ANALYSIS OF MITOGEN-STIMULATED LYMPHOCYTE SUBSET PROLIFERATION AND NITRIC OXIDE PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS OF CAPTIVE ELK (CERVUS ELAPHUS)

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ANALYSIS OF MITOGEN-STIMULATED LYMPHOCYTE SUBSET PROLIFERATION AND NITRIC OXIDE PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS OF CAPTIVE ELK (*CERVUS ELAPHUS*)

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ABSTRACT: Elk (*Cervus elaphus*) are reservoirs for *Brucella abortus, Mycobacterium bovis,* and *Mycobacterium avium* subsp. *paratuberculosis,* each a serious pathogen of domestic livestock. An understanding of the basic immune responsiveness of elk would aid efforts to develop methods to diagnose and prevent these diseases of elk. Peripheral blood mononuclear cells (PBMC) isolated from captive elk were examined for phenotype, lymphocyte subset proliferative capacity, and ability to produce nitric oxide (NO) upon pokeweed mitogen (PWM) stimulation. Although γδ TCR^+ cells represented a high percentage of the peripheral blood lymphocyte pool, these cells responded poorly to PWM stimulation. B cells (i.e., $sIgM^+$ cells), conversely, were responsive to PWM stimulation. Addition of PWM to PBMC cultures also resulted in a significant production of nitrite, the stable oxidation product of NO. Similar to other ruminant species, the majority of elk peripheral blood $sIgM^+$ cells co-expressed MHC class II and B-B4, a B cell lineage marker that varies in expression during B cell development. Findings from the present study provide basic information on several parameters of cellular immunity of elk.

Key words: B cells, cellular immunity, *Cervus elaphus,* lymphocyte subsets, nitric oxide.

INTRODUCTION

Elk serve as reservoirs for several intracellular pathogens (*Brucella abortus, Mycobacterium avium* subsp. *paratuberculosis,* and *M. bovis*) of economic significance to domestic livestock (Whipple et al., 1997; Cheville et al., 1998, Manning et al., 1998). These diseases also threaten the health of wild and captive elk and have resulted in regulations on interstate movements and concern about commingling with domestic species. To combat these diseases, it would be beneficial to develop vaccines that could be used in wild and/or captive herds. Vaccine development for elk is hindered, however, by a paucity of basic information on cellular immunity of this species. Indeed, it has recently been determined that a live *B. abortus* vaccine (strain RB51) proven effective for bison and cattle is ineffectual in prevention of abortion caused by *B. abortus* infection of elk (Kreeger et al., 2000, 2002). Vaccine failure may be due to inadequate cellular immune responses, despite elevated levels of RB51-specific IgG in vaccinated elk (Olsen et al., unpubl. data).

Cell-mediated immune responses are critical in the host defense against intracellular bacterial pathogens (Cheville et al., 1993; Chan and Kaufmann, 1994; Chiodini, 1996). A key component of this response is clonal expansion of lymphocytes and the elaboration of cytokines that activate macrophages for killing of bacteria located within the phagosomal compartment. Potent mediators of intra-phagosomal killing are reactive nitrogen intermediates (e.g., nitric oxide, NO) produced via the induction of inducible NO synthase (NOS), often as a sequalae to interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), or lipopolysaccharide (LPS) stimulation (MacMicking et al., 1997; Kaufmann, 1999). The objective of this study was to determine the phenotype,

proliferative capability, and NO production capacity of mononuclear cells isolated from the peripheral blood of healthy elk in order to obtain baseline data for future studies with antigen specific responses.

MATERIALS AND METHODS

This study was conducted from October 2000 to February 2001 at the National Animal Disease Center (Ames, Iowa, USA). Elk (approximately 8 to 9 mo of age and of both sexes) were obtained from a brucellosis- and tuberculosis-free area (Starkey Experimental Forest and Range, Oregon Department of Fish and Wildlife, La Grande, Oregon, USA). Upon arrival, elk were vaccinated against *Clostridium* spp. (Ultrabac-7, Pfizer, Groton, Connecticut, USA) and treated with ivermectin (Dectomax®, Pfizer, Groton, Connecticut). Elk were fed alfalfa hay and a commercial cervid diet (High-protein Deer Checkers, Purina, St. Louis, Missouri, USA). Prior to sampling, elk were allowed to acclimate to their new environment (a paddock of 1 ha) for 4 wk.

Mononuclear cells were isolated from buffy coat fractions of peripheral blood collected in 2x-acid citrate dextrose using standard procedures (Burton and Kehrli, 1996). For lymphocyte blastogenesis assays, 96-well round-bottomed microtiter plates (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA) were seeded with 2×10^5 mononuclear cells in a total volume of 200 μl per well. The medium was RPMI 1640 supplemented with 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 5×10^{-5}M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Missouri), and 10% fetal bovine sera (FBS). The wells contained either medium plus pokeweed mitogen (PWM, 0.1–10 μg/ml, Sigma Chemical Co.) or medium alone (no stimulation). Cells in wells were then incubated for 2 days at 37 C in 5% CO_2 in air. After 2 days, 0.5 μCi of methyl-[^{3}H] thymidine (specific activity 6.7 Ci mmole^{-1}; Amersham Life Science, Arlington Heights, Illinois, USA) in 10 μl of medium was added to each well, and cells were incubated for an additional 20 hr. The well contents were harvested onto fiber filters with a Wallac 96 well plate harvester (EG & G Wallac, Gaithersburg, Maryland, USA) and the incorporated radioactivity measured by liquid scintillation counting. Treatments were run in triplicate and data are presented as mean counts per minute (cpm) ± SEM.

Nitrite is the stable oxidation product of NO and the amount of nitrite within culture supernatants is indicative of the amount of NO produced by cells in culture. Replicate PBMC cultures (i.e., as prepared for lymphocyte proliferation assays, triplicate wells for each treatment) were established so that supernatants could be harvested after 24, 48, and 96 hr of incubation for analysis of nitrite. Nitrite within the supernatant was measured using the Griess reaction (Rajaraman et al., 1998) performed in 96-well microtiter plates (Immulon 2, Dynatech Laboratories, Inc., Chantilly, Virginia, USA). Culture supernatant (100 μl) was mixed with 100 μl of Griess reagent (0.5% sulfanilamide; Sigma Chemical Co.) in 2.5% phosphoric acid (Mallinckrodt Chemicals, Inc., Paris, Kentucky, USA) and 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma Chemical Co.). The mixture was incubated at 21 C for 10 min. Absorbances of test and standard wells were measured using an automated enzyme linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Menlo Park, California, USA). All dilutions were made using culture medium (RPMI 1640 medium with 2 mM L-glutamine and 10% FBS v/v). Absorbance of standards, controls, and test samples at 550 nm was then converted to nanograms of nitrite by comparison with absorbance values of sodium nitrite (Fisher Chemicals, Fair Lawn, New Jersey, USA) standards within a linear curve fit and presented as ng/ml. N^G-monomethyl-L-arginine (L-NMMA, 1.15mM; equimolar to the amount of L-arginine in the culture medium; Calbiochem, La Jolla, California), a competitive inhibitor of the enzyme NOS, was added to parallel non-stimulated or stimulated cultures in order to verify that the nitrite produced was the result of the specific activity of NOS.

To evaluate lymphocyte subset proliferative responses, PKH67 assay, a flow cytometric proliferation assay was used. The PKH67 proliferation assay was performed according to manufacturer instructions (Sigma Chemical Co.) and as previously described (Waters et al., 2000a). Briefly, 2×10^7 PBMC were centrifuged (400 × G) for 5 min, supernatants aspirated, and cells resuspended in 1 ml of diluent (Sigma Chemical Co.). Cells, in diluent, were added to 1 ml of PKH67 green fluorescent dye (4×10^{-6} M) and incubated 5 min followed by a 1 min incubation with 2 ml of FBS to stop the reaction. Cells were then washed three times with RPMI 1640. The PKH67 stained cells were added to wells (2×10^5/well with six replicates) of a 96-well round-bottomed microtiter plate in medium (no stimulation) or medium plus 1 μg/ml PWM. Cells in wells were then incubated at 37 C in 5% CO_2 humidified air for 3 days. As cells divide, PKH67 staining diminishes resulting in a decreased mean fluo-

rescence intensity of the cell (Ashley et al., 1993). Stability of the dye incorporation into the lipid membrane ensures that when cells divide the dye is distributed equally between daughter cells. At the end of the culture period, cells were harvested and analyzed by flow cytometry for PKH67 staining intensity and cell surface marker expression enabling the determination of proliferation of individual cells of known phenotype. Modfit proliferation wizard (Verity Software House Inc., Topsham, Maine, USA) and CellQuest software (Becton Dickinson, San Jose, California) were used for cell proliferation and phenotype analyses. Proliferation profiles were determined for both gated (i.e., $CD4^+$, $CD8^+$, $\gamma\delta$ TCR^+, $WC1^+$, and $sIgM^+$) or ungated (total PBMC) populations and presented as the number of cells proliferating per 10,000 PBMC ± SEM.

Mononuclear cells were analyzed for expression of cell surface antigens by flow cytometry. Cells (2×10^6/ml) in 100 μl balanced salt solution with 1% FBS and 0.1% sodium azide (FACS buffer) were stained with 100 μl of primary antibody to leukocyte surface antigens 17D1, anti-CD4; ST8, anti-CD8; GB21A, anti-γδ TCR; BAQ4A, anti-WC1; and PIG45A, anti-IgM for B cells (all obtained from VMRD Inc., Pullman, Washington, USA). Following a 15 min incubation, cells were centrifuged (400 × G) for 2 min and resuspended in 100 μl of either phycoerythrin (PE)-conjugated goat anti-mouse IgM or IgG2b (Southern Biotechnology Associates, Inc., Birmingham, Alabama, USA) or peridinin chlorophyll protein (PerCP)-conjugated rat anti-mouse IgG1 (Becton Dickinson). Cells were then incubated for an additional 15 min, centrifuged (400 × G) for 2 min, resuspended in FACS buffer and analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson) with at least 10,000 events analyzed from each sample.

Two color staining of mononuclear cells was also performed to analyze the expression of cell surface antigens by flow cytometry. Cells at a concentration of 1×10^6/ml were suspended in FACS buffer and stained in a 50 μl volume with the following primary antibodies: PIG45A, anti-IgM; TH14B, anti-major histocompatibility complex (MHC) class II; BAQ155A, anti-B-B4 (all obtained from VMRD Inc.). After 15 min of incubation at 4 C, the cells were washed twice in FACS buffer and stained in a 50 μl volume of the following secondary antibodies: goat anti-mouse IgG1 fluorescein isothiocyanate (FITC); goat anti-mouse IgG2a FITC; goat anti-mouse IgG2b PE (Southern Biotechnology Associates, Inc.). Cells were incubated for 15 min at 4 C, washed twice in FACS buffer and then fixed with 2% paraformaldehyde in phosphate buffered saline (PBS). For each sample, 10,000 events were collected using a Becton Dickinson FACScan flow cytometer and data were analyzed using CellQuest software.

Data were analyzed by one-way analysis of variance followed by Tukey-Kramer multiple comparisons test. Differences between groups were considered significant if probability values of $P < 0.05$ were obtained.

RESULTS

In preliminary studies, we determined that PWM stimulation of elk PBMC induces greater proliferation than does concanavalin A or phytohemagglutinin stimulation (data not shown). Thus, in the present study, PWM was used for the study of mitogen-induced proliferation and nitrite production by PBMC. Stimulation of PBMC for 3 days with either 0.1 or 1.0 μg/ml PWM resulted in significantly ($P < 0.05$) greater DNA synthesis as measured by [^{3}H] thymidine uptake than did stimulation with 10 μg/ml PWM (Fig. 1). Stimulation with 0.1 μg/ml PWM, however, did not induce significant production of nitrite as compared to levels produced by non-stimulated cultures whereas stimulation with 1.0 or 10 μg/ml PWM induced significant ($P < 0.05$) nitrite production by PBMC (Fig. 2). Production of nitrite by PBMC over a period of 3 days increased steadily with a significant ($P < 0.05$) increase at 72 hr as compared to the response at 24 or 48 hr (Fig. 3). Addition of L-NMMA to duplicate cultures abrogated nitrite production by PWM-stimulated PBMC indicating nitrite production was due to the specific action of NOS (data not shown).

Pokeweed mitogen stimulation induced a significant proliferative response as measured by [^{3}H] thymidine uptake. To assess the contribution of individual lymphocyte subsets to this proliferative response, PKH67 assay was used. Stimulation of PBMC with 1 μg/ml PWM resulted in a significant ($P < 0.05$) proliferative response compared to the response of non-stimulated cells (Table 1). Analysis of sub-

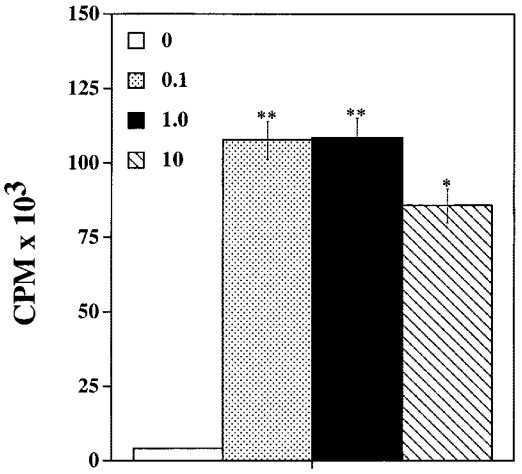

FIGURE 1. DNA synthesis as measured by [^{3}H] thymidine uptake. Elk peripheral blood mononuclear cells were cultured with no stimulation (open bar), 0.1 μg/ml pokeweed mitogen (PWM) (stippled bar), 1.0 μg/ml PWM (closed bar), or 10 μg/ml PWM (hatched bar) for 2 days, pulsed with 0.5 μCi methyl-[^{3}H] thymidine for 20 hr and harvested as described in Materials and Methods. Results are presented as the mean ± SEM counts per minute (n = 8). *DNA synthesis was greater ($P < 0.05$) than in non-stimulated cultures. **DNA synthesis was greater ($P < 0.05$) than in non-stimulated cultures and cultures stimulated with 10 μg/ml PWM.

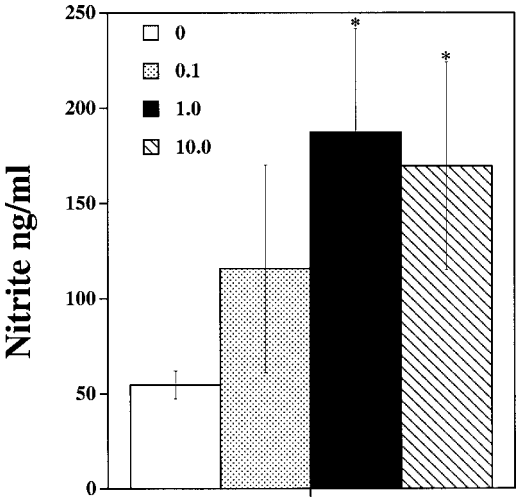

FIGURE 2. Nitrite production of elk peripheral blood mononuclear cells as measured by the Griess reaction. Cells were cultured with no stimulation (open bar), 0.1 μg/ml PWM (stippled bar), 1.0 μg/ml PWM (closed bar), or 10 μg/ml PWM (hatched bar) for 2 days and supernatants harvested for determination of nitrite production. Results are presented as the mean ± SEM nitrite (ng/ml) (n = 8). *Nitrite production was greater ($P < 0.05$) than in non-stimulated cultures.

set proliferative responses revealed that PWM stimulation induced a significant ($P < 0.05$) response by CD4$^+$, CD8$^+$, and sIgM$^+$ cells but not γδ TCR$^+$ cells (Table 1). The majority of cells proliferating in non-stimulated cultures were γδ TCR$^+$ cells. The predominant subset of cells responding to PWM stimulation were sIgM$^+$ cells (greater [$P<0.05$] than responses by other subsets). To further demonstrate this finding, the PKH67 intensity of non- or PWM-stimulated sIgM$^+$ or γδ TCR$^+$ cells were analyzed using Modfit Proliferation Wizard software (Fig. 4). With this software, PKH67 staining intensity of each cell was analyzed and generation (i.e., parent and daughter populations) plots created. The PWM stimulation resulted in numerous sIgM$^+$ cells in daughter generations yet did not induce proliferation of γδ TCR$^+$ cells. A potential concern with this assay is that PKH67 staining of lymphocyte subsets was not uniform. The PKH67 staining of elk PBMC, prior to culture, was uniform throughout all subsets (data not shown). All cells examined, regardless of subset, stained brightly with PKH67 prior to culture.

The final objective of this project was to characterize the phenotype of peripheral blood lymphocytes. The percent (mean ± SEM) expression of leukocyte differentiation markers for elk PBMC were 13.23 ± 0.90 CD4$^+$ cells, 9.37 ± 0.50 CD8$^+$ cells, 18.06 ± 1.86 γδ TCR$^+$ cells, and 30.72 ± 2.47 sIgM$^+$ cells. In addition, we also examined the expression of WC1, a scavenger receptor present on a high percentage of cattle and sheep PBMC. All WC1$^+$ cells were γδ TCR$^+$ (i.e., GB21A$^+$). The γδ TCR$^+$ cells, however, segregated into three distinct populations (WC1^{hi}, WC1^{lo}, and WC1$^-$) based upon WC1 expression with approximately equal percentages of these three populations represented (approximately 5–7% of each subset). The PKH67

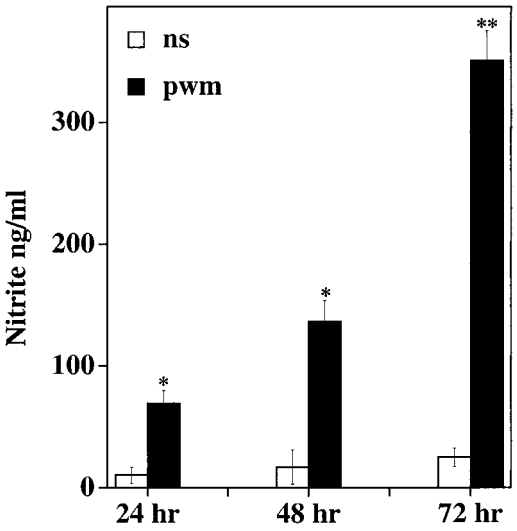

FIGURE 3. Temporal production of nitrite by elk PBMC in response to PWM stimulation. Cells were cultured with no stimulation (open bars) or 1.0 μg/ml PWM (closed bars) for 24, 48, or 72 hours and supernatants harvested for determination of nitrite by the Griess reaction. Results are presented as the mean±SEM nitrite (ng/ml) (n = 11). * Nitrite production was greater ($P < 0.05$) than in non-stimulated cultures. ** Nitrite production was greater ($P < 0.05$) than in non-stimulated cultures and cultures stimulated with 1 μg/ml PWM for 24 or 48 hours.

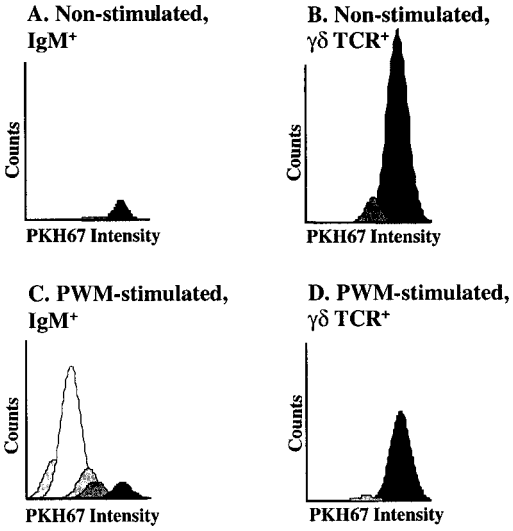

FIGURE 4. Flow cytometric evaluation of sIgM$^+$ and γδ TCR$^+$ cell proliferative capability to pokeweed mitogen (PWM) stimulation. Histograms represent responses from (A) non-stimulated sIgM$^+$ cells, (B) non-stimulated γδ TCR$^+$ cells, (C) PWM-stimulated sIgM$^+$ cells, and (D) PWM-stimulated γδ TCR$^+$ cells. The parent generations (non-proliferative fraction) are indicated by black filled peaks whereas daughter generations (proliferative fraction) are indicated by grey-shaded peaks. Note that multiple daughter generations of sIgM$^+$ cells are induced upon PWM stimulation whereas minimal daughter generations of γδ TCR$^+$ cells are induced by PWM stimulation. Results from one representative elk are shown.

analysis of each of these subsets of γδ TCR$^+$ cells did not reveal a proliferative fraction after 3 days of PWM stimulation.

Expression of sIgM, B-B4, and MHC class II on elk PBMC was evaluated (Fig. 5). The majority of elk peripheral blood sIgM$^+$ B cells co-expressed B-B4. However, there were also two minor populations of elk PBMC that were either B-B4$^+$, sIgM$^-$ or B-B4$^-$, sIgM$^+$. In some elk a distinct population of sIgM^{lo}, B-B4^{lo} cells was evident (Fig. 5, middle panel). As a percent of the total PBMC population, there

TABLE 1. Lymphocyte subset proliferative responses of elk to 1 μg/ml pokeweed mitogen (PWM) stimulation. Data represent the mean number of cells that proliferated per 10,000 cells (within the live gate) ± SEM (n = 11).

Lymphocyte populations	No stimulation	PWM stimulation	PWM stimulation minus no stimulation
Peripheral blood mononuclear cells	2,080 ± 320	5,765 ± 319[a]	3,685 ± 351
CD4$^+$	260 ± 89	1,065 ± 149[a]	805 ± 83
CD8$^+$	345 ± 169	1,289 ± 154[a]	944 ± 159
γδ TCR$^+$	1,652 ± 27[b]	861 ± 249	−791 ± 202
sIgM$^+$	143 ± 45	3,130 ± 443[a,b]	2,987 ± 408[b]

[a] Significantly ($P \leq 0.0005$) greater than no stimulation responses for the same lymphocyte population.
[b] Significantly ($P < 0.01$) greater than responses of other lymphocyte subsets receiving the same stimulation (i.e., vertical comparisons between CD4$^+$, CD8$^+$, γδ TCR$^+$, and sIgM$^+$ cells).

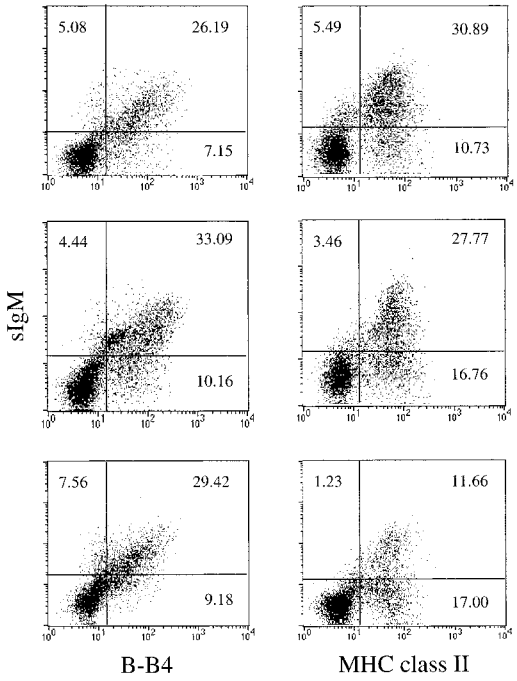

FIGURE 5. Expression of sIgM, B-B4 and major histocompatibility complex (MHC) class II on elk peripheral blood mononuclear cells. Cells were isolated and stained for two-color flow cytometric analysis. Plots in each column shown are from three separate animals and were chosen to illustrate the staining observed for cells isolated from 27 elk. Numbers shown in each quadrant are the percentages of cells within each gate. Gating was determined on the basis of the fluorescence intensity of cells stained with secondary antibody alone (negative control).

were 22.26 ± 1.65 B-B4$^+$, sIgM$^+$ cells (range 9.33–41.79, n = 27) and 10.14 ± 1.04 B-B4$^+$, sIgM$^-$ cells (range 1–24.62, n = 27). Further, the majority of sIgM$^+$ cells co-expressed MHC class II. The MHC class II$^+$, sIgM$^+$ B cells represented 15.99 ± 1.98 (range 4.01–36.53, n = 27) of the PBMC population. There was also a minor subpopulation of sIgM$^+$ cells that did not express MHC class II. The percent of MHC class II$^+$, sIgM$^-$ in the PBMC population ranged from 5.00 to 38.85 (mean 14.84 ± 1.49, n = 27).

DISCUSSION

Recent reports suggested that elk develop a predominant antibody response to vaccination with the live *B. abortus* vaccine, strain RB51 (Kreeger et al., 2002; Olsen et al., unpubl. data). It was hypothesized that this bias of the elk immune response towards a humoral response may have resulted in vaccine failure due to an insufficient cellular immune response. In the present study, we determined that elk PBMC proliferate in response to PWM stimulation. The proliferative response to PWM stimulation was mixed with both T and B cells responding. There was, however, a greater response by sIgM$^+$ cells as compared to other subsets examined. In studies with cattle and pig PBMC, PWM did not induce a significant proliferative response by sIgM$^+$ cells (Quade and Roth, 1999; Waters, unpubl. data). Upregulation of activation markers (e.g., MHC class II antigen and IL-2 receptor) on sIgM$^+$ cells was, however, detected on PWM stimulated cattle PBMC (Quade and Roth, 1999). It was likely that cytokines produced by proliferating T cells induced expression of these activation markers. Examination of sIgM$^+$ cell proliferation (Fig. 4) demonstrated that PWM induced multiple daughter generations, indicating that the sIgM$^+$ cells had cycled through several cell divisions. Thus, it was likely that PWM stimulated proliferation of elk sIgM$^+$ cells directly; however, cell separation studies would be necessary to conclusively rule out a T cell dependent pathway.

Macrophage activation is a necessary component for killing of intracellular pathogens. Macrophages may become activated by contact with bacterial products (e.g., LPS) or cytokines (e.g., IFN-γ, TNF-α, etc.). Detection of reactive oxygen or nitrogen intermediates (e.g., NO) produced by macrophages is a functional measure of this activation. Our findings suggested that elk macrophages were capable of producing reactive nitrogen intermediates upon stimulation with PWM. Because PWM-stimulated cultures also contained lymphocytes, it was likely that cytokine production by T cells induced macrophage activation and subsequent NO production. It was possible, although unlikely, that cell types

other than macrophages also contributed to NO production within PBMC supernatants. It is also possible that PWM induced NO production by macrophages directly. Interestingly, others studying red deer (*Cervus elaphus*) macrophage function have been unable to detect NO in the supernatants of isolated macrophages stimulated with LPS (Cross et al., 1996b). However, superoxide anion and pro-inflammatory cytokine activity by red deer macrophages stimulated with various stimuli were detected (Cross et al., 1996b). Our findings, conversely, suggested that macrophages isolated from elk were fully capable of NO production.

Phenotypic analysis of elk PBMC revealed similar percents of lymphocyte subsets as described previously for red deer (Buchan and Griffin, 1990; Cross et al., 1996a) and other domestic ruminant species (Smith et al., 1994; Wyatt et al., 1994). An unusual finding of the present study was the differential expression of the scavenger receptor, WC1, by γδ TCR^+ cells (e.g., $WC1^{hi}$, $WC1^{lo}$, and $WC1^-$). Approximately one third of γδ TCR^+ cells were $WC1^-$. In cattle, it was estimated that approximately 90% of peripheral blood γδ TCR^+ cells co-express WC1 (Park et al., 1993; Wyatt et al., 1994). The majority of cells proliferating in non-stimulated cultures were γδ TCR^+ cells. Similarly, γδ TCR^+ cells also proliferated in non-stimulated PBMC cultures of cattle (Waters et al., 2000b). In the present study, γδ TCR^+ cells regardless of WC1 expression did not proliferate upon PWM stimulation. Likewise, PWM was a poor stimulant of γδ TCR^+ cell proliferation of cattle and pigs (Quade and Roth, 1999; Waters, unpubl. data). Thus, the relevance of the differential expression of WC1 by γδ TCR^+ cells of elk is unclear at this time. In future studies, analysis of subpopulations of γδ TCR^+ cells in the recall proliferative response to antigens may provide clues to their function.

The cell surface marker, B-B4, is a B cell lineage marker that varies in expression during B cell development. The majority of $sIgM^+$ B cells of elk co-expressed B-B4 similar to that previously described for other ruminant species (Griebel et al., 1992). The minor subpopulations of B cells that were $sIgM^+$, B-$B4^-$ or $sIgM^-$, B-$B4^+$ likely represent B cells in different developmental stages. In some elk a distinct population of $sIgM^{lo}$, B-$B4^{lo}$ cells was evident. Whether these $sIgM^{lo}$, B-$B4^{lo}$ B cells were indicative of a distinct developmental stage or a separate lineage was not known. Thus, further examination of the ontogeny of elk B cell subpopulations is warranted.

In conclusion, we have defined some basic immune parameters of the cellular responsiveness of elk. While it is tempting to speculate that the unusual responsiveness of elk B cells to PWM stimulation is indicative of their reported tendency to generate a biased humoral immune response to certain pathogens, further studies are necessary to clearly define this phenomenon. It was, however, determined that elk peripheral blood-derived mononuclear cells are fully capable of NO production. Thus, this aspect of their cellular immunobiology is intact.

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