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Noradrenergic responses of peripheral organs to cyclophosphamide in mice

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Abstract

To determine if the chemotherapeutic drug cyclophosphamide influences the activity of the sympathetic nervous system, the effects of cyclophosphamide on norepinephrine concentration in the heart, adrenal gland, spleen, and thymus gland were evaluated. Male BALB/cByJ mice were administered a single injection of cyclophosphamide (15, 50, or 100 mg/kg, i.p) or saline-vehicle. Organs were collected 72 or 120 h after injection and norepinephrine concentrations were determined by high pressure liquid chromatography with electrochemical detection. Cyclophosphamide reduced spleen, thymus gland, and heart mass while also elevating spleen and thymus gland norepinephrine concentrations (both pmoles/mg tissue and pmoles/mg protein) in a dose- and time-dependent manner. Norepinephrine concentrations in heart and adrenal gland were not altered by cyclophosphamide at any drug dose or time point. Dose- and time-dependent cyclophosphamide-mediated changes in peripheral norepinephrine levels in the spleen and thymus gland are interesting because subjects administered cyclophosphamide may be more susceptible to opportunistic infections, not only because the drug is antineoplastic, but also because the drug alters nervous system-immune system communication and the neurochemical milieu in which surviving cells interact.

Introduction

Cyclophosphamide is a nitrogen mustard used to inhibit the growth of some tumors, to treat various autoimmune disorders, and to prevent rejection of organ transplants (Singer and McCune, 1998; Colvin, 1999). Most research involving cyclophosphamide has examined the cells targeted by cyclo- phosphamide and the mechanisms through which cyclophosphamide influences these targets. These studies show that cyclophosphamide is activated by liver cytochrome P450 and the resulting bioactive metabolites prevent mitosis of tumor cells or myeloid cells by alkylating DNA and preventing cell division (Ludeman, 1999).

Little work has investigated non-cytotoxic in vivo consequences of cyclophosphamide administration. It is not known to what extent cyclophosphamide alters factors that could influence the activity of cells that survive initial cyclophosphamide exposure or if organs without proliferating cells are responsive to this cytotoxic drug. Changes in the chemical milieu from cyclophosphamide administration could contribute to the side-effects associated with cyclophosphamide treatment (such as fatigue, nausea, headaches, or taste aversion). In addition, non-cytotoxic effects of cyclo- phosphamide could contribute to the physiological mechanisms underlying reports that behavioral conditioning, psychological stress, and environmental (e.g., seasonal) factors influence the ability of cyclophosphamide to inhibit tumor growth and suppress rejection of tissue allografts (Gorczynski, 1990; Perissin et al., 1991; Giraldi et al., 1994; Perissin et al., 1996; Zorzet et al., 1998, 2002). Indeed, in recent studies, cyclophosphamide has been shown interact with anti-angiogenic drugs to reduce tumor metastasis (Mauceri et al., 2002) and to improve the efficacy of cytochrome P-450 based gene therapy (Jounaidi and Waxman, 2001). It is possible that cyclophosphamide-mediated changes in the neurochemical milieu could play a role in the mechanisms subserving these observations.

This experiment investigated the possibility that cyclophosphamide might alter the noradrenergic environment of organs innervated by the sympathetic nervous system. Norepinephrine concentrations following cyclophosphamide treatment were evaluated in organs with ongoing cell proliferation (spleen and thymus gland) and in organs not typically associated with cell proliferation (heart and adrenal gland). These experiments sought to confirm and extend previous observations that cyclophosphamide induces dose- and time-dependent changes in the norepinephrine content of the spleen (Karp and Szczytkowski, 2003). We considered two possible outcomes. First, cyclophosphamide might alter norepinephrine concentrations in all

peripheral organs innervated by the sympathetic nervous system. Alternatively, cyclophosphamide might only alter norepinephrine concentrations in organs with a high percentage of proliferating cells.

Materials and methods

Animals

Male BALB/cByJ mice (n = 64, aged 3–5 wks, The Jackson Labs, Bar Harbor, ME) were the subjects of these experiments. Mice were housed 4 per cage in standard shoe box cages and allowed to acclimate to our colony for 2–3 wks prior to the start of an experiment. Food (Purina rodent chow #5015) and water were available at all times. All the mice in a cage always received the same treatment. The Rider

University Institutional Animal Care and Use Committee approved all animal procedures in accordance with the guidelines established by the National Institutes of Health.

Injections

Cyclophosphamide (Sigma-Aldrich, St. Louis, MO) was solubilized in ethanol, diluted in sterile filtered phosphate buffered saline (GIBCO, Carlsbad, CA), and administered by peritoneal injection (in a total volume of 500 Al). Vehicle-treated animals received an equal volume of the phosphate buffered saline-ethanol solution. The body mass of the mice was determined 3 days before cyclophosphamide or vehicle injections.

Organ harvest and norepinephrine measurements

Mice were killed by rapid cervical dislocation either 72 or 120 h after injection of cyclophosphamide or vehicle (time course selected from Karp and Szczytkowski, 2003). Spleen, thymus gland, heart, and one adrenal gland were immediately harvested, weighed, placed in cold 0.1M HClO4 (spiked with an internal standard of 3,4-dihydroxybenzylamine to a final

concentration 0.25 AM), and stored at 80 8C until assayed. At the time of assay, samples were homogenized (Sonic Dismembrator, Artek Systems Corporation, Farmingdale, NY), centrifuged and aliquots of the supernatants were combined with sodium phosphate buffer (pH 6.1) and 1.5M Tris/EDTA buffer (pH 8.6). Acid-washed alumina was added to each sample and norepinephrine was extracted by shaking for several minutes. The alumina was washed with distilled water, placed into a clean microfilter and the water was removed by centrifugation. Norepinephrine was eluted off the alumina by adding 0.1 M HClO4. Sample extracts in the 0.1 M HClO4 were stored on ice until they were manually injected into the high-performance liquid chromatography (HPLC) machine.

The HPLC system consisted of a Varian ProStar programmable pump and a Varian Res Elut C18 reverse-phase column (150 mm x 4.6, 5 A) located in a temperature controlled housing (27 8C) with the electrochemical detector (Varian Star 9080). The system was operated at a flow rate of 1.0 ml/min with the detector potential set at 0.75 V versus an Ag-AgCl reference electrode. The mobile phase buffer consisted of 0.33 M citrate, 0.67 M phosphate (pH 4.5) with sodium octyl sulfate (1.2 mM) and 12% methanol. The signal from the detector was recorded by a PC computer using Varian Star Chromatography software (Star Chromatography, version 5). The Varian Star Chromatography software measured area under each peak and compared the value obtained to the internal standard (5 pmoles of 3,4-dihydroxybenzylamine).

Protein assays

The protein content of each sample was determined using a commercially available protein assay kit (Pierce, Rockford, IL). This assay is based on the bicinchroninic acid method for the colorimetric detection and quantification of total protein. Directions recommended by the supplier for conducting this assay on 96-well plates were followed and the assay reaction was

read using a plate reader at 570 nm (SpectraCount Microplate Photometer, Packard Bioscience Company, Meriden, CT). Protein content was read off a standard curve of known amounts of bovine serum albumin.

Data analysis

Data were evaluated at each time point by ANOVA followed by the Student-Newman-Keuls post-hoc test (SPSS for Windows version 11.0, SPSS Inc., Chicago, IL). Treatment effects were considered significant at p V 0.05. All data are presented as mean F SEM.

Results

Norepinephrine concentrations in spleen

Spleen mass was reduced by cyclophosphamide administration in a dose-dependent manner (Fig. 1A). At the 72 hr time point, all three cyclophosphamide doses decreased spleen mass ($F(3,31) = 50.1$, p b.01); among the cyclophosphamide-treated groups, the mice administered 100 mg/kg exhibited smaller spleens compared to the mice administered 50 mg/kg and mice in the 50 mg/kg condition exhibited smaller spleens than mice in the 15 mg/kg group. At the 120 hr time point, cyclophosphamide-mediated reductions in spleen mass persisted, as all cyclophosphamide treatments reduced spleen mass compared to saline-vehicle ($F(3,31) = 18.5$, p b .01). Amongst the cyclophosphamide-treated conditions at 120 h, mice administered 100 mg/kg cyclophosphamide exhibited smaller spleens than the mice in the 15 and 50 mg/kg groups.

Cyclophosphamide elevated the concentration of norepinephrine (pmoles per mg spleen) in a dose- and time-dependent manner (Fig. 1B). At 72 h ($F(3,30) = 15.6$, p b .01), mice in the 50 and 100 mg/kg conditions showed a greater concentration of norepinephrine in comparison to

mice the saline-vehicle and 15 mg/kg conditions. At 120 h no statistical differences were observed between the saline-vehicle and any of the cyclophosphamide-treated conditions.

The concentration of norepinephrine (pmoles per mg protein) in the spleen was also elevated by cyclophosphamide in a dose- and time-dependent manner (Fig. 1C). At the 72 hr time point ($F(3,30) = 15.2$, p b .01), spleens from the mice administered 50 and 100 mg/kg cyclophosphamide exhibited significantly more norepinephrine per mg protein than the saline-vehicle and 15 mg/kg groups. By 120 h after injection no statistical differences were observed between the saline-vehicle and any of the cyclophosphamide-treated groups.

The total amount of norepinephrine per spleen was calculated by multiplying the concentration of norepinephrine per mg spleen x the mass of the spleen (Table 1). These calculations allow an estimate of the effects of cyclophosphamide on norepinephrine turnover. These analyses revealed neither an effect of cyclophosphamide dose (0, 15, 50, 100 mg/kg) nor an effect of time (72 or 120 h) on the total norepinephrine content of the spleen.

Norepinephrine concentrations in thymus

Thymus mass was reduced by cyclophosphamide administration in a dose-dependent manner (Fig. 2A). At the 72 hr time point, all three cyclophosphamide doses significantly decreased thymus mass compared to saline-vehicle ($F(3,31) = 39.6$, p b .01). The magnitude of these changes at 72 h were dose- dependent as the 100 and 50 mg/kg groups were less than the 15 mg/kg group, and the 15 mg/kg group was less than only the saline-vehicle treated group. At the 120 hr time point, all cyclophosphamide doses

Fig. 1. Effects of cyclophosphamide (CY) on spleen mass (mg, 1A), norepinephrine (NE) per mg spleen (pmoles/mg, 1B), and norepinephrine per mg protein in the spleen (pmoles/mg protein, 1C). Spleens were harvested 72 or 120 h after an i.p. injection of 0, 15, 50 or 100 mg/kg cyclophosphamide. Asterisk (*) indicates significantly different from saline at same time point. All values are mean F s.e.m. (n = 8/group).

reduced thymus mass compared to saline-vehicle ($F(3,31) = 12.5$, p b .01) and the cyclophosphamide- treated conditions did not differ from each other.

Cyclophosphamide elevated the concentration of norepinephrine (pmoles per mg thymus) in a dose- and time-dependent manner (Fig. 2B). At 72 h ($F(3,31) = 21.0$, p b .01), thymus norepinephrine concentration was elevated among mice in the 50 and 100 mg/kg groups compared to the saline- vehicle and 15 mg/kg groups. The 100 mg/kg group was also elevated compared to the 50 mg/kg group at 72 h. Cyclophosphamide mediated elevations in norepinephrine per mg thymus persisted 120 h after a single cyclophosphamide injection ($F(3,31) = 6.0$, $p = .03$) with only the mice treated with 100 mg/kg cyclophosphamide showing elevated thymus norepinephrine concentrations compared to saline-vehicle.

Table 1

Organ Norepinephrine Content

	total pmoles/organ at 72 h				total pmoles/organ at 120 h			
	saline	CY 15	CY 50	CY 100	saline	CY 15	CY 50	CY 100
spleen	200.7 F 20.0	15 5.0 F 8.6	1 71.9 F 17.2	1 81.2 F 9.4	7 8.6 F 26.5	9 1.4 F 26.9	11 2.4 F 28.6	86. 4 F 25.5
thymus	35.7 F 3.5	5 1.0 F 4.3	6 2.0 F 5.8*	78.7 F 12.6*	2 8.9 F 11.4	2 9.8 F 11.0	3 1.6 F 11.5	77.3 F 11.9*
heart	504.4 F 23.5	55 5.4 F 35.3	6 08.0 F 64.9	5 65.3 F 35.4	60 7.5 F 54.5	52 8.6 F 57.1	47 9.7 F 48.7	63 5.1 F 29.3
adrenal	14655.8 F 1981.0	2070 1.0 F 2144.0	151 52.3 F 984.5	1759 8.6 F 1442.3	2216 5.1 F 1443.9	2474 5.3 F 1905.3	2355 8.5 F 2190.7	18 157.5 F 961.8

Total norepinephrine content was calculated as norepinephrine per mg tissue × the mass of each organ. These calculations estimate the effects of cyclophosphamide (CY) on norepinephrine turnover in each of the indicated organs. Asterisk (*) indicates significantly different from saline at same time point. All values are mean pmoles per organ F s.e.m. (n = 8/group).

Total norepinephrine content was calculated as norepinephrine per mg tissue x the mass of each organ. These calculations estimate the effects of cyclophosphamide (CY) on norepinephrine turnover in each of the indicated organs. Asterisk (*) indicates significantly different from saline at same time point. All values are mean pmoles per organ F s.e.m. (n = 8/group).

Fig. 2. Effects of cyclophosphamide (CY) on thymus gland mass (mg, 2A), norepinephrine (NE) per mg thymus (pmoles/mg, 2B), and norepinephrine per mg protein in the thymus (pmoles/mg protein, 2C). Thymus glands were harvested 72 or 120 h after an i.p. injection of 0, 15, 50 or 100 mg/kg cyclophosphamide. Asterisk (*) indicates significantly different from saline at same time point. All values are mean F s.e.m. (n = 8/group).

The concentration of norepinephrine (pmoles per mg protein) in the thymus was also elevated by cyclophosphamide in a dose- and time-dependent manner (Fig. 2C). At 72 h ($F(3,31) = 12.6$, p b .01), the 50 and 100 mg/kg groups exhibited significantly more norepinephrine per mg protein than the saline and 15 mg/kg groups. An effect of cyclophosphamide on norepinephrine per mg protein in the thymus endured at 120 h ($F(3,31) = 6.5$, p b .01) but by this time only the 100 mg/kg group remained elevated compared to the saline group.

A single injection of cyclophosphamide elevated the total amount of norepinephrine (pmoles per thymus, Table 1) at 72 ($F(3,31) = 5.9$, p b .01) and 120 ($F(3,31) = 4.2$, $p = .01$) h after injection. At

72 h, thymus glands from both the 50 and 100 mg/kg groups exhibited increases in total norepinephrine content compared to the saline-vehicle and 15 mg/kg group. At 120 h, only thymus glands from the 100 mg/kg group still exhibited elevations in total norepinephrine content compared to saline-vehicle.

Norepinephrine concentrations in heart

Cyclophosphamide-mediated reductions in heart mass were evident 72 h ($F(3,31) = 3.2$, $p = .04$) and 120 h ($F(3,31) = 2.9$, $p = .05$) after injection; 100 mg/kg lowered the total mass of the heart compared to

Fig. 3. Effects of cyclophosphamide (CY) on heart mass (mg, 3A), norepinephrine (NE) per mg heart (pmoles/mg, 3B), and norepinephrine per mg protein in the heart (pmoles/mg protein, 3C). Hearts were harvested 72 or 120 h after an i.p. injection of 0, 15, 50 or 100 mg/kg cyclophosphamide. Asterisk (*) indicates significantly different from saline at same time point. All values are mean F s.e.m. (n = 8/group).

saline-vehicle and to the other cyclophosphamide-treated conditions (Fig. 3A). There were no effects of cyclophosphamide treatment on any measure of norepinephrine concentration (Fig. 3B–C) or total norepinephrine per heart (Table 1).

Norepinephrine concentrations in adrenal gland

Adrenal gland mass, norepinephrine per mg adrenal tissue, norepinephrine per mg protein (Fig. 4A– C), and the total amount of norepinephrine per adrenal gland (Table 1) were not altered by cyclophosphamide at either 72 or 120 h after injection.

Fig. 4. Effects of cyclophosphamide (CY) on adrenal gland mass (mg, 4A), norepinephrine (NE) per mg adrenal gland (pmoles/ mg, 4B), and norepinephrine per mg protein in the adrenal gland (pmoles/mg protein, 4C). Adrenal glands were harvested 72 or 120 h after an i.p. injection of 0, 15, 50 or 100 mg/kg cyclophosphamide. Asterisk (*) indicates significantly different from saline at same time point. All values are mean F s.e.m. (n = 8/group).

Discussion

These data confirm and extend observations that cyclophosphamide induces dose- and time- dependent elevations in norepinephrine concentration in the spleen (Karp and Szczytkowski, 2003). We additionally demonstrate that cyclophosphamide elevates norepinephrine concentration in the thymus gland but not in the heart or adrenal gland. It appears therefore that cyclophosphamide does not cause a widespread activation of the sympathetic nervous system as measured by changes in norepinephrine concentration. Instead, cyclophosphamide appears to alter the amount of norepinephr- ine available to influence cell-cell interactions in tissues associated with immune system function. It is therefore possible, though not shown here, that given the effects of norepinephrine on cells of the immune system, that cyclophosphamide is immunomodulatory in addition to being cytotoxic and immunosuppressive.

It is possible that cyclophosphamide-induced reductions in organ mass are responsible for cyclophosphamide-induced elevations in organ norepinephrine content. However reductions in organ mass were not always associated with elevations in norepinephrine in the spleen, thymus gland, or heart (see Figs. 1–3). Additional experiments are needed to determine the mechanism(s) through which changes in organ mass and norepinephrine concentration are associated. It is possible that cyclophosphamide-mediated reductions in organ mass might occur from a transient drug-induced alteration of cell proliferation, from changes in cell metabolism, from changes in vascular tone of the target organs, changes in cell migration, or from another mechanism. It is also possible that cyclophosphamide-induced reduction in heart mass may be associated with the myocarditis and cardiomyopathy associated with some chemotherapeutic interventions (Remme, 1998; Sparano, 1999; Calabrese et al., 2003; Kang, 2003).

As the migration of lymphocytes into the peripheral circulation is a well-known effect of norepinephrine (Kohm and Sanders, 2001; Madden, 2003), it is possible, though not proven here, that norepinephrine stimulated lymphocyte migration from the spleen, the thymus gland, and to a lesser extent the heart without causing damage to surviving tissues. This possibility is supported by a report that examined the effects of cyclophosphamide on the sympathetic nerve fibers in the spleen; Carlson and colleagues (Carlson et al., 1987) reported that cyclophosphamide increases the density of sympathetic nerve fibers around the arterioles of the spleen and, consistent with our results, that these fibers maintained organ norepinephrine levels even as the number of lymphocytes in the spleen decreased (Carlson et al., 1987; Karp and Szczytkowski, 2003). To our knowledge, the structural consequences of cyclophosphamide administration on the sympathetic nerve fibers of the thymus gland have not been directly evaluated, though cyclophosphamide appears to stimulate apoptosis in the thymus (Ishiyama et al., 1999) as well as

reduce the number of PNA+/CD3- and double positive (CD4+, CD8+) thymocytes (Miyauchi et al., 1990).

The current experiments do not allow us to determine if cyclophosphamide directly or its in vivo metabolites (Ludeman, 1999; Roy et al., 1999) are responsible for cyclophosphamide-induced elevations in norepinephrine concentrations in the spleen and thymus gland. In order to inhibit the proliferation of tumor cells, cyclophosphamide acts as a prodrug and is catalyzed by hepatic P450 enzymes to yield cytotoxic phosphoramide mustards (Anderson et al., 1995; Roy et al., 1999). It is not yet determined if cyclophosphamide-induced elevations in norepinephrine concentration alter the activity of P450 enzymes or if elevations in norepinephrine concentration alter other aspects of cyclophosphamide metabolism. It is interesting however that others report that cyclophosphamide can improve the efficacy of cytochrome P-450 based gene therapy (Jounaidi and Waxman, 2001).

What role cyclophosphamide and/or cyclophosphamide-induced changes in norepinephrine have on specific cells not targeted by the drug or its metabolites has not been elucidated. The effects of norepinephrine and beta-receptors on endothelial cells of the blood vessels (Esler, 2000; Von Kanel and Dimsdale, 2000) make it tempting to speculate that the noradrenergic effects of cyclo- phosphamide might contribute to the anti-angiogenic and anti-tumor effects of repeated, low dose cyclophosphamide treatments (Jounaidi and Waxman, 2001; Man et al., 2002; Mauceri et al., 2002). In regard to the cells of the spleen and thymus gland, some research suggests in vivo cyclophosphamide administration preferentially depletes B cells and results in suppression of antibody responses (Austin et al., 1997; Zandvoort et al., 2001). Other research suggests that cyclophosphamide depletes CD4+ T cells which results in a relative enlargement of the CD8+ T cell pool (Lacki et al., 1997); such data are consistent with

cyclophosphamide-induced increases in CD8+ cell infiltration of the heart in an animal model of a parasitic infection (Calabrese et al., 2003). The spleens from cyclophosphamide-treated mice also contain nitric oxide producing myeloid cells which might contribute to the anti-tumor actions of cyclophosphamide (Pelaez et al., 2001). Yet, irrespective of the cell types most affected by cyclophosphamide, it appears as if the drug directly or indirectly alters the relative amount of norepinephrine (and perhaps other neurochemicals) available to influence cell-cell interactions in some of the organs innervated by the sympathetic nervous system.

Cyclophosphamide-mediated changes in norepinephrine could contribute to the side-effects some- times associated with administration of chemotherapeutic drugs. Norepinephrine is generally associated with increases in glucose utilization, increases in heart rate and blood pressure, and central nervous system actions such as motor control, cognition, emotion, memory processing, and endocrine modulation (Lambert, 2001). Norepinephrine is also known for its immunoregulatory properties (Elenkov et al., 2000; Bellinger et al., 2001; Kohm and Sanders, 2001; Sanders and Straub, 2002; Madden, 2003). For example, stimulation of norepinephrine receptors on B cells can drive increases in antibody production (Elenkov et al., 2000; Bellinger et al., 2001; Kohm and Sanders, 2001; Sanders and Straub, 2002; Madden, 2003). Furthermore, both antagonism of noradrenergic beta-receptors and sympathectomy attenuated the conditioned suppression of lymphocyte activity established by pairing a chemotherapeutic drug with saccharin (Exton et al., 2002). Finally, interactions between cyclo- phosphamide administration and animal behavior are evidenced in observations that the ability of cyclophosphamide to retard tumor growth can be reduced by both restraint (Zorzet et al., 1998) and spatial disorientation (Perissin et al., 1991).

Conclusion

Cyclophosphamide is not merely a cytotoxic compound whose effects are limited to cells undergoing mitosis. When it is administered by injection, cyclophosphamide and/or its metabolites can alter the noradrenergic environment of the spleen and thymus gland even though the sympathetic division of the nervous system is not known as a reservoir of actively proliferating cells. As a consequence, cyclophosphamide, through alterations in norepinephrine and perhaps other neurochemicals, has the potential to influence bidirectional interactions between immunological, neurochemical, and behavioral factors. Cyclophosphamide-induced changes in the neurochemical environment could contribute to the efficacy or the side-effects of chemotherapeutic interventions such as lethargy, nausea, vomiting, alopecia, and cognitive alterations (Schagen et al., 2002; Phillips and Bernhard, 2003). It follows that peripheral nervous system responses to cyclophosphamide and perhaps other chemotherapeutic drugs should be considered when treating the side-effects associated with these drugs and perhaps even employed as potential cofactors in the use of these drugs for chemotherapeutic purposes.

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