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IMMUNOTOXICITY STUDIES IN MINK (*MUSTELA VISON*) CHRONICALLY EXPOSED TO DIETARY BLEACHED KRAFT PULP MILL EFFLUENT

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ABSTRACT: The immunotoxic potential of bleached kraft pulp mill effluent (BKME) to cell-mediated immunity in mink (*Mustela vison*) was investigated October 1993 through May 1994. For 26 weeks, 20 mink were fed a diet based upon fish caught within 6 km downstream of a bleached kraft mill in Saskatchewan, Canada. Water for this group contained 25% softwood-run BKME. Twenty control mink were fed nutritionally matched diets based upon fish from lakes receiving no municipal or industrial effluent and tap water. Using in vitro and in vivo immunotoxicity assays, the proliferative response of mink peripheral blood mononuclear cells (PBMC) to mitogens was optimal, at 72 hr with 10 μg/ml Concanavalin A, 1/80 dilution pokeweed mitogen, and 1/80 dilution phytohemagglutinin. Bacterial cell wall *Escherichia coli* lipopolysaccharide did not stimulate mitosis of the mink PBMC. No difference ($P < 0.05$) in PBMC proliferation was seen between the control and BKME-exposed mink with any of the mitogens used. Delayed type hypersensitivity (DTH), a cell mediated response, was assessed in mink vaccinated with live bacille Calmette-Guérin (BCG) and then challenged by intradermal toe web injection with 200 μg of sonicated BCG approximately 6 weeks later. The DTH response in the BKME-exposed mink was impaired based upon assessment using skin thickness measurements, histopathological assessment and image analyzer technology. This decreased response is evidence for suboptimal immune function associated with BKME exposure, which could affect the competitive fitness of piscivorous mammals naturally exposed to BKME.

Key words: Immunotoxicity, mink, *Mustela vison*, delayed type hypersensitivity, lymphocyte proliferation, bleached kraft mill effluent.

INTRODUCTION

The pulp and paper industry world-wide discharges an enormous amount of effluent into the aquatic environment. The bleached kraft mill in this study released approximately 110,000 m^3 of biologically treated effluent into the North Saskatchewan River (53°15′N, 105°05′W) daily. Semi-aquatic mammals, water birds, and aquatic organisms are exposed directly, or indirectly through the food chain, to a vast number of chemical compounds which comprise pulp effluent (Environment Canada, 1991). Little work has been done regarding possible effects of bleached kraft mill effluent (BKME) on aquatic mammals at risk.

The production of bright, white paper by pulp mills entails a bleaching process to remove residual lignin which imparts a brownish color to the pulp. In kraft bleaching operations delignification entails a sequence of chorination followed by alkali extraction (Owens, 1991). The biology of wood plus the pulping process result in a complex mixture of compounds including extractives, organic acids, a range of sulfur-containing compounds, chlorinated phenolics, chlorinated neutral compounds, chlorinated organic acids, and various phytochemicals (Owens, 1991). Pulp effluent is impossible to characterize completely. Besides the hundreds of individual compounds in pulping effluent, the composition of these compounds changes according to the types of wood used, types of pulp being produced, and the treatment of the effluent before it is released into receiving waters. In both field and experimental stream exposures, the BKME com-

pounds most commonly measured in fish have included resin and fatty acids, chlorophenols, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and extractable organic chlorine load (Owens 1991).

The impact of environmental contamination on animals may be dramatic, such as high mortality from direct exposure, or subtle, as by increased incidence of disease, or reproductive dysfunction. Semiaquatic mammals in areas contaminated by industrial effluents are exposed to a mixture of xenobiotics indirectly via bioaccumulation through the food chain, as well as by direct contamination from the water. Organochlorines were more toxic to mink when they were fed contaminated fish, than when they were directly exposed to the technical grade compounds, indicating that bioaccumulated organochlorine metabolites are more toxic than the parent compounds (Aulerich et al., 1986). Environmental toxicants have been found in experimental mink (*Mustela vison*) fed on Great Lakes (Canada, USA) fish (Wren, 1991), in free ranging mink and river otter (*Lutra canadensis*) in New York (USA) (Foley et al., 1988), and in otters in Alberta, Canada (Somers et al., 1987). A causative role between exposure to toxic chemicals and declining populations of mink or otter has been proposed for North America (Henney et al., 1981; International Joint Commission United States and Canada, 1988) and Europe (Mason and MacDonald, 1986).

Many environmental contaminants including organochlorines, heavy metals, alkylating agents, and pesticides other than organochlorines can alter immune function (Wong et al., 1992). Immunosuppression occurs in waterfowl experimentally exposed to concentrations of selenium comparable to those measured in natural environments (Fairbrother and Fowles, 1990). A compromised immune response has been associated with increased levels of glucocorticoids in birds subsequent to environmental or physiological stress (Fowles et al., 1993). Bleavins et al. (1983) demonstrated immunosuppression of mitogen-stimulated mink lymphocyte proliferation by hexachlorobenzene. Of four antigens tested in mink in the early 1970's, only bacille Calmette-Guérin (BCG) immunization with tuberculin-purified protein derivative intracutaneous challenge produced erythema and induration, hallmarks of the delayed type hypersensitivity (DTH) response (Munoz et al., 1974).

Cell mediated immunity (CMI) is dependent upon viable T-lymphocytes. Different aspects of CMI can be assessed through the proliferative response of mitogen-stimulated lymphocytes and DTH tests. The lymphocyte proliferation assay is used to measure the proliferative potential of peripheral blood lymphocytes, an intermediate event required to mount an immune response to a foreign antigen (Abbas et al., 1991).

A DTH response is predicated upon appropriate antigen-presenting cells, CD4+ T-lymphocyte, and macrophage interaction with cytokine-mediated intercellular communication (Abbas et al., 1991). Delayed type hypersensitivity, one aspect of cell mediated immunity, provokes a measurable local immune reaction to an injected antigen in presensitized animals.

Our objectives were to evaluate the effects of dietary BKME on immune function in mink. This required the development of in vitro and in vivo immunotoxicity assays, which were then applied to mink exposed to dietary BKME for 26 wk.

MATERIALS AND METHODS

Mink were chosen as experimental subjects because they are piscivorous semi-aquatic mammals at risk, they are amenable to captive rearing, and are highly sensitive to several organic environmental toxins (Wobeser et al., 1976; Bleavins et al., 1980; Kupfer and Bulger, 1982).

This study was carried out between October 1993 and May 1994. Forty mature demibuff female mink and 20 subadult black male cohorts from a commercial mink ranch in Wetaskiwin, Alberta, Canada, were acclimatized in their new sheds on this farm for a minimum of 1 wk, and had been vaccinated at weaning or 2 mo

before this study with a standard commercial combination mink vaccine against canine distemper, mink viral enteritis, *Pseudomonas* sp. pneumonia and botulism, Distox Plus®, (Burns Biotec, Schering, Pointe Claire, Québèc, Canada). The animals were housed individually in standard steel mink cages with attached wooden nest boxes (20 cm × 20 cm × 25 cm), natural light and ambient temperatures during a 26 wk study of physiological, reproductive, pathological (Smits et al., 1995) as well as immunological parameters.

Diets were formulated to meet or exceed National Research Council recommended levels for nutrition of mink (National Research Council, 1982), with additional nutrients added as advised by the Canadian Mink Breeders Association (Rexdale, Ontario, Canada), considering ambient temperatures and physiological state of the animals. The diets provided similar levels of protein, fat and carbohydrate. Each diet contained 45% whole fish, 55% offal and cereal fortified with minerals (calcium 0.95%, phosphorus 0.8%, copper 35 ppm, zinc 300 ppm, iron 400 ppm, manganese 160 ppm) and vitamins (vitamin A 39,000 IU/kg, vitamin D 7,000 IU/kg, vitamin E 155 IU/kg) (Feedrite Ltd., Winnipeg, Manitoba, Canada). Thirty ml softwood-run BKME per 150 g of food was incorporated into the contaminated diet. An antioxidant at 200 ppm (ethoxyquin 67%) Davis and Lawrence, Division of Canada Packers, Toronto, Ontario, Canada), was added to all feed. The use of raw thiaminase-containing fish such as sucker (*Catostomus* spp.), sauger (*Stizostedion canadense*), and whitefish (*Coregonus clupeaformis*) (National Research Council, 1982) necessitated the addition of 2 mg of thiamine HCl (Hoffman-LaRoche Ltd. Mississauga, Ontario) daily, sprinkled onto each ration.

The diet of the control group was based upon fish caught in Saskatchewan lakes not receiving municipal or industrial effluent. These included Murray Lake (53°10′N, 107°39′W), Jackfish Lake (53°04′N, 108°23′W), Wakaw Lake (52°38′N, 105°39′W), Meeting Lake (53°10′N, 107°39′W) and Lake Lenore (52°30′N, 104°59′W). The exposed group's diet was based upon fish collected within 6 km downstream from the discharge point of a bleached kraft pulp mill on the North Saskatchewan River (53°15′N, 105°05′W), 20 km east of Prince Albert, Saskatchewan, Canada, which uses 80% and 72% chlorine dioxide substitution during hardwood and softwood bleaching, respectively (E. Yee, Weyerhaeuser Canada, pers. comm.). Details of the mill pulping and bleaching technology and effluent treatment are described by Smits et al. (1995). Various species of sucker (*Catostomus commersoni, C. catostomus,* and *C. moxostoma macrolepidotum*) made up greater than 50% of the fish in both control and contaminated diets. The balance consisted of lake whitefish (*Coregonus clupeaformis*), walleye (*Stizostedion vitreum*), sauger (*S. canadense*), goldeye (*Hiodon alosoides*) and northern pike (*Esox lucius*). Whole fish were stored frozen within 8 hr of being caught, later thawed to make up the complete ration, and refrozen until fed to the mink. Samples of the composite diets based upon both control and BKME-exposed fish were analyzed for various chlorinated dioxin and dibenzofuran congeners at the Canada Centre for Inland Waters, Environment Canada (Huestis and Sergeant, 1992). Tissue residue analyses of white sucker and walleye collected in 1987 and 1988 from the same site on the North Saskatchewan River as the fish in this study, had levels of 2,3,7,8-TCDD of ≤8.0 ppt (1987) and ≤1.6 ppt (1988), and 2,3,7,8-TCDF of 17 ppt (1987) and 5.7 ppt (1988) (Saskatchewan Environment and Resource Management, 1989). Drinking water was either municipal tap water (control groups), or tap water to which 25% softwood-run BKME, collected as it entered the discharge pipe from the secondary treatment pond into the river, (exposed group) had been added. This was collected at the beginning of the study and stored frozen until required.

During the autumn fish collection period in 1993, compounds of concern in the effluent were monitored. The average biochemical oxygen demand of the effluent after an 8 day residence time in the secondary treatment aeration lagoon was 20 to 30 mg/l, sulfide was 0.65 ppm and the adsorbable organic halide level was 12 mg/l. Resin acid levels were ≤ 160 μg/l, and fatty acids ranged from 100 to 300 μg/l. (K. Dube, Weyerhaeuser Canada, pers. comm.).

The assay used for lymphocyte proliferation evaluation was a modification of the technique described by Tomar et al. (1988). After 26 wk on the BKME diet, over the course of 1 day, all mink were anesthetized by intramuscular injection of 18 to 20 mg/kg ketamine (Rogar/STB Inc., Montréal, Québèc), and 1 mg/kg xylazine (Bayvet, Etobicoke, Ontario). Blood was collected into 5 ml heparinized vacuutainer tubes (Becton Dickinson, Mississauga, Ontario) by anterior vena caval puncture, and kept on ice until harvesting of the mononuclear cells 1 to 24 hr later. The following assay was carried out in a laminar flow hood using sterile technique. Mink blood was transferred to sterile 15 ml polypropylene tubes (Falcon, Becton Dickinson, Mississauga, Ontario) using 2 to 3 ml of calcium and magnesium free Hank's Balanced Salt Solution (HBSS-Ca, Mg free) (Gibco, Bur-

lington, Ontario) to rinse the blood tubes. This cell suspension was gently mixed. Five ml of Lympholyte®-M (Cedarlane Laboratories, Hornby, Ontario) was added to sterile 15 ml tubes. The mink cell suspensions were layered onto the Lympholyte®-M, and centrifuged at 750 × *G* for 20 min. The mononuclear cell layers were carefully harvested from the interface between the overlying platelet-rich plasma and underlying Lympholyte®-M, and placed into sterile 15 ml tubes. The cells were washed twice with sterile HBSS and centrifuged 6 to 7 min at 400 × *G*, discarding the supernatant each time. Cell pellets were resuspended in 1 ml of sterile Roswell Park Memorial Institute (RPMI) 1640 cell culture medium (Sigma Chemical Co. St. Louis, Missouri, USA) containing 10% heat inactivated fetal bovine serum, and 1% penicillin-streptomycin. Twenty µl of this cell suspension was added to 180 µl of 0.04% trypan blue in 1% acetic acid in isotonic saline (Gibco) and the viable cells were counted using a hemacytometer (Spencer Brightline Hemacytometer, Canlab, Edmonton, Alberta). Sterile complete RPMI was added to each tube to achieve a final concentration of 5×10^6 cells/ml.

We added 100 µl of cell suspension into each well of a 96-well flat bottom microtiter plate (Linbro, International Chemical Nuclear Pharmaceuticals Ltd., Horsham, Pennsylvania, USA); 100 µl of RPMI only was added to the double control wells. Working in triplicate, 100 µl RPMI was added to the control wells, and 100 µl of three mitogens, Concanavalin A (ConA), pokeweed mitogen (PWM) (both Sigma Chemical Co.), and phytohemagglutinin (PHA-P) (Gibco) were added to the test wells to produce predetermined optimal concentrations per well of 10 µg/ml, 1/80 dilution, 1/80 dilution, respectively. These concentrations were determined from the proliferative responses of PBMC from four healthy normal mink stimulated with ConA, PWM, PHA-P and bacterial cell wall lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (Difco, Detroit, Michigan, United States) with dextran (LPS/Dextran). Plates were incubated at 37 C in 5% CO_2, 95% relative humidity for 72 hr. After 54 hr, 0.5 µCurrie (Ci) (20µl) [^{3}H] methyl thymidine (New England Nuclear, Boston, Massachusetts, USA) was added to all wells and incubation was completed. Well contents were harvested onto glass microfiber filters (Cambridge Technology, Watertown, Massachusetts, USA) using a cell harvester (PHD Cell Harvester, Cambridge Technology, Watertown, Massachusetts). Drying of the filters was enhanced by using 95% ethanol for the final rinse. Filters were deposited into individually labelled scintillation vials (Beckman, Mississauga, Ontario), and, once dry, 3 ml of liquid scintillation cocktail (Ready Safe, Beckman) was added to each vial. Radioactivity was measured using a β-scintillation counter (Beckman LS-3800), and expressed as counts per minute (CPM).

A pretrial screen was set up in which two naïve mink from the same cohort, but not part of the experimental group, received 50 µl intradermal injections of sonicated BCG (Connaught Laboratories Ltd., Toronto, Ontario) 200 µg (2 µl in 48 µl phosphate buffered saline pH 7.3), in the left hind interdigital toe web between the second and third phalanx. Skin thickness at the injection site was measured with a manual micrometer (Oditest 0–10 mm, Kröetlin, H. C. gmbH, Germany) at 0, 24, 48 and 72 hr. This preliminary step was taken to ensure that the mink were naïve to BCG antigen and to eliminate the possibility of a nonspecific inflammatory response to the injected compound. In order to determine the optimal time to measure DTH response in the experimental animals, two normal non-experimental mink immunized subcutaneously with live BCG (10^5), were given 50 µl intradermal injections with sonicated BCG 4 wk later, and skin thickness was again measured.

All 40 experimental female mink were immunized subcutaneously with 10^5 live BCG in 200 µl phosphate buffered saline with 0.05% Tween 80 (PBST) (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, USA) approximately 3 wk post-breeding, after the vernal equinox, and hopefully, after blastocyst implantation. Six weeks later, mink were given a 50 µl intradermal injection of 200 µg (2µl in 48 µl PBS) of sonicated BCG (100 mg/ml) in the left hind interdigital toe web between the second and third phalanx, 48 hr before euthanasia. Mink were anesthetized with 5 ml halothane (Halocarbon Laboratories, River Edge, New Jersey, USA) in a bell jar, then exsanguinated by cardiac puncture.

The DTH response was evaluated using toe-web thickness, histopathology, and inflammatory response. Toe-web thickness was measured using a spring operated micrometer, the injection site, plus the same uninjected site on the other hind foot were measured. The experimental sites were measured at 48 hr post-intradermal injection, at the time of necropsy, just prior to the tissues being formalin-fixed for histopathological examination. Histopathological reaction was assessed with the pathologist being unaware of the treatment group or the animal from which the tissues came. The inflammatory response was quantified using image analyzer technology (Zeiss ultraphot microscope, Hamamatsu C2400 camera, software

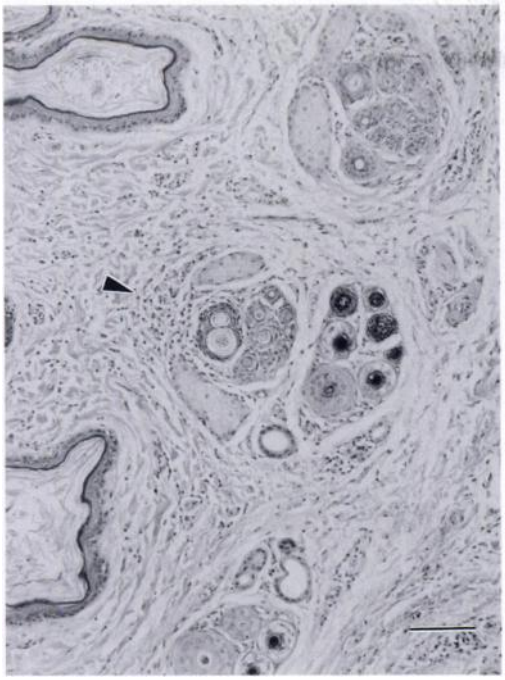

FIGURE 1. Intradermal injection site from the delayed type hypersensitivity test in bleached kraft mill effluent-exposed mink. Arrow indicates a sparse population of mononuclear cells. H&E. Bar = 100 μm.

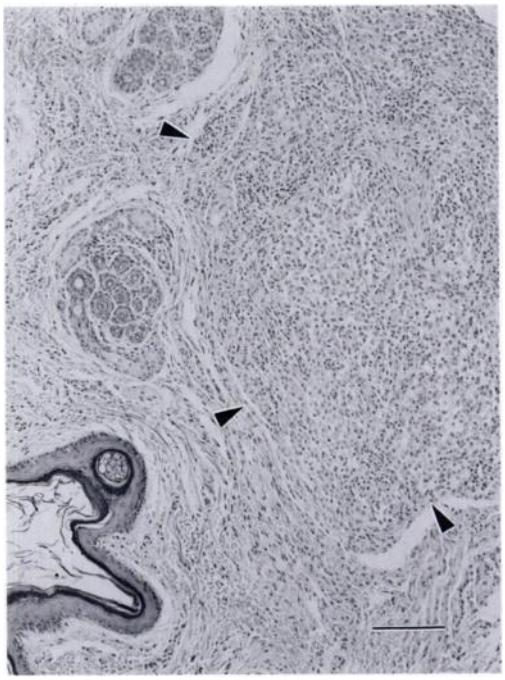

FIGURE 2. Intradermal injection site from the delayed type hypersensitivity test in control mink. Arrows indicate mononuclear cell-rich inflammatory focus. H&E. Bar = 100 μm.

Image 1 by Universal Software Corporation, West Chester, Pennsylvania). The image analysis entailed defining the inflammatory focus on a projected image of the histopathological section, and measuring in mm^2.

At necropsy, both the injection site and the same site on the other hind foot were measured with a micrometer, excised, and fixed in neutral buffered saline for histological assessment. The nature and intensity of the inflammatory response at the injection site were used to score the reaction: 0, a few scattered lymphocytes and macrophages in the perivascular dermal tissue; 1, a small area or thinly populated foci of mononuclear cells (Fig. 1); 2, dense focus of mononuclear cells and macrophages, with or without granulocytes, occupying <60% of a 10× magnified field; 3, a densely populated focus of mononuclear cells and macrophages, with or without granulocytes, covering ≥60% of a 10× field (Fig. 2).

Statistical analyses were done using SPSS-X® (McGraw-Hill Book Co. New York, New York, USA). Lymphocyte blastogenesis assay results were analyzed by one-way analysis of variance (ANOVA). For statistical analysis of the stimulation indices (SI), only results within 2 standard deviations (SD) of the mean for the exposed or control group, respectively, were included in the data set. This resulted in the exclusion of one control and three exposed individuals' data. A response was considered positive if the SI was at least 2.5. Both a median test and Kruskal-Wallis analysis were applied to the ranked histological DTH results, and unpaired *t*-tests were applied to the skin thickness, as well as image analysis measurements. Spearman's correlation coefficient was applied to the histological ranking, relative to each of the other methods used in DTH assessment, while a Pearson's correlation coefficient was used to compare the image analyzer and micrometer measurements, with significance set at $P < 0.05$.

RESULTS

The radioactivity (as CPM) released from cell cultures is directly related to the incorporation of [^{3}H] thymidine and represents a mean of lymphocyte proliferation. The stimulation index (SI) for each animal with the different mitogens was calculated as the ratio of CPM of mitogen stimulated cell culture/CPM of unstimu-

TABLE 1. Proliferative response, expressed as mean counts per minute (CPM) ± S.E. of normal mink peripheral blood mononuclear cells to concanavalin A (ConA), pokeweed mitogen (PWM), phytohemagglutinin (PHA), and lipopolysaccharide with dextran (LPS and DXT).

	CPM[a] (±SE) (n=4)	Stimulation index[b]
Control[c]	286 ± 50.6	NA[d]
ConA[e]	67,325 ± 18,578	235 ± 65
PWM[f]	25,304 ± 7,330	88 ± 26
PHL[g]	8,027 ± 5,488	28 ± 19
LPS and DXT[h]	350 ± 121	1.7 ± 0.8

[a] CPM = Counts per minute (± SE) from [^{3}H] thymidine release by mononuclear cells.
[b] Stimulation index (mitogen stimulated cell CPM/control cell CPM).
[c] No mitogen.
[d] NA, not applicable.
[e] ConA at 10 μg/ml.
[f] PWM at 1/80 dilution.
[g] PHA at 1/80 dilution.
[h] LPS and DXT at 100 μg/ml and 20 μg/ml respectively.

lated, background cell culture. From the four normal mink, cell proliferation was optimal using the following mitogen concentrations per well; ConA 10 μg/ml, PWM 1/80 dilution, and PHA 1/80 dilution (Table 1). The cellular response was greatest with ConA, and lowest with PHA, while LPS/Dextran at concentrations (μg/ml) of 100/20, 50/10, or 25/5 did not consistently induce proliferation of mink PBMC (Table 1). Neither the blastogenic response of mink PBMC (Table 2), nor the stimulation indices were altered by exposure to BKME (Table 3).

No response was detected to the pretrial DTH test in mink which had not been presensitized to BCG. The BCG-immunized, non-experimental mink had an increased mean ± SE skin thickness at the injection site compared with the reference site at 24 hr (0.05 ± 0.0 mm), 48 hr (0.17 ± 0.11 mm) and 72 hr (0.10 ± 0.14 mm). The DTH response of BKME-treated mink was less than control mink when comparisons were made using each of the three methods of evaluation (Table 4). The toe web thickness of the control group was greater than that of the BKME-exposed group. Histopathological reactions were more cellular and larger in the control group (Fig. 2) than in the BKME-exposed group (Fig. 1), and the control group had a greater area of reaction than did the BKME-exposed mink. The micrometer measurement had a low correlation coefficient with the histopathological data (r = −0.22, P = 0.17) while the image analyzer data had a high correlation coefficient (r = 0.88, $P < 0.001$) with the histopathological ranking, and a low correlation with the micrometer measurement (r = 0.22, P = 0.18).

DISCUSSION

Two components of cell mediated immunity were evaluated in our experiments.

TABLE 2. Proliferative response of peripheral blood mononuclear cells from mink exposed to bleached kraft mill effluent, to concanavalin A (ConA), pokeweed mitogen (PWM) and phytohemagglutinin (PHA), expressed as mean counts per minute (CPM ± standard error).

	Control[a] CPM (n=20)	BKME-exposed[b] CPM (n=20)	Percent of control	Probability[c] of no treatment effect
No mitogen	261 ± 50	229 ± 30	88	0.61
ConA[d]	3,460 ± 594	3,002 ± 783	87	0.64
PWM[e]	2,028 ± 367	1,772 ± 425	87	0.65
PHA[f]	1,579 ± 194	1,379 ± 298	87	0.57

[a] Diet contained no BKME.
[b] Both food and water contained BKME (water had 25% BKME; food had 45% BKME-contaminated fish).
[c] Probability of no treatment effect using a one way analysis of variance.
[d] ConA at 10 μg/ml.
[e] PWM at 1/80 dilution.
[f] PHA at 1/80 dilution.

TABLE 3. Stimulation index (SI) of peripheral blood mononuclear cells from mink exposed to bleached kraft mill effluent using three mitogens, Concanavalin A (ConA), pokeweed mitogen (PWM) and phytohemagglutinin (PHA).

	Control[a] SI[d] (n=20)	BKME[b] SI (n=20)	Percent of control	Probability[c] of no treatment effect
ConA[e]	17 ± 3.3[f]	17 ± 5.0	100	0.92
PWM[g]	8.7 ± 1.4	8.4 ± 2.0	96	0.81
PHA[h]	7.1 ± 1.0	7 3 ± 1.8	103	0.91

[a] Diet contained no BKME.
[b] Both food and water contained BKME (water had 25% BKME; food had 45% BKME-contaminated fish).
[c] Probability of no treatment effect using one way analysis of variance.
[d] SI = mitogen stimulated cell count per minute (CPM)/control cell CPM.
[e] ConA at 10 μg/ml.
[f] Mean ± SE.
[g] PWM at 1/80 dilution.
[h] PHA at 1/80 dilution.

Concanavalin A and PHA specifically stimulate T-lymphocyte mitosis, while PWM is a T- and B-lymphocyte mitogen. The mink used to establish the appropriate conditions for blastogenesis assay in mink (Table 1) were four animals kept at the animal care unit in this veterinary college. Their cells were cultured within 1 to 3 hr of being collected. Logistics were such that blood from the 40 experimental mink (7 hr away from the veterinary college) was collected over one day, and cultured the following day. This would likely have affected the viability of the mononuclear cell population, and thus be responsible for the overall decreased response seen in the experimental population (Tables 2 and 3).

Concanavalin A, at the concentration used here, produced the greatest proliferation in hexachlorobenzene-exposed mink lymphocytes (Bleavins et al., 1983), as well as in these BKME-exposed mink. Bacterial cell wall lipopolysaccharide is described as a B-lymphocyte mitogen in many species (Anderson et al., 1972). However, it induced no proliferation of PBMC in these mink; thus, either B-lymphocytes in peripheral circulation of mink were not at an appropriate stage of maturity to respond to mitogenic stimulation, or there were too few B-lymphocytes in circulation to mount a detectable response, or mink B cells did not respond to LPS. No difference between BKME-exposed and control mink PBMC was evident with any of the three mitogens used, evidence that PBMC of both BKME-exposed and control mink were undergoing normal pro-

TABLE 4. Comparison of three methods of evaluation of the delayed type hypersensitivity (DTH) response in mink exposed to bleached kraft mill effluent (BKME).

	Control[a] (n=20)	BKME-exposed[b] (n=20)	Percent of control	Probability[c] of no treatment effect
Histopathology rank	24.85[d]	16.15[d]	65	0.014
Micrometer (mm)	0.12 ± 0.07[e]	0.06 ± 0.05	50	0.003
Image analyzer (mm^2)	0.57 ± 0.14	0.34 ± 0.25	60	0.022

[a] Food and water contained no BKME.
[b] Both food and water contained BKME (water had 25% BKME; food had 45% BKME-contaminated fish).
[c] Probability of no treatment effect using a Kruskal-Wallis analysis of ranked data for histopathology, and unpaired t-tests for both micrometer and image analyzer measurements.
[d] Ranked score values from Kruskal-Wallis analysis.
[e] Mean ± SE.

liferation. Thus, the intermediate stage of cellular immune response, lymphocyte proliferation, was not affected by exposure to BKME.

These findings were in contrast to work by de Swart et al. (1994) in which seals (*Phoca vitulina*) fed fish from the heavily polluted Baltic Sea for 2 yr had depression of proliferative response to ConA, PWM, and PHA. There was no change, however, with LPS-stimulated proliferation, demonstrating B lymphocytes of seals to be relatively insensitive to damage in this system compared with T lymphocytes. The proliferative response of mink PBMC followed the same pattern as did seal PBMC; maximum proliferation with ConA and PWM, less with PHA, and least with LPS (de Swart et al., 1993). Even though the seal PBMC were cultured for 24 hr longer with LPS than with the other mitogens, the proliferative response remained the lowest. It may be of value to extend the cell culture time for mink PBMC to allow a longer response time, before excluding LPS as a potential mink B lymphocyte mitogen.

Reports of the effect of BKME on immune function in fish are equivocal. Förlin et al. (1991) reported changes in the white blood cell profile, implying a weakened immune system in exposed fish, while others found no difference in white blood cells in fish exposed to BKME (Haley et al., 1995). Immune alteration associated with exposure to pulp mill effluent was suggested in a study in which effluent-exposed fish had a dose-dependent, increased frequency of gill, fin and skin parasites (Axelsson and Norrgren, 1991). Immunosuppression attributed to pulp mill effluent exposure is likely dependent upon the type of exposure as well as the particular effluent involved.

The compromised DTH response in the BKME-exposed mink is an expression of immunotoxicity. Delayed type hypersensitivity is an integrated systemic response based upon several cell populations and their secretory products. It is mediated by CD4+ T-lymphocytes after being activated by an antigen bound to antigen presenting cells. Activated T-lymphocytes and macrophages secrete effector molecules including tumor necrosis factor (TNF), interleukin 1 (IL-1), and gamma interferon (INF γ) during the DTH response (Abbas et al., 1991). These cytokines induce activation of vascular endothelial cells which become leaky and contribute to the local inflammation (Abbas et al., 1991). The decreased DTH response in the mink was evidence for a deficit in the cell mediated immune response. In light of the normal response of T lymphocytes to mitogenic stimulation, BKME did not affect clonal proliferation. If the in vitro lymphocyte proliferation assay reflected the proliferative potential of lymphocytes in vivo, the mechanism of immunotoxicity must have been at the level of post-proliferation T-lymphocyte function, tissue macrophage function, or the interaction among either of these cell types and antigen presenting cells.

Ross et al. (1995) demonstrated depression of the DTH response in captive harbor seals (*Phoca vitulina*) fed 2 yr on a diet of Baltic Sea fish contaminated with polychlorinated biphenyls (PCB), chlorinated dioxins, and dibenzofurans. Dietary BKME fed to the mink in this study contained very low levels of 2,3,7,8-TCDD and -TCDF, implicating other components in the effluent to be involved in immunomodulation. Although PCBs have not been detected in pulp mill effluent, numerous organochlorine compounds, 2,3,7,8-TCDD included, may occur in BKME, along with organic acids, chlorinated phenolics, chlorinated neutral compounds, chlorolignins, and other phytochemicals (Owens, 1991).

The histopathological assessment of DTH was used as the standard to which the micrometer and image analysis measurements were compared. We concluded that image analysis was the second most reliable method. There are difficulties associated with cellular definition on the

projected image using this technology. The hand-pressure controlled micrometer was the least reliable, proving quite awkward to use on the small, lightly haired interdigital space of mink toes. There are no similar studies comparing methods of DTH response evaluation in mink.

Delayed type hypersensitivity is a relatively noninvasive in vivo measure of cell-mediated immunity. Although numerous infectious, environmental, nutritional, seasonal, age, and sex related stressors can affect immune function, in situations where specific contaminants are of concern, and with the existence of either good baseline data, or reference populations, immunotoxicity testing provides another useful tool for monitoring environmental health. While the DTH provides a more integrated test of immune function, the mitogen assay tests a specific response. This makes it a less encompassing measure of the potential impact of pollutants on exposed individuals or populations, but it allows investigation of the mechanism of immune dysfunction.

The decreased response in BKME-exposed mink is important, as it may be evidence for a defect which could ultimately affect the competitive fitness of animals in their natural environment. The relationship between a decreased DTH response under experimental conditions, and the ability of mink to deal with natural challenges from pathogenic organisms in the wild is not possible to define at this time. However, the mechanisms involved with a DTH response are similar regardless of the origin of the antigen, so this in vivo evaluation of immunocompetence should have relevance in the natural setting.

Wild mink consume up to 50% fish and crustaceans, while otter and other semi-aquatic mammals such as seals, are almost entirely piscivorous (Gilbert and Nancekivell, 1982). This would increase any risk associated with bioaccumulation or biomagnification of xenobiotics through the aquatic food chain. Immunotoxicity may occur at levels of exposure to xenobiotics well below that required to produce overt signs of toxicity, so the endpoints described in this study may be useful monitors of subclinical toxicity.

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