Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 118 (2007) 105–112
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Early, microbially driven follicular reactions in the neonatal piglet do not contribute to expansion of the immunoglobulin heavy chain V-D-J repertoire

Stephen Wilson [a,*], Pam Norton [b], James Leigh [b], Michael Bailey [a]

[a] *Division of Molecular and Cellular Biology, Department of Clinical Veterinary Science, University of Bristol, Langford House, Bristol, BS40 5DU, United Kingdom*
[b] *Institute of Animal Health, Compton, Newbury, Berkshire, RG20 7NN, United Kingdom*

Received 17 February 2007; received in revised form 18 April 2007; accepted 26 April 2007

Abstract

Selective microbial colonisation of germ-free piglets is reported to result in expansion of immunoglobulin V_H- and D_H-segment usage from an initially limited repertoire. Here, the response of the palatine tonsil to microbial colonisation was compared in age-matched conventionally reared and germ-free piglets. At 3 and 5 days after birth an expansion in the B-cell follicle area was observed in the conventional, microbially colonised animals, which was not seen in the germ-free piglets. Consistent with this observation, sequencing of re-arranged heavy chain V-D-J units demonstrated accumulation of point mutations indicating somatic hypermutation in the conventional, microbially colonised piglets but not in the germ-free animals. However, V_H- and D_H-segment usage and CDR3 length did not differ between the groups. The results suggest that the follicle reaction observed occurs in response to microbial challenge, involves proliferation and somatic hypermutation of B-cells but does not expand repertoire or generate classical, isotype-switched memory B-cells. We suggest that microbial colonisation of neonatal piglets drives immunological competence in two stages: first, an antigen non-specific, follicular reaction which expands immunological compartments; and second, microbe driven changes in V-segment usage which expand immunological repertoire.

Keywords: Mucosa; Pig; Repertoire development

1. Introduction

The characteristic feature of the adaptive immune system in higher vertebrates is the generation of antibody molecules that recognise a multitude of antigenic epitopes. Antibody diversity is generated by V-D-J re-arrangement, by pairing of different combinations of heavy and light chains V-regions, by introduction of N- and P-nucleotides (Tonegawa, 1983) and by somatic hypermutation (Wagner and Neuberger, 1996). However, although the V-D-J re-arrangement processes are common to all vertebrates, not all species share the same degree of germ-line sequence variability. In contrast to humans and mice, there are only a limited number of germ-line V_H gene segments either present or used in a many species, such as the pig (Sun et al., 1998), rabbit (Friedman et al., 1994), cattle (Saini et al., 1997; Sinclair et al., 1997) and the chicken (Reynaud et al., 1989). These species rely on other mechanisms for the diversification of their immunoglobulin V(D)J gene sequences. For example, rabbits have been shown to use a combination of gene conversion and somatic point mutation (Winstead et al., 1999) while sheep appear to diversify their repertoire by antigen-independent somatic

* Corresponding author.
E-mail address: s_m_wilson@hotmail.com (S. Wilson).

0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2007.04.018

hypermutation (Reynaud et al., 1995), although a large number of sequences in sheep previously thought to be due to somatic hypermutation are actually coded within the germline (Jenne et al., 2003).

Previous studies have shown that foetal and neonatal piglets use only four V_H gene segments and two D_H gene segments (Sun et al., 1998), and possess only one J_H gene segment (Butler et al., 1996). The V_H- and D_H-segment usage expands with age and microbial colonisation, but the use of low numbers of V, D and J gene segments in immunoglobulin re-arrangement implies that the combinatorial heavy chain pre-immune repertoire is comprised of only eight to ten re-arrangements before mutation. Restricted combinatorial diversity could be offset by a number of mechanisms including high light chain diversity, junctional diversity in CDR3, gene conversion of foetal V_H genes or somatic hypermutations. As with a number of other species, such as rabbits (Lanning et al., 2000), microbial colonisation of the pig intestinal tract expands the level of immunoglobulin diversity (Butler et al., 2000). Butler and others have shown that colonisation of germ-free piglets with a defined microbial flora selectively diversifies the immunoglobulin V_H and D_H gene segment usage in mucosal tissues by 6 weeks after colonisation. That this is associated with repertoire expansion is suggested by further studies demonstrating that germ-free pigs make poor responses to thymus-dependent and independent antigens prior to colonisation (Butler et al., 2002).

The association between microbial colonisation and expansion and competence of the neonatal immune system is increasingly relevant to human populations, in which infant allergic disease has been linked to early exposure to commensal micro-organisms. We have previously shown that the pig palatine tonsil develops with age and is also affected by the presence of micro-organisms or microbial antigens. Here we demonstrate a transient expansion of immunological compartments, specifically B-cell follicles, and in order to determine whether this reflected antigen-specific reactions by mucosal B-cells or an antigen non-specific expansion of repertoire, we have directly examined B-cell repertoire by sequencing re-arranged V-D-J units of immunoglobulin heavy chain genes.

2. Materials and methods

2.1. Animals

SLA defined NIH mini-pigs (c/c MHC haplotype) (Sachs et al., 1976) kept under conventional conditions and large white/landrace pigs kept under germ-free conditions were obtained from the Institute for Animal Health, Compton. Conventional animals were killed at birth ($n = 2$), at 3 days old ($n = 2$), 5 days ($n = 3$), 15 days ($n = 2$), 30 days ($n = 2$), 90 days ($n = 2$), 180 days ($n = 3$) and 270 days ($n = 3$). Germ-free animals were killed at 3 days old ($n = 3$), 5 days ($n = 3$) and 15 days ($n = 3$). Germ-free animals were not kept for longer than 15 days due to ethical constraints imposed by the national regulatory body. Tonsils were removed and mounted onto cork discs in a defined orientation embedded in OCT (Bright Instruments, UK) and snap frozen in isopentane cooled over liquid nitrogen. Serial sections of 5 μm were cut and mounted on multi-spot glass slides (Hendley, UK), air dried for 1 h and fixed for 10 min in cold acetone. Slides were stored at −20 °C until use. All animal work complied with institutional and Home Office ethical guidelines.

2.2. Immunohistology

Five micron sections were cut from embedded, frozen tissues and stained for multiple-colour immunofluorescence histology as previously described (Haverson et al., 2000). Briefly, Fc receptors were blocked for 40 min with PBS containing 5% pig serum and 5% goat serum. Combinations of pre-titrated monoclonal antibodies (CD3, clone STH164; CD21, clone BB611C9; IgG, clone 254 G8; CD45RC, clone MIL 15) were applied in PBS for a minimum of 2 h. Slides were washed thoroughly in three changes of PBS for 3–5 min each. Secondary reagents were isotype specific goat anti-mouse antibodies (Southern Biotechnology, Birmingham, AL) conjugated to Texas Red, or fluorescein isothiocyanate (FITC), applied together at optimal concentrations. After a final wash, the sections were mounted in Fluoromount (Vector Laboratories) and sealed with nail varnish. Stained slides were examined with a Leica fluorescence microscope fitted with a multiple-colour excitation and emission filter block. 24-bit colour images were recorded on a digital camera (Photonic Science, Milham Mountfield, UK). For each antibody combination four sections were stained and from these sections seven or more images were analysed. Area measurement was quantified by analysing representative images for each tissue section using the GEM software package (ME Electronics, Reading, UK), with at least 10 follicles or T-cell areas measured per animal.

2.3. DNA extraction

Tonsil from 3-, 5- and 15-day-old conventional and germ-free animals were cryosectioned (25 μm) and the