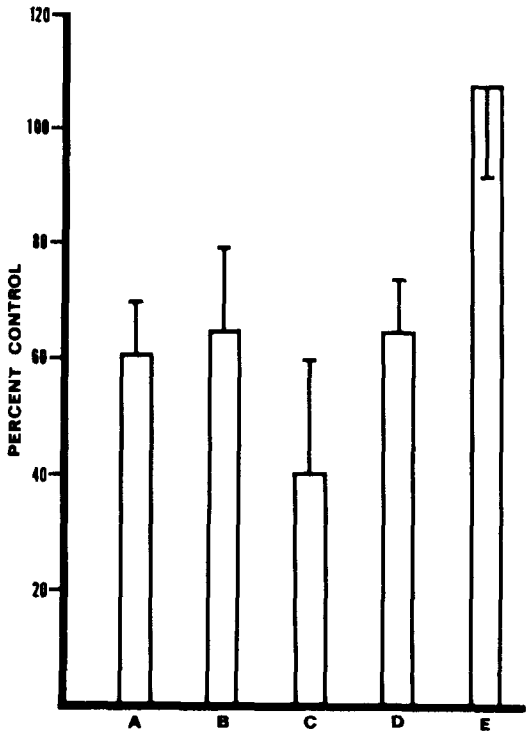

Fig. 2. Inhibition of benzoate hydroxylation expressed as percent products formed versus paired control. Key: (A) DMSO, (B) mannitol; (C) azide; (D) SOD; and (E) catalase.

strated that the hydroxyl radical produced by these cells oxidizes benzoic acid. This observation predicted that the $OH^{\cdot}$ produced by granulocytes would also cause ring hydroxylation of this drug. This possibility was tested in our current experiments. We were able to demonstrate the production of three monohydroxybenzoate isomers—ortho-, meta- and para-hydroxybenzoate—after solutions of benzoate were incubated with stimulated granulocytes. These data provide additional evidence that human PMNs generate $OH^{\cdot}$. Of importance is that our observations suggest that stimulated PMNs may chemically modify xenobiotic materials by ring hydroxylation and thereby their activity at a site of inflammation *in vivo*.

Departments of Hematology and Oncology, and Clinical Pharmacology
The Ohio State University
Columbus, OH 43210, U.S.A.

MICHAEL S. ALEXANDER
ROSE M. HUSNEY
ARTHUR L. SAGONE, JR.*

* Address all correspondence to: Dr. Arthur L. Sagone, Jr., Division of Hematology and Oncology, The Ohio State University, 10 North Doan Hall, 410 West 10th Ave., Columbus, OH 43210.

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Chlorphentermine-induced alterations in the response of human lymphocytes to mitogens

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In recent years numerous studies have shown that the administration of certain cationic amphiphilic drugs (CADs) to animals, and in some instances humans, results in the induction of a lysosomally-derived phospholipidosis in many tissues of the body [1–3]. The hallmark feature of the disorder is the development of phospholipid-rich lamellar inclusions in association with lysosomes in the cell [4]. Of the CADs reported to induce this disorder, chlorphentermine (CP) has been studied the most extensively [1, 5, 6]. One cell type that has been reported to be quite sensitive to the induction of phospholipidosis by CP is the lymphocyte of peripheral blood [6]. The administration of a single dose of this drug to rats results in the appearance of lamellar inclusions in the cell. Other CADs including citalopram [7] and a number of tricyclic antidepressants [8] are capable of inducing the disorder in lymphocytes present in peripheral blood or lymph nodes. At present, we are aware of no studies investigating the consequences of exposure to CP, or any other CAD reported to induce phospholipidosis in this cell, on lymphocyte function. We have initiated studies to address this problem, and in this communication report on the effects of CP exposure *in vitro* on mitogen-induced blastogenesis in human peripheral blood lymphocytes.

Materials and methods

Peripheral blood (30 ml) was drawn from normal, healthy males by venipuncture into heparinized Vacutainer blood collection tubes (Becton Dickinson, Rutherford, NJ). All subjects were volunteers who signed a consent form approved by the West Virginia University Institutional Review Board for the Protection of Human Subjects.

All cultures were carried out in RPMI 1640 medium (MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, 5×10^{-5} M mercaptoethanol, 10 mg pyruvate/100 ml, 2 μg asparagine/100 ml (Sigma, St. Louis, MO), 100 units penicillin/100 ml (Squibb, Princeton, NJ), 100 μg

streptomycin/100 ml and 0.25 mg fungizone/100 ml (Gibco, Grand Island, NY).

Peripheral blood was diluted 1:7 using Hanks' Balanced Salt Solution (HBSS) supplemented with 0.35 g of $NaHCO_3$/l, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 10 mM glucose, 1% FBS, 200 units penicillin/l, and 200 μg streptomycin/l and the pH was adjusted to 7.4 using 1 ml of 4 N NaOH. Lymphocyte-enriched samples were obtained by underlaying 10 ml of Percoll, diluted to a density of 1.077 g/ml with HBSS containing 1% HEPES, below 25 ml of diluted blood. Preparations were centrifuged at 400 *g* for 30 min at 4°. The enriched lymphocyte fraction, located at the interface, was removed and washed with 10 vol. of supplemented HBSS. Pelleted cells were resuspended in 2 ml of culture medium, and an aliquot was counted on a hemocytometer using Turk's Solution. Cellular viability was assessed using 0.2% trypan blue.

Slides were prepared from samples of the lymphocyte-enriched fraction isolated by Percoll separation, using a Cytospin 2 (Shandon, Cheshire, England). Slides were stained by exposure to 1.0 ml of Wright–Giemsa stain (Harleco, Gibbstown, NJ) for 2 min followed by a 4-min exposure to 1.25 ml of buffer (from a stock consisting of 4 ml of 0.067 M Na_2HPO_4 and 11 ml of 0.067 M KH_2PO_4, pH 6.5). Cellular morphology was assessed using oil immersion light microscopy. The percentage of lymphocytes, monocytes, erythrocytes and other cells within each sample was determined.

The lymphocyte suspension was adjusted to a concentration of 5×10^5 cells/ml. CP was dissolved in saline and the appropriate concentration was added. (Chlorphentermine was obtained from Warner-Chilcott, Morris Plains, NJ.) Then 0.2-ml aliquots of the suspension were dispensed in triplicate into plastic, flat-bottomed, 96 well microtiter plates (Corning Glass Works, Corning, NY) for each experiment. The appropriate mitogens [phytohemagglutinin (PHA), Wellcome, Beckenham, England, 1 μg/ml; concanavalin A (Con A) 2.5 μg/ml; or pokeweed mitogen (PWM), Gibco, Grand island, NY, 1:100 dilution of a 5 ml stock] were added. (Experiments were initially performed using four different mitogen concentrations. Only results from the optimal mitogen concentration are presented.) The cultures were incubated without shaking at 37° in an atmosphere of 5% CO_2 in air (Queue CO_2 Incubator, Parkersburg, WV). Eighteen hours before harvesting, 10 μl of 20 μCi/ml [^{3}H]thymidine (sp. act. 6.7 Ci/mmole, New England Nuclear, Boston, MA) was added to each well to quantify cellular blastogenesis. An 18-hr pulse, as opposed to the standard 6-hr pulse, was chosen because investigations have shown that longer labeling times with low specific activity isotopes increase the sensitivity of assessing [^{3}H]thymidine incorporation [9]. Cells were harvested at 24, 48 and 72 hr using a Titertek Cell Harvester (Flow Laboratories, Rockville, MD). Filter disks were placed into miniscintillation vials (VWR, Bridgeport, NJ), and 5 ml of scintillation fluid (Scinti Verse, Fisher Scientific, Fairlawn, NJ) was added to each vial. Radioactivity incorporated into the cells was measured using a Packard model C2425 Liquid Scintillation Spectrometer.

Electron microscopy was carried out on lymphocytes cultured for 3 days with 10^{-5} M CP and Con A present. Cells were isolated, washed in phosphate-buffered saline (PBS, pH 7.4) and then resuspended in 3% glutaraldehyde in PBS for 1 hr at 4° and post-fixed in 1% OsO_4 in PBS for 1 hr. Cells were then dehydrated through graded ethanol, cleared in propylene oxide, and embedded in Epox 812 Resin (E. Fullam, Inc., Schenectady, NY). Thin sections were cut on a DuPont-Sorvall MT-5000 Ultramicrotome, stained with 3% uranyl acetate (w/v) for 20 min, followed by Reynolds' lead citrate [10] for 5 min. Sections were viewed on a JEM-1000 CX TEMSCAN electron microscope.

To assess the formation of CP metabolites, lymphocytes were exposed to 10^{-5} M [^{14}C]chlorphentermine (10 μCi/ml) and PHA for 3 days in culture. [^{14}C]Chlorphentermine was obtained from Dr. Alan G. E. Wilson, Monsanto Co., and purified as previously described [11]. After incubation, the lymphocyte suspension was isolated and enough 4 N NaOH was added to alkalinize the sample to make it 1 N NaOH (final pH = 13). Diethyl ether was then added to extract organic-soluble material. The mixture was shaken using a vortex, and then centrifuged (300 *g*) to separate the solution into two layers. The upper (organic phase) layer was found to contain greater than 98% of the ^{14}C label. The upper label was isolated, and thin-layer chromotography was performed using two different solvent systems (CH_3OH–$CHCl_3$–concentrated NH_4OH, 88/9/3, by vol., or CH_3CH_2OH–concentrated NH_4OH, 80/20, v/v) to assess for metabolites.

The effect of CP on mitogen-induced agglutination was determined by the method of Pfeifer and Patterson [12]. Lymphocytes were diluted to a concentration of 1.33×10^6 cells/ml. The cellular suspension was pre-incubated for 1 hr at 37° with either 10^{-5} M chlorphentermine (CP) or 10^{-6} M colcemid (microfilament inhibitor). Following preincubation, 1.5 ml of the cells was dispensed into 16 × 125 mm test tubes, and either 0.5 ml saline, 0.5 ml PHA (20 μg/ml), 0.5 ml Con A (50 μg/ml) or 0.5 ml PWM (10 μg/ml) was added. The cells were centrifuged at 300 *g* for 1 min and then incubated at 37° for 45 min. Following incubation, the tubes were gently mixed and the turbidity was determined as the absorbance at 620 nm.

Experiments were performed to determine the time frame during which CP must be present in the incubation medium to impair blastogenesis. Aliquots of cells were preincubated with 10^{-5} M CP for either 30 or 60 min and then stimulated with PHA (1 μg/ml). Additional aliquots of cells were stimulated with PHA (1 μg/ml); then at distinct time intervals (0, 10, 20, 30, 30, 60 min) the samples were exposed to 10^{-5} M CP. With all samples, blastogenesis was quantified 2 days later using [^{3}H]thymidine as previously described.

Data were compared using an analysis of variance appropriate for the experimental design (two-way with repeated measures on one factor). The significance of difference between treatment means was tested using the Fisher least significant difference test [13]. Statistical significance was established at $P < 0.05$.

Results and discussion

The cell suspensions used for each treatment were isolated from a single individual and consisted, on an average, of 67% human peripheral blood lymphocytes, 28% monocytes, 4% erythrocytes, and 1% other cells.

In the presence of 10^{-5} M CP and Con A, a phospholipidosis was induced in lymphocytes following 3 days of culture. One to two lamellar inclusions were found in a number of lymphocytes.

Lymphocytes were responsive to all three mitogens during the culture period. In the absence of drug, uptake of [^{3}H]thymidine was small on day 1 but increased markedly on days 2 and 3 with the average stimulation index (cpm in the presence of mitogen/cpm in the absence of mitogen) calculated at 235, 80 and 70 for lymphocytes responding to PHA, Con A and PWM, respectively, after 3 days in culture.

Compared with controls the blastogenic response of drug-exposed human peripheral blood lymphocytes to all three mitogens was lower on day 1, but because of the low stimulation index on this day, the extent of inhibition was quite variable and we, therefore, focussed our attention on the responses on days 2 and 3. On day 2, blastogenesis induced by all three mitogens was inhibited by CP at doses from 10^{-6} M to 10^{-4} M (Fig. 1). At 10^{-6} M the responses to

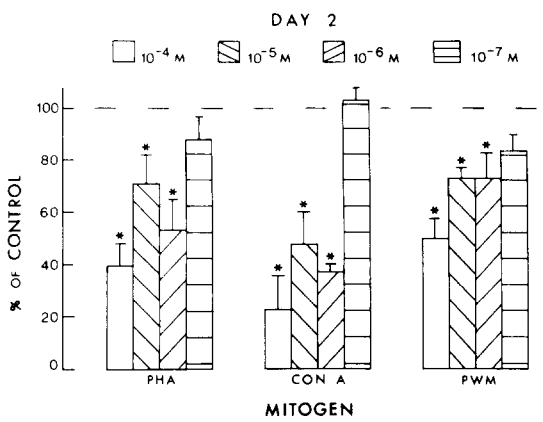

Fig. 1. Blastogenic response of human peripheral lymphocytes exposed to chlorphentermine for 2 days in culture. Values (N = 5 experiments) are expressed as a percent of control (mitogenic response in the absence of drug) ± S.E. and reflect the amount of [^{3}H]thymidine taken up by cultures originally plated with 10^5 cells. Control values were 34,058 ± 4,299 cpm in response to PHA, 5,605 ± 1,227 cpm in response to Con A, and 7,697 ± 1,254 cpm in response to PWM. An asterisk (*) indicates $P < 0.05$ compared to the control value (no drug).

PHA, Con A and PWM were inhibited 45, 60 and 25 respectively. No significant inhibition occurred with any mitogen at lower doses of CP. By day 3, lymphocyte responses to mitogenic stimulation were at control levels (Fig. 2). It appeared from these results that the normal time course of blastogenesis had been delayed by 24 hr. The lower day 2 response was not due to cell death as drug exposure had no effect on cellular viability (data not shown), and the response would not otherwise have reached control levels on day 3.

We examined the possibility that "recovery" in the blastogenic response was caused by CP metabolism or breakdown during the incubation period. It was postulated that metabolism of CP would have reduced drug levels enough to allow the recovery on day 3 to occur. Using thin-layer chromatographic analysis, only CP was detected in cultures when the response was at control levels (day 3); no metabolites were found. Therefore, recovery was occurring even with continuous exposure to CP.

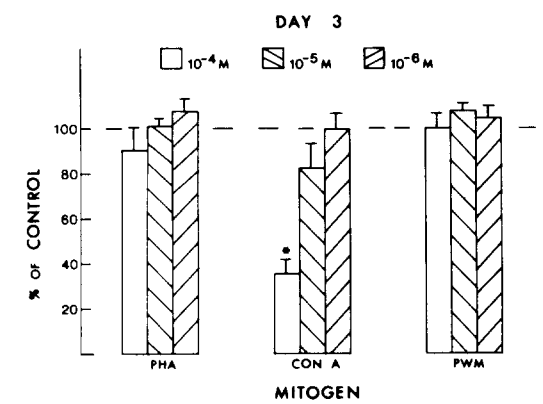

Fig. 2. Blastogenic response of human peripheral blood lymphocytes exposed to chlorphentermine for 3 days in culture. Values (N = 5 experiments) are expressed as a percent of control (mitogenic response in the absence of drug) ± S.E. and reflect the amount of [^{3}H]thymidine taken up by cultures originally plated with 10^5 cells. Control values were 56,337 ± 4,170 cpm in response to PHA, 25,271 ± 4,812 cpm in response to Con A, and 16,126 ± 2,158 cpm in response to PWM. An asterisk (*) indicates $P < 0.05$ compared to the control value (no drug).

Our results show that inhibitory effects of CP on the blastogenic response of human peripheral blood lymphocytes to PHA on day 2 were dependent on the time during activation that the lymphocytes were exposed to the drug. If lymphocytes were preincubated with CP for 30 min, then stimulated with PHA, the blastogenic response was decreased 45%. Preincubation of the cells with CP for 60 min did not result in further inhibition. When lymphocytes were exposed to CP and PHA simultaneously, a lesser inhibition of blastogenesis was observed (18% inhibition). If cells were stimulated with PHA, then exposed to CP 10 min later, the blastogenic response was not significantly different from that of controls. No inhibition was noted when CP was added at times later than 10 min after exposure to PHA. We did not examine this time-dependency in detail with the other mitogens.

In the presence of PHA, Con A and PWM, human peripheral blood lymphocytes will maximally agglutinate within 45 min. Our results show that CP significantly potentiated Con A-induced agglutination, and caused an apparent potentiation of PHA-induced agglutination although this was not statistically significant (Table 1). The effects of CP on PWM-induced agglutination were negligible. Colcemid, a microfilament inhibitor, was used as a positive control and was effective in totally or partially inhibiting the ability of all the mitogens to cause agglutination.

Table 1. Mitogen-induced agglutination of human peripheral blood lymphocytes in the presence and absence of CP and colcemid

Agglutination assay condition	O.D. (620 nm)
Cells	0.233 ± 0.015
Cells/Con A	0.176 ± 0.031*
Cells/Con A/CP	0.144 ± 0.020*†
Cells/Con A/Colcemid	0.204 ± 0.028*
Cells/PHA	0.145 ± 0.015*
Cells/PHA/CP	0.129 ± 0.015*
Cells/PHA/Colcemid	0.194 ± 0.015*
Cells/PWM	0.177 ± 0.016*
Cells/PWM/CP	0.168 ± 0.015*
Cells/PWM/Colcemid	0.219 ± 0.014

Values (mean of five experiments ± S.E.) are presented as the O.D. at 620 nm.

* $P < 0.05$ compared to "cells" value. † $P < 0.05$ compared to "cells/Con A" value.

CP has been shown previously to induce phospholipidosis in the circulating lymphocytes of rats [6]. Using electron microscopy, we have shown that this disorder can be induced *in vitro* in human lymphocytes from peripheral blood. Moreover, when incubated for 2 days in culture with CP, the cells demonstrate a significantly depressed ability to respond to the T-cell mitogens, PHA and Con A, and the B-cell mitogen, PWM. Therefore, day 2 blastogenesis of both classes of lymphocytes appears to be impaired by CP. In contrast to these findings, we observed that after 3 days in culture with CP, the human lymphocytes recovered from the drug effects, demonstrating a blastogenic response similar to that of non-drug treated controls. The results indicated that CP caused a delay in blastogenesis. We examined the possibility that recovery was due to metabolism of the drug or its breakdown during the 3-day incubation leading to a reduction in the effective concentration of CP in the culture. Neither metabolism nor breakdown

of CP occurred as only the parent drug was present in the incubation medium after 3 days. It has been shown previously that phospholipidosis induced by CADs in lymphocytes is reversible upon termination of drug treatment [7, 14]. Our observation is unique in that functional recovery occurred in the presence of the drug at a time when lamellar inclusions were present.

Investigations into the mechanism behind the delay in blastogenesis indicated that the apparent inhibition produced by CP on day 2 depended on the time at which lymphocytes were exposed to drug in relation to the time they were stimulated with mitogen. If lymphocytes were exposed to CP prior to or concurrently with stimulation by PHA, a significant reduction of blastogenesis was observed. On the other hand, if lymphocytes were stimulated with PHA and then exposed to CP 10 min later, no inhibition of blastogenesis resulted. This finding is consistent with those of others who reported inhibition of lymphocyte blastogenesis by the CADs, chlorpromazine [15] and nortriptyline [16]. Accordingly, if these drugs are added after the mitogen, inhibition is diminished or does not occur. In none of these reports however, was loss of inhibition shown in as short a time as our data indicate. Therefore, CP appears to affect an early event occurring during the first 10 min of mitogen-induced lymphocyte activation. There are a myriad of events occurring in the first 10 min of lymphocyte activation. These include an interaction of the mitogen with its surface membrane receptor [17], cellular depolarization [18], Ca^{2+} influx [18], changes in intracellular cyclic nucleotide levels [19], and alterations in membrane phospholipids [20].

In an attempt to gain insight into which event was affected by CP, we examined the effects of CP on mitogen-induced agglutination. We have shown that CP significantly potentiated Con A-induced agglutination of human peripheral blood lymphocytes and caused an apparent potentiation of PHA-induced agglutination. Although the mechanism behind mitogen-induced agglutination is unknown, it has been shown that agglutination will increase as the plasma membrane fluidity increases [21–24]. Together, these results suggest that CP alters a component of the lymphocyte plasma membrane architecture. Other investigators [21–23] have observed that the drugs ketamine, lidocaine, diazepam and dibucaine potentiate agglutination and increase membrane fluidity. In addition, these drugs also inhibit the blastogenic response of lymphocytes to mitogens [25, 26]. These observations suggest that there may be a link between potentiation of agglutination, increased membrane fluidity, and alteration in blastogenesis.

The appearance of lamellar inclusions within cells incubated with CP indicates that an inhibition in phospholipid metabolism occurred as a result of drug exposure [27]. It is therefore possible that the delayed blastogenic responses were linked in some manner to an initial impairment in phospholipid metabolism involved in a critical step of cell activation. As a result of this impairment, the kinetics of activation were shifted as observed. It is of interest that the chronic treatment of humans with another CAD, amiodarone, induces lamellar inclusions in peripheral blood lymphocytes [28]. At present, it is unknown as to whether patients using this drug, or other CADs, demonstrate alterations in lymphocyte function. Based upon our results, however, such a possibility should be examined.

In summary, CP has an ability to inhibit or delay mitogen-induced blastogenesis. It appears that this drug exerts its toxicity by inducing alterations at an early and critical time during cellular activation. Interestingly, the cells are able to overcome this impairment in function in the presence of the drug by a yet unidentified mechanism. Finally, a possible relationship between the development of phospholipidosis and the alteration in lymphocyte function induced by CP is proposed but is speculative at present. Additional studies are required to gain a better understanding of these initial observations.

*Departments of *Pharmacology and Toxicology and †Anatomy, West Virginia University Medical Center, Morgantown, WV 26506, U.S.A.*

LEONARD J. SAUERS*
DANIEL WIERDA*
ELIZABETH R. WALKER†
MARK J. REASOR*‡

‡ Author to whom all correspondence should be sent.

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