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Somatic hypermutation in peritoneal B1b cells

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ABSTRACT

Murine B1 cells have been shown to be able to switch to IgA *in vitro*. In agreement, we could demonstrate in the peritoneum of mice the presence of IgA producing B1 cells. Interestingly, enzyme-linked immunospot assays of lipopolysaccharide stimulated cultures revealed that only the B1b cell subpopulation contained high numbers of such cells while IgA producing B cells were rare amongst the B2 and B1a cell populations. This was confirmed by RT-PCR on sorted peritoneal B cell subpopulations. In addition, the variable regions associated with IgA of peritoneal B1b cells displayed extensive variation due to somatic hypermutation. In contrast, mutations were found only at low frequencies in VH regions associated with IgM of both B1 cell populations. Thus, peritoneal B1b cells display many similarities to B2 cells. This finding is consistent with the idea of a layered immune system in which peritoneal B1a and splenic follicular B2 cells appear at the two extremes and peritoneal B1b and B2 cells represent intermediates.

1. Introduction

The mature B cell populations of the mouse can be divided into several subpopulations of which B1 and splenic follicular B2 cells have been studied most intensively. Follicular B2 cells are known for their exquisite receptor specificity. They represent the dominating B cell population in the spleen and are continuously generated in the adult bone marrow. Upon antigen encounter in conjunction with appropriate T cell help, these B cells migrate to germinal centers where they proliferate. In addition, they initiate differentiation that might drive them into memory B cells or antibody secreting plasma cells. Along this route, germinal center B cells will up-regulate the enzyme activation-induced cytidine deaminase (AICDA) that will result, under the influence of appropriate cytokines, in class switching. In parallel, AICDA expression will result in the introduction of somatic mutations into the variable region of the H and L chain genes. This, due to selection by antigen, will cause an increase in the average affinity of the BCRs and consequently in affinity maturation of secreted antibodies (Kosco-Vilbois et al., 1997; McHeyzer-Williams et al., 2001; Tarlinton, 1998). These events provide the basis for humoral immunity against pathogens and other antigens.

In contrast, B1 cells dominate the peritoneal and pleural cavities but are also found to a small extent in the spleen. They were originally believed to be mainly responsible for the production of natural antibodies i.e. IgM that is found without antigen challenge, like in germ free or antigen free mice (Hayakawa et al., 1984; Kantor and Herzenberg, 1993; Stall et al., 1996). Antibodies derived from B1 cells have been shown to often exhibit weak anti-self-reactivity or reactivity against conserved carbohydrates on bacterial pathogens. In addition, some antibodies derived from B1 cells have been shown to recognize multiple antigens (Berland and Wortis, 2002). B1 cells often respond to antigens without the requirement of T cell help and were thought not to undergo class switching and affinity maturation.

In the meantime, the picture is more differentiated. Peritoneal B1 cells can be divided into B1a and B1b subpopulations according to their cell surface phenotype, origin and function (Herzenberg et al., 1992; Stall et al., 1992). Phenotypically, B1a cells are characterized as $B220^{lo}CD19^{hi}IgM^{hi}IgD^{lo}CD43^{+}Mac\text{-}1^{+}CD5^{int}$. They were shown to be mainly generated during fetal life and to be maintained by self-renewal. B1b cells share all the markers with B1a cells except CD5, thus being $B220^{lo}CD19^{hi}IgM^{hi}IgD^{lo}CD43^{+}Mac\text{-}1^{+}CD5^{-}$. They are believed to be mainly generated during neonatal life in the bone marrow although recently a precursor of such cells has been characterized in the bone marrow of adult mice (Montecino-Rodriguez et al., 2006).

B1 cells are now known to be involved in the earliest specific reaction against pathogens either by providing specific natural

Abbreviations: AICDA, activation-induced cytidine deaminase; CDR, complementarity determining region; ELISPOT, enzyme-linked immunospot technique; H chain, heavy chain; L chain, light chain; LPS, lipopolysaccharide; mRNA, messenger RNA; RT-PCR, reverse transcriptase polymerase chain reaction; VH region, heavy chain variable region.

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antibodies (Ochsenbein et al., 1999) or by rapidly differentiating into antibody secreting plasma cells (Martin et al., 2001). In addition, B1b cells after immunization were able to produce specific IgM and protect mice against *Streptococcus pneumoniae* infection (Alugupalli et al., 2004; Haas et al., 2005). Similarly, B1b cells were shown to provide long lasting T cell independent humoral immunity against *Borrelia hermsii* (Alugupalli et al., 2004; Haas et al., 2005).

B1 cells have also been claimed to make a significant contribution to IgA producing plasma cells in the lamina propria of the gut (Fagarasan et al., 2000; Kroese et al., 1989; Macpherson et al., 2000). In line with this claim, peritoneal B1 cells were shown to rapidly and preferentially switch to IgA under appropriate stimulatory conditions *in vitro* i.e. in the presence of LPS, Blys and TGF-β (Baumgarth et al., 2005; Kaminski and Stavnezer, 2006; Martin et al., 2001). In the present work, we wanted to extend these findings and to investigate in more detail whether peritoneal B1 cells are able the switch to IgA producing cells also *in vivo*. Here we show that the isotype switch is mainly found in B1b cells and such cells display high frequencies of somatic mutations in their IgA associated VH regions.

2. Materials and methods

2.1. Mice

Wild type BALB/c mice, 8–14 weeks old, were used for the experiments. All experiments were performed in accordance with the German Law on Care and Use of Laboratory Animals and were approved by the local ethics committee (LAVES).

2.2. Cell sorting

Monoclonal antibodies against mouse CD19, CD5, CD43 and Mac-1 conjugated with FITC, PE, APC, PE-Cy7 or biotin were obtained from Pharmingen (Heidelberg, Germany) or eBioscience (Frankfurt, Germany). Biotinylated antibodies were revealed by Streptavidin–FITC (Pharmingen, Heidelberg, Germany) or Streptavidin–PE (SouthernBiotech, Eching, Germany). Cell sorting was performed using FACSAria® (Becton Dickinson, Heidelberg, Germany). Doublets from the lymphocyte gated population were excluded by applying appropriate scatter gate. Reanalysis revealed that cells were >90% pure.

2.3. RT-PCR

Total RNA from bulk-sorted cells was prepared with TRIzol™ Reagent (GIBCO, Karlsruhe, Germany) according to the manufacturer's protocol. DNase1 (Amersham Biosciences, Munich, Germany) treated RNA was reverse transcribed using oligo-d$(T)_{12-18}$ (Amersham Biosciences, Munich, Germany) and Superscript II RNaseH$^-$ reverse transcriptase (Invitrogen, Karlsruhe, Germany). For semi-quantitative PCR analysis, amplification of threefold serial dilutions was performed using HotstarTaq™ DNA polymerase (Qiagen, Hilden, Germany) and the following primers: RPS9, forward (for) 5′-TTGACGCTAGACGAGAAGGAT-3′ reverse (rev) 5′-AATCCAGCTTCATCTTGCCCT-3′; Igμ/α heavy chain variable (VH) region, for VHcons 5′-GAGGTGCAGCTGCAGGAGTCTGG-3′ rev Cμ2 5′-CATTTGGGAAGGACTGA-3′ or Cα2 5′-GAGCTGGTGGGAGTGTCAGTG-3′; Igα constant region for 5′-GTGATAATCGGCTGCCTGATT-3′ rev 5′-TCTCAGGCCATTCAGAGTACA-3′; Igμ constant region for 5′-TCTGATAAGAATCTGGTGGCCAT-3′ rev 5′-GAAGTTCGTGGCCTCGCAGAT-3′. PCR conditions were: 94 °C for 20 s, annealing at various temperatures for 40 s, 72 °C for 40 s; 35–37 cycles.

2.4. Real-time PCR

Real-time PCR to quantify the Igα and Igμ transcript levels was conducted using a SYBR Green PCR master mix kit (Applied Biosystems, Darmstadt, Germany) with the 7500 Real Time PCR System (Applied Biosystems, Darmstadt, Germany). Results were normalized using the housekeeping gene RPS9. Primer pairs for Igα for 5′-CCCGTCCAAGAATTGGATGTG-3′ rev 5′-ATCTGAACCCAGGAGCAGGTC-3′; Igμ for 5′-CACAGGGGTCTCACCTTCTTG-3′ rev 5′-GAGGAAGATGTCGGCAAAGGA-3′, and RPS9 (as above) were used for the amplification of the cDNA.

2.5. Sequencing of Ig VH chain and sequence analysis

RT-PCR products of IgH chain transcripts from bulk-sorted cells were cloned using the TOPO TA Cloning® kit (Invitrogen, Karlsruhe, Germany) following the manufacturer's protocol. Plasmids were isolated using the GFX™ Micro Plasmid Prep Kit (Amersham Pharmacia Biotech, Munich, Germany) or QIAprep Spin Mini Prep Kit (Qiagen, Hilden, Germany) and the insertion of the DNA fragments was tested by EcoRI restriction digestion of plasmid DNA aliquots prior to sequencing. Sequencing was carried out using M13 Reverse primers by the department of Genome Analysis of Helmholtz Centre for Infection Research, Braunschweig. Sequences were compared using SEQUENCHER™ Version 4.1 for Macintosh software (Gene Codes Corporation) and aligned for the best match to one of the four J_H gene segments. Assignment of VH chain was done by using VBASE2 database (http://www.vbase2.org/). For the determination of CDR3 the longest D element matches ≥5 were assigned. A second D element was assigned only if there was a ≥5 nucleotide match. After assignment of all possible P nucleotides, the remaining nucleotides were considered N nucleotides. To avoid any bias due to cloning, identical sequences isolated repeatedly from the same sorting experiment were included only once in the data set.

2.6. LPS stimulation and ELISPOT

LPS (*Escherichia coli*, Sigma, Munich, Germany) at 50 μg/ml concentration was used for the stimulation of sort purified peritoneal cells resuspended in IMDM at a density of 2×10^4–2×10^5 cell per ml. Detection of antibody secreting cells amongst cultured cells was done using the standard protocol for ELISPOT assay. Briefly single-cell suspensions at serial dilutions of 1:5 were plated on plates coated with goat-anti-mouse IgA (α chain specific, Sigma, Munich, Germany) or rat-anti-mouse IgM (Pharmingen, Heidelberg, Germany). Cells were incubated overnight at 37 °C in 5% CO_2 and 95% humidity. After washing with PBS 0.01% Tween 20, biotin conjugated goat-anti-mouse IgA (α chain specific, Sigma, Munich, Germany) or biotin conjugated rat-anti-mouse IgM (heavy chain specific, SeroTec, Düsseldorf, Germany) was added. Bound antibodies were developed with Streptavidin–horseradish peroxidase using AEC (3-amino-9-ethyl-carbazole; Sigma, Munich, Germany) in DMF (N,N-dimethylformamide; Sigma, Munich, Germany) diluted in 0.1 M acetate solution and added with H_2O_2 as substrate. Spot development was stopped by washing plates with double distilled water and spots marking antibody secreting cells were counted after air drying.

2.6.1. Sequence data

IgA VH sequences derived from sorted peritoneal cavity B1b cells and IgM VH sequences derived from B1a and B1b cells were submitted to GenBank under the accession numbers EU934835–EU934859, EU934860–EU934882 and EU934883–EU934901 respectively.