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Pyridostigmine Bromide (PYR) Alters Immune Function in B6C3F1 Mice

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ABSTRACT

Pyridostigmine bromide (PYR) is an anticholinesterase drug indicated for the treatment of myasthenia gravis and neuromuscular blockade reversal. It acts as a reversible cholinesterase inhibitor and was used as a pretreatment for soldiers during Operation Desert Storm to protect against possible nerve gas attacks. Since that time, PYR has been implicated as a possible causative agent contributing to Gulf War Illness. PYR's mechanism of action has been well-delineated with regards to its effects on the nervous system, yet little is known regarding potential effects on immunological function. To evaluate the effects of PYR on immunological function, adult female B6C3F1 mice were gavaged daily for 14 days with PYR (0, 1, 5, 10, or 20 mg/kg/day). Immune parameters assessed were lymphoproliferation, natural killer cell activity, the SRBC-specific antibody plaque-forming cell (PFC) response, thymus and spleen weight and cellularity, and thymic and splenic CD4/CD8 lymphocyte

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subpopulations. Exposure to PYR did not alter splenic and thymus weight or splenic cellularity. However, 20 mg PYR/kg/day decreased thymic cellularity with decreases in both CD4+/CD8+ (20 mg/kg/day) and CD4−/CD8− (10 and 20 mg/kg/day) cell types. Functional immune assays indicated that lymphocyte proliferative responses and natural killer cell activity were normal; whereas exposure to PYR significantly decreased primary IgM antibody responses to a T-cell dependent antigen at the 1, 5, 10 and 20 mg/kg treatment levels for 14 days. This is the first study to examine the immunotoxicological effects of PYR and demonstrate that this compound selectively suppresses humoral antibody responses.

Key Words: Pyridostigmine bromide; IgM antibody responses; Gulf War Illness; Immunotoxicity.

INTRODUCTION

Pyridostigmine bromide (PYR) (Fig. 1) is a quaternary dimethyl carbamate that has been historically used to treat myasthenia gravis; a neuromuscular disorder characterized by muscle weakness due to the development of autoantibodies against nicotinic acetylcholine receptors.[1,2] More recently, four hundred thousand Desert Storm soldiers were administered PYR tablets as a pretreatment against possible nerve gas attack.[3–5] PYR acts as a reversible cholinesterase (ChE) inhibitor; meaning once bound it can disassociate thereby restoring cholinesterase function.[3,6] Due to this reversible nature, PYR was selected by the US Armed Forces and some North Atlantic Treaty Organization nations as a pretreatment drug for Gulf War military personnel to guard against organophosphorous (OP) nerve gases.[3]

Several reports document that PYR causes few adverse effects in humans. In a retrospective study, 34,000 of 41,650 soldiers in the XVIII Airborne Corps were instructed to self-administer PYR tablets for durations of 6–7 days.[3] Following the immediate use of this drug, 483 clinic visits were noted with adverse reactions described as gastrointestinal and urinary disturbances, headaches, worsening of acute bronchitis, rashes, and two episodes of hypertension.[3] However, few side-effects were reported when healthy soldiers were exposed to PYR for 7 or 14 days in conjunction with various training exercises or unique environmental conditions.[6–10]

With the surfacing of Gulf War Illness in the early 1990's, additional studies exploring combinations of drugs, environmental agents, or underlying pathologies that could alter the toxicity of PYR were undertaken. Toxic synergism was reported when PYR was combined with adrenergic agonists and caffeine.[11] Marked increases in neurotoxicity were also demonstrated when PYR was concurrently administered with DEET (*N*,*N*-diethyl-*m*-toluamide) and permethrin[12,13] or with DEET and chlorpyrifos.[14] Moreover, it was learned that PYR exacerbated preexisting cases of asthma.[15]

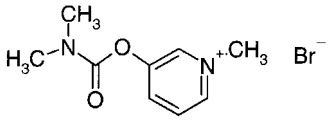

Figure 1. Chemical structure of pyridostigmine bromide (PYR).

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A number of reports documenting various effects of PYR in humans or in animal models are available.[16–26] Yet, none of the studies to date have thoroughly considered potentially subtle effects of PYR on immunological function. Some reports suggest that Gulf War Illness may have a common immunological basis proposing disruptions in Th1/Th2 responses or the development of autoimmunity.[27–29] However, data evaluating potential alterations in immunity with agents implicated in Gulf War Illness are lacking. Due to the extensive use of PYR and its potential applications in future military environments, examination of its effects on immunological health were conducted.

MATERIALS AND METHODS

Chemicals, Antibodies, and Supplies

Unless otherwise specified, all chemicals, including mitogens, were purchased from Sigma (St. Louis, MO). 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Radian International (Austin, TX). Sheep erythrocytes (SRBCs) in Alsever's solution were obtained from BioWhitaker Inc. (Walkersville, MD). Lyophilized guinea pig complement (GPC), GPC restoring solution, non-essential amino acids (NEAA; 10 mM 100X) and sodium pyruvate (100 mM) were obtained from GIBCO Laboratories (Grand Island, NY). RPMI-1640 (with L-glutamine and sodium bicarbonate), phosphate buffered saline (PBS; with or without Ca^{+2} and Mg), and penicillin/streptomycin (5,000 I.U./mL, 5,000 μg/mL) were purchased from Cellgro (Mediatech; Herndon, VA). The radio-isotopes, sodium chromate (^{51}Cr), and tritiated thymidine were acquired from ICN (Costa Mesa, CA). Fetal bovine serum was from Hyclone (Logan, Utah). The fluorescent antibodies, rat anti-mouse IgG2a (isotype control), fluorescein isothiocyanate (FITC) conjugated rat anti-mouse CD4 (monoclonal), and phycoerythrin (PE) conjugated rat anti-mouse CD8 (monoclonal) were purchased from Caltag (Burlingame, CA). Luma Plates™, Unifilters®, and Microscint 20™ were procured from Packard (Meriden, CT). YAC-1 cells were purchased from ATCC (Manassas, VA).

Animal Care and Dosing

Mice were housed in plastic shoebox cages and received food (Tek Lab Sterilizable Rodent Diet, formula no. 8656) and water ad libitum. Prior to starting these exposure studies, 7- to 8-week old female B6C3F1 mice were homogeneously distributed by weight in cages and maintained in a treatment room of an accredited animal facility (Association of Assessment and Accreditation of Laboratory Animal Care) at the Medical University of South Carolina. They were acclimated to the conditions of the treatment room (12 hour light/dark cycle, 22 ± 2°C, 60–65% relative humidity) for 1 week before the experiments began. Four concentrations of PYR (1, 5, 10, and 20 mg PYR/kg/day) and a distilled water carrier control (0 mg PYR/kg/day) were used. Mice were dosed daily for 14 days via oral gavage with either PYR or the carrier control. There were six or seven animals per treatment group. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (15 μg/kg/day gavage for 3 days) or dexamethasone (40 mg/kg gavage for 3 days) were used as positive controls. All experiments were repeated at least twice.

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Body Mass, Organ Mass, and Splenic and Thymic Cellularity

Body mass was measured one day prior to initial exposures and at the termination of each experiment. Spleen, thymus, and liver were collected and weighed following euthanization in a CO_2-saturated environment. Organ mass was normalized for body weight and reported as a somatic index {(organ weight/body wt) $\times$ 100}. Spleen and thymus were aseptically processed into single cell suspensions with the use of frosted, sterile microscope slides. A Coulter Counter (Model ZF; Hialeah, FL,) was used to obtain cell counts. The counter was calibrated using cell suspensions that were verified with a hemacytometer.

Hematology

Whole blood was collected into EDTA coated microtainers at the time of sacrifice. Blood was mixed immediately to avoid clot formation, stored in the refrigerator, and sent by courier on the same day of collection to Antech Diagnostics (Farmington, NY) for complete blood count (CBC) analysis with white blood cell (WBC) differential. CBC analysis reported erythrocyte and leukocyte number, hemoglobin, hematocrit, and red blood cell indices.

Lymphocyte Proliferation

A standard ^{3}H-thymidine incorporation assay was optimized and used to assess lymphocyte proliferative responses. Spleens were processed and diluted to 2.0×10^6 cells/mL in Supplemented RPMI-1640 (RPMI-1640, 10% fetal bovine serum, 1% nonessential amino acids, 10 mM HEPES, 1% pen-strep, and 10 μM 2-mercaptoethanol, pH 7.4). One hundred microliter aliquots of the resulting cell suspensions were dispensed into 96-well plates (2.0×10^5 cells/well) containing triplicate wells of 100 μL of either 2.5 μg/ml culture of concanavalin A (Con A, type IV-S), 5 μg/ml culture of lipopolysaccharide (LPS; *E. coli* 0111:B4), or supplemented RPMI-1640 (unstimulated wells). Plates were incubated for 40 hours at 37°C and 5% CO_2. After 40 hours, 100 μL of a 0.5 μCi/mL stock of tritiated thymidine in RPMI-1640 was added to each well. Six hours later, cells were harvested onto Unifilter plates using a Packard Filtermate™ 96-well plate harvester and the plates were allowed to dry. Once dry, 25 μL of Microscint 20™ was added to each well and the samples were analyzed using a Packard Top Count™-NXT scintillation counter. Results are reported as the stimulation index (stimulated CPM/unstimulated CPM).

Natural Killer (NK) Cell Activity

An in vitro cytotoxicity assay using ^{51}Cr-labeled Yac-1 cells (ATCC, TIB 160, Manassas, VA) was used as described previously.[30,31] To minimize radioactive waste, the procedure was adapted to 96-well plates read on a Packard TopCount scintillation counter. Splenocytes were adjusted to 1×10^7 cells per mL in complete medium (RPMI, 10% fetal calf serum, 50 IU penicillin and 50 μg streptomycin). Splenocyte and Yac-1 cells were prepared in ratios of 100:1, 50:1 and 25:1 and replicated thrice. After the 4-hour incubation at 37°C and 5% CO_2, 25 μL of supernate was transferred to a 96-well plate containing solid scintillant (LumaPlate, Packard, Meriden, CT). Plates

were air dried overnight, and then, after a 10-minute dark delay on the Packard Top Count, were counted for 5 minutes. Maximum release was determined by lysing ^{51}Cr-labeled Yac-1 cells with 0.1% Triton X in complete medium. Spontaneous release was determined by incubating Yac-1 tumor cells only, in complete medium. The results are expressed in lytic units per 10^7 splenocytes using 10% lysis as the reference point and effector to target (ET) ratios of 100:1, 50:1, and 25:1.[32]

Antibody Plaque-Forming Cell (PFC) Assay

The number of plaque forming cells (PFCs) was determined using the Cunningham modification of the Jerne plaque assay.[33,34] Five days prior to euthanasia, mice were administered 0.1 mL of a 25% sheep red blood cell (SRBC) suspension in PBS via intraperitoneal injection.[35] All sheep red blood cells for the experiments were drawn from a single, donor animal. Mouse spleens were processed, cell numbers counted, and suspensions diluted to 2×10^6 cells/mL in Supplemented RPMI-1640 (as previously described). Aliquots of the cell suspension were added to eppendorf tubes containing Supplemented RPMI-1640 and SRBCs. Lyophilized guinea pig complement (GPC) was reconstituted with GPC restoring solution as per manufacturers directions and was then diluted 1:2 (v/v) in GPC restoring solution. Fifty μL of the diluted, reconstituted lyophilized guinea pig complement was then added on a tube by tube basis, aliquots of the solution were placed in Cunningham chamber slides. The slides were sealed with a wax mixture and were incubated at 37°C for 1 hour. Results are reported as PFCs/million splenocytes.

Splenic and Thymic CD4/CD8 Subpopulations

Spleen or thymus cells were labeled with fluorescent (phycoerythrin or fluorescein isothiocyanate) rat IgG_2 monoclonal antibodies specific for murine CD4 or CD8. In this procedure, single cell suspensions of thymocytes and splenocytes were washed and resuspended in phosphate buffered saline (pH 7.4 containing 0.1% sodium azide and 1% bovine serum albumin). Monoclonal antibodies were incubated with cells for at least 30 minutes at 6°C in the dark. Red blood cells were lysed and removed by several washings with the phosphate buffered saline. Lastly, the cells were fixed with 1% paraformaldehyde and stored at 6°C in the dark. Flow cytometric analysis was performed using a Becton Dickinson flow cytometer (FACSCalibur™; San Jose, CA). Non-stained cells and isotypic antibody controls were used to establish gates for the CD4/CD8 subpopulations in thymic and splenic cells. Absolute values were determined by multiplying the percent value of gated lymphocytes by the total number of nucleated cells enumerated by the Coulter particle counter.

Statistics

Data were tested for normality (Shapiro Wilks W-test) and homogeneity (Bartlett's test for unequal variances). A one-way ANOVA was used to determine differences among doses for each endpoint using JMP 4.0.2 (SAS Inst. Inc., 2000). When significant differences were detected by ANOVA, Dunnett's comparison was used to compare treatment groups to controls.

RESULTS

Body and Organ Mass, Organ Cellularity and Peripheral Blood Counts

There were no overt signs of toxicity as evidenced by fur condition, activity, or eye clarity following PYR treatment at doses ranging from 0–20 mg/kg/day over the 14-day experiment. Although the amount of body mass gained over the 14-day experiment was significantly less in the 20 mg PYR/kg/day treatment, relative spleen, liver and thymic mass were not altered (Table 1). Total splenic cellularity was not altered by PYR treatment (Table 2) but thymic cellularity was significantly decreased at the 20 mg/kg/day dose (Table 3). Additionally, no effect was observed in peripheral white blood cell counts, differential lymphocyte counts, or in erythrocyte indices following PYR treatment (data not shown).

Splenic Lymphocyte Proliferation and NK Cell Function

Oral exposure to PYR for 14 days did not result in alterations in T- or B-cell mitogen-induced lymphocyte proliferation (Fig. 2). Additionally, natural killer (NK) cell activity was not altered by PYR exposure (Fig. 3).

IgM Antibody Plaque-Forming Cell Response

Humoral immunity was significantly suppressed following exposure to PYR. Specific IgM antibody responses to a T-cell dependent antigen was suppressed in mice treated with PYR at all levels (Fig. 4). Suppression beginning with the 1 mg/kg treatment level ranged from a 25% decrease at 1 mg/kg/day to an 85% decrease at 20 mg/kg/day.

Splenic and Thymic CD4/CD8 Subpopulations

Splenic T-cell subpopulations were not significantly altered at any exposure level utilized in this study (Table 2); however, there was a decreasing trend noted in the

Table 1. Body mass change and normalized spleen, thymus and liver mass in adult female B6C3F1 mice exposed orally to pyridostigmine bromide (PYR) for 14 days.

PYR (mg/kg/day)	Body mass change[a] (g)	Normalized[b] spleen mass	Normalized[b] thymus mass	Normalized[b] liver mass
0	0.983 ± 0.13	0.473 ± 0.01	0.157 ± 0.01	5.01 ± 0.11
1	0.817 ± 0.28	0.448 ± 0.02	0.229 ± 0.01	5.08 ± 0.08
5	1.24 ± 0.12	0.495 ± 0.02	0.187 ± 0.01	4.97 ± 0.23
10	1.25 ± 0.14	0.445 ± 0.03	0.214 ± 0.02	4.93 ± 0.12
20	0.467 ± 0.12*	0.393 ± 0.03	0.184 ± 0.03	5.04 ± 0.18

Data are reported as mean ± SEM. Sample size for all treatment groups is 6.
[a](Final mass−start mass).
[b]Normalized mass was calculated as the (organ mass (g)/body mass (g)) × 100). Significantly different from water control (*).

Table 2. Splenic total cellularity and T-lymphocyte subpopulations in adult female B6C3F1 mice following a 14-day oral exposure pyridostigmine bromide (PYR) in a distilled water carrier.

PYR mg/kg/d	Splenic cellularity (cells $\times 10^7$)	% CD4+	%CD8+	%DN	%DP	Ratio CD4/CD8	Absolute CD4+ (cells $\times 10^6$)	Absolute CD8+ (cells $\times 10^6$)	Absolute DN (cells $\times 10^6$)	Absolute DP (cells $\times 10^6$)
0	7.33 ± 0.62	1.71 ± 0.65	11.74 ± 0.51	65.56 ± 0.04	0.27 ± 0.93	1.93 ± 0.08	16.5 ± 1.56	8.4 ± 0.51	48.2 ± 4.35	0.190 ± 0.03
1	9.09 ± 0.87	1.59 ± 0.60	11.68 ± 0.38	65.71 ± 0.04	0.28 ± 0.88	1.92 ± 0.05	20.3 ± 2.11	10.5 ± 0.93	59.7 ± 5.85	0.246 ± 0.03
5	8.7 ± 0.78	1.42 ± 0.54	12.18 ± 0.49	63.78 ± 0.02	0.22 ± 0.91	1.97 ± 0.06	20.8 ± 1.98	10.5 ± 0.50	55.5 ± 5.15	0.184 ± 0.01
10	8.4 ± 0.93	2.94 ± 1.11	11.66 ± 0.96	65.28 ± 0.03	0.19 ± 1.96	2.00 ± 0.10	18.7 ± 1.43	9.3 ± 0.67	55.8 ± 7.50	0.165 ± 0.04
20	7.89 ± 0.55	2.61 ± 0.99	11.49 ± 0.64	65.48 ± 0.01	0.19 ± 1.51	2.01 ± 0.08	17.9 ± 1.15	8.9 ± 0.45	51.9 ± 4.45	0.154 ± 0.01

Data are presented as mean ± SEM. Sample size for all groups is 7. Absolute values are reported as (mean ± SEM) $\times 10^6$. $P \leq 0.05$.
DN = CD4−/CD8−, DP = CD4+/CD8+.

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Table 3. Thymic total cellularity and T-lymphocyte subpopulations in adult female B6C3F1 mice following a 14-day oral exposure pyridostigmine bromide (PYR) in a distilled water carrier.

PYR mg/kg/d	Thymic cellularity (cells $\times 10^7$)	% CD4+	%CD8+	%DN	%DP	Ratio CD4/CD8	Absolute CD4+ (cells $\times 10^6$)	Absolute CD8+ (cells $\times 10^6$)	Absolute DN (cells $\times 10^6$)	Absolute DP (cells $\times 10^6$)
0	5.62 ± 0.56	9.26 ± 0.39	2.25 ± 0.11	3.62 ± 0.19	84.86 ± 0.50	4.18 ± 0.29	5.22 ± 0.60	1.27 ± 0.16	1.99 ± 0.15	47.7 ± 4.77
1	6.29 ± 0.29	9.48 ± 0.22	2.31 ± 0.06	3.41 ± 0.12	84.81 ± 0.28	4.12 ± 0.14	5.94 ± 0.23	1.44 ± 0.04	2.13 ± 0.10	53.4 ± 2.54
5	4.17 ± 0.58	10.33 ± 0.49	2.49 ± 0.15	3.58 ± 0.19	83.61 ± 0.67	4.18 ± 0.16	4.72 ± 0.44	1.11 ± 0.07	1.65 ± 0.19	38.8 ± 4.28
10	3.80 ± 0.29	10.90 ± 0.90	2.73 ± 0.19	3.46 ± 0.21	82.91 ± 1.22	3.98 ± 0.18	4.46 ± 0.53	1.11 ± 0.38	1.42 ± 0.18*	34.7 ± 5.08
20	4.63 ± 0.49*	10.77 ± 0.37	2.51 ± 0.04	3.83 ± 0.12	82.88 ± 0.47	4.29 ± 0.12	4.08 ± 0.30	0.949 ± 0.06	1.44 ± 0.09*	31.5 ± 2.46*

Data are presented as mean ± SEM. Sample size for all groups is 7. Absolute values are reported as (mean ± SEM) $\times 10^6$.
DN = CD4−/CD8−, DP = CD4+/CD8+.
*Significantly different from respective control. $P \leq 0.05$.

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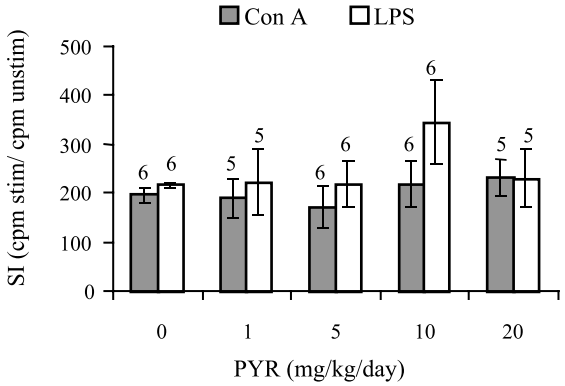

Figure 2. Mitogen-induced lymphocyte proliferation in adult female B6C3F1 mice following treatment with pyridostigmine (PYR) via oral gavage for 14 days. Data are representative of two trials and presented as a stimulation index (SI = average cpm of stimulated triplicate/ average cpm of unstimulated triplicate). Each bar represents mean SI ± SEM with sample size indicated above and an asterick to represent significance ($P \leq 0.05$). Mitogens used were 2.5 μg/mL final concentration of concanavalin A to stimulate T-cell proliferation and 5 μg/mL final concentration of lipopolysaccharide to stimulate B-cell proliferation. Unstimulated wells averaged 413 cpm and were not significantly different between treatment groups.

CD4+/CD8+ lymphocyte population and a slight increase in the CD4+ lymphocytes. Changes were more apparent in the thymus where the absolute number of thymic CD4+/CD8+ cells was significantly decreased at the highest treatment level (20 mg/kg/day) (Table 3). Additionally, the total number of CD4−/CD8− cells was significantly decreased compared to control at the 10 and 20 mg/kg/day exposure levels (Table 3). Although these significant differences in the thymus were not found with the corresponding percentage data, similar tendencies were evident. No alterations were noted in the ratio of CD4+:CD8+ cells in either the thymus or spleen (Tables 2 and 3). Decreases

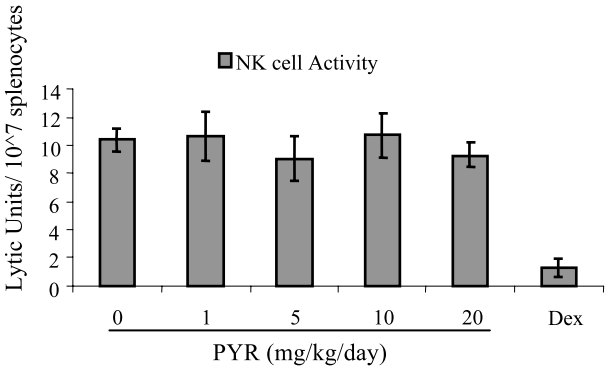

Figure 3. NK cell activity in adult female B6C3F1 mice following treatment with pyridostigmine (PYR) via oral gavage for 14 days. Data are representative of two trials and presented as mean ± SEM. Sample size for all groups is 6, $P \leq 0.05$. Dexamethasone (Dex) was used as a positive control (40 mg/kg for 5 days, n = 2).

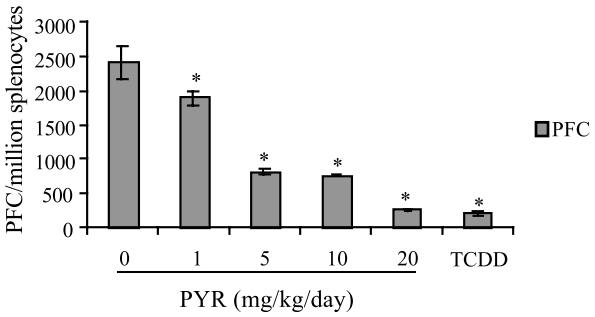

Figure 4. PFC response in adult female B6C3F1 mice following treatment with pyridostigmine (PYR) via oral gavage for 14 days. Data are representative of two trials and presented as mean ± SEM. Sample size for all PYR and control groups is 7. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was used as a positive control (15 μg/kg orally for 3 days, n = 5). *Significantly different from control, $P \leq 0.05$.

in thymic cellularity at the higher PYR exposure levels were consistent with decreases in immature T-cell populations.

DISCUSSION

Information regarding the immunotoxicity of carbamate compounds is limited and of the available data, there is no consistent pattern of immunotoxicity demonstrated among this class of compounds. PYR, however, shares a common mode of action with anticholinesterase insecticides. This class of compounds consists of carbamates and organophosphates that function by occupying the serine residue on the active site of acetylcholinesterase by carbamylation or phosphorylation, respectively. Due to this action, these compounds are considered potential inhibitors of serine hydrolase-dependent immune mechanisms that for instance, are involved in interleukin-2 (IL-2) signaling.[36]

Select cholinergic carbamates and organophosphates have been shown to inhibit T-cell proliferative functions following IL-2 stimulation.[36] In these studies, however, PYR did not alter T-cell proliferation. This might be explained by the fact that the Con A lectin is a polyclonal T-cell activator that binds to multiple surface glycoproteins[37] and can act independently of IL-2.[38] Therefore, deficits in IL-2 production and signaling are not likely to affect this endpoint.

When examining NK function, diverse effects exist after exposure to different cholinomimetic carbamates. For instance, carbaryl suppressed NK cytolytic activity,[39] whereas PYR did not. This difference, however, can be attributed to the fact that studies with carbaryl were performed with human NK cells that require IL-2, while the murine NK assay is not dependent on IL-2. Therefore, an interruption in IL-2 signaling pathways is not likely to interfere with the murine chromium release assay. However, alterations in IL-2 signaling do not seem to account for differences with other carbamates such as urethan (ethyl carbamate) that suppresses NK cell activity,[40,41] while methyl carbamate does not.[41]

The most remarkable immunological effect was a deficit in humoral immunity. Again, differences exist among cholinomimetic carbamates in regard to this endpoint. For instance, ethyl carbamate and carbofuran decrease SRBC-specific IgM production, while methyl carbamate does not.[41–43] Inhalation exposure to carbaryl resulted in suppression of the PFC response in male CD rats, but oral and dermal exposures do not.[44] Furthermore, contradictory results have been reported with aldicarb which has been shown to suppress antibody responses to SRBC, in a study by Olson et al.[45] Yet, Thomas et al.[46,47] report no effects on this endpoint.

A mechanism accounting for the suppression of humoral immune responses by PYR is not known. Alterations in macrophage uptake and processing are not likely a contributing factor as it has been shown that peritoneal macrophage numbers, bacterial phagocytosis, or nitric oxide production were not altered after exposure to PYR (2 or 5 mg/kg/day for 14 days) in MRL lpr/lpr mice.[48] Furthermore, a reduction in antibody responses is not likely attributed to a reduction in splenic B-cells.[49,50] Major deficiencies were not apparent in antigen presenting macrophages, and T- and B-cells suggesting that the cytokine network coordinating IgM antibody responses may be a factor in contributing to the observed decrease in primary IgM responses.

Nevertheless, it is clear that PYR suppresses humoral immunity. Of concern is the fact that this deficit was detected at treatment levels comparable to doses reported for military personnel. Soldiers were administered 30 mg every 8 hours for upwards of a week which is approximately 1.3 mg/kg per day assuming a 70 kg person. Based on dose per body weight comparisons, the human exposure level is within the range evaluated in this study that correspond to significant deficits in humoral immunosuppression. Interestingly, these same exposure levels were several fold less than prescribed dosages self-administered by myasthenia gravis patients (180–900 mg/day or 200–1400 mg/day).[1,2,51] Perhaps this study suggests an additional mechanism by which PYR effectively manages myasthenia gravis, as suppression of humoral immune function might lessen autoantibody production in this disease.

Although levels of blood cholinesterase inhibition were not quantitated in this study, the range of PYR dosages was expected to include levels with negligible to substantial inhibition of blood cholinesterase. For instance, 30 mg/kg of PYR in rats was determined to be the maximum tolerated dose and this exposure inhibited whole blood cholinesterase activity by less than 50% for a period of 24 hours.[52] Based on this report, it might be speculated that cholinesterase activity was inhibited for much of the 24-hour period following each daily exposure, at least for the higher PYR dosages used in this study. Regarding the lower PYR treatment levels, it has been reported in mice that 3 mg/kg/day administered by slow infusion produced no change in blood butyrylcholinesterase and decreased acetylcholinesterase activity after 7 days of treatment, whereas 1 mg/kg/day did not alter either of these enzymes.[53] This is consistent with a report by Somani and colleagues[54] indicating that daily oral exposure to 1.2 mg/kg/day PYR for 14 days did not alter butyrylcholinesterase or acetylcholinesterase activity in mice. Taken together, these reports suggest that cholinesterase activity may not have been largely affected by exposure to PYR dosages at the lower end of this dose–response study. For that reason, supplementary studies would be necessary to confirm a role, if any, regarding cholinergic influences on humoral immunosuppression by PYR.

The etiology of Gulf War Illness continues to be examined. As some studies suggest that this illness is linked with immune dysfunction,[27–29] other studies report

no abnormal immune function in veterans to include NK cytolytic activity and T-cell proliferation.[55,56] This does not preclude the fact, however, that immunity may have been compromised during exposure to PYR while in the Gulf War environment, thereby permitting the exacerbation of underlying pathologies or increasing the opportunity for others. This study demonstrates that PYR can modulate immunological function by suppressing humoral responses. As PYR was extensively used during the Gulf War and applications are possible in future military deployments, further examination of the effects of PYR on immunological function are necessary to determine potential human health effects.

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