The analysis of expressed genes in kidney of Japanese flounder, *Paralichthys olivaceus*, injected with immunostimulant peptidoglycan

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Abstract

Immunostimulants are widely being used in aquaculture. But there are few reports that genes expressed by their stimulation. Therefore, in this study, we analyzed expressed genes in kidney of Japanese flounder *Paralichthys olivaceus* injected with immunostimulant peptidoglycan.

The results of single-pass sequencing of ESTs from 198 clones (AU090255-AU090451, AU090935) from kidney cDNA are presented. Sequences of the cDNA clones were compared with sequences in the GenBank database. One hundred and six clones (53.5 %) appeared to be completely unknown and are likely to represent newly described genes, whereas 92 clones (46.5%) were identified based on matches to sequences in the database. The results contain the genes such as alpha globin (AU090287), several ribosomal proteins (AU090-263, 274, 299, 351, 365, 375, 377, 382, 434, 445), heat shock protein 90 (AU090374) and cytochrome oxidase subunit (AU090385). Immune related cDNAs identified from the kidney were immunoglobulin heavy (AU090291) and light chain (AU090352), β_2-microglobulin (AU090280), macrophage inflammatory protein 1-α precursor (AU090535), thymosin β-10 (AU090391), lysozyme (AU090322) and MHC class II α (AU090435). We thought that macrophage inflammatory protein 1-α of those was activating macrophage.

I. Introduction

Immunostimulants can increase resistance to infectious diseases, not by promoting the specific immune responses, but by enhancing non-specific defense mechanisms. Use of immunostimulants is an effective means of increasing the immunocompetency and disease resistance in fish.

Research on fish immunostimulants such as lactoferrin, glucans, chitin has recently been initiated and many agents are currently in use in aquaculture industry. Peptidoglycan(PG) is an excellent immunostimulant and is widely used in Japan. The immunostimulant effects of PG have already been reported by Matsuo et al. (1995), Boonyaratpalin et al. (1995) and Itami et al. (1996). Itami and Miyazono (1993) reported that PG stimulates phagocytosis in leucocytes of yellowtail.

Usually, fish treated with immunostimulants show enhanced phagocytosis (Sakai, 1999). Immunostimulants also enhance the mitogen activities of lymphocytes induced by concanavalin A or lipopolysaccharides and produce macrophage activating factors (Hardie et al., 1991; Siwicki et al., 1996). There are reports that carp injected sodium alginate and acleroglucan expressed interleukin-1 β , CC-chemokine, allograft inflammatory factor-1, and natural killer cell enhancing factor (Fujiki et al., 1999, 2000). Expressed sequenced tags (ESTs), which survey sequences contained in cDNA libraries, has been shown to be a powerful tool

in identifying the homologues of reported genes and the gene expression in tissues or cells (Adams et al., 1995; Okubo et al., 1997). In fish, the approach using EST has already been reported in Japanese flounder, *Paralichthys olivaceus* (Inoue el al., 1997; Nam et al., 1999; Aoki, et al., 1999), and rainbow trout (Dixon et al., 1998).

In this study, the sequences of immune related genes expressed in the kidney of Japanese flounder injected with PG are reported.

II. Materials and Methods

Fish and immunostimulants

A total of 20 flounder (mean weight 120 g) were maintained in outdoor tanks with running fresh water at 16 ℃ for two weeks and fed commercial diets twice a day.

Peptidoglycan (PG) was kindly donated by Kyowa Hakkou Co.Op and KagakuShiryou Kenkyujyo Co.Op. PG was suspended in physiological saline and used for administration.

Administration of PG

Flounder was anaesthetised with 0.02 % MS-222 and 1 mg PG was injected for each fish intraperitoneally (i.p.). Five days post injection, five fish of each group were sampled for the RNA isolation and nitroblue

tetrazolium (NBT) assay. Control fish were injected with equal dosage of bovine serum albumin (BSA, Sigma, USA) in saline.

Of the fish treated with 1 mg of PG, five were killed 5 days post injection.

Macrophage isolation

The head kidney phagocytic cells of flounder were isolated according to the modified method described by Braun-Nesje et al. (1982). The cells were isolated from the fish and pushed through a nylon mesh with RPMI 1640 medium (Nissui, Japan) containing 1 % streptomycin/penicillin (S/P, Gibco, USA), 0.2 % heparin (Sigma, USA) and 10 % flounder serum (FS). Viable phagocytic cells, including neutrophils (about 10 %) and macrophages (about 90 %), were counted by trypan blue exclusion. Five individual fish were used in this experiment.

Detection of superoxide anion in phagocytic cells

The superoxide anion from phagocytic cells was determined by the reduction of NBT as described by Sakai et al. (1996). The viable cells were adjusted to 10^7 cells ml^{-1} in Hank's balanced salt solution (HBSS) (Nissui, Japan), and 100 μl of this suspension was added to wells of microtitre plates (Nunc, USA). After incubation at 18 ℃ for 2 h, unattached cells were washed off with HBSS and monolayers were fed with 1ml RPMI 1640

supplemented with 10 % fetal bovine serum (FBS), 1 % P/S and incubated at 18 ℃ overnight. The cell number in the monolayer was about 2.5×10^5 cell ml^{-1}. The phagocytic cell monolayer was washed 2 times with HBSS, after hormone treatment, and then 100 ml of NBT solution (1 mg ml^{-1} in RPMI 1640 medium) and 1 ng ml^{-1} phorbol myristate acetate (PMA, Sigma) was added to each well and incubated at 20 ℃ for 60 min. The NBT reduction was stopped by the addition of methanol, after the removal of the medium from the cells. NBT solution containing PMA without cells was used as blank. The formazan in each well was dissolved in 120 μl of 2 M KOH and 140 μl of DMSO, and the optical density was measured by a multiscan spectrophotometer (Phamacia, Sweden) at 620 nm. Triplicate wells were used for each variable investigated.

Construction of cDNA libraries

Five days post PG injection, the anterior kidney pooled from sampled fish was used for NBT assay and for the construction of cDNA library.

The mRNAs were isolated from the kidney by using micro mRNA purification kit (Pharmacia, Sweden). cDNA synthesis was performed using a cDNA synthesis kit (Pharmacia) with an oligo (dT) primer. The cDNA library was constructed in λZAP II vectors according to the manufacturer's instructions (Stratagene, U. S. A.).

Determination of DNA sequencing

Conversion of the recombinant λZAPIIs into pBluescript plasmids was carried out by *in vivo* excision according to the manufacturer's protocol (Stratagene, U.S.A.). Recombinant plasmid DNA was isolated by the alkaline lysis method (Sambrook et al., 1989).

cDNA clones were sequenced using ThermoSequenase (Amersham, U.K.) with M13 forward or M13 reverse primers or both and an automated DNA sequencer LIC-4200L (Li-Cor, U.S.A.).

Data analysis

Sequence data were compared with GenBank (Re. 97.0) including dbEST for identification using the BLAST 2 (Blue 1) program (blast@genome.ad.jp) (Altschul et al., 1990).

Ⅲ. Results

The production of superoxide anion in kidney leucocytes of Japanese flounder injected with 1 mg of PG is shown in Fig. 1. The production of superoxide anion in kidney cells was significantly increased by the treatment of PG ($P < 0.05$).

In this study, 198 cDNA clones isolated from a cDNA library of kidney

cells of Japanese flounder injected with PG were partially sequenced. Of the 198 clone sequences, 92 demonstrated a significant similarity to previously reported genes according to the blastn and blastx programs. Sixty seven clones were similar to the EST of Japanese flounder reported previously. A total of 106 distinct genes did not match the database.

A summary of the identified genes is shown in Table 1. Among all identified sequenced clones, 40 were represented only once and 13 were present more than once. The most frequently identified clones were α-globin (n =17) (AU090287). Five different genes involved in cell structure and motility were identified, like α-tubulin (AU090310) or Kinesin light chain (AU090275). Similar genes were found in Japanease flounder and are registered in Gene Bank. A single gene activated protein kinase C receptor (AU090290) involved in cell signalling and cell communication was cloned, total 29 genes were cloned for gene and protein expression. Four genes such as ribosomal protein S21 (AU090294) or L9 (AU090365) were newly identified in this study.

For genes related to cell/organ defence, β_2-microglobin (AU090280), MHC class II α (AU090435), heat shock protein 90β (AU090374), hepatic lectin (AU090439), immunoglobulin heavy (AU090291) and light chains (AU090352), lysozyme (AU090322), CC-chemokine (AU090535) and thymosin β-10 (AU090391) were identified. Aoki et al. (1999) and Nam et al. (1999) already reported the β_2-microglobin, MHC class II α, heat shock protein

90β, lysozyme and thymosin β-10 genes from Japanese flounder. At the DNA sequence level the genes reported here are more than 95 % identical to the previously reported study on Japanese flounder (Inoue et al., 1997; Hikima et al., 1997; Nam et al., 1999). However, hepatic lectin, immunoglobulin heavy and light chains, and CC-chemokine reported in this study have not been reported in Japanese flounder. A comparison of the deduced amino acid sequence of Japanese flounder immunoglobulin light chain constant region with previously described sequences of immunoglobulin light chain constant (CL) region from other vertebrates is shown in Fig. 2. The deduced amino acid sequence of Japanese flounder immunoglobulin light chain constant region had 31.5 %, identity with that of carp, 31.2 % identity with that of catfish F, 31.2 % identity with that of catfish G, 31.5 % identity with that of trout IgL1 and 48.7 % identity with that of trout IgL2.

Fig. 3 compares the amino acid sequences of immunoglobulin heavy chain CH 4 region from Japanese flounder and other fish. The amino acid sequences of immunoglobulin heavy chain CH 4 region of Japanese flounder revealed the highest identity with that of rainbow trout (47.8 %), and 42.3, 40.7, 38.5 and 37.1 (%) identities with those of the carp, catfish, lady fish and cod, respectively.

Fig. 4 compares the amino acid sequences of CC-chemokine from Japanese flounder and other vertebrates. The amino acid sequences of CC-

chemokine of Japanese flounder revealed the highest identity with that those of human eotaxin (37.11%), and 36.7, 36.0, 34.5, 34.4, 33.3, 33.0, 32.6, 30.9, 29.8 and 29.4 (%) identities with those of the human MCP-1, human MIP-alpha, mouse MIP1-β, mouse MIP1-α, chicken MIP, mouse eotaxin, human MIP-beta, mouse RANTES, human RANTES and rainbow trout CK1, respectively.

IV. Discussion

Macrophage of fish treated with immunostimulant is activated, and the fish resistance against disease increased. (Sakai, 1999) In the NBT assay carried out in this study, the production of superoxide anion increased significantly , in kidney cells of fish injected with PG than in control fish. Japanese flounder injected with PG were used for EST analysis and expected the expression of biodefence related genes due to the immunostimulant treatment. We couldn't identify the genes stimulating macrophage such as interleukin 1β, macrophage activating factor and TNF but nine kinds of biodefence related genes were identified.

One of those, IgH gene constant region has been already reported in

carp (Nakao et al., 1998), Atlantic salmon (Hordvic et al., 1992), Atlantic cod (Bengten et al., 1991), channel catfish (Ghaffari & Lobb, 1989) and ladyfish (Amemiya & Litman, 1990). In this study, IgH had not full length but CH4 domain was preserved. Comparison of their primary structures revealed that the Japanese flounder IgH was closest to the Atlantic salmon IgH, as in other teleosts.

The CL region contains the characteristic three cysteines (Cys-28, Cys-85, Cys-103), which are likely to form the intradomain disulphide bridge (Cys-28, Cys-85) or the interchain disulphide linkage (Cys-103). It has been reported that both channel catfish (Ghaffari & Lobb, 1997) and rainbow trout (Partula et al.,1996) had two isotypes of IgL chains, designated as F and G, and as IgL1 and IgL2, respectively. It has also been reported in carp (Tomana et al., 1999). The amino acid sequence comparison showed that the the Japanese flounder (clone M-134) CL chain was closest to the rainbow trout IgL2 isotype. In this study, although only one isotype of IgL chain could be identified, it is possible that another isotype is present as in other teleosts.

Fish treated with immunostimulants are likely to have activated macrophages and lymphocytes. However, in this study, only a CC-chemokine gene was identified and was to related the activation of macrophages or lymphocytes. Chemokine genes family are well characterised in mammals, but in fish they have been identified only in carp (Fujiki et al., 1999) and

rainbow trout (Dixon et al., 1998). We have isolated a cDNA clone from Japanese flounder which encodes a protein having structural features typical of CC-chemokine. There are two types of CC-chemokine. One has four conserved cysteins and the other has six conserved cysteins in sequence, except in signal sequence. Isolated cDNA clone was type conserved with four cysteins. Rainbow trout chemokine gene was type conserved with six cysteins (Dixon et al., 1998), and was different from isolated cDNA clone. But carp chemokine was type conserved with four cysteins, and was closest to rainbow trout CK1. Isolated cDNA clone was closest to human eotaxin, which is different from carp chemokine. Thus, isolated cDNA clone was CC-chemokine of a new type in teleost fish.

There is other report that the analysis of expressed genes in carp injected with sodium alginate and scleroglucan by suppression subtractive hybridization (Fujiki et al., 1999, 2000). They analyzed immune-related genes that interleukin-1 β, serum amyloid A, monocyte chemotactic protein-2 and CXC chemokine receptors. We used PG defferent from sodium alginate and scleroglucan. We analyzed immune-related genes that heat shock protein 90 β, hepatic lectin, immunoglobulin light & heavy chain, MHC class II α, lysozyme, and thymosin β-10. We are of the opinion that the difference in expression of genes by injected with immunostimulants depends on the kind of immunostimulants and the time of sampling. In the present study, mRNA was isolated, only 5 days post injection of peptidoglycan.

Considering the expression of cytokines, which are immediate and for a short duration, standardisation of the timing for sampling needs to be performed.

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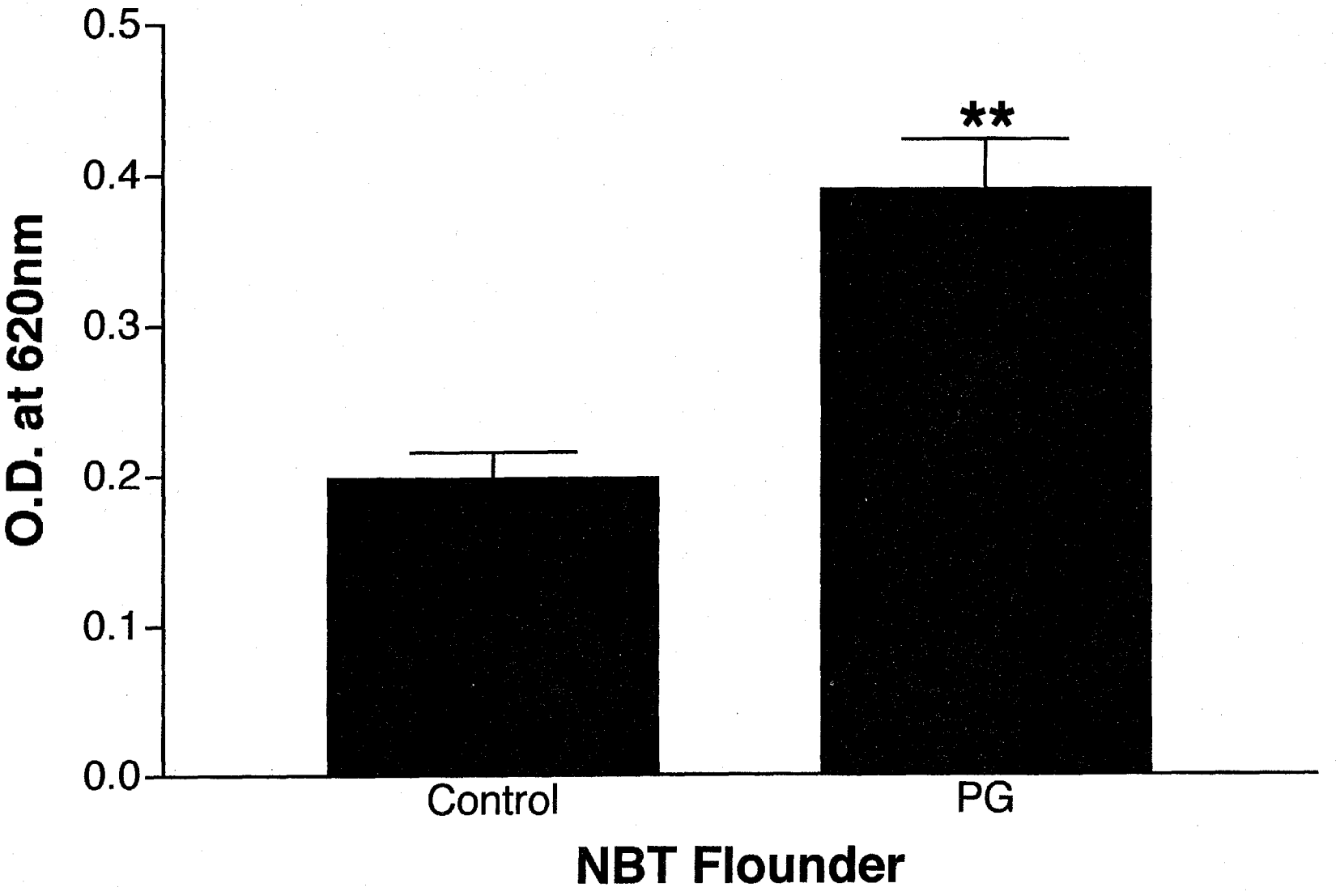
0.5
0.4
0.3
0.2
0.1
0.0
O.D. at 620nm
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Control
PG
NBT Flounder

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M-134          1:GSSLSPPVLTVFPPSSAELR-SNKATLLCLSSQSVP-FAEVTWLVGGSPVSSGISTSTAS 58

Catfish F      1:TGPTVKPSVSLLPPSSLQLS-EGSASLLCLLSAYSPQGALVSWTVDGSEVKDGVLTSAEE 59
Catfish G      1:--RLTQPSVTVLPPSSVELQ-QEKVTLVCVAYKGFPSDWRLSWKVDGSSWSSGESRSSAV 57
Cod            1:--GVVQPTLSVLPPSRVELE-QGSATLLCVASGGFPSDWKLGWKVGGSSRSGGVSDSLGV 57
Horse kappa    1:--DDAKPSAFIFPPSSEELS-SGSASVVCLVYGFYPSGATINWKVDGLAKTSSFHSSLTE 57
Horse ramda    1:--PTSAPSVSLFPPSSEELS-ANKATVVCLISDFSPSDLTVSWKVNGAAISQGVQTTKPS 57
Human kappa    1:--ADAAPTVSIFPPSSEQLT-SGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTD 57
Human ramda    1:--PKANPTVTLFPPSSEELQ-ANKATLVCLISDFYPGAVTVAWKADGSPVKAGVETTKPS 57
Mouse kappa    1:--ADVAPTVSIFPPSSEQLT-SGGASVVCFMNNFYPRDINVKWKIDGSERQGGVLNSWTD 57
Mouse ramda    1:--PKSSPSVTLFPPSSEELE-TNKATLVCTITDFYPGVVTVDWKVDGTPVTQGMETTQPS 57
Sheep kappa    1:--SDAQPSVFLFKPSEEQLR-TGTVSVVCLVNDFYPKDINVKVKVDGVTQNSNFQNSFTD 57
Sheep ramda    1:--PKSAPSVTLFPPSKEELD-TNKATVVCLISDFYPGSVNVVWKADGSIINQNVKTTQAS 57
Trout IgL1     1:--SNSAPTLTVLPPSSEELSSTTTATLMCLANKGFPSDWTLSWKVDGTSQK--QETSSTV 56
Trout IgL2     1:DSTLPPPVLTILPPSSDELKSS-KVTLVCLASQMAMGYADVSWTAGGTPVTGGIATSGPV 59
Carp IgL       1:--SVTRPKVSVLPPSSAEISSKKTATLMCVASEGFPSDWSLSWKVDGSSRSQ--ESSAGL 56
                        *     **          *                *

Catfish F     60:RKK-DG-YTHSSTLTLSKALWEKGEEFVCKVSHDN--VDHPVTF-RKSQCEV-       106
Catfish G     58:LQA-DGLYSWSSTLSLHPEQWRNK-VVTCEASKDNQ-PPVVSTVNTEQC----       103
Cod           58:LGK-DGHYSWSSTLTLPADQWRKAGSVSCEASKNGQTQPVTQTLNPGECSE--       107
Horse kappa   58:QDSKDNTYSLSSTLTLPKADYEAHNVYACEVSHKTLSSPLVKSFNREDC----       106
Horse ramda   58:KQS-NGKYAASSYLTLTPAQWKSSSSVSCQVTHQG--KTVEKKLSPSECS---       104
Human kappa   58:QDSKDSTYSMSSTLTLTEDEYERHNSYTCEATHKTSTSPIVKSFNRNEC----       106
Human ramda   58:KQS-NNKYAASSYLSLTPEQWKSHRSYSCQVTHEG--STVEKTVAPTECS---       104
Mouse kappa   58:QDSKDSTYSMSSTLTLTKAEYEQHNSYTCEATHRTSASPIVKSFNRNEC-----       106
Mouse ramda   58:KQS-NNKYMASSYLTLTARAWERHSSYSCQVTHEG--HTVEKSLSRADCS---       104
Sheep kappa   58:QDSKKSTYSLSSTLTLSSSEYQSHNAYACEVSHKSLPTALVKSFNKNEC----       106
Sheep ramda   58:KQS-NSKYAASSYLTLTGSEWKSKSSYTCEVTHEG--STVTKTVKPSECS---       104
Trout IgL1    57:LEK-DGLYSWSSTLTLTTQEWTKAGEVTCEAQQKSQ-TPVTKTLRRADCSG--       105
Trout IgL2    60:PQA-DKTFQLSSCLTVDTSEWNQDKVFSCKVT-VGS-KFAEKDIKKSECSTE-       108
M-134         59:TDRTRLPNKQLSGL-QT-SDWNVDKIYTCKVSH-GSQTS-EKNINKSVCTTEE       107
Carp IgL      57:LEK-DGLYSWSSSLTLSEQEWMESVSVSCEATRSGQ-PALTGHVTRQQCSE--       105
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M56          1:TGVQTQRPSVFMMPPVEHVKKDTVTLTCYVKDFSPPEVFVSWLVDDE-------YPSGYKF 54
Catfish      1:NGN-PEFPKVYLLAPPESS-GESVTLTCYVKDFYPKEVAVSWLVNDK---QVEEVVGYEQ 55
Cod          1:NGRNRVPPSVYLLPPVDDLSCTNMTLTCFVKDFYPADILVHWLVDN---LTIDGNALYSH 57
Cpl          1:NGKKPKKPTVFVLAPPEHKKGEPMTLTCYVKDFYPKEVFVSWLADD--------EPVTSKY 53
Lady fish    1:CGGKWQSPTVFILAPAEQRNLSTVTLICYAKDFYPEQVLISWLVDD--------QPVETDV 53
Salmon       1:TGGDPQRPSVFLLAPAEKTSDNTVTLTCYVKDFYPKEVLVAWLIDDEPVERTSSSALYQF 60
                *     * *    *          ** *  *** *      **

M56         55:NTTNPIESNGSYSAYGQLSLDLEQWKKEGVMYSCVVYPQSVVN-NTKAIVRSIGPKTFET 113
Catfish     56:NTTAVIDRNNLFSVYSQLIIKTADWNSG-SVFSCLVYHESIKD-CVRHISRSIAKDSK-T 112
Cod         58:KTTNVIENGDLFSTYGQLTFSSDGWKDG-RVFRCEVYHMSMDS-KNQPIVKLITEKSSGN 115
Cpl         54:STSLPIQKDQTFSVYSQLTVNDSKWTNG-TVFSCVVYHEAIDE-KMRVLTRSITDNIE-K 110
Lady fish   54:PTTEVVKTEGTYSVFSQLTIPASDWDSG-VVYSCAVHHETVMESVVKTIVRTTDSVSK-K 111
Salmon      61:NTTSQIQTGRTYSVYSQLTFSNDLWKNKEVVYSCVVYHESMIK-STKILMRTIDRTSN-Q 118
                *          *   **      *        * *

M56        114:TNLVNLNMNIP--CKAQ--                                          128
Catfish    113:PTLVNLTLTNPQSCSCSTY                                          131
Cod        116:VNIINMNLG-PSTCLPQ--                                          131
Cpl        111:AGVINLSMNTPAFCK-P--                                          126
Lady fish  112:PTTVSLDLNVPQTCKV---                                          127
Salmon     119:PYLVNLSLNVPQSCRAQ--                                          135
                     * *
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M-160                1:      MRPLQVLLLCILGAALLAPVLCNNA....LGPDDCCFNFYSRRVKKTLKS.Y..Y  48

Carp                 1:..METR-I-MRS-AVAVVI-SVIWTTTA...ADTAVVYS--TKVTTAE-TDPILNIRL.Q  54
Rainbow Trout CK1    1:MISCRVCV-AA-FSLLIITLIPTTQSA..........---LK-TR-P-HCRWLKG-TFQ  49
Human eotaxin        1:.....-KVSAA--WLL-I--AFS-QGLA...GPASV-TT----LAN-KIPLQRLES-R.R  51
Mouse eotaxin        1:.....-QSSTA--FLL-TVTSFTSQVLA...HPGSI-TS---IMT-KKIPN--LKS-K.R  51
Chicken MIP          1:.....-KVSVAA-AVL-..IAICYQTSA.-PVGSDP-TS---TYI--QLPFSFVAD-..-  50
Human MIP-alpha      1:.....-QVSTAA-AVL-CTMA-CNQFSA..SLAADT-TA---SYT--QIPQNFIAD-..F  51
Human MIP-beta       1:.....-KLCVTV-SLLMLV-AFCSPALS.-PMGSDP-TA---SYTA-KLPRNFVVD-..-  52
Mouse MIP1 alpha     1:.....-KVSTTA-AVL-CTMT-CNQVFS.-PYGADT-TA---SYS.-KIPRQFIVD-..F  51
Mouse MIP1 beta      1:.....-KLCVSA-SLL-LV-AFCAPGFS.-PMGSDP-TS---SYT--QLHRSFVMD-..-  52
Human RANTES         1:.....-KVSAAA-AV--I-TA-CAPASA.SPYSSDT.TP---AYIA-PLPRAHIKE-..F  51
Mouse RANTES         1:.....-KISAAA-TI--T--A-CTPAPA.SPYGSDT.TP---AYL-LELPRAHVKE-..F  51
                                                              **

M-160               49:MTDSRCSKMGVILVTQKSHHICADPDVQWVQGIMKFLDEKNF                  90

Carp                55:RESLP-V-.A--FK-KQG.EF-S--KLR--KEKV-QFFK-TPAASSISD           101
Rainbow Trout CK1   50:DIT-S-DLNA--FQNLRNKFV-----SQD-TKRVQRC-RKRQEKKSQLKKRV       100
Human eotaxin       52:I-SGK-PQKA--FK-KLAKD------KKK---DS--Y--QRSPTPKP            97
Mouse eotaxin       52:I-NN--TLKAIVFK-RLGKE------KKK---DAT-H--QRLQTPKP            97
Chicken MIP         51:E-N-Q-PHA--VFI-R-GRFV--N-END---DY-NK-FLN                    90
Human MIP-alpha     52:E-S-Q---P---FL-KR-RQV-----SEE---KYVSD-FLSA                  92
Human MIP-beta      53:E-S-L--QPA-VFQ-KR-KQV-----SES---EYVYD-FLN                   92
Mouse MIP1 alpha    52:E-S-L--QP---FL-KRNRQ-----SKET---EYITD-ELNA                  92
Mouse MIP1 beta     53:E-S-L---PA-VFL-KRGRQ---N-SEP--TEY-SD-ELN                    92
Human RANTES        52:Y-SGK--NPA-VF--R-NRQV--N-EKK--REYINS-FMS                    91
Mouse RANTES        52:Y-S-K--NLA-VF--RRNRQV--N-EKK---EYINY-EMS                    91
                           *                *      *
```

Legend of Figure

Fig. 1. The production of superoxide anion in kidney leucocytes of Japanese flounder injected with 1mg peptidoglycan / fish. Samples were collected 5 days after peptidoglycan injection and five fish were examined. ** $P < 0.05$

Fig. 2. Alignment of amino acid sequence of M134 with IgL of different vertebrate. Dashes(-) indicate identify with clone M134 included in this alignment, while dots(.) indicate deletion. The asterisks(*) indicate the 8 amino acids which are conserved in IgL CL region.

Fig. 3. Alignment of amino acid sequence of M56 with IgH of different vertebrate. Dashes(-) indicate identify with clone M56 included in this alignment, while dots(.) indicate deletion. The asterisks(*) indicate the 22 amino acids which are conserved in IgH CH4 domain.

Fig. 4. Alignment of amino acid sequence of M160 with CC chemokine of different vertebrate. Dashes(-) indicate identify with clone M160 included in this alignment, while dots(.) indicate deletion. The asterisks(*) indicate the 9 amino acids which are conserved in CC-chemokine.