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The response of grey seal pups to intradermal phytohaemagglutinin injection

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Abstract

The intradermal injection of phytohaemagglutinin (PHA) into the rear flipper web of grey seal (*Halichoerus grypus*) pups resulted in an acute, dose-dependent inflammatory response which was maximal between 12 and 24 hr post injection. The reaction was characterized macroscopically by swelling and, in some individuals, by induration and erythema and microscopically in two animals by a perivascular to diffuse inflammatory cell infiltrate, which comprised of polymorphs and mononuclear cells. In 1991 and 1992 26 weaned pups were injected with 50 and 100 µg PHA dissolved in phosphate buffered saline (PBS) and of these, 23 individuals responded to both doses. The magnitude of the response was significantly higher at the 100 µg dose. No animals responded to control injections of PBS. A second study in 1995 indicated there was also a significant age-related response, which was higher in post (n=16) than in pre-weaned pups (n=19). The magnitude of the response was significantly and linearly related to body mass.

Introduction

It is well known that peripheral blood lymphocytes (PBL) from several mammalian and avian species proliferate in response to mitogenic stimulation in vitro (Mumford *et al*., 1975; Bertram *et al*., 1997) and recently this has been demonstrated in the harbour seal (*Phoca vitulina*) (Ross *et al*., 1995; Dewart *et al*., 1993). Administered intradermally, mitogens can also be used to assess the response in vivo. The non-specific activation of lymphocytes at the injection site also appears to be related to the capacity of individuals to elicit a cell-mediated immune response. Mitogen skin test responses in rats (Mendenhall *et al*., 1989) treated with chemical immunosuppressants were found to correlate well with other measures of immune function. This was the first suggestion that the technique could be used to estimate in vivo immune reactivity in mammals. Since then, the response has developed into a useful test for assessing cellular immunocompetence in many species, such as pigs, turkeys, and seabirds (Vanheugten *et al*., 1994; Scott and Siopes, 1994; Grasman *et al*., 1996). Dexamethasone-treated ducks had significantly suppressed skin responses to phytohaemagglutin (PHA) (Schrank *et al*., 1990) and studies in dogs naturally infected with canine distemper virus (Krakowka *et al*., 1977) indicated that intradermal mitogen tests could be used as rapid method for in vivo assessment of cell-mediated immunity in canid species. In humans, the capacity of the test as an indicator of immunocompetence appears to be age-specific. Blaese *et al*. (1973) concluded that PHA skin testing provided useful information in the evaluation of cellular immunodeficiency in children and infants but that in adults it was not as effective in indicating the presence of clinical immunosuppression. However, Casseb *et al*. (1995) indicated that PHA skin tests could be useful in the immune evaluation of HIV infected patients.

Concerns over the immunosuppressive effects of marine environmental contaminants, such as polychlorinated biphenyls (PCBs) and DDT has increased the need to find techniques and assays that can measure immune responses in free-living marine mammals. Studies on captive seals have shown that harbour seals in particular may be susceptible to the immunosuppressive effects of organochlorines (Ross *et al*., 1995; DeSwart *et al*., 1994) but some of the assays of immune function used in these studies cannot easily be translated to animals in the wild. Grasman *et al*., (1996) found a strong exposure-response relationship between PHA skin testing to assess T-lymphocyte function and exposure to organochlorine contaminants in prefledgling herring gulls (*Larus argentatus*) and Caspian terns (*Sterna caspia*). As part of a study to investigate the immunological effects of the transfer

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of PCBs from grey seal females to their pups during lactation, (Hall *et al*., 1997) we included a PHA skin response test. In this study we investigate further the dose-dependent and age-related nature of the response in grey seal pups.

Materials and methods

The population of grey seal pups born at the Isle of May, in the Firth of Forth, Scotland (longitude 02°33′W latitude 56°11′N) provided the study animals. In November 1991, 1992 and 1995, the response of 53 pups (14, 12 and 27 respectively) to intradermal (i.d.) injections of PHA was investigated. In 1991 and 1992 a dose-response study was carried out and in 1995 the magnitude of the response was compared between pre- and post-weaned animals.

Dose-response study

In 1991 and 1992, single intradermal (i.d.) injections of 50 and 100 μg of sterile PHA-P (L-8754 Sigma Chemical Co., St. Louis, MO) in 100 μl PBS were given into a rear flipper web of 26 weaned pups. Injection sites were pre-shaved to enhance the accuracy of the measurement of the swelling. Control injections of 100 μl PBS were given into the corresponding web of the other rear flipper. Animals were manually restrained in a pup bag and the thickness of the web measured when stretched (i.e. the digits opened) to within 0.1 mm using a thickness gauge (Mitutoyo thickness gauge, RS Components Ltd, Northampton). The means of 5 measurements taken before injection and at 6, 12, 24 hr post injection (p.i.) were used to assess reactivity. A total of 26 pups were injected with 50 μg PHA, 10 were injected at the same time with 100 μg PHA, in the web between the adjacent digit. The skin thickness of 4 of the latter, at both doses, was also measured at 48 hr. All animals were weighed in bags using Salter spring balance scales to the nearest 0.5 kg.

Sites of induced inflammation were biopsied sterilely in two pups, 16 hr after the i.d. injection of 50 μg PHA. The samples (approximately 6 mm in diameter) were taken using a sterile disposable scalpel and fixed in 10% formol saline for 24 hr before being transferred to PBS. Tissues were embedded in paraffin wax and sectioned at 4 mm before being stained with haematoxylin and eosin (H and E).

Age-related study

In 1995, 27 animals were injected with PHA as in the previous study, at a dose of 100 μg in 100 μl PBS. 19 pups were neonates, between 2 and 4 days of age, 16 were post-weaned animals whose mothers had returned to the sea after a lactation period of between 18 and 21 days (Fedak and Anderson, 1982). The difference in the magnitude of the response (as the mean of 5 measurements at each site, PHA treated web thickness minus PBS control web thickness) was compared between these two age groups.

Results

Dose-response study

Of the 26 seals studied in 1991 and 1992, 23 (88%) reacted to the i.d. injection of PHA-P. A reaction was defined as a significant increase in flipper web thickness at the PHA treated site compared to the control site. Swelling at the PHA injection site was accompanied, in some individuals, by induration and erythema. Erythema was particularly apparent at the higher dose but was difficult to assess in all animals because of the dark colour of the flipper webs. Control injections of PBS resulted in slight swelling but with no evidence of induration or erythema.

The mean web thickness was significantly increased at all times after the i.d. injection of 50 μg PHA compared to contralateral control PBS injected webs (n=26, Fig. 1a). All increases in web thickness were significantly greater than the controls at 6-24 h p.i. (separate variance t-tests, $0.014>P>0.0001$).

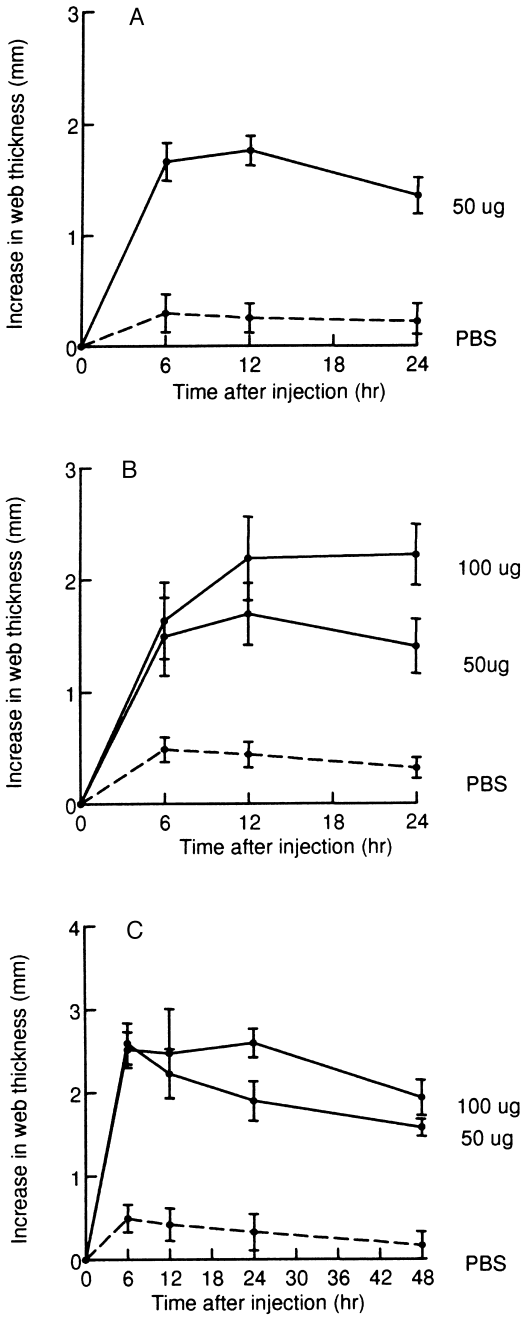
Ten animals were also injected i.d. with 100 μg PHA in adjacent webs on the same flipper. There was a virtually identical increase in web thickness during the first 6 hr following the injection of both doses (Fig. 1b). Although the mean thickness was not statistically different from that induced by the 50 μg dose at 12 hr, they were significantly different at 24 hr (t-test separate variances $P=0.01$).

The skin thickness in four of the weaned pups, which received i.d. injections of both doses of PHA, was also measured at 48 hr p.i. (Fig. 1c) and showed a decrease for both doses, although still statistically significantly different from control sites (t-test separate variances, $P<0.0001$).

Fig. 2 shows the responses of individual pups to i.d. PHA injection, plotted as the mean of the increase in web thickness when measured at 12 and 24 hrs p.i. At the 50 μg dose, this increase in 3 weaned pups was low compared to the remaining 23 (increases in web thickness were <1 mm). These increases were not significantly different from the controls. Two of these low responding weaned pups were also injected with the higher dose, and the increase in web thickness in one of these was also much reduced. In the second weaned pup, the increase was delayed, with no significant increase observed until 12 hr p.i.

Histological studies of the biopsied inflammatory sites revealed that the stratum corneum of

PHA-injected skin was normal. The epidermis, the sebaceous glands, sweat glands and hair follicles, appeared normal. The main finding was an infiltrate of neutrophils with smaller numbers of lymphocytes and other mononuclear cells at the site of PHA injection. This infiltrate ranged from being perivascular to diffuse in its distribution, was located mainly in the middle dermis and in some areas extended into the superficial dermis. In places, the infiltrate extended around adnexal structures but did not directly involve them. The connective tissues of the dermis appeared edematous in the affected areas. Many neutrophils were seen around blood vessels and some were marginated on the vascular endothelium, the cells of which appeared plump. Pycnotic nuclei were widespread. Control sections of flipper taken from the same area of two untreated seals had no significant dermal inflammatory component.



Age-related study

In order to determine if the response to PHA was age-specific, in 1995 two groups of pups of different ages were challenged with 100 µg doses. Responses were measured at 18 hr p.i., again as the mean of 5 repeat measurements. The magnitude of the response was determined as the mean web thickness at PHA treated site minus the mean thickness at the control site.

Responses were significantly higher in post-weaned (n=16, >18 days old) than neonate (n=19, 2–3 days old) pups (Fig. 3a), separate variance t-test $P<0.0001$. This comparison excluded two pups (one in each group) which did not respond, i.e. web thickness' at the treatment site were no different from the control. The response was also significantly linearly related to mass ($P=0.01$; Fig. 3b).

Discussion

We have demonstrated that the i.d. injection of PHA into the rear hind flipper web of grey seal pups results in an acute inflammatory response. The reaction, as measured by increased thickness of web skin, was dose and age-dependent. For both concentrations used it was maximal at between 6 and 24 hr p.i. but had decreased by 48 hr. At all times the mean increased thickness remained significantly greater than control skin injected with PBS.

Figure 1. The response of weaned grey seal pups to the i.d. injection of PHA and control injection of PBS into the rear flipper web as measured by increase in skin thickness at 6, 12, 24 and 48 hr p.i.

Figure 1(a). The mean ± S.E. increase in skin thickness (n=26) after the injection of 50 µg PHA.

Figure 1(b). The mean ± S.E. increase in skin thickness (n=10) after the injection of 50 and 100 mg PHA. For a and b skin thickness was measured at 6, 12 and 24 hr p.i.

Figure 1(c). The mean ± S.E. increase in skin thickness (n=4) after the injection of 50 and 100 µg PHA 6, 12, 24 and 48 hr p.i.

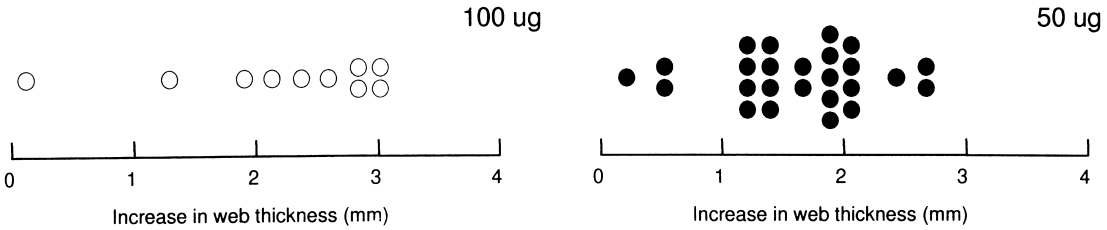
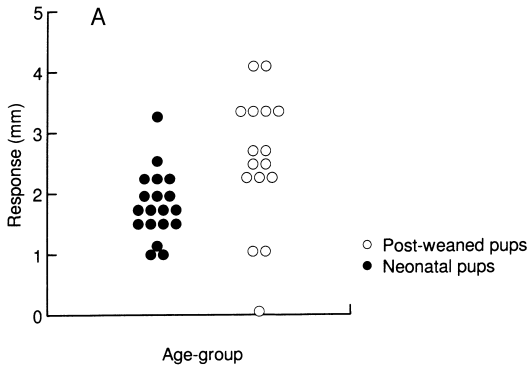


Figure 2. Increase in web thickness in individual weaned grey seal pups after i.d. injection of PHA. 26 pups were injected i.d. with 50 μg PHA and 10 of these pups were also injected at the same time with 100 μg PHA. The data are plotted as the mean of the increase in web thickness at between 12 hr and 24 hr p.i.

The reaction was characterized by edema and, in some individuals by induration and erythema, particularly at the higher concentration (100 μg). The relatively small degree of erythema would appear to be atypical compared to other mammalian (pigs, Binns *et al*., 1990; dogs, Krakowka *et al*., 1977) and avian species (ducks, Schrank *et al*., 1990) but was difficult to assess accurately against the dark skin of the flipper webs. It is perhaps worth noting that in the present study the skin reactions were first observed 6 hr p.i. and that any earlier reaction would therefore have been missed. In fact Binns *et al*., (1990) noted that in some species, especially in calves, there was a more pronounced early reaction and much less at 24 hr. The results also indicate that erythema was only observed when animals were injected with 100 μg. In dogs Krakowka *et al*., (1977) found the most pronounced response using 150 μg.



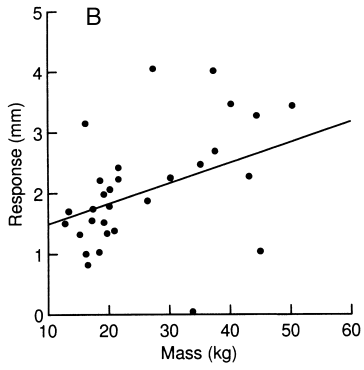


Figure 3(a). The response of neonatal and weaned grey seal pups to the i.d. injection of 100 μg PHA. The mean difference in the increase of the web thickness between the control and injection sites at 18 hr p.i. is shown.

Figure 3(b). The response of neonatal and weaned grey seal pups to the i.d. injection of 100 μg PHA versus mass in kg.

Of particular interest is the fact that four weaned pups and one neonate pup showed little response to PHA injection. Whilst a wide range of reactivity within a population is to be expected, these individuals were distinctly different. This difference was confirmed at the higher PHA dose, with two out of the four weaned pups and one neonate injected showing a low response and a further weaned pup a normal but delayed response. The mechanism of this inhibition remains unclear. Firstly, it is well established in a number of species that increased levels of corticosteroids, such as occur in illness and stress, can result in the failure of lymphocytes to respond in vitro to a variety of mitogenic stimuli (Kunicka *et al*., 1993; Ekkel *et al*., 1995). In vitro lymphokine production is often affected (Kunicka *et al*., 1993) and in the tissue, localisation properties of circulating lymphocytes altered (Chung *et al*., 1986). It is perhaps noteworthy that of the non-responders, three weaned and one neonatal pup had lower body weights and higher numbers of white blood cells and polymorphs than the other animals in the group (data not shown), possibly indicative of subclinical infection. In fact, one animal which failed to respond to i.d. PHA injection at 6 hr had died of an undiagnosed respiratory infection before the second time point at 12 hr. In our study of the uptake of PCBs by grey seal pups from maternal milk there was no relationship between PCB exposure and PHA response in weaned grey seal pups

(Hall *et al*., 1997). However, non-responders may have been immunosuppressed for other reasons, including nutritional or physiological stress. Future studies in this and other phocid species will more directly address the question as to whether the lack of cutaneous PHA reactivity results from generalised immune suppression by also studying in parallel, mitogen responsiveness and cytokine production in vitro, relating this to physiological and external stress factors.

As well as being dose-dependant, the response of pups to PHA was age-specific. The magnitude of the response in neonatal pups was significantly lower than in weaned pups. Five of the 19 (26%) neonatal pups studied did not survive to weaning. This was similar to previously observed pre-weaning mortality rates at the Pilgrims Haven breeding beach on the Isle of May of between 16 and 31% (Wylie, 1988). Although one of these animals did not respond to PHA, the skin response test at 2–3 days of age is clearly not a good overall predictor of pre-weaning mortality, the major causes of which appear to be starvation following abandonment by the mother and infection (Baker and Baker, 1988). In general, the response on the day of challenge among those that did survive was no different from the response among the non-survivors. The difference in the magnitude of the response between the two age groups suggests immune function was more rigorous in the older than in the younger animals. This is corroborated by other studies that indicate that pre-weaned pups are less immunocompetent than post-weaned animals (Hall, 1998). The PHA skin test in yearling and adult animals would determine whether the response is maximal in pups at weaning.

In conclusion, this study has demonstrated that the i.d. injection of PHA-P in grey seal pups results in an acute inflammatory response, which is age-related, in the majority of individuals tested. That certain individuals did not respond suggests that this technique, which can readily be carried out in the field, could be of some value as rapid method for assessing immune responsiveness.

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