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Proliferation effects of phenylboronic acid and boric acid on canine peripheral blood mononuclear cells

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Abstract: The effects of phenylboronic acid (PBA) and boric acid (BA) on the proliferation of canine peripheral blood mononuclear cells (PBMCs) were investigated. Treatment of PBMCs from mixed breed dogs with PBA or BA (0.05 µg/mL) alone elicited blastogenic responses of 0.478 ± 0.438 and 0.499 ± 0.366, respectively. We observed an additive effect on proliferation when purebred Kangal PBMCs were cultured in a combination of BA (0.001 µg/mL) and the mitogenic lectin concanavalin A (1 µg/mL). However, the effects were not statistically significant. We detected varied IL-2 and IL-4 secretion in response to stimulation with boron-containing compounds. Our findings suggest that PBA induces canine PBMC proliferation, albeit less potently than lectins. However, boronic acid compounds may still prove beneficial as adjuvants to elicit cell proliferation and boost canine immune responses.

Key words: Boric acid, canine, mitogen, phenylboronic acid, proliferation

1. Introduction

Lectins are carbohydrate-binding proteins that can attach to cell membranes. Concanavalin A (Con A) and pokeweed mitogen (PWM) are lectins that have been used to induce lymphocyte blastogenesis in vitro. The main obstacles to the use of lectins in immune therapy are that they can be toxic and nonimmunogenic in vivo (1–3). Therefore, the discovery of novel nontoxic immune modulators would increase treatment options (4). Boron compounds have found widespread use in various fields, including medicine, aeronautics, agriculture, environmental science, and energy saving. Medical uses of boron compounds are particularly varied. The compounds are used in germicidal and antibiotic products, agents that protect against ischemic damage, and agents that enhance the effectiveness of prostate cancer treatment, wound-healing, and organ preservation. In addition, boronic acids have proven valuable in a variety of medical applications, including the treatment of cancer and infection, and the removal of biofilm in vitro (5–9). Furthermore, boron compounds have been reported to be immunogenic, although studies on how boron affects immunity have largely focused on the effects of supplemental dietary boron. Fry et al. (10) reported that supplementation of dietary boron had minimal effects on immune function in cattle. Armstrong and Spears (11) demonstrated that oral administration of boron (2.1 mg/kg) in pigs increased their titers of specific IgM and IgG. In addition, boric acid (BA) (0.5 mg/kg, administered orally) augmented tumor necrosis factor (TNF)-α responses against lipopolysaccharide from *Escherichia coli*. Higher TNF responses in the presence of boron have also been documented in chicken and human cell cultures (12,13). However, boron compounds have varied effects on lymphocyte blastogenesis. In a study of cell proliferation, borax (0.15 mg/mL) was shown to be toxic to human peripheral blood cells (14). On the other hand, Miyazaki et al. (15) suggested that boronic acids can play a lectin-like role in mouse lymphocyte cultures. This finding was one of the most dramatic reported effects of boron on immune cells. Further evidence of the mitogenic potential of phenylboronic acid (PBA) on mouse lymphocytes in vitro has been reported (16). If this effect on immune cell proliferation is consistent across species, PBA could potentially be used as an artificial mitogen in vitro. In addition to PBA, there is also evidence that BA has a similar effect on cell proliferation (17). On the other hand, development of optimal vaccine adjuvants is ongoing. PBA, alone or in combination with other adjuvants, could be a candidate for enhancing vaccination-induced immune responses.

In this study, we investigated the effects of the boron-containing compounds PBA and BA on canine peripheral blood mononuclear cell (PBMC) proliferation.

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2. Materials and methods

2.1. Compliance with ethical standards

This study was carried out after approval by the Local Ethics Committee of the Selçuk University Veterinary Faculty in Konya, Turkey (No. 79/2014)

2.2. Animals and sampling

Twelve dogs (six purebred Kangal and six mixed breed dogs; 0.5–3 years old) were used in the study. The animals were provided by the Canine Research Unit of the Faculty of Veterinary Medicine, Selçuk University. Prior to blood sampling for the experiments, stool and blood from each dog were examined for parasites in the Laboratory of Parasitology, according to Knott's and native methods. Only animals found negative for parasites were included in the study. Heparinized peripheral blood was collected from the dogs by venipuncture of the antebrachial vein.

2.3. PBMC isolation

PBMCs were isolated according to the method reported by Wagner et al. (18), with slight modifications. In brief, we diluted two parts blood with one part RPMI-1640 medium (Sigma-Aldrich, R8758, USA) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, F9665) and 1% each penicillin-G (Sigma-Aldrich, P3032) and streptomycin (Sigma-Aldrich, 9137). Lymphocytes were separated by one-step centrifugation on a 57.5% Percoll gradient (Sigma-Aldrich, P1644) at 400 × *g* for 30 min. The PBMCs were washed three times, then resuspended in supplemented RPMI-1640 medium. Isolated cells were counted by light microscopy, using a Neubauer chamber. Viability was determined by the trypan blue exclusion test (19).

2.4. PBMC culture

Cell suspensions were adjusted to a concentration of 3 × 10^6 viable cells/mL with supplemented RPMI-1640 formulated as above. PBA-free 96-well flat-bottom tissue-culture microplates were used in this study (Corning, CLS3585, USA). Aliquots of 100 µL of cell suspension per well were plated in triplicate in tissue-culture microplates. We then added 98 µL of the culture medium to all wells, followed by 2 µL of the specified mitogen (0.1 mg/mL Con A and 1 mg/mL PWM). Finally, we added 2 µL of various concentrations (10 µg/mL, 5 µg/mL, and 0.1 µg/mL) of PBA (Sigma-Aldrich, AC13036010) and BA (Sigma-Aldrich, 6768) for final concentrations of 0.1, 0.05, and 0.001 µg/mL for each compound. All wells contained 200 µL; the volumes in control wells were adjusted with supplemented RPMI-1640. The cells were incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2.

2.5. Quantification of cell proliferation

Proliferation was measured by analysis of bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) incorporation, using the Cell Proliferation ELISA, BrdU (Roche, 11647229001, Switzerland). Briefly, following 48 h of culture, BrdU labeling solution (20 µL) was added to each well of the microculture plates, which were incubated for an additional 2 h. Then the microplates were centrifuged at 300 × *g* for 10 min at 15 °C, and the pellets were dried and DNA was denatured. We coated plates with anti-BrdU monoclonal antibody for 90 min. After the incubation, we washed the plates three times to remove unbound antibody and incubated them with horseradish peroxidase-conjugated goat antimouse antibody for 90 min. We washed the plates three times, then added substrate solution 3,3',5,5'-tetramethylbenzidine (TMB) to each well and incubated them in the dark at room temperature for 30 min. Stop solution was added to each well in the same sequence as the previous addition of substrate solution. We measured the absorbance at dual wavelengths (450 and 630 nm), using a spectrophotometric microplate reader (Biotek ELX 800, USA).

2.6. Cytokine measurement

We measured the levels of IL-2 or IL-4 in the supernatants of the cell cultures by ELISA according to the manufacturer's instructions (Sunred Biotechnology Co., Canine IL-2 ELISA, 201-15-0140 and Canine IL-4 ELISA, 201-15-0127, China). Supernatant samples were collected before BrdU labeling and stored at - 80 °C until use. The results were analyzed in accordance with the manufacturer's instructions.

2.7. Statistical analysis

Statistical analyses were performed with SPSS 6.0 for Windows (SPSS Inc., Chicago, IL, USA). Wilcoxon's test was performed to compare mitotic activity of PBMCs between groups. Differences were considered significant when $P < 0.05$.

3. Results

All the animals included in the study were found negative for parasites. The mean viability of the PBMCs was 92.8 ± 1.8%. There was no difference in proliferations in either breed group between mitogen-free cultures ($P > 0.05$). We found that both Con A and PWM alone induced significantly greater proliferation than unstimulated controls in both breed groups ($P < 0.05$) (Table). We did not detect an increase in blastogenesis in non-purebred cells treated with BA (0.001 µg/mL) or PBA (0.1, 0.05, and 0.001 µg/mL) alone ($P > 0.05$).

BA or PBA in combination with either Con A or PWM affected PBMC proliferation in each breed group, although none of these combinations induced as strong a response in non-purebred PBMC as Con A alone. Similarly, none of the combinations induced proliferation of non-purebred PBMCs as potently as PWM alone.

Con A (1 µg/mL) alone induced a statistically significant increase in proliferation in PBMCs isolated from purebred

Table. Effects of boronic acid compounds on cell proliferation and cytokine production.

Stimulators	Non-purebred*			Purebred*		
	Proliferation	Cytokine (pg/mL) (mean ± SD)		Proliferation	Cytokine (pg/mL)	
	(OD; mean ± SD)	IL-2	IL-4	(OD; mean ± SD)	IL-2	IL-4
Control	0.280 ± 0.137	44.59 ± 2.18	76.53 ± 4.72	0.208 ± 0.071	63.73 ± 2.8	78.55 ± 8.45
Con A (1 µg/mL)	1.469 ± 1.001**	57.22 ± 1.51	82.42 ± 1.19	1.045 ± 0.319**	69.67 ± 3.98	78.37 ± 1.52
PWM (10 µg/mL)	1.136 ± 0.757**	55.54 ± 0.32	82.18 ± 3.5	0.857 ± 0.168**	65.94 ± 3.42	70.31 ± 4.29
PBA (0.1 µg/mL)	0.387 ± 0.245	56.47 ± 1.97	78.74 ± 3.38	0.254 ± 0.092	61.73 ± 1.87	79.33 ± 0.61
PBA (0.05 µg/mL)	0.478 ± 0.438	57.42 ± 6.61	74.16 ± 5.52	0.302 ± 0.107	50.71 ± 1.57	80.46 ± 0.86
PBA (0.001 µg/mL)	0.365 ± 0.264	55.64 ± 2.94	60.75 ± 8.49	0.301 ± 0.139	69.31 ± 0.48	67.48 ± 8.11
BA (0.1 µg/mL)	0.314 ± 0.160	54.7 ± 3.64	82.17 ± 0.54	0.233 ± 0.087	63.52 ± 1.63	71.52 ± 3.21
BA (0.05 µg/mL)	0.499 ± 0.366	60.92 ± 1.3	79.05 ± 4.56	0.262 ± 0.055	60.1 ± 5.47	76.4 ± 1.63
BA (0.001 µg/mL)	0.353 ± 0.183	61.82 ± 1.35	72.32 ± 1.47	0.290 ± 0.066	54.41 ± 7.1	100.56 ±14.06
Con A (1 µg/mL) + PBA (0.1 µg/mL)	0.764 ± 0.411	59.17 ± 3.06	77.21 ± 0.42	0.707 ± 0.395	75.3 ± 0.61	75.99 ± 2.61
Con A (1 µg/mL) + PBA (0.05 µg/mL)	1.130 ± 0.727	57.06 ± 9.96	85.66 ± 8.21	1.069 ± 0.367	80.23 ± 19.4	85.96 ± 5.92
Con A (1 µg/mL) + PBA (0.001 µg/mL)	1.166 ± 0.847	46.01 ± 7.74	74.69 ± 0.92	1.169 ± 0.484	89.3 ± 11.53	83.11 ± 0.54
Con A (1 µg/mL) + BA (0.1 µg/mL)	1.177 ± 0.937	61.02 ± 2.37	73.11 ± 1.77	1.040 ± 0.316	82.76 ± 13.93	77.79 ± 0.85
Con A (1 µg/mL) + BA (0.05 µg/mL)	1.231 ± 0.438	62.23 ± 2.64	80.74 ± 4.44	1.089 ± 0.337	59.09 ± 3.31	66.8 ± 3.46
Con A (1 µg/mL) + BA (0.001 µg/mL)	1.309 ± 0.899	60.0 ± 2.74	79.23 ± 2.09	1.178 ± 0.575	54.39 ± 8.35	75.82 ± 2.54
PWM (10 µg/mL) + PA (0.1 µg/mL)	0.387 ± 0.179	55.89 ± 2.49	74.44 ± 3.87	0.385 ± 0.138	68.39 ± 1.68	86.04 ± 1.6
PWM (10 µg/mL) + PBA (0.05 µg/mL)	0.772 ± 0.471	54.82 ± 3.08	73.92 ± 1.66	0.518 ± 0.109	74.78 ± 0.64	80.45 ± 4.6
PWM (10 µg/mL) + PBA (0.001 µg/mL)	1.061 ± 0.571	60.95 ± 3.71	75.40 ± 2.08	0.814 ± 0.155	65.34 ± 0.59	78.86 ± 0.4
PWM (10 µg/mL) + BA (0.1 µg/mL)	0.981 ± 0.396	66.61 ± 1.37	79.55 ± 13.46	0.629 ± 0.169	67.17 ± 2.21	79.03 ± 3.46
PWM (10 µg/mL) + BA (0.05 µg/mL)	0.963 ± 0.571	44.84 ± 2.77	76.42 ± 1.34	0.865 ± 0.148	75.88± 10.31	86.84 ± 7.9
PWM (10 µg/mL) + BA (0.001 µg/mL)	0.996 ± 0.505	59.31 ± 2.64	74.11 ± 2.02	0.779 ± 0.226	71.12 ± 0.67	79.02 ± 0.91

* n = 6. ** Significant difference vs. control ($P < 0.05$) by Wilcoxon test. All the conditions were assayed in triplicate. OD: Mean optical density. Effect of PBA and BA on proliferation of PBMC and on production of interleukin (IL)-2 and IL-4 by 2 days culture alone or simultaneously exposed to Con A and PWM.

dogs (1.045 ± 0.319 versus 0.208 ± 0.071). On the contrary, we found very limited increases in blastogenic responses over Con A alone when Con A was used in combination with either PBA or BA (Table).

The cytokines measured in the cell culture supernatants were observed to be synthesized in variable amounts as induced by the type of mitogens (Con A or PWM) (Figure). We observed high levels of IL-4 induced by BA (0.001 µg/mL) alone in purebred PBMC cultures (100.56 ± 14.06 pg/mL).

PBA (0.05 µg/mL) induced the most potent mitogenic response in PBMCs isolated from purebred dogs, whereas BA (0.05 µg/mL) induced the greatest proliferation in non-purebred PBMCs in PBA and BA groups (0.302 ± 0.107 and 0.499 ± 0.366, respectively).

4. Discussion

Boron is a trace element (B, atomic number 5) that can be found in soil, water, rocky ground, and atmosphere (5). It is necessary for human and animal cell functions, as it binds to cis-hydroxyl groups on cell membranes (20,21). Boron has increasingly been studied in new areas of research (7,8,22). Based on their principal mechanism of action, one of two groups of vaccine adjuvants is called "immune potentiators" (23). Studies with mouse cells indicated that a boron-containing polymer is an effective immunoadjuvant for the induction of lymphokine-activated killer (LAK) cells (4). In this study, we investigated the effects of two boron compounds, BA and PBA, on canine immune cell proliferation.

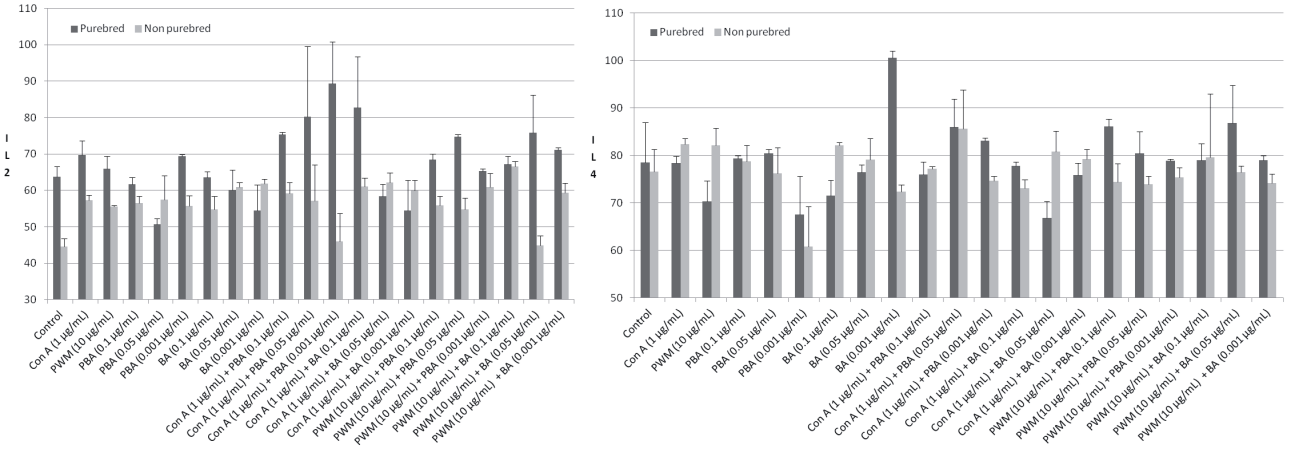

Figure. Quantities of cytokines in supernatants.

Kangals (or Kangal shepherd dogs) are purebred dogs native to Turkey (24). Our study provides data on Kangal immune responses, which have not been thoroughly investigated. The proliferation values (expressed as OD units) for Kangal PBMCs found in this study were higher than those reported for cells from four dogs from a different breed under modified culture conditions (12). Wagner et al. (18) reported that the mean OD values for wells containing control and stimulated canine cells were 0.073 and 0.421, respectively. Their cells were cultured in supplemented RPMI-1640 medium with sodium pyruvate and 1% FCS, although the number of cells per well, mitogen concentrations, and incubation conditions were the same as those in our study. In the present study, we used RPMI-1640 without sodium pyruvate supplemented with 10% FCS. In addition to the differences in breed and sample size, differences in the composition of the RPMI-1640 medium may also account for differences in the OD values between the two studies.

PBA, one of the boron compounds that we investigated, was reported to have a different effect on murine cells. Otsuka et al. (16) showed that boronic acid-containing solid phase polymers (present in the structure of the microplates) mediated mitogenic effects on mouse lymphocytes via cross-linking of boronic acid with cell membrane glycoproteins. In the study by Uchimura et al. (4), who demonstrated that a copolymer of 3-acrylamidophenylboronic acid effectively induced murine LAK cells, proliferating cytotoxic splenocytes had increased expression of IL-2 receptors on their surfaces. In our study, very mild proliferation in cultures with PBA or BA in the absence of either mitogen, together with the use of non-PBA-containing microculture plates, may affect the mitogenic potency of PBA or BA on canine PBMC. Therefore, we believe that PBA-free plates should be used to obtain reliable lymphocyte proliferation data that can be compared across species. The statistically significant proliferative response of Kangal cells to the known mitogens Con A and PWM confirmed that they are stimulants for this breed's immune cells. We observed low variation among the blastogenic responses of purebred PBMC to both known mitogens. On the contrary, there was greater variation in the responses of cells from the mixed breeds. This can be attributed to the fact that non-purebred dogs carry genetic material from various breeds, which may be differentially responsive to the stimuli. Furthermore, the low, varied mitogenic potential of the two boron compounds BA and PBA may also be a result of genetic diversity.

In non-purebred PBMCs, we observed modest proliferative response in the presence of PBA (0.05 µg in PBA groups). Since there were no other mitogens or boronic acid compounds in the cultures, these results suggest that PBA induces lymphocyte proliferation. In cultures using Con A to stimulate non-purebred cells, we observed an OD of 1469. Therefore, the ratio of the proliferative activity of PBA to Con A, a typical T cell mitogen at its optimum concentration, was greater than 1 to 2. This was also the case for BA (0.05 µg/mL) under the same culture conditions. In mouse lymphocytes, this ratio was reported as 1 to 7 when the cells were cultured in the presence of AAm-coPBA and Con A (15).

In the present study we also investigated the effects of combination treatment with PBA or BA and Con A or PWM. We found small, varied effects on proliferation, related to the concentration of the boron compound.

The findings from the non-purebred cell cultures in the presence of BA and Con A together revealed that their effects are not additive; the measured response (1.231 ± 0.438) of Con A (1 µg/mL) plus BA (0.05 µg/mL) was lower than that of Con A alone in cells from the same animals (1.469 ± 1.001). We found a similar effect with Con A in the presence of PBA. However, we observed the opposite in PBMC cultures from purebreds when PWM

was used in combination with the boron compounds; in comparison with that with PWM alone (0.857 ± 0.168), culture in combination with BA (0.05 or 0.001 µg/mL) induced similar proportion of proliferation. However, these effects did not reach statistical significance, which may be due to a variety of factors, including complex interactions of mitogens and boron compounds with the various cells in the PBMC milieu. Thus, it is not clear if PBA or BA triggered the release of factors that could promote the recruitment and maturation of immune cells, although the boron compounds, alone or in combination with a mitogen, induced little secretion of IL-2 and IL-4. However, these cytokines act through autocrine and/or paracrine mechanisms after release, so we believe that a time-dependent measurement of these cytokines, as well as other immune mediators, would be instructive.

Valli et al. (25) reported an increase in IL-2 levels in 48-h culture supernatants after stimulation of canine PBMCs with Con A. In our study, which utilized different culture conditions, we detected variable increases in IL-2 release. It is difficult to draw conclusions about T lymphocyte proliferation based solely on IL-2 levels. Such assessments could be strengthened by measuring additional cytokines. BA in the Kangal cell cultures may predominantly drive proliferation of B lymphocytes, since IL-4 is also referred to as B cell growth factor (BCGF) (7). More comprehensive measurements of additional cytokines over a time course should be undertaken in the future.

In conclusion, BA and PBA, which are used in various medical and healthcare fields, were found to have a partial stimulating effect on canine immune cell proliferation, although these effects did not reach statistical significance. With further study, use of these boron compounds in vivo as immune stimulants may still be revealed.

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