A Functional C5a Anaphylatoxin Receptor in a Teleost Species[1]

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The anaphylatoxins are potent, complement-derived low m.w. proteins that bind to specific seven-transmembrane receptors to elicit and amplify a variety of inflammatory reactions. C5a is the most potent of these phlogistic peptides and is a strong chemoattractant for neutrophils and macrophages/monocytes. Although lower vertebrates possess complement systems that are believed to function similarly to those of mammals, anaphylatoxin receptors have not previously been characterized in any nonmammalian vertebrate. To study the functions of C5a in teleost fish, we generated recombinant C5a of the rainbow trout, *Oncorhynchus mykiss* (tC5a), and used fluoresceinated tC5a (tC5aF) and flow cytometry to identify the C5a receptor (C5aR) on trout leukocytes. Granulocytes/Macrophages present in cell suspensions of the head kidney (HKL), the main hemopoietic organ in teleosts, showed a univariate type of receptor expression, whereas those from the peripheral blood demonstrated either a low or high level of expression. The binding of tC5aF was inhibited by excess amounts of unlabeled tC5a or tC5a^{desArg}, demonstrating that sites other than the C-terminal of tC5a interact with the C5aR. Both tC5a and tC5a^{desArg} were able to induce chemotactic responses in granulocytes in a concentration-dependent manner, but the desArg derivative was at least 10-fold less active. Homologous desensitization occurred after HKL were exposed to continuous or high concentrations of tC5a, with a loss of tC5aF binding and an 80% reduction in chemotactic responses toward tC5a. Pertussis toxin reduced the migration of HKL toward tC5a by 40%, suggesting only a partial involvement of pertussis toxin-sensitive G_i proteins in tC5a-mediated chemotaxis. ***The Journal of Immunology,*** **2004, 172: 349–355.**

The anaphylatoxins C3a, C4a, and C5a are important mediators of inflammation (1, 2). They are low molecular mass cleavage fragments (~10 kDa) of the α-chain of the complement components C3, C4, and C5, respectively, that are liberated during complement activation. They regulate the inflammatory process by increasing vascular permeability, stimulating smooth muscle contractions, and inducing the release of histamine from mast cells and secretory granules from granulocytes and macrophages. In addition, C5a, one of the most potent phlogistic peptides, is a powerful chemoattractant for neutrophils and other leukocytes. No chemoattracting properties have yet been attributed to C4a, while those of C3a appear to target primarily eosinophils, mast cells, and hemopoietic stem cells (2, 3).

To date in humans, three types of seven-membrane-spanning receptors have been identified that mediate the actions of the anaphylatoxins: the C3aR, which binds C3a specifically (4); the C5aR (CD88), which binds C5a (5); and the C5L2, for which all three anaphylatoxins may be ligands (6, 7). The first two receptors couple to regulatory G proteins (8, 9), whereas the C5L2 receptor has been shown to couple poorly to G protein-mediated signaling pathways (6, 10). The distribution of these receptors is not restricted to leukocytes; they are also expressed on many types of nonmyeloid cells, including hepatocytes, lung epithelial cells, endothelial cells, brain astrocytes, and microglial cells, where they may mediate the involvement of the anaphylatoxins in several vascular, pulmonary, regenerative, and degenerative neurological conditions (11–15). If not bound to their receptors, the anaphylatoxins are rapidly digested by plasma carboxypeptidases, which remove the C-terminal arginine residue from each peptide (16). The desArg derivatives are either inactive or have 10- to 1000-fold lower activities than those of the native peptides (2).

Lower vertebrates, such as reptiles, amphibians, and fishes, possess complement systems that are in many ways considered to be functionally similar to those of mammals (reviewed in Refs. 17–19). Complement-mediated immune protective responses, such as cytolysis and opsonization, have been demonstrated in some of these animals. In addition, the chemoattracting and leukocyte-activating properties of complement-activated fish serum are well documented (20, 21). However, information about complement receptors, which are believed to mediate many of these events, is sparse to nonexistent for poikilothermic vertebrates.

Recently, the C3a of the protochordate tunicate *Ciona intestinalis* has been shown to possess chemotactic activities for tunicate hemocytes, suggesting the presence of a C3aR in these animals (22). Protochordates may not possess C4a and C5a, because the classical complement activation pathway, which yields C4a, and the lytic pathway, which generates C5a, are believed to be absent in these animals (18, 23). Gnathostome fishes, however, possess all known complement activation pathways, and the C3, C4, and C5 molecules of various fish species have been identified. Interestingly, fish have multiple isoforms of several of the complement components, including C3, C2/Bf, C4 (reviewed in Refs. 17 and 19), and C5 (24). Although different functions for the C3 isoforms have been suggested (25), it remains to be established whether different receptors exist for these isoforms.

In the present study, we describe the presence of a specific and functional receptor for the C5a anaphylatoxin in a lower vertebrate species. Using ligand-binding studies and the rainbow trout (*Oncorhynchus mykiss*) as a model, we examined the binding of homologous fluoresceinated C5a (C5aF)[3] to leukocytes isolated

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[3] Abbreviations used in this paper: C5aF, fluoresceinated C5a; HK, head kidney; HKL, HK leukocyte; mC5a, mouse C5a; MF, mean fluorescence; PTx, pertussis toxin; tC5, trout C5; ZAS, zymosan-activated serum; PBLk, peripheral blood leukocyte.

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from the peripheral blood (PBLk) and head kidney (HKL), the main hemopoietic organ of teleost fishes. Unlike another teleost fish, the common carp (*Cyprinus carpio*), from which two different C5 isoforms have been cloned (24), the trout is thought to have only one C5 molecule (26). Through functional studies, we also demonstrated that some of the mammalian-type functions of C5a, such as the capacity to induce migration of neutrophils, are already established early in evolution.

Materials and Methods

Reagents, plasmids, and strains

All materials were obtained from Sigma-Aldrich (St. Louis, MO), except where otherwise indicated. Percoll was purchased from Amersham Biosciences (Piscataway, NJ), FITC (Isomer I) from Molecular Probes (Eugene, OR), restriction enzymes from New England Biolabs (Beverly, MA), and Leibovitz medium (L-15) from Life Technologies (Rockville, MD).

The pET-32a vector, Origami D(DE3) *Escherichia coli* host cells, and recombinant enterokinase were obtained from Novagen (Madison, WI). The pGem T-easy cloning vector and JM109 *E. coli* host cells were purchased from Promega (Madison, WI). The Qia-expressionist kit containing the pQE-30 vector and M15[pRep] *E. coli* host cells, and the Ni-NTA were obtained from Qiagen (Stutio, CA).

Expression and purification of trout C5a (tC5a)

The C5a fragment (77 aa) was amplified by PCR from a cloned tC5 fragment (26), using the primers 5′-GGATCCGCCCTCTCTTATGAC-3′ and 5′-AAGCTTTTAACGTGCCAAGACATG-3′. These primers added a *Bam*HI site at the 5′ end and a *Hin*dIII site at the 3′ end of the C5a sequence. The obtained fragment was cloned into *Bam*HI and *Hin*dIII of the expression vector pET-32a$^+$ (Novagen), and the sequence was verified. The pET-32a$^+$ vector added a cleavable 109-aa protein containing a Trx-Tag, a His-Tag, and a S-Tag to the C5a sequence. The C5a fusion protein was expressed and purified from Origami B(DE3) *E. coli* host cells, according to the manufacturer's instructions (Novagen). The frozen cell pellets were treated with lysozyme (1 mg/ml) in 0.05 M NaH_2PO_4 with 0.3 M NaCl and 0.01 M imidazole (pH 8.0), and the cleared lysate was mixed with Ni-NTA agarose (Qiagen) for 1 h at 4 C. The Ni-NTA was transferred to a disposable column and washed with 0.05 M NaH_2PO_4 containing 0.3 M NaCl and 0.02 M imidazole (pH 8.0). The fusion protein was eluted with the same buffer containing 0.25 M imidazole and dialyzed overnight against 0.02 M Tris-HCl with 0.05 M NaCl and 0.002 M $CaCl_2$ (pH 7.4). The N-terminal tag was cleaved from the fusion protein by incubating the protein with recombinant enterokinase (Novagen; 4 U/mg fusion protein) for 5 h at room temperature. Immediately after cleavage, C5a was separated from the contaminants using a reversed-phase Resource RPC 3-ml column (Amersham Biosciences), as previously described for recombinant human C5a (27). The purity and identity of recombinant tC5a were assessed by HPLC, SDS-PAGE, and mass spectrometry.

Recombinant mouse C5a (mC5a; 77 aa) was expressed using the pQE-30 vector and M15[pREP] *E. coli* host cells (Qiagen) and was purified under denaturing conditions, as previously described for human C5a and newt C5a (28, 29) (D. Mastellos and J. D. Lambris, unpublished results).

Generation of tC5a^{desArg}

The desArg derivative of tC5a was generated by incubating 200 μg tC5a with carboxypeptidase B in a ratio of 1:100 (w/w) for 1 h at room temperature. The tC5a^{desArg} was immediately separated from the enzyme by HPLC using a C4 column and a linear gradient of 0.1% trifluoroacetic acid and acetonitrile. The identity of the protein was analyzed by mass spectrometry.

Fluoresceination of tC5a

Aliquots of 200 μg tC5a in 200 μl of 0.5 M $NaHCO_3$, pH 8.0, were conjugated to FITC (Molecular Probes) in a 1:1 molar ratio by incubating the mixture at room temperature for 1 h under rotation. tC5aF was immediately separated from unconjugated protein and free FITC by HPLC using a C4 column (Waters, Milford, MA) and a linear gradient of 0.1% trifluoroacetic acid and acetonitrile. The HPLC system was equipped with both UV and fluorescence detectors, and the tC5aF-containing fractions were collected based on the absorbance at 214 nm (protein) and 495 nm (FITC). Fractions were frozen, lyophilized, and resuspended in 0.01 M NaH_2PO_4 with 0.15 M NaCl, pH 7.4 (PBS). The binding capacity of each fraction was tested by FACS using isolated trout leukocytes.

Fluoresceination of mC5a was conducted as described for tC5a, and the binding capacity of the mC5aF-containing HPLC fractions was tested by FACS using isolated mouse neutrophils (D. Mastellos, M. C. H. Holland, and J. D. Lambris, unpublished results).

Isolation of trout leukocytes

Rainbow trout were obtained from Clear Springs Foods (Buhl, ID) and were maintained in 180-gallon recirculating tanks until used for experimentation. All procedures were conducted in compliance with the regulations of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Fish (100–300 g) were anesthetized using 2-phenoxyethanol (0.25 ml/L) and bled from the caudal vein. Blood was diluted immediately (1/3) in ice-cold L-15 medium (Life Technologies) supplemented with 0.02 M sodium citrate (pH 8.0). The head kidney (HK) was removed and transferred to a sterile tube containing 5 ml ice-cold L-15 with 0.02 M sodium citrate. The tissue was forced through a 100-μm pore-size cell strainer (BD Falcon, Bedford, MA), and the cells were collected and washed twice with L-15 containing 0.02 M sodium citrate. The HK cells and the diluted blood were layered over a discontinuous 35–51% and 48–57% Percoll gradient, respectively, and centrifuged for 25 min at 400 $\times$ *g*. Cells above the 51% (for HKL) or 57% (PBLk) interphase were collected and washed three times with L-15. The cell pellets were resuspended in L-15 with 0.25% BSA, with or without 0.1% NaN_3 (see below), counted, and adjusted to 5×10^6 cells/ml. Cell viability was greater than 98%, as determined by trypan blue exclusion.

Flow cytometric analysis of FITC-conjugated tC5a binding

For the binding assays, HKL or PBLk were resuspended in L-15 containing 0.25% BSA and 0.1% NaN_3 (5×10^5 cells/ml). The cells (25 μl) were incubated with an equal volume of medium in the absence or presence of 4×10^{-6} M unlabeled ligand, at room temperature. After 10 min, 50 μl of 0.2×10^{-6} M tC5aF or mC5aF was added to each tube, and the cells were incubated in the dark for 25 min at room temperature. Cells were washed by adding 2 ml of PBS, followed by centrifugation at 500 $\times$ *g* for 5 min. The resuspended cells were analyzed on a FACScan flow cytometer (BD Biosciences). Using forward and sideward scattering characteristics as a means of identifying cell populations, the binding capacity of each cell population was determined separately by setting appropriate gates. Analyses were performed on 10,000 gated cells per sample. The increase in mean fluorescence (MF) was expressed as the MF of the tC5aF-binding cells divided by that of cells treated with the control protein (mC5aF).

Leukocyte chemotaxis assays

Leukocyte chemotaxis was measured using the transwell assay (6.5 mm diameter, 3 μm pore size; Costar, Cambridge, MA). Isolated HKL were resuspended at a concentration of 5×10^6 cells/ml in L-15 medium containing 0.25% BSA. The bottom chambers were filled with 600 μl medium containing various concentrations of tC5a or tC5a^{desArg} (1–100 $\times$ 10^{-9} M). Cells (100 μl) were added to the top chambers. Medium without chemotactic agent served as a negative control, while 100 μl cells plus 500 μl medium placed in the bottom well were used to obtain the total number of cells that could be recovered. After 2 h of incubation at 21°C, the bottom chambers were harvested and the transmigrated cells were counted by FACS. Dead cells and debris were excluded by gating. Cells were counted by recording the events for 60 s, and migration was expressed as a percentage of the total number of cells. All assays were performed in duplicate, and migration toward tC5a and tC5a^{desArg} was tested simultaneously.

The chemotactic response of leukocytes to zymosan-activated serum (ZAS) was also examined. Normal trout serum was incubated with activated zymosan for 30 min at 37°C, then centrifuged to remove the zymosan particles and frozen in aliquots. Preliminary studies showed that medium containing 1% ZAS gave an optimum leukocyte chemotactic response.

To examine the effects of pre-exposure to tC5a or pertussis toxin (PTx) on leukocyte chemotactic response, freshly isolated HKL were resuspended in L-15, 10% FBS, penicillin, and streptomycin, with or without tC5a (1×10^{-8} M) or PTx (0.1 and 1.0 μg/ml). The cells were transferred to a 24-well tissue culture plate (3×10^5 cells/well) and incubated for 5 h at 21°C. Cells were collected, washed, resuspended in L-15 and 0.25% BSA, and used in chemotaxis assays.

All assays were repeated at least three times, each time using cells from different fish.

Histochemical analyses of the tC5a-responsive cells

Cells that had migrated toward C5a (1×10^{-8} M) were collected from the bottom wells of the chemotaxis plate, and deposited onto microscope slides using the Cytospin III (Shandon, Pittsburgh, PA). Cells were fixed, stained

for myeloperoxidase and nonspecific esterase, and counterstained with hematoxylin and methylene blue, respectively, according to the manufacturer's instructions (Sigma-Aldrich; procedure 181-B and 390). Macrophages/Monocytes from mammals and fish can be identified by positive staining for esterase and negative staining for myeloperoxidase, while granulocytes stain positive for myeloperoxidase and negative for esterase (30, 31).

Results

Characterization of recombinant tC5a and tC5a^{desArg}

The recombinant tC5a preparation was >95% pure, because no contaminating proteins could be detected in the HPLC chromatogram (Fig. 1*A*) or the SDS-PAGE gel (Fig. 1*B*). Despite the lower m.w. of the tC5a^{desArg}, which was obtained after enzymatic removal of the Arg residue at the C-terminal of tC5a, it ran slightly slower than tC5a in the SDS-PAGE gel. This discrepancy between molecular size and migration may reflect a lower incorporation of SDS by tC5a^{desArg}. Mass spectrometric analysis confirmed the identity of both proteins (Fig. 1*C*): the observed mass of tC5a was 9519, and that of tC5a^{desArg} was 9363, both values very close to their theoretical mass values of 9524 and 9368, respectively.

Flow cytometric analysis of tC5aF binding to trout leukocytes

Based on the forward and sideward scatter of HKL and PBLk, one cell population in each preparation was identified that bound tC5a in a specific and concentration-dependent manner. In both cases, this population consisted of the larger and more granular cells, most likely the granulocytes (HKL and PBLk) and macrophages (HKL). To examine the tC5aF-binding characteristics of this cell population, the cells were gated, and changes in fluorescence were examined (Fig. 2). As indicated by the increase in fluorescence, trout HKL and PBLk bound tC5aF, but not mC5aF (Fig. 2, *B* and *E*). The tC5aF binding could be completely inhibited by excess amounts of unlabeled tC5a, but not mC5a (Fig. 2, *C* and *F*), thus demonstrating that the binding was specific. Binding of tC5aF to HKL and PBLk could also be inhibited by excess amounts of tC5a^{desArg} (Fig. 3). The HKL population displayed a univariate distribution of tC5aF binding, with a 2.5-fold increase in MF. The PBLk, in contrast, showed a bivariate distribution, with one population of cells expressing undetectable or low levels of the receptor (1.1-fold increase in MF), and a second population showing a high level of expression (5.5-fold increase in MF). For both HKL and PBLk, the tC5a binding was concentration dependent, with the greatest increase in fluorescence taking place between 1.0×10^{-9} and 2.5×10^{-8} M tC5aF (data not shown).

A single injection (i.p.) of LPS (*E. coli*, 111:B4; 1 mg/kg) did not affect tC5aF binding to trout leukocytes, when cells were harvested 6 h after the injection (data not shown). Also, in vitro exposure of cultured HKL to LPS (100 μg/ml) or PHA (10 μg/ml) for 6 h did not result in a change in tC5aF-binding capacity (data not shown). However, exposure of cultured HKL to tC5a (1×10^{-8}) for 5 h, followed by two washes, resulted in a complete loss of binding (Fig. 6*A*). A shorter exposure of the cells to tC5a (30 min) also abolished tC5aF binding (data not shown).

Chemotaxis of trout leukocytes to tC5a and tC5a^{desArg}

tC5a and tC5a^{desArg} stimulated the migration of HKL in a dose-dependent manner (Fig. 4). Although the leukocyte chemotactic

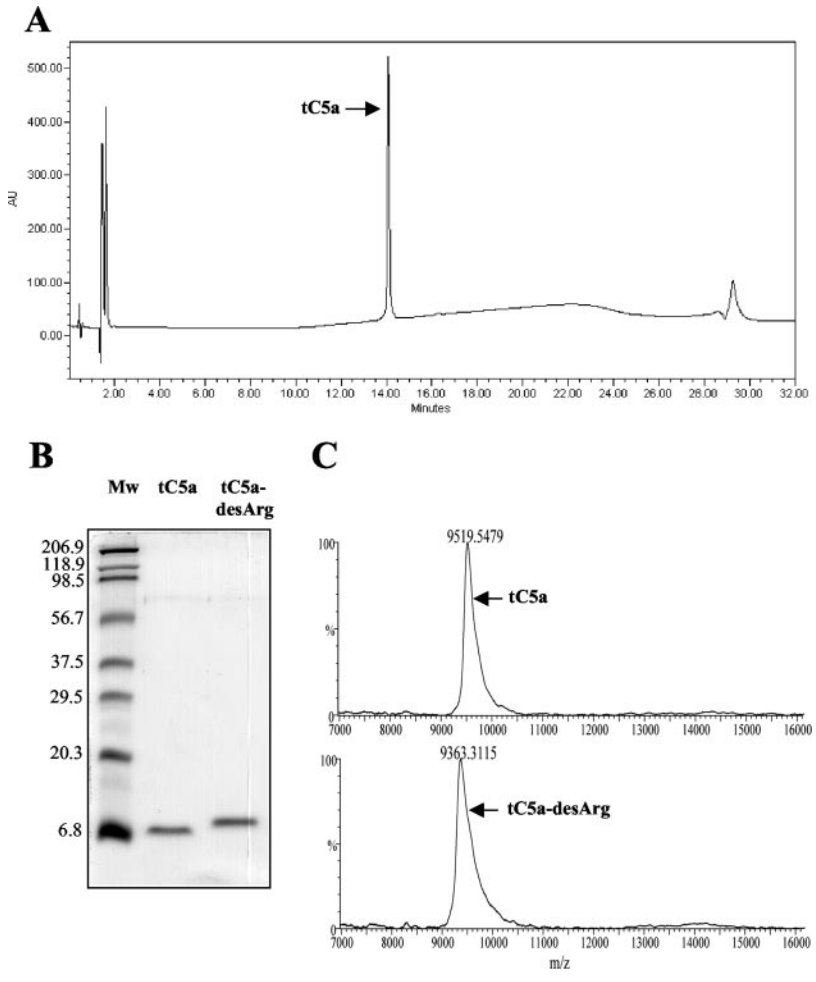

FIGURE 1. Characterization of the expressed tC5a and tC5a^{desArg} proteins. *A*, HPLC chromatogram of purified recombinant tC5a; *B*, Coomassie-stained 14% SDS-PAGE gel showing tC5a (*middle lane*, 0.5 μg) and tC5a^{desArg} (*right lane*, 0.5 μg) under reducing conditions. Numbers on the *left* indicate the m.w. of the markers (Mw, *first lane*); *C*, mass spectrometric analysis of tC5a (*top panel*) and tC5a^{desArg} (*lower panel*).

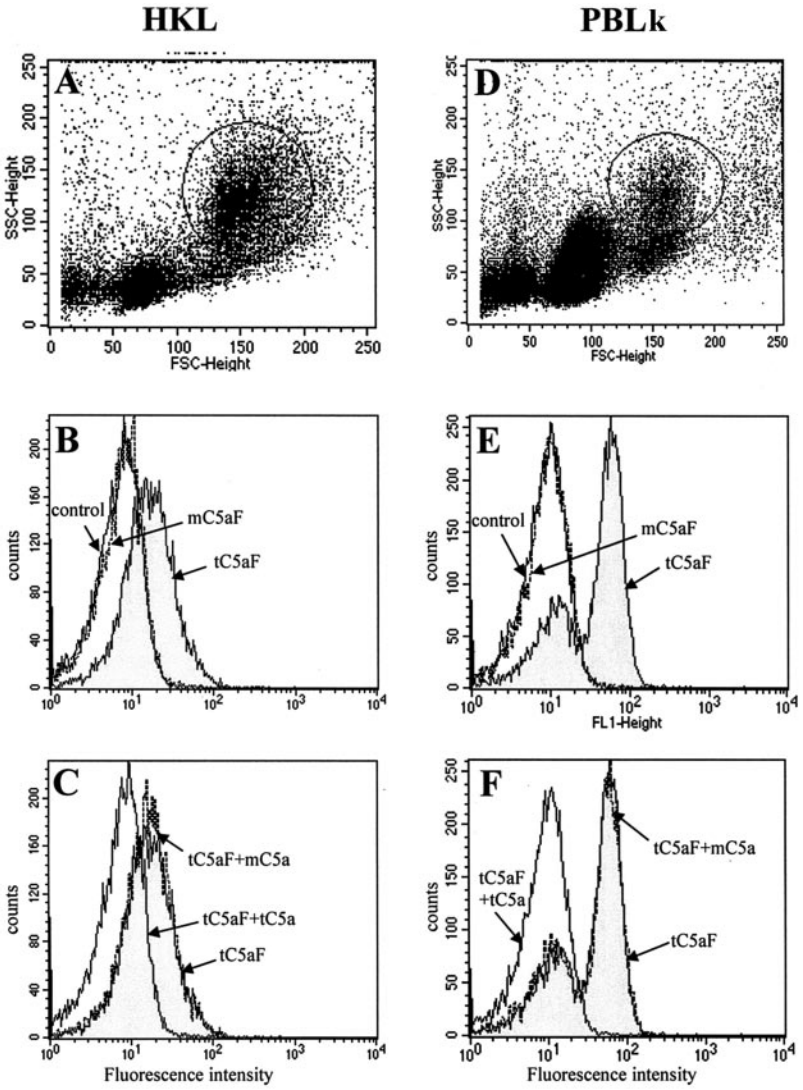

FIGURE 2. Specific binding of tC5aF to trout leukocytes. Leukocytes isolated from the head kidney (HKL; *A–C*) and peripheral blood (PBLk; *D–F*) were incubated with tC5aF or a similar fluoresceinated control protein (mC5aF), and analyzed by FACS. *A* and *D*, Dot plots of forward light scatter vs side scatter. The gated population contained the tC5aF-binding cells. *B* and *E*, Overlay histograms showing binding of tC5aF (1×10^{-7} M; shaded area), but not mC5aF (1×10^{-7} M; dashed line). Control indicates the autofluorescence of the cells (solid line). *C* and *F*, Overlay histograms showing the inhibition of tC5aF binding by an excess amount (2 μM) of unlabeled tC5a (tC5aF + tC5a; filled line), but not mC5a (tC5aF + mC5a; dashed line).

response toward tC5a varied slightly among individual animals, the optimum concentration for chemotaxis appeared to be consistently between 1×10^{-8} and 2×10^{-8} M tC5a. Concentrations of 1×10^{-7} or higher were generally ineffective (data not shown). The cells displayed a lower mobility toward $tC5a^{desArg}$ than to tC5a (Fig. 4). Based on the data presented in Fig. 4 as well as other unpublished data, the desArg derivative was estimated to be at least 10% less active than the intact molecule.

Chemotaxis of HKL was also observed when medium supplemented with ZAS was used. Medium containing nontreated or heat-inactivated trout serum (1–5%) was devoid of any chemotactic activity (data not shown). The responsiveness of trout leukocytes to ZAS was, depending on the animal, either similar (Fig. 4) or greater (Fig. 6) than that to tC5a.

The scattering characteristics of the cells that bound tC5aF were similar to those of the cells that responded chemotactically to tC5a (see Figs. 4 and 6), suggesting that granulocytes and macrophages are the cell types most likely to express the C5aR (i.e., high forward and sideward scatter). Myeloperoxidase staining demonstrated the presence of both granulocytes and macrophages in the HKL suspensions, but the migrated cell populations appeared to be comprised of granulocytes only (Fig. 5). These findings were

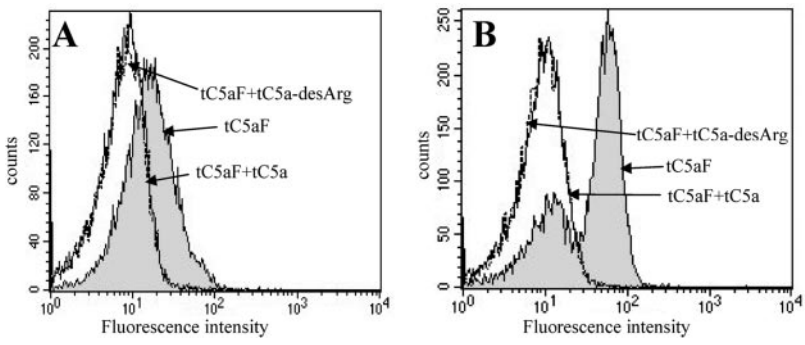

FIGURE 3. $tC5a^{desArg}$ and tC5a are competing ligands of the tC5aR. *A*, Overlay histogram demonstrating complete inhibition of tC5aF binding to HKL by 2 μM of unlabeled tC5a (filled line) and $tC5a^{desArg}$ (dashed line). *B*, Inhibition of tC5aF binding to peripheral blood leukocytes (PBLk) by 2 μM unlabeled tC5a and $tC5a^{desArg}$.

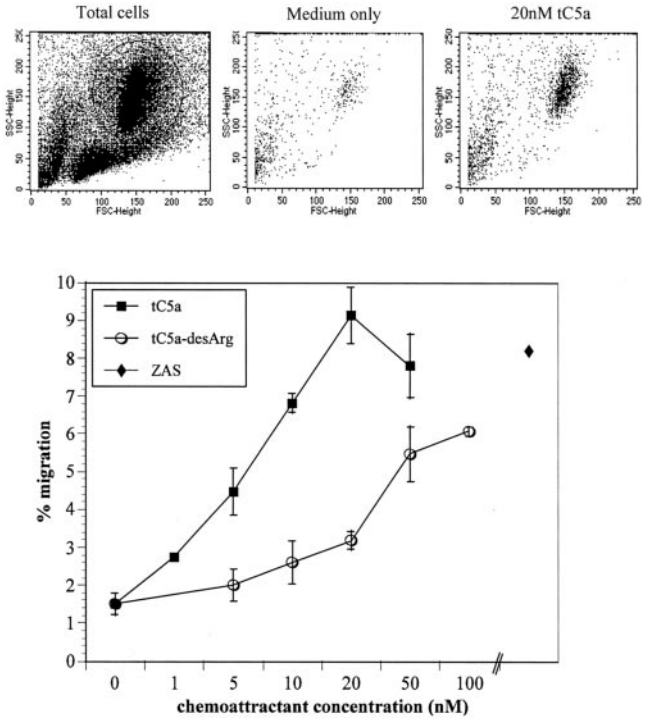

FIGURE 4. tC5a is a chemoattractant for leukocytes. Trout HKL migrated toward tC5a (squares) and tC5a^{desArg} (circles) in a concentration-dependent manner, with the latter being less potent in mediating chemotaxic responses than intact tC5a. Optimum migration was achieved with 2×10^{-8} M tC5a. In addition, ZAS (1%) stimulated the migration of HKL (diamond). Dot plots at the top show the forward light scatter vs side scatter of the cells as they were added to the wells (total cells; *left*), cells collected from a well that received medium only (*middle*), and those collected from a well that contained medium and 2×10^{-8} M tC5a (*right*). The migrating cell population has been gated. The data points represent the number of migrated cells expressed as a percentage of the total cells (means of duplicate values ± SEM).

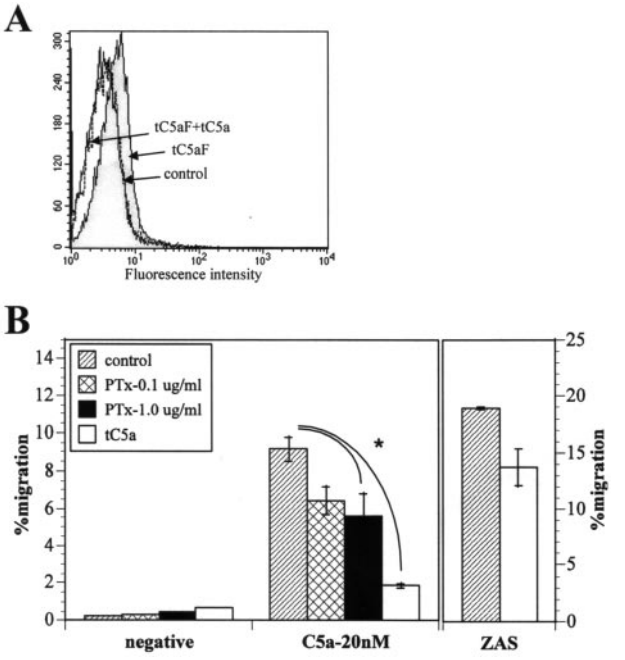

FIGURE 6. Desensitization of the tC5aR. *A*, Flow cytometric analysis showing a loss of tC5aF-binding capacity after HKL were pretreated with tC5a (1×10^{-8} M) for 5 h at room temperature, followed by two washes (tC5aF + tC5a). Sham-treated cells showed normal binding (tC5aF), and the control represents the autofluorescence of the cells. *B*, Chemotaxis of trout HKL toward 2×10^{-8} M tC5a or ZAS (1%). Cells were preincubated with PTx (0.1 and 1.0 μg/ml) or tC5a (1×10^{-8} M), or were sham treated (control) for 5 h at room temperature, followed by two washes. Medium without chemoattractant served as a negative control (negative). Migration was significantly inhibited by pre-exposure to PTx (1 μg/ml) and 1×10^{-8} M tC5a (*, $p < 0.05$, Duncan New Multiple Range Test). The bars represent the number of migrated cells expressed as a percentage of the total number of cells (means of duplicate values ± SEM).

confirmed by nonspecific esterase staining, which showed positive cells in the HKL, but not in the migrated cell cytospin preparations (data not shown).

Effects of tC5a and PTx on chemotactic response

HKL preincubated with 1×10^{-8} M tC5a for 5 h, then washed twice, showed an 80% reduction in chemotactic response toward 2×10^{-8} M tC5a and a 30% reduction toward ZAS-supplemented medium, when compared with sham-treated controls (Fig. 6). Because the C5aR, CD88, is linked to a PTx-sensitive heterotrimeric G protein on mammalian leukocytes, the susceptibility of cultured HKL to PTx was determined. The effect of PTx on tC5a-induced HKL migration was dose dependent, with concentrations equal to or less than 50 ng/ml being ineffective, and concentrations of 100 and 1000 ng/ml resulting in a maximum reduction of ~30–40%, when compared with the sham-treated controls (Fig. 6). The tC5a desensitization and PTx inhibition studies were conducted simultaneously using the same cell preparations. The limited reduction in migration obtained with PTx-treated trout cells was not due to limitations of the assay or reagents, because the migration of isolated human neutrophils toward mC5a (1×10^{-8} M) was inhibited by 40 and 81%, when neutrophils were treated with 100 and 1000 ng/ml PTx, respectively (data not shown).

Discussion

In the present study, we have demonstrated, for the first time, the presence of specific receptors for the C5a anaphylatoxin in a teleost species. Using recombinant tC5aF, we identified a leukocyte

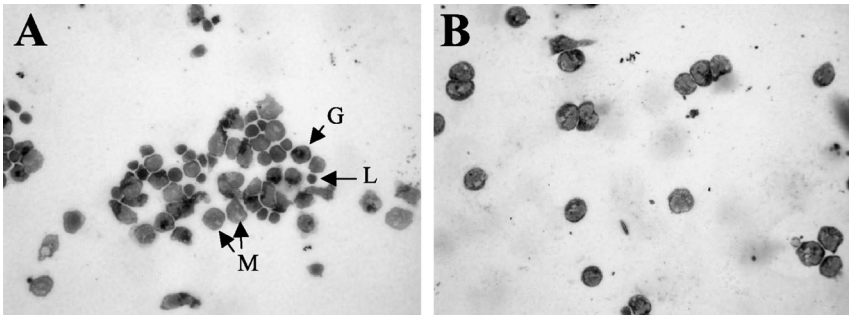

FIGURE 5. Myeloperoxidase staining to identify granulocytes (G) in cell suspensions before (*A*) and after (*B*) tC5a-mediated chemotaxis. *A*, Positive (brown-black cytoplasmic staining) and negative staining cells were present in cytospin preparations of HK cell suspensions. Negative staining cells included lymphocytes (L) and macrophages (M). *B*, Only positive staining cells were observed in cytospin preparations of cells collected after migration toward tC5a (1×10^{-8} M). Magnification, ×400.

population among HK and peripheral blood cells that bound tC5aF in nanomolar concentrations. The concentration used for these binding studies (1×10^{-7} M tC5aF) was at saturation for both HKL and PBLk and was ~10- to 20-fold higher than that commonly reported for mammalian binding studies using C5aF (32, 33). The observed tC5aF binding was considered specific, because mC5aF did not bind to trout leukocytes, and tC5aF binding could be completely inhibited by excess amounts of unlabeled tC5a, but not mC5a. Like other anaphylatoxins, mC5a and tC5a possess six cysteine residues that are responsible for creating the tightly packed core of the molecules. The free-moving C-terminal, with the residues QLGR at positions 74–77 in the mC5a molecule, is important for biological function and appears to be conserved in mammalian C5a molecules. In trout, this sequence has been replaced by the residues VLAR. This difference in the C-terminal sequence, together with the overall low amino acid identity of mC5a and tC5a (26.5%), are likely causes for the lack of cross-reactivity observed between mC5a and tC5aR.

The tC5a-binding PBLk population consisted of two subpopulations: 1) cells with a high level of C5aR expression, and 2) non-receptor- or low receptor-expressing cells. No evidence was found for a C5aR-negative subpopulation in the gated HKL population. The PBLk in the first category had a ~2-fold higher level of receptor expression than the tC5a-binding HKL. It is possible that the difference in C5aR expression on PBLk and HKL is due to a difference in maturational stage, because the granulocytes in the circulation are terminally differentiated, whereas those in the HK can be in various stages of differentiation.

The expression of the tC5aR could not be increased by in vivo or in vitro treatment with LPS or PHA. In mammals, LPS treatment has been shown to increase the expression of the C5aR in lung, liver, kidney, and thymus cells (11, 12). However, on leukocytes, the regulation of the C5aR may differ from that on non-myeloid cells, with the receptor being constitutively expressed. Further studies are needed to address whether the C5aR in trout is up-regulated under certain inflammatory or noninflammatory conditions.

In addition to binding to its receptor, tC5a was capable of inducing chemotactic responses in HKL. Concentrations of 1 to 2×10^{-8} M were optimal for tC5a-induced migration, whereas higher concentrations resulted in a reduced migration or a total loss of response. The concentration needed to induce a maximum response in cell migration was equal to or slightly higher than the optimum concentrations reported for mammalian neutrophils and monocytes (32, 34). The desArg derivative of tC5a was biologically active, because it was able to bind to the C5aR (excess amounts of unlabeled $tC5a^{desArg}$ completely inhibited tC5aF binding) and induce chemotaxis in HKL. However, $tC5a^{desArg}$ was a weaker chemotactic stimulus for HKL than was tC5a. This finding is similar to that reported for mammalian C5a and $C5a^{desArg}$, with the desArg derivative being ~10-fold less active than C5a in inducing leukocyte chemotaxis (35, 36). Taken together, these findings support the notion that the C-terminal portion is important for the biological function of C5a. However, they also suggest that sites other than the C terminus are involved in C5a-C5aR interactions. To date, three discontinuous regions of the human C5a molecule have been identified that interact with the C5aR (5).

As is true for its mammalian counterpart, CD88, the tC5aR showed ligand-induced desensitization. Preincubation of HKL with tC5a (1×10^{-8} M), followed by two washes, abrogated the binding of tC5aF to cultured HKL and reduced the chemotactic response by 80%. Human neutrophils can recycle resensitized receptors back to the surface within 30–60 min (37, 38), while human monocytes re-express the C5aR within 90 min (33). The duration of the trout chemotaxis assay was 2 h, and cells had ample time to recycle the receptor. However, the continuous presence of C5a in the assay may have prevented the re-expression of the already desensitized cells. Preincubation of HKL with tC5a reduced the migratory response of the cells toward ZAS-supplemented medium by 30%, while nontreated serum or heat-inactivated serum was without activity at the concentrations tested (1–5%). Zymosan-activated serum is likely to contain all three anaphylatoxins, C3a, C4a, and C5a, in addition to other cell-activating factors. The 30% reduction in migration of tC5a-desensitized cells demonstrates that only part of the ZAS-mediated migration could be attributed to tC5a. Although it is not yet known whether the trout anaphylatoxins bind to the same receptor, the present data suggest that receptors other than the one described in this study are involved in ZAS-mediated HKL migration.

Due to the lack of cell surface markers for fish leukocytes, the identity of the tC5a-binding cell populations could not be determined. However, based on the high forward and sideward scatter (i.e., size and granularity) of the tC5a-binding cells in flow cytometry, it can be assumed that, similar to the situation in mammals (32, 35, 39), granulocytes and macrophages/monocytes are the predominant cell types expressing the C5aR in trout. Histochemical analyses of the cells that migrated toward tC5a revealed a cell population almost exclusively comprised of granulocytes (mostly neutrophils). Therefore, HK-derived neutrophils, rather than macrophages, are capable of responding chemotactically to tC5a.

In mammalian leukocytes, C5a causes chemotaxis in a G_i-dependent, PTx-sensitive fashion. Treatment of trout HKL with PTx (0.1 or 1.0 μg/ml) for 5 h at room temperature resulted in a 30–40% reduction of tC5a-mediated migration. This limited effect of PTx suggests that the tC5a-mediated effects may only partially involve the classical G_i proteins. Transfection studies have shown that the mammalian C5aR, CD88, is capable of coupling to the PTx-insensitive G_z and the $G\alpha_{16}$ proteins, as well as to the PTx-sensitive G_i proteins (9, 40). Whether the tC5aR couples to any of these G proteins remains to be investigated.

In conclusion, the C5aR present on trout leukocytes displays many of the properties of mammalian C5aR (CD88): 1) it binds tC5a specifically and in nanomolar concentrations; 2) $tC5a^{desArg}$ is a competing ligand; 3) it mediates chemotactic responses of neutrophils upon binding to tC5a and $tC5a^{desArg}$, the latter being less potent; and 4) it rapidly desensitizes in response to C5a overstimulation. However, the tC5aR differs from the CD88 with regard to its sensitivity to PTx, because PTx only partially inhibited the tC5a-mediated chemotactic response of HKL. Further studies are needed to reveal whether the tC5aR binds other anaphylatoxins, as has been suggested for the C5L2, or whether it is specific for C5a and $C5a^{desArg}$, like the CD88. The fact that fish, unlike mammals, possess structurally (and possibly functionally) different isoforms of C3 and C5 (24, 25, 41) raises interesting questions about the number of anaphylatoxin receptors present in these animals, as well as their ligand specificities. Studies addressing these questions will lead to a better understanding of the functions of the anaphylatoxins throughout evolution.

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