



Contaminant-related Suppression of Delayed-type Hypersensitivity and Antibody Responses in Harbor Seals Fed Herring from the Baltic Sea

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Recent mass mortalities among several marine mammal populations have led to speculation about increased susceptibility to viral infections as a result of contaminant-induced immunosuppression. In a 2.5-year study, we fed herring from either the relatively uncontaminated Atlantic Ocean or the contaminated Baltic Sea to two groups of captive harbor seals and monitored immune function in the seals. Seals fed the contaminated fish were less able to mount a specific immunological response to ovalbumin, as measured by *in vivo* delayed-type hypersensitivity (DTH) reactions and antibody responses. The skin reaction to this protein antigen was characterized by the appearance of mononuclear cells which peaked at 24 hr after intradermal administration, characteristic of DTH reactions in other animals studied. These DTH responses correlated well with *in vitro* tests of T-lymphocyte function, implicating this cell type in the reaction. Aryl-hydrocarbon (Ah) receptor-dependent toxic equivalent (TEQ) profiles in blubber biopsies taken from the seals implicated polychlorinated biphenyls rather than dioxins or furans in the observed immunosuppression. Marine mammal populations currently inhabiting polluted coastal environments in Europe and North America may therefore have an increased susceptibility to infections, and pollution may have played a role in recent virus-induced mass mortalities. **Key words:** delayed-type hypersensitivity, harbor seals, immunosuppression, organochlorines. *Environ Health Perspect* 103:162–167 (1995)

The immunosuppressive potential of organochlorine chemicals has been well established in studies of laboratory animals (1), but little is known of the effects of environmentally occurring mixtures on immune function in free-ranging animals. Because organochlorine chemicals bioaccumulate in many wildlife species occupying high trophic levels, these animals may serve as early warning indicators for problems of ecosystem health. Classes of chemicals that are of particular concern include the ubiquitous and highly immunotoxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related dioxins, furans, and polychlorinated

biphenyls (PCBs) (1). Fish-eating animals including gulls, cormorants (2), seals (3), carnivorous whales (4,5), and certain groups of humans (6,7) can be exposed to high levels of such contaminants and may therefore be expected to be the first to exhibit symptoms of toxicological stress. There is accumulating evidence that these organochlorines have adverse biological impacts in free-ranging animals, including skeletal malformations in gulls, terns, cormorants (2), and seals (8,9) and reproductive impairment in seals (10,11).

Mass mortalities among harbor (*Phoca vitulina*) and grey (*Halichoerus grypus*) seals in Europe in 1988 (12–14), Baikal seals (*Phoca sibirica*) in 1987–88 (15,16), bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico in 1987–88 (17), and striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea in 1990–91 (18), led to speculation that environmental pollution was impairing the immunocompetence of these marine mammal populations and had rendered them more susceptible to viral infection. The 1988 phocine distemper virus (PDV) epizootic among European harbor seals was particularly devastating, resulting in the deaths of approximately 20,000 seals (13,19). Although no studies could directly link pollution to the PDV epizootic, the speed at which the infection passed through the population and the high mortality rate has fueled a continuing debate. Since the PDV epizootic, studies have provided additional clues: harbor seals surviving the epizootic had lower organochlorine burdens than seals that died (20); PDV or a similar virus had infected Canadian harbor seals before the European disaster, with no apparent mortality (21,22); and harbor seals living in less contaminated areas of Britain had apparently lower mortality rates during the PDV epizootic than those from polluted areas (23). In addition, we recently demonstrated that lymphocytes isolated from harbor seals fed herring from the contaminated Baltic Sea were functionally impaired compared to those isolated from seals fed herring from the relatively unpolluted Atlantic Ocean, as

measured by *in vitro* T-cell mitogen stimulation (24) and natural killer cell activity tests (Ross et al., submitted). Here, we extend these *in vitro* results by evaluating the *in vivo* immune response of these harbor seals, as measured by delayed-type hypersensitivity (DTH) and antibody responses to ovalbumin.

Methods

Captive Harbor Seals

Two groups of 11 healthy young harbor seals (*Phoca vitulina*) were housed at the Seal Rehabilitation and Research Centre in Pieterburen, The Netherlands, as described in detail elsewhere (24). They had been captured as recently weaned pups from the relatively uncontaminated northeast coast of Scotland, and all seals were fed relatively uncontaminated herring from the Atlantic Ocean for an acclimation period of 1 year. The 22 seals were matched for body weight and sex and subsequently divided at random between two feeding groups. At the start of the feeding experiment in October 1991, the control group continued to receive Atlantic Ocean herring and the treatment group received herring originating from the relatively contaminated Baltic Sea. Both groups received weekly vitamin supplements to compensate for nutrient losses during storage of the fish at -20°C. Estimated daily intakes of potentially immunotoxic compounds analyzed in the herring were 3 to 10 times higher in the Baltic group of seals, as compared to the Atlantic group, and are summarized elsewhere (24). The average daily intakes of Ah receptor-defined organochlorine contaminants in the Baltic Sea group were 288 ng toxic equivalents (TEQ) per seal, compared to 29 ng TEQ per seal in the Atlantic group (24). Similarities in the

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nutritional quality of the two diets, clinical chemistry profiles, and weight gain of the animals suggested that other than the differences in intake of environmental contaminants, the two groups of seals were comparable (24; De Swart et al., submitted). All animals were handled in accordance with institutional guidelines in the Netherlands, and their care was supervised by a Veterinary Consultant and the Veterinary Advisory Committee of the Seal Rehabilitation and Research Centre in Pieterburen.

Skin Test

After approximately 2 years on the respective diets (week 100), seals of both groups were tested for DTH reactivity to ovalbumin. In this prescreen, aimed at ensuring that the seals were immunologically naive to the test antigen, we prepared a sterile solution of 250 µg/ml ovalbumin (grade V; Sigma Chemicals, St. Louis, Missouri) in physiological saline solution. After cleansing of the skin with Betadine (Mundipharma, Basel, Switzerland), seals were injected with 100 µl of the ovalbumin solution (25 µg per injection) intradermally in the flipper webbing between two toes. We marked the site by placing a drop of waterproof paint 2 cm above the injection. We measured the thickness of the skin before and 48 hr after the injection using a Mitutoyo digital micrometer (Mitutoyo Corp., Tokyo).

After the prescreen, all seals were immunized intramuscularly in the gluteal region (week 105) with 100 µg ovalbumin and 800 µg dimethyldioctadecylammonium bromide (DDA; Eastman Kodak, Rochester, New York) as adjuvant in 2 ml physiological saline solution.

We began the DTH recall skin test 9 days later (week 106) using the same ovalbumin stock, concentration, and intradermal route of administration as the prescreen. In addition, a control injection of physiological saline was administered to assess the aspecific inflammation induced by the injection process alone. Sites were marked and skin thickness measured before and at 24, 48, and 72 hr after injection.

We took a skin biopsy from three seals of each group at 72 hr after injection to assess the cellular infiltrate responsible for the observed swelling. Biopsies of the swelling were taken using a 6-mm biopsy plug (Codman and Shurtleff, Randolph, Massachusetts) and scalpel after cleansing the area using Betadine and sterilizing surgical instruments in 95% isopropyl alcohol. Skin samples were immediately placed in 4% formaldehyde solution. Samples were later embedded in paraffin and 5-µm sections stained using hematoxylin and eosin and mounted on glass microscope

slides for evaluation. We assessed the cellular infiltrate by identifying and qualitatively ranking cell types observed using light microscopy.

Toxicological Analyses of Seal Blubber

We took blubber biopsies from the study seals for toxicological analyses at week 104 of the experiment. After cleansing the skin surface in the dorsal region approximately 10 cm lateral to the spinal column, a 1.5-cm incision was made. A 6-mm biopsy plug (Codman) was inserted into the incision, and a sample of approximately 200 mg blubber was removed and frozen in glass vials at -20°C until analysis. Congener-specific analyses of planar (IUPAC numbers 77, 126, and 169) and mono- (IUPAC numbers 118, 156, and 189) and di-ortho (IUPAC number 180) PCBs were undertaken as described elsewhere (25,26). We measured 7 dioxin (2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, and OCDD) and 10 furan (2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, and OCDF) congeners as described elsewhere (27). 2,3,7,8-TCDD TEQs were calculated on the basis of toxic equivalent factors (TEFs) designated for dioxins and furans (28) and for PCBs (29). Concentrations of dioxin, furan, and planar PCB congeners measured around the detection limit suffered from variation due to an analytical source of error, which is not expected to appreciably alter total levels as presented here or after the relationship between the two groups of seals.

Detection of Serum Antibodies

Blood was sampled 5 weeks after the DTH test (week 111), and serum antibodies against ovalbumin were detected by an enzyme-linked immunosorbent assay (ELISA) as follows. ELISA plates (Costar, Cambridge, Massachusetts) were coated with 1 µg/well ovalbumin in carbonate buffer (pH 9.6). Plates were washed in water-Tween (0.05%) and blocked at 37°C using ELISA buffer (phosphate-buffered saline, PBS; 0.01 M) containing 0.3% milk powder, 5% NaCl, 0.1% Tween 20 (Merck, Munich), and 0.1% Triton X 100 (Merck). Plates were again washed, and sequential twofold dilutions of seal serum were made in ELISA buffer, starting at 1:1000. Plates were incubated at 37°C and washed. We used a conjugate preparation of protein A-horseradish peroxidase (Amersham Life Sciences, Little

Chalfont, UK) at a 1:2500 dilution and developed plates using a solution 0.1% tetramethylbenzidine dimethylsulfoxide (10 mg/ml TMB in DMSO) and 0.001% H₂O₂. Antibody titers were expressed as the reciprocal of the dilution at 50% of the maximum optical density at 450 nm.

Correlation between DTH and *in vitro* Test Results

As part of the routine monitoring of immune function in the two groups of harbor seals, mitogen-induced proliferative responses of peripheral blood mononuclear cells (PBMC) were measured and reported elsewhere (24). Here, we correlate results of these *in vitro* responses with the *in vivo* DTH responses in the same animals. Briefly, PBMC were isolated from heparinized blood and stimulated with the T-cell mitogens concanavalin A (ConA; 5 µg/ml) and phytohemagglutinin-M (PHA; 20 µg/ml), the T- and B-cell mitogen pokeweed mitogen (PWM; 2.5 µg/ml), or the B-cell mitogen lipopolysaccharide from *Salmonella typhimurium* (LPS; 100 µg/ml). We measured proliferation after 4 (ConA, PHA, and PWM) or 5 (LPS) days in culture as ³H-thymidine incorporation, expressed in counts per minute (cpm). For each animal, proliferative responses measured from seven blood samplings (weeks 67, 75, 80, 93, 104, 111, and 121) were averaged after subtraction of medium controls. These proliferation data were log-transformed before correlation to the 24-hr DTH values obtained at week 106 of the experiment.

Results

Baltic Sea seals had higher average Ah receptor-binding contaminant burdens than North Sea seals, with 3.4 times higher TEQ levels in blubber samples (Table 1). Also apparent was the predominant contribution of the PCBs to these levels, representing 93% ± 0.6% (SE) of the total TEQ values, compared to only 7% ± 0.6% for the dioxins and furans.

Table 1. Ah receptor-defined contaminant concentrations in blubber biopsies at week 104 from seals fed herring from the relatively uncontaminated Atlantic Ocean or from the contaminated Baltic Sea for a period of 2 years^a

| | ng TEQ/kg lipid | |
|-------------------------|-----------------|---------------|
| | Atlantic seals | Baltic seals |
| PCB (mono and di-ortho) | 35.5 ± 3.66 | 140.0 ± 7.95 |
| PCB (planar) | 22.2 ± 1.00 | 51.1 ± 3.04 |
| Dioxins and furans | 4.1 ± 0.19 | 17.7 ± 4.14 |
| TEQs (total) | 61.8 ± 4.13 | 208.7 ± 11.60 |

Abbreviations: PCB, polychlorinated biphenyl; TEQ, toxic equivalent.

^aValues represent the means of 11 seals per group ± SE.

In a prescreen of seals of both groups, there was no significant skin swelling 48 hr after-intradermal injection with ovalbumin (paired *t*-test; $p > 0.05$; results not shown), indicating that the seals were immunologically naïve to this antigen. After the post-immunization recall skin test, both Atlantic and Baltic seals responded significantly to the antigen (univariate repeated-measures *F*-test; $p < 0.01$; Fig. 1), with a localized and palpable swelling. After intradermal injection, the seals fed the Baltic Sea herring had significantly lower responses than those fed the Atlantic herring (repeated-measures analysis of variance with grouping factors; $p < 0.01$), as the former had a mean swelling of 47% of that observed in the Atlantic group at the peak response time of 24 hr. In addition, an inverse relationship was found between DTH swelling and Ah receptor-binding contaminant levels (total TEQ) in blubber samples of the same seals ($r = -0.64$, $p < 0.01$).

In microscope preparations of skin biopsies taken from the ovalbumin reaction sites of 6 of the 22 seals 72 hr after injection, cellular infiltrates in the dermis were characterized as typical DTH reactions by the presence of perivascular mononuclear cells (likely lymphocytes) and a limited presence of polymorphonuclear cells (Fig. 2).

Before immunization and the recall skin test, seals of both groups had no detectable antibodies against ovalbumin, confirming the immunological naïveté to this antigen. In a blood sampling 4 weeks

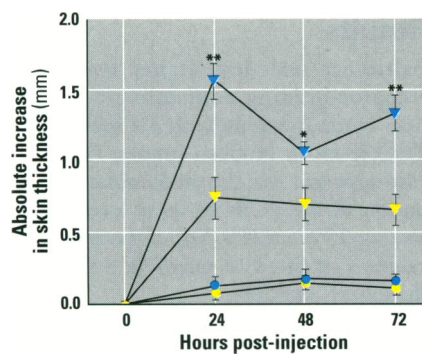


Figure 1. Nine days after immunization with ovalbumin and the adjuvant DDA, harbor seals of both Atlantic (blue triangles) and Baltic (yellow triangles) groups exhibited a delayed-type hypersensitivity (DTH) response to an intradermal skin challenge using ovalbumin. Seals fed the relatively contaminated Baltic Sea herring for a 2-year period had a significantly lower response to the antigen (repeated-measures ANOVA with grouping factors; $p < 0.01$). The peak swelling occurred 24 hr after injection. A control injection of 100 μ l saline resulted in only a very small swelling for both Atlantic (blue circles) and Baltic (yellow circles) seals. Data points represent the means of 11 seals \pm SE. Significant differences between the two groups at each measured time point were analyzed by an independent *t*-test (* $p < 0.05$; ** $p < 0.01$).

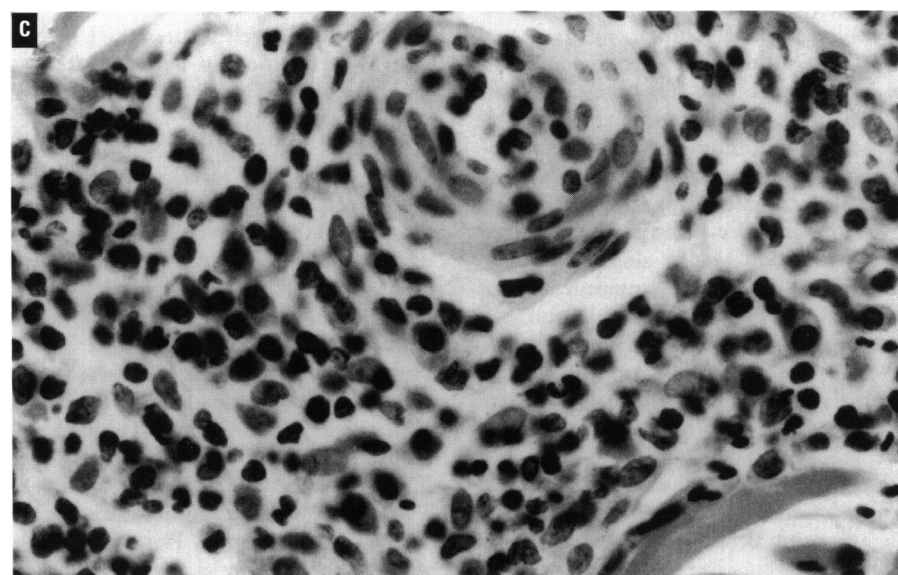
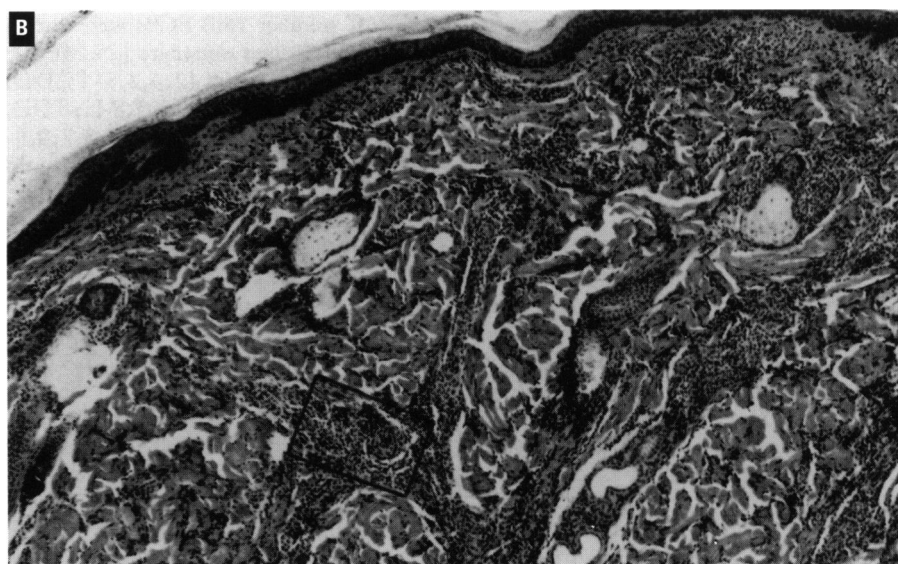
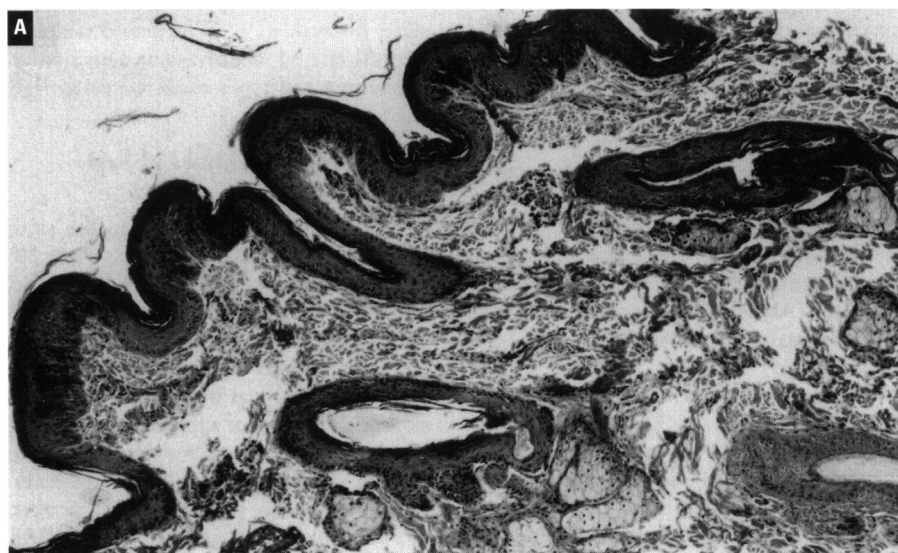


Figure 2. Biopsies of seal skin from (A) a control sample (40 \times , H&E), (B) a positive skin test 72 hr after intradermal injection with the recall antigen ovalbumin (40 \times , H&E), and (C) an enlargement of an indicated section (see outlined square) of the same cut (200 \times , H&E). The enlarged section reveals a perivascular infiltrate characterized by mononuclear cells, with the presence of some polymorphonuclear granulocytes. Biopsies of three seals from each group were examined.

after the immunization, seals of both groups had mounted antibody responses to ovalbumin, with titers about 37% lower in the Baltic group than the Atlantic group (independent *t*-test; $p < 0.01$; Fig. 3).

In correlation analyses between *in vitro* tests of immune function and the DTH response, the DTH response correlated best with lymphocyte stimulation by ConA ($r = 0.62$, $p < 0.01$) and PHA ($r = 0.57$; $p < 0.01$), and less with PWM ($r = 0.35$; $p > 0.05$) and LPS ($r = 0.29$; $p > 0.05$) (Fig. 4).

Discussion

Exposure to contaminants occurring at levels found in the Baltic Sea herring impaired the ability of captive harbor seals to mount a specific immune response to the T-lymphocyte-dependent antigen ovalbumin with DDA as adjuvant. This was evidenced by impaired DTH and serum antibody responses. DDA was selected as an adjuvant because it is particularly effective in stimulating the induction of DTH responses (30). The skin reaction to ovalbumin was characterized by a mononuclear infiltrate in the dermis, as observed in classical DTH responses in other species studied (31). In addition, the correlation between the mean DTH response and the *in vitro* lymphocyte stimulation tests with the mitogens ConA and PHA, and not PWM and LPS, support the notion that T-lymphocytes are involved in the mechanism of DTH swelling in our study seals. ConA and PHA specifically stimulate T-lymphocytes *in vitro*, whereas PWM stimulates T- and B-lymphocytes, and LPS stimulates B-lymphocytes in many species (32), including the harbor seal (33). These results not only strengthen our previous evidence of a contaminant-induced suppression of T-lymphocyte function in the harbor seals (24), but also lend support to the use of *ex-vivo/in vitro* tests of immune function.

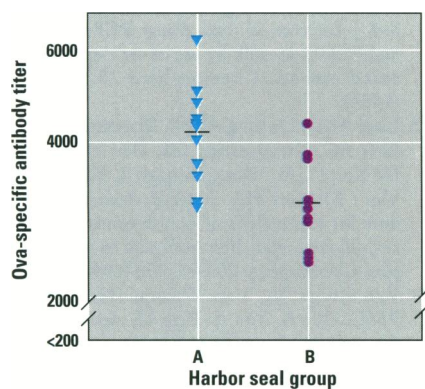


Figure 3. Serum antibody titers mounted against ovalbumin 4 weeks after immunization were significantly lower (independent *t*-test; $p < 0.01$) in the Baltic Sea seals (circles) as compared to the Atlantic group (triangles). Seals of both groups had no detectable antibodies to ovalbumin before immunization.

The DTH skin test represents the only practical *in vivo* test for cellular immunity. Moreover, it reflects a system-wide immune response, ranging from antigen processing and presentation after immunization, to the T-helper cell response which coordinates a secondary response in the skin reaction. It is difficult to extrapolate from the immunological responses using ovalbumin as an antigen to a seal's ability to mount a specific immune response against a pathogen in the natural environment, though the DTH response does provide an overview of an animal's ability to mount a response to a foreign protein in a manner similar to which it would defend itself against infection by a viral agent.

Impairment of DTH reactions after exposure to TCDD has been observed in guinea pigs receiving eight weekly doses of 0.04 $\mu\text{g}/\text{kg}$ (34); C57BL/6 mice receiving four weekly doses of 4 $\mu\text{g}/\text{kg}$ (35) or a one-time dose of 50 $\mu\text{g}/\text{kg}$ (36); and Fischer-344 rats exposed pre- and postnatally with four doses of 5 $\mu\text{g}/\text{kg}$ or postnatally alone via nursing with three doses of 5 $\mu\text{g}/\text{kg}$ (37). Guinea pigs exposed to 50 ppm of a dietary PCB mixture (Clophen A60) had suppressed DTH responses to purified protein derivative and antibody responses to tetanus toxoid (38). Vos and Moore (39) noted that rats must be exposed to TCDD during ontogenesis of the immune system

for immunosuppression to take place, whereas the adult immune system is less sensitive to suppression of thymus-dependent immunity. Although the results of several studies of human exposure to organochlorines have been difficult to interpret, the accidental exposure of people in Taiwan to rice oil contaminated with PCBs (most likely contaminated with dioxins and furans) led to significant impairment of DTH responses (40). Impairment of B-cell responses has been observed in adult animals, with lower antibody responses to various antigens reported following exposure to PCBs (1) and dioxins (34). Because few studies have examined the effects of environmentally occurring mixtures of anthropogenic contaminants on immune function in mammals, it is difficult to relate results of other studies to those reported here.

Although our results suggest an impairment in the function of the T-cell-mediated immune response of the Baltic seals, we cannot conclude that the T-lymphocyte or its precursor are the targets of immunotoxic action by the contaminants. Possible effects of the contaminants at the antigen presentation level or a multidirected action (e.g., at both T- and B-lymphocytes) are conceivable. However, the concurrent and similar results in the DTH and the antibody responses in the Baltic Sea group as

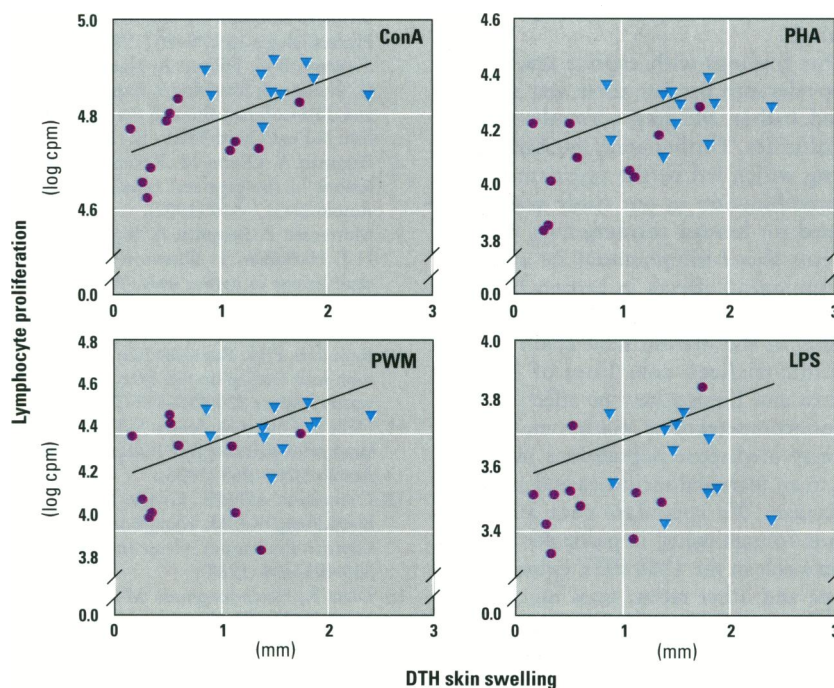


Figure 4. Correlations between *in vitro* tests of immune function conducted previously and delayed-type hypersensitivity (DTH) responses. Good correlations existed between DTH and lymphocyte stimulation by the mitogens concanavalin A (ConA; $r = 0.62$, $p < 0.01$) and phytohemagglutinin (PHA; $r = 0.57$, $p < 0.01$), but not pokeweed mitogen (PWM; $r = 0.35$, not significant) and lipopolysaccharide (LPS; $r = 0.29$, not significant), implicating T-lymphocytes in the DTH response. Mean counts per minute were calculated for *in vitro* lymphocyte stimulation tests from five samplings before and two after the DTH test and then log-transformed before plotting against skin thickness at the peak 24-hr swelling after intradermal ovalbumin injection (triangles) Atlantic seals; (circles) Baltic seals.

compared to the Atlantic group point to a common site of action. This is consistent with the findings of Lundberg et al. (36), among others, who observed reduced DTH responses, antibody responses, and specific lymphocyte stimulation to ovalbumin in mice and normal function of antigen presentation cells. In addition, the thymus is a sensitive target for TCDD-induced immunosuppression, leading to impaired T-lymphocyte responses (1).

Many different classes of potentially immunosuppressive contaminants bioaccumulate in the Baltic Sea ecosystem, making it impossible to identify any one contaminant as responsible for the impaired immune responses in our study. Evidence for the mediation of immunotoxicity via the Ah receptor in animals and the high affinity of TCDD and its dioxin and PCB analogs for this cytosolic receptor (41) suggest a cumulative effect of the many contaminants found in the Baltic Sea herring. The observed impairment of DTH and antibody responses suggests that the mixture of contaminants in the Baltic Sea herring has immunosuppressive properties. Assuming that the observed effects are mediated by Ah receptor-binding contaminants, the blubber profile of TEQ values suggests that the PCBs are largely responsible for these effects, as opposed to the dioxins and furans. However, we cannot rule out an immunotoxic contribution from non-Ah receptor binding classes of chemicals.

Our findings with captive seals have direct relevance because three seal species (ringed, harbor, and grey) currently inhabit the Baltic Sea. Furthermore, the Baltic Sea herring which led to the impairment of immune function in our study seals was destined for human consumption, raising concerns about the potential for adverse immunological effects in certain human consumer groups. Current levels of contaminants in the marine food chain along the industrialized coastlines of North America and Europe may be affecting the immunocompetence of marine mammals and may predispose populations in these areas to an increased incidence and severity of disease. We speculate that anthropogenic contaminants, in particular PCBs, played a role in the 1988 PDV epizootic in Europe and other recent mass mortalities of marine mammals caused by virus infections.

Efforts to detect possible immunosuppression in free-ranging populations of marine mammals is fraught with difficulties due to the complexity of the mammalian immune system and the difficulty in obtaining reliable samples. However, field studies have demonstrated that it is possible to obtain useful immunological

information from free-ranging seals (42,43), and correlative approaches similar to those used in wildlife toxicology (44) may provide the best direction for research in the future.

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