observed with VC exposure. The effect of this chemical after in vitro addition were obvious only at relatively high concentrations of 2.5×10^{-4} M. It is unlikely that the effects of vinyl chloride on lymphocyte transformation are produced via this metabolite. The effect of thiodiglycolic acid may be independent

of its origin from vinyl chloride since several sulfur containing compounds and a glycol have been shown to possess immunostimulatory effects (Renoux et al., 1976; Renoux et al, 1976, Goodman and Weigl, 1977).

Direct evidence that metabolism of vinyl chloride is a determining factor for its lymphocyte stimulating property is derived when its metabolism is altered. Ethanol is known to decrease the metabolism of VC (Hefner et al., 1975; Watanabe et al., 1977 a) and simultaneous administration of ethanol interfered with the immunostimulating property of VC in splenic cultures of exposed mice. Use of phenobarbital also exhibited a similar phenomenon. In studies reported previously, vinyl chloride metabolism was not enhanced by microsomes obtained from phenobarbital treated animals (Kappus et al., 1976). Similarly, although the rate of VC metabolism was not altered, its binding in hepatic tissue was increased (Watanabe et al., 1977 b). This suggests that phenobarbital may induce metabolism through another pathway leading to the formation of a product which more readily binds to macromolecules in liver and thus have little effect on lymphocytes. A slight reduction of lymphocyte stimulating activity in VC exposed animals by simultaneous administration of phenobarbital may be explained on this basis.

The suggestion that the epoxide metabolite of VC, chloroethylene oxide, may be responsible for stimulated lymphocyte transformation is also

supported by our other studies (unreported previously, Sharma et al., 1977 a, 1977 c) with chemicals possessing a similar chemical structure as that of vinyl chloride. Vinyl benzene (styrene monomer), has no effect on lymphocyte transformation when added directly to lymphocyte cultures in vitro. Addition of styrene oxide, on the other hand, in concentrations as low as 2.5×10^{-7} M showed a remarkable increase in such mitotic activity of lymphocytes in splenic cultures.

These studies, together with our other reports that vinyl chloride substantially increases lymphocyte mitosis in splenic cultures (Sharma et al., 1976 b) provide credence to previous reports by Ward et al. (1976) that vinyl chloride syndrome may involve an immunologic phenomenon.

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Table 1

LYMPHOCYTE TRANSFORMATION IN MOUSE SPLENIC CULTURES FROM ANIMALS EXPOSED TO VINYL CHLORIDE AND CULTURES INCUBATED IN THE PRESENCE OF VINYL CHLORIDE (VC) ATMOSPHERE[a]

Animal Group	Incubation Atmosphere	DPM/10^6 Cells (without mitogen)	Stimulation index with PHA	PWM
Control	Normal	6699±1473	8.8±1.5	7.6±1.1
	VC	5080±1804	11.3±2.2	8.2±1.6
VC exposed	Normal	10262±1107	8.5±3.2	10.8±2.0
	VC	6385±822	9.1±3.3	9.3±0.6

[a] Splenic cells from either control or vinyl chloride exposed (1000 ppm, 6 hrs/day, 5 days/wk, for 8 weeks) mice were cultured in appropriate chambers containing either 5% CO_2 in air or 1000 ppm vinyl chloride in CO_2:air atmosphere. The values are mean±SE for 3 animals, each culture run in quadruplicate.

Table 2

INFLUENCE OF IN VITRO ADDITIONS OF VINYL CHLORIDE METABOLITES ON SPLENIC LYMPHOCYTE TRANSFORMATION[a]

Metabolite	Concentration	DPM/10^6 Cells (No mitogen)	Stimulation index with PHA	PWM
Thiodiglycolic acid	0 (Control)	53408	4.4	5.2
	$2.5x10^{-7}$ M	62899	4.1	4.6
	$2.5x10^{-6}$ M	37733	7.3	6.9
	$2.5x10^{-5}$ M	58212	4.8	5.2
	$2.5x10^{-4}$ M	102786*	4.4	3.8
N-acetyl-S-(hydroxy ethyl)-cysteine	0 (Control)	163757	2.8	2.7
	$2.5x10^{-7}$	144416	2.2	2.3
	$2.5x10^{-6}$	154484	2.5	2.4
	$2.5x10^{-5}$	153957	2.5	2.3
	$2.5x10^{-4}$	113485	3.1	3.3

[a]Mean of 4 replicates in each case. Pooled spleens were used in separate experiments for each chemical tested.
*indicate difference from control at $p<.05$.

Table 3

CHANGE IN LYMPHOCYTE TRANSFORMATION IN MICE SPLENIC CULTURES PRODUCED BY THE INHALATION OF 1000 ppm VINYL CHLORIDE FOR ONE WEEK AND IN MICE TREATED WITH PHENOBARBITAL AND ETHANOL TO ALTER THE METABOLISM OF VINYL CHLORIDE

Animals	Δ DPM/10^6 cells[a] in the presence of		
treated with	No mitogen	PHA	PWM
Saline	43,113	121,976	167,844
Phenobarbital	27,770	26,147	55,478
Ethanol	4,929	-147,786	-78,668

[a] (DPM in animals exposed to vinyl chloride)-(DPM in animals held in control chamber). Each value is obtained from an average of 4 animals and cultures of each animal run in triplicate. Negative values shown in last 2 columns were obtained because ethanol treated mice had lower counts in those treated with ethanol and also exposed to vinyl chloride.

LEGEND TO FIGURE 1

Fig. 1 Uptake of ^{3}H-thymidine in splenic lymphocyte cultures of mice exposed to thiodiglycolic acid in drinking water for 14 days. Average of 4 animals each, where each culture was run in triplicate. *indicate significant difference from control at $p<0.05$. The increases in stimulation indices by PHA and PWM were not significant at any dose level of thiodiglycolic acid.

Figure 1