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Abstract

Chemotherapeutic drugs may not only kill rapidly dividing cells but may also alter the extracellular environment of surviving cells. We investigated the possibility that cyclophosphamide might alter the noradrenergic environment of the spleen. Male BALB/cByJ mice were administered a single injection of cyclophosphamide (0, 15, 50, or 100 mg/kg). Seventy-two hours after injection animals receiving 50 or 100 but not 15 mg/kg experienced elevated norepinephrine concentrations (pmol/mg) compared to animals given 0 mg/kg. The time course of changes in norepinephrine concentration was investigated 24–216 h after administration of 50 mg/kg cyclophosphamide; norepinephrine took 48 h to elevate, remained elevated for 48–96 h, and returned to vehicle-treated levels by 120 h. Cyclophosphamide in both experiments reduced spleen mass but did not alter total norepinephrine/spleen. These results suggest that low doses of cyclophosphamide can increase the norepinephrine available to influence cell–cell interactions in the spleen.

Keywords

Norepinephrine

Spleen

Neuroimmunology

Cyclophosphamide

Chemotherapy

The integration of nervous and immune system function is an important component for understanding susceptibility to disease. Through innervation of immune organs and shared chemical messengers, the nervous system influences the cells that are involved in generating immune responses. Sympathetic nervous system innervation of lymphoid organs provides a direct and rapid route for the central nervous system to influence the immune system [2], [14]. Neurochemicals released by sympathetic nervous system activity include norepinephrine, substance P, neuropeptide Y, vasoactive intestinal peptide, somatostatin, purines, and calcitonin gene-related peptide, among others [2], [7]. Norepinephrine is recognized as one of the most relevant to neuroimmunomodulation [7], [12], [14]. Norepinephrine is among the neurochemicals localized in the nerve fibers of lymphoid organs, noradrenergic receptors are found on many types of immune cells, and removal of norepinephrine-producing nerve fibers changes characteristics of immune responses [2], [14]. Comprehensive reviews indicate that sympathetic nervous system manipulations and norepinephrine can either stimulate or inhibit specific immune parameters depending upon the immune status of the subject, the type and dose of antigen used to activate the immune system, the type of animal studied, and the timing of antigen exposure relative to sympathetic nervous system activation [7], [12], [16], [17]. Indeed, understanding effects of norepinephrine on the immune system is significant because pharmacological manipulation of sympathetic nervous system activity is commonly used in clinical medicine [17].

An examination of changes in norepinephrine content following cyclophosphamide treatment is warranted because cyclophosphamide is used as part of the therapy for autoimmune disorders (such as lupus erythematosus, chronic polyarthritis, myasthenia gravis), as part of chemotherapeutic treatments for some malignant tumors (e.g. non-Hodgkin's lymphoma, metastatic breast cancer, lymphatic leukemia), and for preventing rejection after organ or bone marrow transplantation [1], [18]. Cyclophosphamide is used clinically because the drug and its metabolites are antineoplastic [13]. The antineoplastic effects of cyclophosphamide and its metabolites might occur not only because of direct alkylation of DNA but also because these drugs change the neurochemical milieu in which surviving cells interact. For instance, alterations in norepinephrine concentration as a result of cyclophosphamide administration could directly or indirectly influence cyclophosphamide-mediated cytotoxicity and/or could alter signaling between the nervous system and the immune system.

The spleen was selected as the initial place to study the effects of cyclophosphamide on norepinephrine concentration because the spleen functions as an interface between the nervous system and the immune system [7], [9]. Removal of sympathetic innervation of the spleen blocks the ability of behavioral conditioning to prolong heart allograft survival [8], attenuates the effects of stress exposure on antibody production [19], and alters some but not all characteristics of an immune response [3].

Male BALB/cByJ mice (aged 3–5 weeks, The Jackson Labs, Bar Harbor, ME) were used in these experiments. Mice were housed 3–5/cage in standard shoe box cages for 3–4 weeks prior to the start of the experiments. Food and water were available at all times. All the mice in a

single cage always received the same treatment. All procedures were approved by the Rider University Institutional Animal Care and Use Committee in accordance with the regulations of the Animal Welfare Act.

Cyclophosphamide (Sigma-Aldrich, St. Louis, MO) was solubilized in ethanol and administered i.p. (in 500 μl of sterile filtered (0.1 μ) phosphate buffered saline, GIBCO, Carlsbad, CA). Vehicle-treated animals received an equal volume of the phosphate buffered saline–ethanol solution. Spleens were harvested, weighed, and frozen on dry ice, and stored at −80 °C until assay. The norepinephrine content of spleen samples was measured by high-performance liquid chromatography (HPLC) with electrochemical detection. Briefly, samples (50–75 mg wet weight) were sonicated in 0.1 M HClO4 (10 μl/mg tissue) spiked with an internal standard of 3,4-dihydroxybenzylamine (final concentration 0.25 μM). Homogenates were centrifuged and aliquots of the supernatants were combined with 1 ml of sodium phosphate buffer (pH 6.1) and 1 ml 1.5 M Tris/EDTA buffer (pH 8.6). Acid-washed alumina (50 mg) was added to each sample and norepinephrine was extracted by shaking for 5 min. The alumina was washed twice 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 to the microfilter and centrifugation was used to collect the extract in a new microcentrifuge tube. For each sample, extract (20 μl) was manually injected into the HPLC.

The HPLC system consisted of programmable pumps (Varian ProStar) and a C18 reverse-phase column (150 mm×4.6, 5 μ) located in temperature controlled housing (27 °C) 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 (Dell Optiplex) running Windows98 fitted with an ADC controller card using Varian Star Chromatography software (Star Chromatography, version 5). The software measured the area under each peak and compared the value obtained to the internal standard (5 pmol of 3,4-dihydroxybenzylamine). All data were evaluated initially by ANOVA followed by the Student–Newman–Keuls post-hoc test (Statview version 5.0, SAS Institute Inc., Cary, NC) on a PC computer. Treatment effects were considered statistically significant at $P<0.05$.

To investigate the effects of cyclophosphamide on the noradrenergic environment of the spleen, mice were administered a single injection of cyclophosphamide (15, 50, or 100 mg/kg) or vehicle. Data analysis from spleens harvested 72 h after injection revealed dose-dependent changes in spleen mass and in norepinephrine content per mg spleen; cyclophosphamide induced a reduction in spleen mass (Fig. 1A, $F3,32=13.3$, $P<0.001$) and an elevation in spleen norepinephrine content (Fig. 1B, $F3,32=20.2$, $P<0.001$). For both measures, post-hoc analysis revealed that vehicle-treated spleens were not different from spleens exposed to 15 mg/kg while the 50 and 100 mg/kg groups were statistically different from the vehicle treatment. Total spleen norepinephrine content (calculated as spleen mass×norepinephrine/mg) was not influenced by cyclophosphamide treatment (Fig. 1C, dose effect, $P=0.08$).

Fig. 1. Dose-dependent effects of cyclophosphamide on spleen mass (A), spleen norepinephrine content (B), and total norepinephrine per spleen (C). Spleens from mice injected with cyclophosphamide (0, 15, 50, or 100 mg/kg) were collected 72 h after injection. The number of mice in each group is indicated within each bar. The * indicates a statistical difference ($P<0.05$) from the 0 mg/kg group.

To investigate the time course of cyclophosphamide-mediated changes in spleen mass and norepinephrine concentration, mice were injected with 50 mg/kg cyclophosphamide and spleen samples were collected 24, 48, 72, 96, 120, 168, or 216 h after injection. ANOVA revealed a dose×time interaction (Fig. 2A, $F_{6,106}=29.3$, $P<0.001$); post-hoc comparisons showed that mice administered cyclophosphamide displayed reductions in spleen mass compared to vehicle-treated mice 24, 48, 72, and 96 h after injection. Evaluation of changes in spleen norepinephrine content also revealed a dose×time interaction (Fig. 2B, $F_{6,106}=2.3$, $P=0.04$); post-hoc analysis showed that spleen norepinephrine concentrations were elevated in cyclophosphamide-treated mice compared to vehicle-treated mice 48, 72, and 96 h after injection. At no time after injection was the total amount of norepinephrine per spleen different between vehicle- and cyclophosphamide-treated mice (Fig. 2C, time×dose, $P=0.77$).

Fig. 2. Time-dependent effects of cyclophosphamide on spleen mass (A), spleen norepinephrine content (B), and total norepinephrine per spleen (C). Mice were injected with 0 or 50 mg/kg cyclophosphamide and spleens were harvested 24, 48, 72, 96, 120, 168, or 216 h

after injection. There were 8, 9, 11, 7, 7, 8, and 6 mice administered the 0 mg/kg dose and 9, 10, 14, 8, 7, 8, and 8 mice administered 50 mg/kg cyclophosphamide at each time point, respectively. The * indicates a statistical difference ($P<0.05$) from the 0 mg/kg group at the indicated time point.

These results suggest that cyclophosphamide or one of its in vivo metabolites [13] induces dose- and time-dependent elevations in norepinephrine content per mg spleen. Interestingly, reductions in spleen mass were not always associated with elevations in spleen norepinephrine content; at 24 h a decrease in spleen mass was observed without a concomitant elevation in spleen norepinephrine content (Fig. 2A,B). The total amount of norepinephrine in the spleen did not vary as a result of cyclophosphamide administration (Fig. 1, Fig. 2) suggesting that sympathetic activity in the spleen compensated for or was unaffected by the changes in cellularity induced by cyclophosphamide [4]. Cyclophosphamide may also alter norepinephrine turnover in the spleen but the current experiments were not designed to address this possibility. The migration of lymphocytes into the peripheral circulation is a well-known effect of norepinephrine [12], [14] and it is possible, though not proven here, that norepinephrine stimulated cell migration from the spleen and that such a migration could be responsible for reductions in spleen mass. Cyclophosphamide-mediated reductions in spleen mass might also occur from a transient drug-induced inhibition of spleen cell proliferation or from changes in norepinephrine metabolism in the spleen. Additional experiments are needed to address these possibilities. Irrespective of the mechanism, it is clear that cyclophosphamide administration does make more norepinephrine available in the spleen to influence cell activity.

Reductions in spleen mass, elevations in spleen norepinephrine content, and a lack of change in the total norepinephrine per spleen are consistent with an earlier report [4]. The current experiments extend these findings to a different mouse strain (BALB/cByJ versus C3H/HeJ) using a different range of cyclophosphamide doses (0, 15, 50, and 100 mg/kg compared to 50 and 250 mg/kg). The dose range used in the current experiments also establishes a threshold for cyclophosphamide-mediated changes in spleen norepinephrine (between 15 and 50 mg/kg). Finally, the current studies describe the kinetics for cyclophosphamide-mediated changes in the spleen (from 24 to 216 h after injection) whereas the earlier report examined only a single time point (48 h after injection).

In regard to immunity, there is an emerging literature indicating that cyclophosphamide is potentially immunomodulatory. Sympathetic nerve fibers increase in density around arterioles of the spleen after cyclophosphamide administration [4] and maintain organ norepinephrine levels even as the number of lymphocytes in the spleen is decreased [4]. The spleens of cyclophosphamide-treated mice develop early myeloid cells capable of producing large amounts of nitric oxide from T cell-derived signals [15]. Cyclophosphamide can also alter the cell surface markers of immune cells in the marginal zone of the spleen [20]. In addition, aged mice treated with cyclophosphamide exhibit increased numbers of immune cells with a ‘young-like’ profile of cell proliferation and antibody responses [10]. Functionally, cyclophosphamide can increase or decrease delayed type hypersensitivity responses depending on the dose administered and on the timing of its administration relative to antigen exposure [5], [6]. What role, if any, cyclophosphamide-induced changes in norepinephrine have on specific cells or immune effector responses remains to be determined. Interestingly, we observed that a dose of cyclophosphamide

that did not elevate spleen norepinephrine levels (15 mg/kg, Fig. 1) and did not alone influence antibody production [11] was able to augment antibody production when administered in combination with a psychological stressor (restraint) that presumably activates the sympathetic nervous system [11]. Such observations imply that cyclophosphamide can influence interactions between immunological, neurochemical, and behavioral manipulations.

Observations that cyclophosphamide alters the noradrenergic environment of the spleen are intriguing because subjects administered chemotherapeutic agents such as cyclophosphamide may be more susceptible to opportunistic infections, not only because the drug alkylates the DNA of mitogenic cells, but also because the drug alters the neurochemical milieu in which immune responses occur. As norepinephrine is a major immunomodulatory product of the sympathetic nervous system that is known to be involved in lymphocyte proliferation, cytokine production, and migratory behavior of immune cells [7], [12], [16], [17], it is possible that cyclophosphamide-mediated changes in norepinephrine could impact upon the ability of the immune system to successfully combat infectious agents. Some have suggested that norepinephrine could stimulate humoral immunity by simultaneously augmenting antibody production by B cells and inhibiting cytokine production that would stimulate cell-mediated immune processes [7], [12], [16], [17].

In the future, it will be important to ascertain if cyclophosphamide effects on norepinephrine concentrations are limited to tissues where cells of the immune system interact and if all tissues innervated by the sympathetic nervous system are influenced by cyclophosphamide in the same way as the spleen. Similarly, as the nervous system and the

immune system are engaged in reciprocal communication, it will be of interest to examine the central nervous system consequences of peripheral cyclophosphamide administration.

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