



Light chain variable region diversity in Atlantic cod (*Gadus morhua* L.)

Helena Widholm^a, Ann-Sofie Lundbäck^a, Annika Daggfeldt^a,
Bergljot Magnadottir^b, Gregory W. Warr^c, Lars Pilström^{a,*}

^aDepartment of Medical Immunology and Microbiology, Uppsala University, Box 582, 751 23 Uppsala, Sweden

^bInstitute of Experimental Pathology, University of Iceland, Reykjavik, Iceland

^cDepartment of Biochemistry and Molecular Biology, Medical School of South Carolina, Charleston, SC, USA

Received 22 January 1998; received in revised form 16 November 1998; accepted 18 November 1998

Abstract

This study was undertaken to determine if a lack of V_L domain variability could explain, in part, the failure of Atlantic cod to respond to immunization with the production of specific antibodies. The variability of cod V_L regions was studied in 33 cDNA and two genomic clones. The variability of the CDRs was estimated by the Shannon entropy method and compared with that in other species. It was found to be lowest in the little skate (*Raja erinacea*), higher in cod, and highest in *Xenopus* and mouse. While the variability of the CDRs is slightly lower in cod than in *Xenopus* and mouse, it is spread over broader areas of the amino acid sequence. The length of CDR1 and CDR3 in cod is equal to or exceeds that found in skate, *Xenopus*, chicken and mammals. Isoelectric points and hydrophobicity vary substantially among the studied Ig light chain domains. Thus, neither the length, nor the variability, nor the physicochemical properties (pI and hydrophobicity) of the L chain CDRs can explain the absence of antibody response to immunization in cod. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Immunization of bony fish generally gives good antibody responses [1–3], but the Atlantic cod (*Gadus morhua* L.) are an exception. Several

studies have shown that model antigens like bovine serum albumin, hemocyanin from *Limulus polyphemus*, SRBC (haptened or unhaptened) and vaccines of killed bacteria are all unable to induce antibody levels higher than those found in non-immunized controls [4,5]. The serum immunoglobulin concentration is rather high in cod ([6], Magnadottir, unpublished data) so failure to synthesize Ig cannot be an explanation of the poor response. The immunoglobulins also seem to be 'sticky'; high levels of antigen binding have been observed in non-immunized cod. However,

Abbreviations: CDR: complementarity determining region; FR: framework region; H- & L-chain: immunoglobulin heavy and light chain; V: variable.

* Corresponding author: Tel.: +46-18-471-42-18; fax: +46-18-55-75-79.

E-mail address: lars.pilstrom@imim.uu.se (L. Pilström)

when immunized cod are challenged with live pathogenic bacteria, it is clear that immunity has been acquired as a result of the immunization, even in the absence of an obvious specific antibody response [5].

The gene organization and mechanisms for generation of variability of the H-chain in teleost fish are basically the same as in mammals [7,8] and there is no deficiency in the number of V_H genes in the cod [9] that could explain its poor antibody response. Studies of the variability of the cod heavy chain indicate that there are at least four different V_H families, each with some 20–30 members, the variability of which should provide the basis for an excellent specific antibody response ([9] and unpublished observations).

In those teleost species where the L-chain has been studied, at least two isotypes have been described. They are called F and G in channel catfish [10,11], and L1 and L2 in rainbow trout [12,13] respectively in Atlantic cod ([12], Wermenstam, personal communication). The L-chain loci in teleosts are organized in multiple repeated clusters of either (V–V–J–C) or (V–J–C) [10–15]. This organization could restrict variability if recombination were to take place only within a cluster. The V-segments of the teleost L-chain clusters, unlike those of elasmobranch fish [16] are in opposite transcriptional orientation to the J and C genes [10,15] which might facilitate rearrangement between clusters and, thereby, increase the potential L-chain variability.

Here, we report an analysis of the variability of the light chain isotype L1 of Atlantic cod, undertaken to determine whether low L-chain variability might contribute to the poor antibody response seen in this species.

2. Materials and methods

2.1. Animals and tissues

Atlantic cod (*Gadus morhua* L.) were raised from eggs and cultivated at the Marine Research Institute of Iceland. They were acclimatized at 9°C for four weeks and then immunized. The in-

dividual described here was immunized with 0.3 mL Freund's complete adjuvant with phosphate buffered saline (PBS). Fifteen weeks later, the fish (male, body weight 306 g) was anesthetized in MS-222 (Sandoz, Basel, Switzerland) and the spleen and head kidney were rapidly removed and snap frozen in liquid nitrogen and stored at -70°C until used.

2.2. Isolation of RNA and preparation of a cDNA library

Total RNA was prepared from approximately 30 mg of tissue by the method of Chirgwin et al. [17] and poly(A)+ve RNA was isolated by chromatography on an oligo-dT column. cDNA was prepared with a kit (Pharmacia LKB Biotechnology, Uppsala, Sweden), and ligated and packaged into the bacteriophage λ GEM-2. A total of 2.8×10^6 pfu was obtained.

2.3. Screening

Approximately 240,000 pfu of the unamplified library were plated on *E. coli* LE392 on LB agar plates. Duplicate nitrocellulose filters were lifted and hybridized with a ^{32}P labeled V probe [18], washed at high stringency and exposed to X-ray film (Kodak BiomaxMR) for 72 h at -20°C . 64 positive clones were isolated and plaque purified. The PCR-generated V probe extended from the leader region to the FR3 of a genomic clone described by Bengtén [15].

2.4. Sequencing

The inserts could not be released by enzyme digestion from the lambda GEM2 and were PCR-amplified with primers specific for the CL domain and for the phage arm, then subcloned into pUC19. They were sequenced according to the Sanger dideoxy nucleotide chain termination method [19] with modified T7 DNA polymerase. Some clones were sequenced using an ABI377 automated sequencer in the MUSC Nucleic Acid Analysis Facility. All clones were sequenced on both strands and the sequences were analysed with the DNASTar[®] programs. Sequences

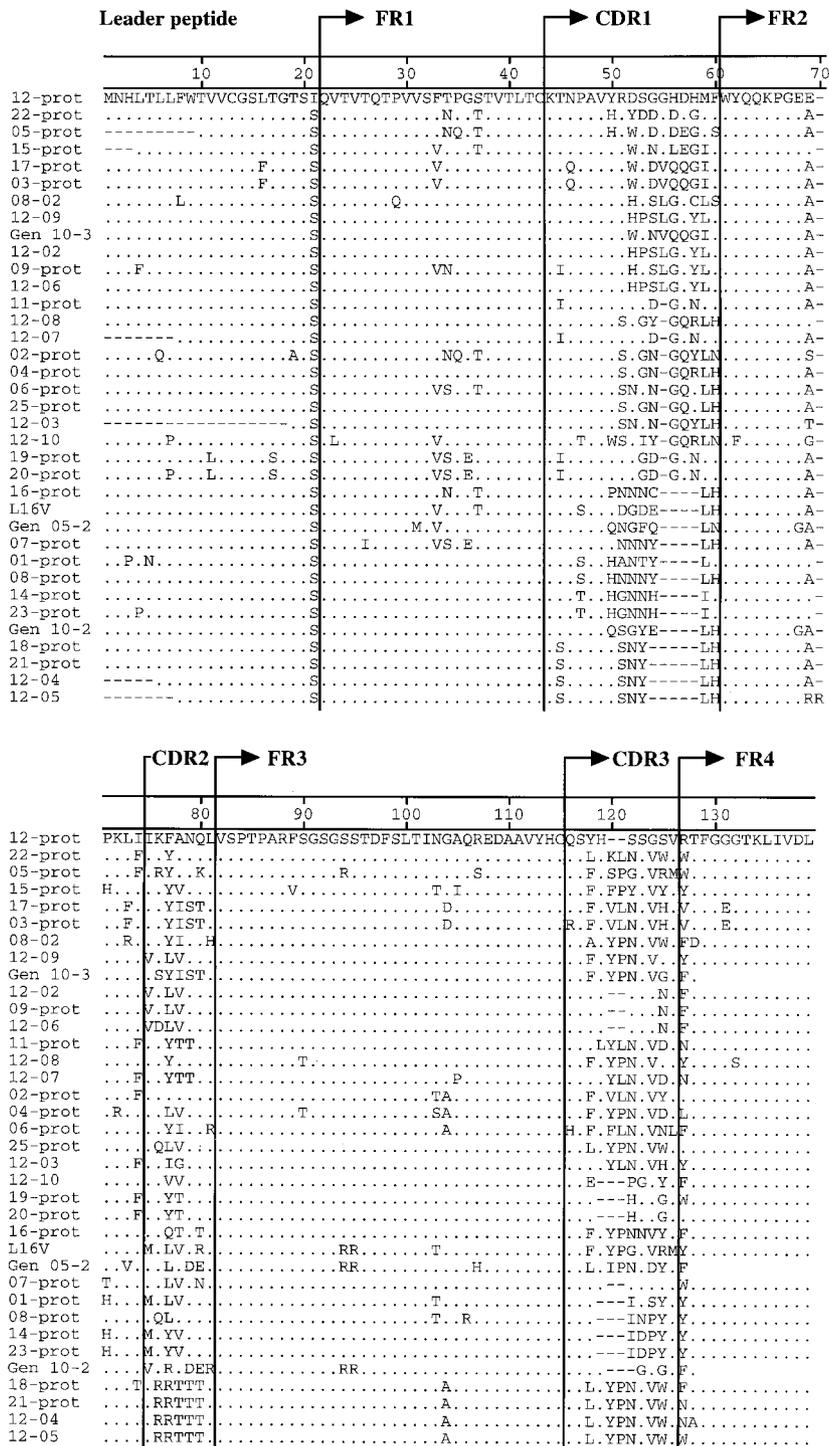


Fig. 1. Multiple alignment of the amino acid sequences of V domains from Atlantic cod. The borders between frameworks (FR) and complementarity determining regions (CDR) correspond to those described by Kabat *et al.* [29]. The sequences are arranged according to the size of the CDRs. The corresponding nucleotide sequences are available at EMBL/GenBank data base under the accession nos. X76517, AJ225237–AJ225268, AF104898 and AF104899.

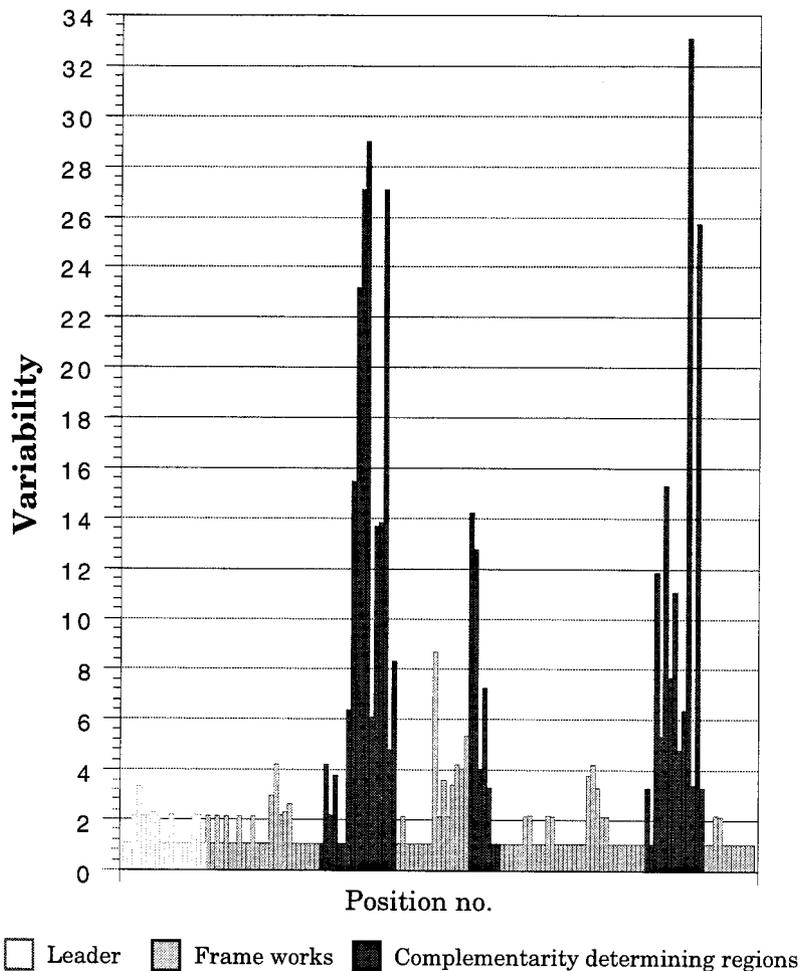


Fig. 2. Variability plot of the amino acid sequences of V domains from Atlantic cod. The plot is based on the data from Fig. 1. The calculations were performed with the formula for variability in [20].

reported in this paper can be found at EMBL/GenBank database under the accession nos. X76517 and AJ225237–AJ225268. Three unpublished V_L and 5 J_L segments from two genomic clones were also included in the analysis (kindly supplied by E. Bengtén, acc. nos. AF104898 and AF104899). Variability has usually been expressed according to the method of Kabat and Wu [20] but, recently, a method based on Shannon entropy was introduced [21,22] which has the advantage of being additive and allowing statistical evaluation to be performed. Shannon

entropy (H) was estimated by a computer programme generously supplied by S. Litwin, Philadelphia, PA, USA. Both methods have been used for analyses of the sequences. A total of 36 V_L sequences of cod isotype L1 were included in the final analyses. They all belong to the same family and no second family has been described. In comparisons with other species, 40 V_L sequences of *Raja erinacea* were obtained from [23,24]. Three of these sequences seem to belong to a separate family. Fifty-three V_L sequences of *Xenopus laevis* were obtained from [25–27] and

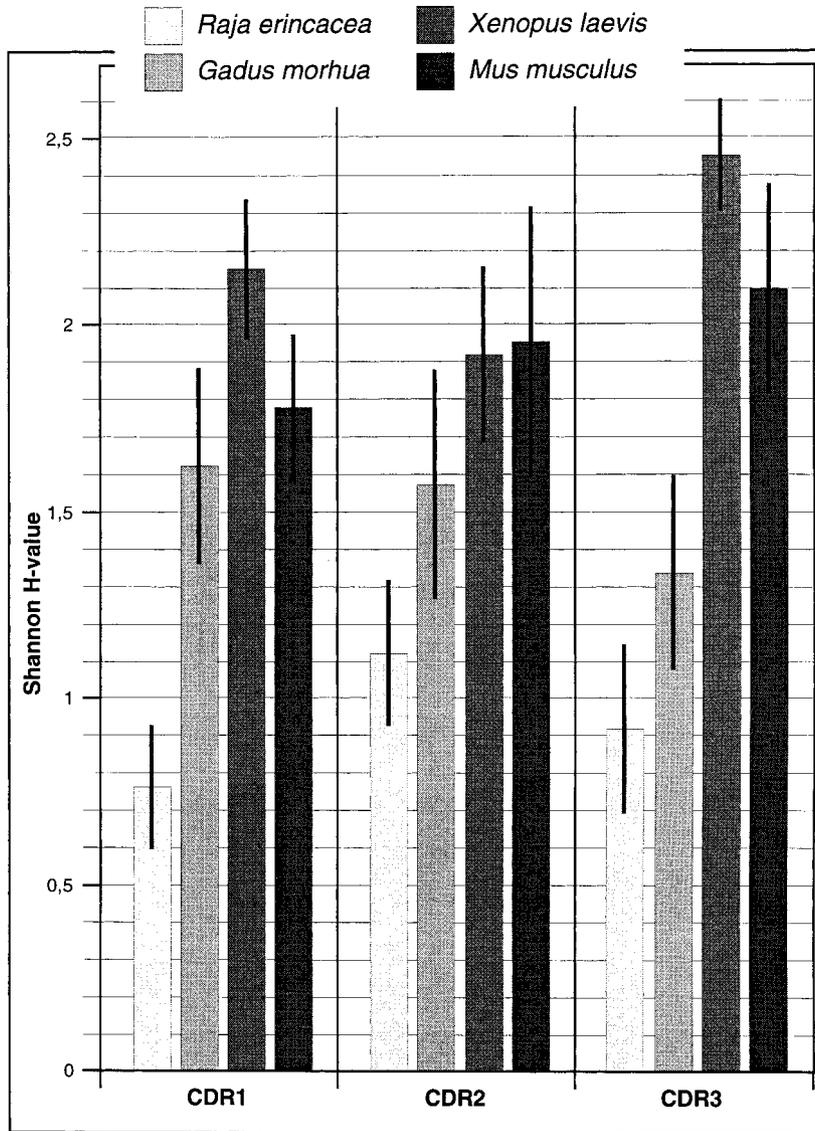


Fig. 3. Variability of CDR of *Raja erinacea*, *Gadus morhua*, *Xenopus laevis* and *Mus musculus* estimated by the Shannon entropy method [21,22]. The bars indicate mean \pm S.E.M. The statistical analysis was performed by ANOVA in StatView[®] with a significance limit of $p < 0.05$. The following differences are statistically significant: For CDR1: *Raja* vs. the three other species; CDR2: *Raja* vs. *Xenopus* and *Mus*; CDR3: *Raja* and *Gadus* vs. *Xenopus* and *Mus*. Data were collected as described in Materials and methods.

comprise the three known isotypes, belonging to several families. In total, 2674 V segments (incl. the six families) of *Mus musculus* kappa chain were obtained from [20,22].

3. Results

Sixty-four clones from a spleen cDNA library of a single Atlantic cod were isolated with a

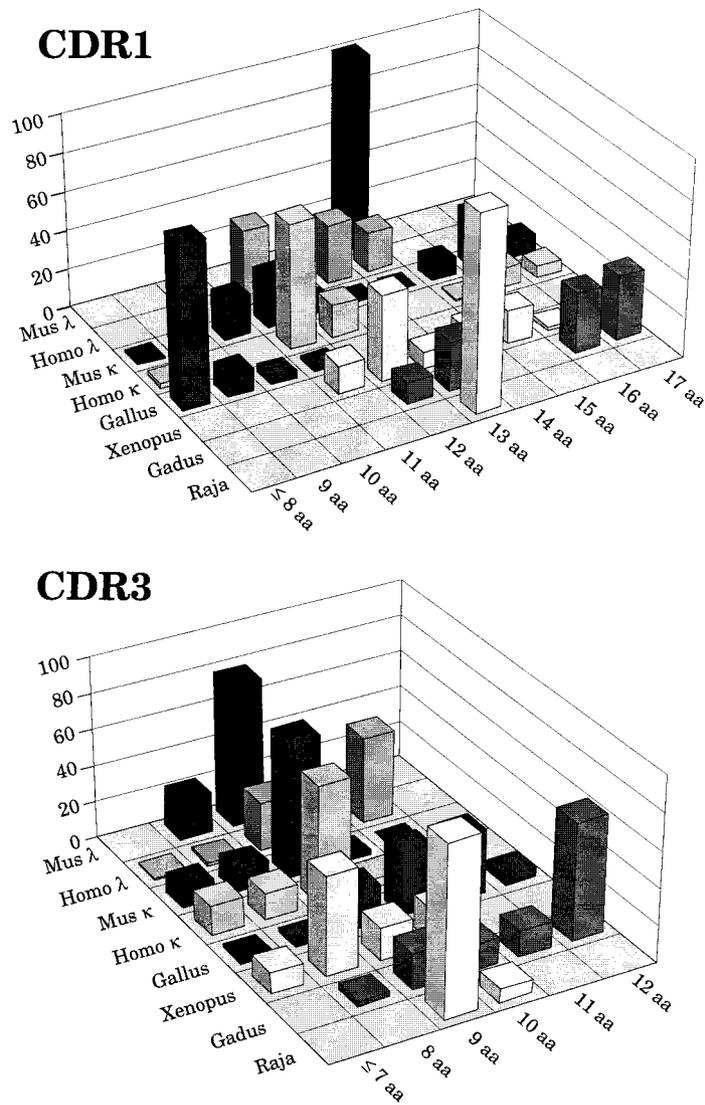


Fig. 4. Frequency distribution of the size of CDR1 and CDR3 of selected species. The data for *Raja* are derived from [23,24], for *Xenopus* from [25–27] and for the mammalian and avian species from Kabat *et al.* [29]. Data compiled from 40 Vs from *Raja*, 39 Vs from Atlantic cod, 48 from *Xenopus*, 53 from chicken, 707 and 62 from κ and λ in mouse and 98 and 83 from κ and λ in human.

probe specific for the Ig L-chain. Of the clones sequenced, 31 contained the full length V-domain with different nucleotide sequences. Two cDNA clones from two other individual fish were added to the final analysis, as well as three V-segments from two genomic clones (Fig. 1). The inferred V region amino acid sequences were easily aligned and the variability was, as expected, mostly con-

fined to the CDR regions. The overall amino acid identity between clones ranged from 72–99%; they all belong to a single family. In order to examine the degree of variability in the cod V sequences, a plot of the variability according to Kabat *et al.* [20] was constructed (Fig. 2). The highest variability was found in the CDRs but a substantial variability could also be found in the

amino acids in FR2 upstream of the CDR2. Thus, the variability is spread over a broader area in cod than in other animals like *Xenopus* or mouse. In order to compare the variability of V_L of cod, the Shannon entropy (H) was calculated from the V_L of cod and three other species—*Raja erinacia*, *Xenopus laevis* and *Mus musculus*—and the mean values of H in the CDRs were compared. Variability in *Raja* V_L is consistently the lowest of the 4 species and variability in the cod V_L is always less than that seen in *Xenopus* and mouse (Fig. 3).

The length of the CDRs is considered important for the variability and capacity to bind antigen [28], so a comparison of the CDR1 and CDR3 for 8 light chains from different species was performed (Fig. 4). CDR2 was omitted from the study since all L-chain CDR2s are considered to have the same number of amino acids [29]. The results show that the frequency of V_L s with 16 and 17 amino acids in CDR1 are the highest in cod, while CDRs with 12 amino acids are most common in *Xenopus*. In *Raja*, as in mouse λ , all CDR1s contain 13 amino acids. Cod has longer CDR1 and CDR3 than the three homeotherm species. The highest length of CDR3 (12 amino acids) is found in cod and this length is only found in low frequency in chicken, while all the other studied species have 8–11 amino acids as the most common CDR3 length. Interestingly, all kappa related sequences, including those of cod and *Xenopus* L1 (see [26]) have a bimodal distribution of the size of CDR1 length. The data of Figs. 3 and 4 should be interpreted with some caution as the data from *Xenopus* are based on three different L-chain isotypes and on only a single isotype in the other species. The L-chain of cod presented here (L1) seems (like the murine kappa) to be the main expressed isotype [30].

As the overall variability of the IgL CDRs in cod is generally a little lower than in *Xenopus* (Fig. 3) but spread over broader regions of the domain, it might be that the antigen binding sites are either markedly hydrophilic or hydrophobic and, thereby, limited in their ability to contribute to functional variability. Hydrophobicity plots of the V sequences show that the degree of hydrophobicity/hydrophilicity differs between the

domains, being most pronounced in CDR3 (data not shown). Clearly, there should be a capacity for the cod V_L s to bind to antigen epitopes by both hydrophobic or charge interactions. In examining the charged residues, an analysis of the pI of the CDRs (data not shown) shows that the median pI in CDR1 is a little less than 9 but that there are several sequences with lower pI values. CDR2 has a limited range of pI and most of the values are 5.5. As the variability associated with CDR2 extends into FR2, an estimation of pI was also performed using CDR2 and the closest five amino acids of FR2. This led to an increase of pI to around 9 but it also increases the variation of pI in this area. In CDR3, the median pI is 7.15, with variations between 3.2 to 10.4.

The clustered type of organisation of the L-chain locus of cod with one J-segment, together with each V-segment [15], suggests that the J-segments may contribute to the variability of the V-domain. In order to find out whether, and to what extent, such a contribution occurs, the J-segments were aligned (not shown). The 39 J-segments encode 7 different amino acids at the NH_2 -end of CDR3. Tyrosine/phenylalanine are represented in half the sequences at a site whose overall character is hydrophobic. All the other nucleotide exchanges induced a change in encoded amino acids (*cf.* Fig. 1), except the two T to C exchanges at the 3' end of clone 08-02.

4. Discussion

The capacity of an animal to generate specific antibodies is limited by the combination of diversity-generating mechanisms that are available to it. These mechanisms have been summarized in several reviews e.g. [7,8]. As cod do not respond to immunization by production of specific antibodies [4,5], we can ask, first, if there are defects in the number of germ-line encoded elements (V, (D), J) in their rearrangement, and in the mechanisms that introduce additional diversity during and after rearrangement. It was first thought that the poor antibody response of cod might be caused by the lack of some of the mechanism(s)

for generating variability. For this reason, the Ig heavy chain locus was studied but no evidence was found that any of the known mechanisms were absent ([9] and unpublished results). Studies of the light chain locus revealed that it has an organization very similar to that seen in elasmobranch fish with V–J–C segments clustered in the genome [9,12,16,31]. Such clusters might restrict diversity and give the antibodies their non-specific ‘sticky’ properties. However, closer analysis of the V clusters of teleost fish showed that the V-segments have an opposite transcriptional orientation in relation to the J- and C-segments. This might allow rearrangement between clusters to take place by means of inversion, as is known to occur in mouse [32]. The results presented here show that the variability of the Ig light chain of cod is nearly as great as in *Xenopus* (Fig. 3) and perhaps even comparable to that in mice. This is true concerning the size of the CDRs, which is at least equal to but also often exceeds that found in mammals (Fig. 4). An explanation of the ‘stickiness’ of the cod immunoglobulin as an effect of the paratopes being either predominantly hydrophilic or hydrophobic [33] can also be ruled out as different degrees of hydrophobicity/hydrophilicity are found in the CDRs. The pI values of the CDRs also vary to such an extent that they can hardly explain the lower or non-existing specificity of cod antibodies. These results, together with those for the heavy chain, give no reasonable genetic or biochemical explanation of the absence of a good antibody response in Atlantic cod. Thus, likely explanations relate to the physiology of the immune response in this species.

In channel catfish, there is a report of a second, IgD-like immunoglobulin, which appears in low amounts and the importance of which for antibody defence is still unknown [34]. There is certainly no evidence that IgD is a major antibody in cod; to date, only IgM has been reported in blood. However, in normal mice, the concentration and the repertoire of serum IgM is independent of external antigenic contact [35] and the authors suggest that IgM production is driven by autoreactivity. Mouthon et al. [36] observed a similar autoreactive IgM repertoire in humans.

In both these investigations, the reactivities to external antigens were considered to be cross-reactivities. If a similar situation occurs in cod, there might not be a need for specific reactivity because the level of cross-reacting antibodies is already so high that they will bind to and eliminate any antigen entering the body.

In conclusion, the variability of the immunoglobulin light chain of cod does not explain why cod do not respond with an increased antibody titer to immunization. A more extensive study of the variability of the Ig heavy chain, combined with knowledge about the physiological regulation of B cell development and activation, will perhaps give a more detailed picture of the functional antibody repertoire in this species.

Acknowledgements

This work has been supported by grants from The Swedish Council for Forestry and Agricultural Research (No. 40.0484/96) and from The Nordic Ministry Council (No. 66080200). G. Warr was the recipient of a Visiting Research Fellowship from Uppsala University. We want to thank Dr. Eva Bengtén for permission to use her unpublished data and Dr. S. Litwin for the generous supply of a computer programme.

References

- [1] Yocum D, Cuchens M, Clem LW. The hapten carrier effect in teleost fish. *Journal of Immunology* 1975;114:925–7.
- [2] Rombout JWHM, Blok LJ, Lamers CHJ, Egberts E. Immunization of carp (*Cyprinus carpio*) with a *Vibrio anguillarum* bacterin: indications for a common mucosal immune system. *Developmental and Comparative Immunology* 1986;10:341–51.
- [3] Espelid S, Hjelmeland K, Jørgensen T. The specificity of Atlantic salmon antibodies made against the fish pathogen *Vibrio salmonicida*, establishing the surface protein VS-P1 as the dominant antigen. *Developmental and Comparative Immunology* 1987;11:529–37.
- [4] Pilström L, Petersson A. Isolation and partial characterization of immunoglobulin from cod (*Gadus morhua* L.). *Developmental and Comparative Immunology* 1991;15:143–52.
- [5] Bjørgan Schröder M, Espelid S, Jørgensen TØ. Two ser-

- otypes of *Vibrio salmonicida* isolated from diseased cod (*Gadus morhua* L.); virulence, immunological studies and vaccination experiments. *Fish & Shellfish Immunology* 1992;2:211–21.
- [6] Israelsson O, Petersson A, Bengtén E, Wiersma EJ, Andersson J, Gezelius G, Pilström L. Immunoglobulin concentration in Atlantic cod, *Gadus morhua* L., serum and cross-reactivity between anti-cod-antibodies and immunoglobulins from other species. *Journal of Fish Biology* 1991;39:265–78.
- [7] Warr GW. The immunoglobulin genes in fish. *Developmental and Comparative Immunology* 1995;19:1–2.
- [8] Pilström L, Bengtén E. Immunoglobulin in fish—genes, expression and structure. *Fish & Shellfish Immunology* 1996;6:243–62.
- [9] Bengtén E, Strömberg S, Pilström L. Immunoglobulin V_H regions in Atlantic cod (*Gadus morhua* L.): their diversity and relationship to V_H families from other species. *Developmental and Comparative Immunology* 1994;18:109–22.
- [10] Ghaffari SH, Lobb CJ. Structure and genomic organization of immunoglobulin light chain in the channel catfish. An unusual genomic organizational pattern of segmental genes. *Journal of Immunology* 1993;151:6900–12.
- [11] Ghaffari SH, Lobb CJ. Structure and genomic organization of a second class of immunoglobulin light chain genes in the channel catfish. *Journal of Immunology* 1997;159:250–8.
- [12] Daggfeldt A, Bengtén E, Pilström L. A cluster type organization of the loci of the immunoglobulin light chain in Atlantic cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) indicated by nucleotide sequences of cDNA and hybridization analysis. *Immunogenetics* 1993;38:199–209.
- [13] Partula S, Schwager J, Timmusk S, Pilström L, Charlemagne J. A second immunoglobulin isotype in the rainbow trout. *Immunogenetics* 1996;45:44–51.
- [14] Pilström L. Immunoglobulins. In: Immunology of Fishes. Pastoret P-P, Griebel P, Bazin H, Govaerts A, editors. *Handbook of vertebrate immunology*, vol.2. London: Academic Press, 1998. p. 15–23.
- [15] Bengtén E. The immunoglobulin genes in Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*). In: *Comprehensive summaries of Uppsala Dissertations from the Faculty of Science and Technology*, vol. 19. Uppsala University, 1994.
- [16] Shambloott MJ, Litman GW. Genomic organization and sequences of immunoglobulin light chain genes in a primitive vertebrate suggest coevolution of immunoglobulin gene organization. *EMBO Journal* 1989;8:3733–9.
- [17] Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979;18:5294–9.
- [18] Feinberg P, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* 1983;132:6–13.
- [19] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Science USA* 1977;74:5463–7.
- [20] Kabat EA, Wu TT. Attempts to locate complementarity-determining residues in the variable positions of light and heavy chains. *Annals of NY Academy of Science* 1971;190:382–93.
- [21] Litwin S, Jores R. Shannon information as a measure of amino acid diversity. In: Perleson AS, Weisbuch G, editors. *Theoretical and experimental insights into immunology*. Berlin/Heidelberg: Springer Verlag, 1992. p. 279–87.
- [22] Stewart JJ, Lee CY, Ibrahim S, Watts P, Schlomchik M, Weigert M, Litwin S. A Shannon entropy analysis of immunoglobulin and T cell receptor. *Molecular Immunology* 1997;34:1067–82.
- [23] Rast JP, Anderson MK, Ota T, Litman RT, Margittai M, Shambloott MJ, Litman GW. Immunoglobulin light chain class multiplicity and alternative organizational forms in early vertebrate phylogeny. *Immunogenetics* 1994;40:83–99.
- [24] Anderson MK, Shambloott MJ, Litman RT, Litman GW. Generation of immunoglobulin light chain chain gene diversity in *Raja erinacea* is not associated with somatic rearrangement, an exception to a central paradigm of B cell immunity. *Journal of Experimental Medicine* 1995;182:109–19.
- [25] Schwager J, Bürckert N, Schwager M, Wilson M. Evolution of immunoglobulin light chain genes: analysis of *Xenopus* IgL isotypes and their contribution to antibody diversity. *EMBO Journal* 1991;10:505–11.
- [26] Stewart SE, Du Pasquier L, Steiner LA. Diversity of expressed V and J regions of immunoglobulin light chains in *Xenopus laevis*. *European Journal of Immunology* 1993;23:1980–6.
- [27] Haire RN, Ota T, Rast JP, Litman RT, Chan FY, Zon LI, Litman GW. A third light chain gene isotype in *Xenopus laevis* consists of six distinct V_L families and is related to mammalian λ genes. *Journal of Immunology* 1996;157:1544–50.
- [28] Hsu E, Steiner LA. Primary structure of immunoglobulin through evolution. *Current Opinion on Structural Biology* 1992;2:422–31.
- [29] Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest. U.S. Department of Health and Human Services, Public Health Service, Bethesda, MD: National Institute of Health, 1991.
- [30] Pilström L, Lundqvist ML, Wermestam NE. The immunoglobulin light chain in poikilothermic vertebrates. *Immunological Reviews* 1998;166:123–32.
- [31] Shambloott MJ, Litman GW. Complete nucleotide sequence of primitive vertebrate immunoglobulin light chain genes. *Proceedings of the National Academy of Science USA* 1989;86:4684–8.

- [32] Zachau HG. Immunoglobulin light-chain genes of the κ type in man and mouse. In: Honjo T, Alt FW, Rabbitts TH, editors. Immunoglobulin genes. London: Academic Press Ltd, 1989. p. 91–109.
- [33] Casali P, Schettino EW. Structure and function of natural antibodies. *Current Topics in Microbiology and Immunology* 1996;210:167–79.
- [34] Wilson M, Bengtén E, Miller NW, Clem LW, Du Pasquier L, Warr GW. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. *Proceedings of the National Academy of Science USA* 1997;94:4593–7.
- [35] Haury M, Sundblad A, Grandien A, Barreau C, Coutinho A, Nobrega A. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. *European Journal of Immunology* 1997;27:1557–63.
- [36] Mouthon L, Lacroix-Desmazes S, Nobrega A, Barreau C, Coutinho A, Kazatchkine MD. The self-reactive antibody repertoire of normal human serum IgM is acquired in early childhood and remains conserved throughout life. *Scandinavian Journal of Immunology* 1996;44:243–51.