Developmental and Comparative Immunology 77 (2017) 166–173

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Changes in mucosal and serum immunoglobulin levels of California sea lions during early development

W. Espinosa-de Aquino [a], M. Bailey [b], K. Acevedo-Whitehouse [a, c, *]

[a] Unit for Basic and Applied Microbiology, School of Natural Sciences, Autonomous University of Queretaro, Queretaro, 76230 Mexico
[b] School of Veterinary Sciences, University of Bristol, Bristol, United Kingdom
[c] The Marine Mammal Center, 2000 Bunker Road, Sausalito, CA 94965, United States

ARTICLE INFO

Article history:
Received 20 April 2017
Received in revised form
25 July 2017
Accepted 10 August 2017
Available online 12 August 2017

Keywords:
California sea lion
Immunoglobulins
Isotypes
Mucosal immunity
Secretory antibodies
Zalophus californianus

ABSTRACT

To date, most studies on pinniped immunoglobulins have focused on circulating antibodies. However, systemic and local immune activities differ in terms of maturation, intensity, and types of effectors that participate. Here, we examined levels of three immunoglobulin isotypes, IgG, IgM and IgA, in the blood and mucosal membranes of free-living California sea lion pups. We investigated whether age, body condition and sex influenced their concentration. Isotype levels varied among tissues, with age-related patterns that could be indicative of differential regulation along development. Serum IgG and IgA increased linearly with age, reaching adult levels at five months of age, while IgM remained unchanged. Mucosal isotypes tended to be low in newborns and remained so until five months of age. Regardless of age, pups with better condition tended to have higher anal IgG levels and higher genital IgA levels, suggesting that their synthesis and transport to the mucosal membranes is costly. Intersex differences were only observed in the genital mucosa, where all isotypes differed between male and female pups, regardless of age, presumably due to histological and anatomical differences.

1. Introduction

Amongst the various immune effectors of vertebrates, immunoglobulins are essential components of long-lasting humoral protection from antigenic challenges. Mammals have various immunoglobulin isotypes, each of which has a different primary location in particular tissues (Chaplin, 2010). Adequate synthesis of immunoglobulins takes time to mature fully (Adkins et al., 2004; Niewiesk, 2014), so during early stages of development offspring are largely protected by maternal antibodies (Grindstaff et al., 2006). The endotheliochorial placenta of carnivores allows some maternal IgG to be transferred to the developing fetus (Day and Schultz, 2014), although the bulk of maternal IgG is delivered to suckling neonates via the colostrum (Dall'Ara et al., 2015).

Compared to what is known about immunoglobulin transfer and synthesis in terrestrial carnivores, our understanding of these processes in pinnipeds is scarce, and has mostly focused on phocids. Early work showed that at least three Ig isotypes (IgG, IgM and IgA) are present in pinnipeds (Cavagnolo and Vedros, 1978). Since then, IgG has been shown to be transferred to phocids pups through the placenta and via the colostrum, and its levels increase rapidly during lactation, nearly reaching adult levels two weeks after birth (Ferreira et al., 2005; King et al., 1998). In contrast to cats and dogs, newborn phocids can develop highly specific antibody responses after an antigenic challenge (Ross et al., 1994). There is also evidence that fetal synthesis of IgM occurs *in utero* (Marquez et al., 1998). Such early maturity of the phocid immune system most likely is a reflection of their early life history characterized by a short nursing period (Perrin et al., 2009). As otariids have longer nursing periods, commonly spanning one year across different species (Riedman, 1990; Heath, 2002), it would be expected that neonatal *de novo* immunoglobulin synthesis would take longer to occur. There is some evidence of this: serum IgG levels of five month-old New Zealand sea lion, *Phocarctos hookeri*, pups are roughly four times lower than that of adult individuals (Castinel et al., 2008), and serum IgG in pups of Galapagos sea lions, *Zalophus wollebaeki*, do not reach adult levels until one year of age (Brock et al., 2013a). A study of Northern fur seals, *Callorhinus ursinus*, showed that the onset of *de novo* synthesis of IgG and IgA took longer in pups than in terrestrial mammals, although serum

* Corresponding author. Unit for Basic and Applied Microbiology, School of Natural Sciences, Autonomous University of Queretaro, Queretaro, 76230 Mexico.
E-mail address: karina.acevedo.whitehouse@uaq.mx (K. Acevedo-Whitehouse).

http://dx.doi.org/10.1016/j.dci.2017.08.006
0145-305X/

IgM increased rapidly and reached 70% of adult levels 5-weeks after birth (Cavagnolo and Vedros, 1979).

Here we studied age-related changes in levels of IgG, IgM and IgA in apparently healthy free-living California sea lion, *Zalophus californianus* (hereafter, CSL) pups. We were interested in comparing the levels in serum and different mucosal membranes, as particular immunoglobulin isotypes are more common in different tissue types (Cerutti et al., 2011) and the immune processes of mucosae are different from those of the blood (Singh and Lillard, 2008). This spatial difference in isotype distribution is likely to be due to differences in the level and type of local exposure to pathogen associated molecular patterns (PAMPs) and antigens owing to close contact of mucosal tissues with the environment, and the constant presence of microbiota (Brandtzaeg, 2009; Cerutti et al., 2011). Thus, not only do they face different challenges; also, mucosal immune effectors need to detect pathogen associated 'non-self' without inducing damaging responses against the local microbiome (Gill et al., 2010; McGhee and Fujihashi, 2012) and responses must be carefully controlled to avoid a constantly activated immune status that could harm the organism (Beineke et al., 2010; Best et al., 2012).

We also aimed to explore the effects of body condition on CSL pup immunoglobulin levels. As adequate maturation of the immune system of mammalian carnivores depends on the bioavailability of nutrients, micronutrients, antibodies, and other immune effectors found in maternal milk (Adkins et al., 2004; Grindstaff et al., 2006; Niewiesk, 2014), we predicted that antibody synthesis of pups would rely on their body condition (as an indicator of maternal milk consumption), as has been observed for inflammatory responses in two month-old CSL pups (Vera-Massieu et al., 2015). Finally, based on life history theory (see Lochmiller and Deerenberg, 2000); we predicted that antibody levels would differ between sexes. There were two potential outcomes for this scenario: *i*) if maternal allocation of resources were biased towards male offspring as a way to maximize evolutionary success (Cockburn et al., 2002), and use of resources were equal between sexes, male pups would expectedly tend to have higher antibody levels than females; and *ii*) if both sexes receive equal amounts of maternal resources, but male pups allocate more resources for growth than for immune development than females (see French et al., 2009; van der Most et al., 2011), female pups would expectedly tend to have higher antibody levels than males.

2. Materials and methods

2.1. Sample collection

We captured 84 California sea lion pups born at Granito rookery (113°34′ W, 29°34′ N), within the Gulf of California, Mexico, at four points in time during the 2012 and 2013 pupping seasons (see details in Table 1). Pups were captured with hoop nets and were kept in a shaded pool prior to handling. Manual restriction was used during the entire sampling protocol, which lasted between 6 and 9 min. Each pup was weighed using a vertical hanging scale (max. 60 kg; 0.1 kg precision), and its total length (nose to the tip of the tail) was recorded with a tape measure (1 mm precision). Each sampling cohort was composed of naive (not recaptured) pups. Capture and sampling of the pups was conducted under permit SGPA/DGVS/03991 issued by the Secretariat of the Environment and Natural Resources of Mexico. The Bioethics Committee of the Autonomous University of Queretaro approved this study.

Table 1
Number of male and female pups captured at Granito rookery at four stages of development.

Age	Male pups	Female pups
Neonates (<2 weeks)	11	16
2 months	8	21
5 months	9	9
12 months	1	9

We used sterile cytological brushes to collect a sample of the genital, rectal and oral mucosae of each pup. The epithelium was scraped lightly for 2–3 s and the brush head was placed in a vial containing RNAlater (Ambion, USA). For each pup we also collected blood from the caudal gluteal vein using a 1.5 inch 21G needle attached to a vacuum tube (Vacutainer, USA) with no preservative. Due to handling and safety difficulties associated with manual containment of the animals, for 12-month-old pups we were unable to collect oral swabs.

Mucosal samples were placed in a cooler with ice packs immediately after collection and were transferred to a container with dry ice 6–8 h later, where they remained during the field trip. Blood samples were kept upright within a cooler with ice packs for 30 min and were centrifuged at 3200 rpm for 10 min to separate the serum, which was stored immediately in a liquid nitrogen container. Once in the laboratory all samples were transferred to an −80 C freezer where they remained until processing (within six months).

2.2. Estimation of body condition

We used mass and total length to calculate the scaled mass index, SMI (Peig and Green, 2010) as a surrogate measure of body condition for each pup. This measure has been used previously to estimate body condition of otariid pups (Brock et al., 2013b; Vera-Massieu et al., 2015).

2.3. Protein extraction

Proteins were extracted from the swabs and the serum samples using a Trizol protocol as reported previously (see Espinosa-de Aquino et al., 2017). Protein quantitation was performed using a DC Protein Assay kit (Bio-Rad) according to the manufacturer's microassay protocol. Samples were incubated at room temperature for 15 min before reading on an iMark (Biorad) microplate spectrophotometer at 750 nm. All measurements were performed in duplicate. Absorbance values were interpolated onto a standard curve of bovine serum albumin (BSA) in order to calculate protein concentrations. The curve was constructed with five serial dilutions that ranged from 1.5 mg/mL to 0.25 mg/mL. All serum and mucosal swab samples were then diluted to contain 10 μg/mL of protein. For the serum, this implied diluting ~1:50,000.

2.4. Immunoglobulin quantitation

We initially chose to use Western Blot for semiquantitation of Ig isotypes. However, the amount of protein needed to adequately perform this assay exceeded that of the majority of our samples. We also attempted to run Sandwich ELISA using anti-IgA, anti-IgM and anti-light chain antibodies, but cross-reactivity was very high. Thus we chose to perform indirect ELISA to quantify levels of isotypes IgM and IgA. For these assays, we used 96 well microtiter plates and added 50 μL of the extraction into each well for a 2 h incubation at 37 °C. Three washes with PBS-T 0.05% were conducted prior to blocking with 5% skim milk for 1 h at 37 °C. After three washes with PBS-T 0.05%, we added either mouse anti-dog IgA (1:2000) or mouse anti-dog IgM (1:4000) as the primary antibody. The wells were incubated overnight at 4 °C, and washed thrice with PBS-T 0.05% before adding the secondary antibody (goat anti-mouse IgG