CiC3-1a-Mediated Chemotaxis in the Deuterostome Invertebrate *Ciona intestinalis* (Urochordata)[1]

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Deuterostome invertebrates possess complement genes, and in limited instances complement-mediated functions have been reported in these organisms. However, the organization of the complement pathway(s), as well as the functions exerted by the cloned gene products, are largely unknown. To address the issue of the presence of an inflammatory pathway in ascidians, we expressed in *Escherichia coli* the fragment of *Ciona intestinalis* C3-1 corresponding to mammalian complement C3a (rCiC3-1a) and assessed its chemotactic activity on *C. intestinalis* hemocytes. We found that the migration of *C. intestinalis* hemocytes toward rCiC3-1a was dose dependent, peaking at 500 nM, and was specific for CiC3-1a, being inhibited by an anti-rCiC3-1a-specific Ab. As is true for mammalian C3a, the chemotactic activity of *C. intestinalis* C3-1a was localized to the C terminus, because a peptide representing the 18 C-terminal amino acids (CiC3-1a$^{59-76}$) also promoted hemocyte chemotaxis. Furthermore, the CiC3-1a terminal Arg was not crucial for chemotactic activity, because the desArg peptide (CiC3-1a$^{59-75}$) retained most of the directional hemocyte migration activity. The CiC3-1a-mediated chemotaxis was inhibited by pretreatment of cells with pertussis toxin, suggesting that the receptor molecule mediating the chemotactic effect is G$_i$ protein coupled. Immunohistochemical analysis with anti-rCiC3-1a-specific Ab and in situ hybridization experiments with a riboprobe corresponding to the 3′-terminal sequence of CiC3-1, performed on tunic sections of LPS-injected animals, showed that a majority of the infiltrating labeled hemocytes were granular amebocytes and compartment cells. Our findings indicate that CiC3-1a mediates chemotaxis of *C. intestinalis* hemocytes, thus suggesting an important role for this molecule in inflammatory processes. *The Journal of Immunology,* 2003, 171: 5521–5528.

I n mammals, leukocyte migration and accumulation from the blood to sites of infection, tissue damage, or inflammation is a well-described and characterized process. It is regulated by various exogenous and endogenous chemoattractant molecules, including bacterial LPS and *N*-formyl peptides (fMLP), lipid mediators, various chemokines, and the highly potent bioactive fragments C3a, C4a, and C5a, which are released during the activation of the complement cascade (1, 2).

The anaphylatoxin C3a is a 74- to 78-aa peptide derived from the N-terminal end of the C3 α-chain by proteolytic cleavage after the activation of either the classical, lectin, or alternative pathway of the complement system (3, 4). C3a mediates a variety of biological responses in vivo and in vitro, such as histamine release and mobilization of intracellular calcium ions from human mast cells, lysosomal enzyme release from leukocytes, smooth muscle contraction, guinea pig platelet aggregation, and modulation of cellular and humoral immune responses (5, 6). More recently, it has been demonstrated that, next to C5a, C3a is the most potent chemotactic proinflammatory molecule for both human eosin-

ophils (7) and mast cells (8, 9), and together with the stromal cell-derived factor 1 enhances the homing-related responses of hemopoietic stem/progenitor cells (10). Eosinophils display concentration-dependent polarization, chemotaxis, and enzyme release upon stimulation by C3a, human rC3a, and synthetic C3a peptides representing the C-terminal sequence of C3a (7). The proinflammatory activity of C3a is elicited via the specific binding to the G$_i$ protein-coupled seven-transmembrane receptor (C3aR), which is broadly expressed in various tissues and organs (2, 6, 7, 11–13).

The recent finding of complement components in deuterostome invertebrates has suggested the presence of a complement system that operates via an alternative pathway in echinoderms and tunicates and via a mannose-binding lectin-mediated pathway that has to date been identified only in tunicates (14). The presence of C3 homologues has been demonstrated in the sea urchin *Strongylocentrotus purpuratus* (15), the cephalochordate amphioxus (16), and the urochordates *Styela plicata* (17) and *Halocynthia roretzi* (18). More recently, two C3-like genes, CiC3-1 and CiC3-2, have been isolated from hemocyte RNA of the ascidian *Ciona intestinalis*. The deduced amino acid sequences of both *Ciona* C3-like proteins exhibit a canonical processing site for α- and β-chains, a thioester site with an associated catalytic histidine, and a convertase cleavage site in CiC3-1, thus showing an overall similarity to the C3 molecules of other species (19). Recently, sequencing of the *C. intestinalis* genome has suggested the presence of several complement genes, including C1q-like and C6-like genes (20); however, the functional identity of these encoded proteins has not yet been established.

Our knowledge of the functional aspects of the complement system in deuterostome invertebrates is sparse. In *H. roretzi*, a first functional characterization provided evidence for the presence of an opsonic activity in its body fluid that enhances phagocytosis of

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yeast by ascidian hemocytes; this activity is C3 mediated, because it is inhibited by an Ab against *H. roretzi* C3 (18). Very recently, in *Pyura stolonifera*, an enhancing hemocyte migration activity, found in partially purified hemolymph, has been attributed to the generation of a C3a-like fragment (21).

To address the issue of the presence of an inflammatory pathway in the ascidian *C. intestinalis*, we have now expressed in *Escherichia coli* the fragment of CiC3-1 corresponding to mammalian C3a and have assessed its chemotactic activity on *C. intestinalis* hemocytes. We found that rCiC3-1a, the 18-aa synthetic peptide representing the C terminus, and the corresponding desArg peptide all promoted hemocyte chemotaxis that could be inhibited by an anti-rCiC3-1a-specific Ab.

Materials and Methods

Hemocyte and cell-free hemolymph preparation

Specimens of *C. intestinalis* were collected in the Gulf of Napoli and maintained in circulating seawater until use. The hemolymph was drained from the heart and/or collected from the perivisceral cavity with a 10-ml syringe.

To prepare hemocytes, hemolymph was immediately diluted 1:2 with ice-cold artificial seawater, Ca^{2+} and Mg^{2+} free, containing 10 mM EDTA (pH 7.0). Following centrifugation at $500 \times g$ for 10 min at 4°C, hemocytes were resuspended in marine solution (MS)[3]: 0.45 M NaCl, 26 mM MgCl₂, 11 mM KCl, and 12 mM CaCl₂ (pH 7.4). The cell number was evaluated in a Neubauer chamber, and the volumes were adjusted to give a final concentration of 2.2×10^6 cells/ml.

To obtain cell-free hemolymph (CFH) for use in chemotaxis experiments, hemolymph was centrifuged immediately after collection at $1250 \times g$ for 10 min at 4°C. The cell pellet was discarded, and the CFH was harvested into chilled tubes and stored on ice.

LPS treatment of hemocytes

Hemocytes, prepared as described above, were resuspended in MS containing 20 μg/ml LPS, at the concentration of 8×10^6/ml. Following a 3-h incubation at 20°C, cell suspension was centrifuged for 5 min at $500 \times g$. The supernatant was recovered and centrifuged again for 15 min at $12,000 \times g$. The supernatant, cell-free medium (CFM), was used undiluted in chemotaxis experiments.

Preparation of rCiC3-1a and specific Ab production

Hemolymph from *C. intestinalis* was collected by syringe in the presence of ~10 mM EDTA (pH 8.0) to prevent cell clotting. After centrifugation for 20 min at $1250 \times g$, the cell pellet was immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted with the Promega kit SV Total RNA isolation system (Promega, Madison, WI). Random-primed single-strand cDNA was synthesized from hemocyte RNA with an RT-PCR kit and PCR amplified (SuperScript; Invitrogen, San Diego, CA). The two gene-specific primers were: sense primer, 5′-CTCACAACTTC GATACAACTGGAAC-3′, and antisense primer, 5′-GTTTTATCAGT GCTTGTTCCCGCTTT-3′, complementary to two nucleotide sequences, respectively, 1311 and 2567 bp downstream of the 5′ end. Thirty cycles of amplification were conducted, using the following parameters: 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. The PCR product was gel purified with the Prep-A-Gene DNA Purification Kit (Bio-Rad, Hercules, CA), ligated into the pCRII-TOPO vector (Invitrogen, San Diego, CA), and controlled by sequencing.

The *Ciona* C3-1a fragment corresponding to amino acid residues 675–750 of the CiC3-1a sequence was amplified by PCR from the cloned C3-1 fragment (1311–2567 bp) using the primers 5′-GGATCCCAGATA CAAGTTGAA-3′ and 5′-AAGCTTTTATCTTCCTTGGACCCT-3′. These primers added a *Bam*HI site at the beginning of the C3 sequence and a *Hin*dIII site at the end of the cDNA. For convenience, the fragment was cloned into pGEM-T Easy vector, and the sequence was verified. The fragment was excised with *Bam*HI and *Hin*dIII and cloned into the expression vector pQE-30 (Qiagen, Stutio, CA).

The CiC3-1a protein was expressed and purified from the *E. coli* strain M15 (pREP4; Qiagen) using the method recently described by Kimura et al. (22). The *E. coli* was first treated with a lysogen solution (1 mg/ml), and the pellet was then resuspended in a solution of 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl containing 10 mM 2-ME and 8 M urea (pH 8.0). The cleared

lysate was mixed with Ni-NTA agarose (Qiagen) for 1–2 h and loaded onto a disposable column. The column was washed with 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl containing 8 M urea (pH 8.0), followed by the same solution at pH 6.3 and then pH 5.9, and finally eluted at pH 4.5. The recombinant protein was refolded by dialysis overnight into 0.1 M Tris-HCl with 2 mM reduced glutathione, 0.2 mM glutathione, and 0.005% Tween 80 (22). Contaminating proteins were removed by a reversed-phase column using a RESORSE RPC 3-ml column (Amersham Pharmacia Biotech, Piscataway, NJ), as previously described (23, 24). The purity and identity of rCiC3-1a protein were assessed by SDS-PAGE and mass spectrometry.

To prepare anti-CiC3-1a-specific Abs, rabbits were immunized by repeated s.c. injections at multiple sites with 30 μg of purified rCiC3-1a emulsified in CFA, followed by a booster injection at 3 wk. The IgG fraction of the immune serum was prepared by a combination of caprylic acid and ammonium sulfate precipitation and, after extensive dialysis against PBS, was stored at −20°C.

Peptide synthesis

The 18-aa peptide, CiC3-1a[59−76] (IALARLNSGTRRQRVQGR) (underlined in Fig. 1), and the 17-aa peptide, CiC3-1a[59−75] (IALAR LNSGTRRQRVQG), representing part of the CiC3-1a-deduced amino acid sequence, were synthesized using an Applied Biosystems 430A peptide synthesizer (Foster City, CA), as previously described (25).

Chemotaxis assays

Chemotaxis experiments with *C. intestinalis* hemocytes were performed in single Blind Well Chambers, model BW200L (NeuroProbe, Gaithersburg, MD). Polycarbonate filters (13 mm diameter, 5.0 μm pore size; Whatman, Clifton, NJ) were used to separate the upper and lower wells. The lower well contained 200 μl of the chemoattractant, represented by undiluted CFH, undiluted CFM, or MS containing a range of concentrations of rCiC3-1a from 15.6 to 750 nM or the synthetic peptide CiC3-1a[59−76] or CiC3-1a[59−75] at 0.62–30 μM. Hemocytes (300 μl, 2.2×10^6 cells/ml) were added to the upper well and allowed to migrate for 2 h at 20°C. At the end of the incubation time, the fluid in the upper chamber and the nonmigrated cells on the top surface of the filter were gently wiped off with a cotton swab. The number of cells that had migrated to the lower well was estimated by counting 16 randomly chosen fields in a hemocytometer; values were expressed as a percentage of the total number of cells added to the chemotaxis chamber.

Inhibition of chemotaxis by specific Ab

For each inhibition experiment using anti-rCiC3-1a Abs, 2.6 μl of the 10 μM recombinant protein was added to 20 μl of the 2.4 mg/ml anti-rCiC3-1a rabbit IgG. Following a 90-min incubation at 20°C, 190 μl of MS was added, and this mixture was used in the chemotaxis inhibition assay, as specified above. Control experiments were run with an equal amount of the corresponding preimmune rabbit IgG. The same procedure was used to inhibit *Ciona* CFH and CFM chemotactic activity: 20 μl of the immune or preimmune rabbit IgG was added to 190 μl of *Ciona* CFH or CFM, and after a 90-min incubation at 20°C, the CFH or CFM was used in chemotaxis experiments following the procedure described above.

Pertussis toxin treatment

Hemocytes were treated with pertussis toxin by incubating 600 μl of hemocyte cell suspension (2.2×10^6 cells/ml) for 2 h at 20°C with pertussis toxin at 0.5 μg/ml (Calbiochem, La Jolla, CA). The cells were recovered as a pellet by centrifugation at $500 \times g$ for 5 min, and then washed twice with MS. The cell pellet was resuspended in MS and counted, and the concentration was adjusted to 2.2×10^6 cells/ml. The hemocyte viability was checked by the trypan blue exclusion assay. This cell suspension was used in chemotaxis experiments with 500 nM rCiC3-1a. Control samples, in which an equivalent amount of MS was substituted for pertussis toxin, were run in parallel.

Immunohistochemistry reaction after LPS injections

To induce an inflammatory reaction, 0.2 ml of 2.5 mg/ml LPS (*E. coli*, serotype 055:B5; Sigma-Aldrich, St. Louis, MO) in PBS was injected into the tunic of five *C. intestinalis* individuals between the outer layer and the epidermis. An equal number of control animals was injected with the same volume of PBS. Animals were maintained in tanks containing aerated seawater at 18°C and sacrificed after various periods of time, from 1 to 48 h. A large fragment (~1 cm²) of the injured tissue was fixed in Bouin's fluid (saturated picric acid:formaldehyde:acetic acid 15:5:1) for 24 h. To remove completely the fixative, the specimens were rinsed with 75% ethanol until

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[3] Abbreviations used in this paper: MS, marine solution; CFH, cell-free hemolymph; CFM, cell-free medium.

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Ciona        ----QIQVEQELSIEA--KLHKCGEDGKKKSFSECDT---CEMQRDRVIYTFD-----DAIPGCSERFYAECIALARLNSGTRRQR--VQGR
Human        SVQLTEKRMDKVGKYP-KELRKCCEDGMRENPMRFS----CQR-RTRFIS---------LGEACKKVFLDCCNYITELRRQHARASHLGLAR
Hagfish      -ELVLEIAIEKASTYP-AELRKCCRDAAIESPLRLS----CEE-RTKHIHD--------EGEGCQETFLECCKHVEEELLIAMEEEDEDLGR
Amphioxus    --DLQNSLEAKVMEFN-ETLQPCCMDGQQWDPLGRS----CLQ-RAKLNST-------SQDECYFAFLTCCNHARSLRRLG-R----GRGR
Lampetra     RQLSMLQIRREAEKYT-QEFRKCCVDGLKMSPTGQG----CEE-RLKRVT--------GPKECVDAFLQCCKKAEEYRKSESLGAKTVLRR
Halocynthia  ----AANPNRDFQLVAAAKVEKCRLDGQRLNLIHLT----CEQ-RLAIISANMQHKDENLRNSCMTAFEEACVALENDFYTQES-----VSR
Sea urchin   -----SIDRDQLCLYDPTYLADCLADKPRLRRVLTEGGWLCPR-RARVLEAECN-----LEPDQTQVYRDCCINSMNPVVTA-------TSR
Swiftia      ------------STDASVDPQCCILGEDLDPAT------CLV-RAMKFSVSNISSSFHSLDACIIEFYKCCYRKFEMDW---------RTR
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FIGURE 1. C3a sequence alignment. The amino acid sequence of the CiC3-1a fragment was aligned, using the Clustal X program, with the human anaphylatoxin sequence and the C3 regions from lower vertebrate or invertebrate species corresponding to the mammalian C3a. The highly conserved amino acid residues of the complement anaphylatoxin domain present in the CiC3-1a sequence are designated by *. The underlined amino acid residues correspond to the synthetic peptide CiC3-1a$^{59-76}$ used in chemotaxis experiments. Accession numbers: *C. intestinalis*, CAC85959; human, P01024; hagfish, P98094; amphioxus, BAB47146; lampetra, Q00685; *H. roretzi*, BAA75069; sea urchin, T14074; *Swiftia exserta*, AAN86598.

the brownish color disappeared. After dehydration through a graded alcohol series, the samples were embedded in paraffin.

The immunohistochemical staining with anti-rCiC3-1a IgG was conducted on 6-μm sections using the Vectastain Elite ABC Kit and the DAB Substrate Kit for Peroxidase (Vector Laboratories, Burlingame, CA). The anti-rCiC3-1a IgG was used at a protein concentration of 2.4 μg/ml. Controls were run with the preimmune rabbit IgG at the same concentration. Staining was observed under a Leica DMLB microscope (Solms, Germany).

In situ hybridization

In situ hybridization experiments were conducted on 6-μm sections of tunic from LPS-injected animals, with digoxigenin-11-UTP-labeled riboprobes (Roche Diagnostics, Basel, Switzerland), according to the instructions of the manufacturer and as previously described (19). The antisense cRNAs corresponded to the CiC3-1 nucleotide fragment 3673–5161 (accession number AJ320542); 100 ng of probe per slide at 60°C was used. Control experiments were run in parallel using the corresponding sense cRNAs.

Results

Hemocyte migration toward cell-free hemolymph

The ability of *C. intestinalis* CFH to promote the migration of hemocytes was assessed using Boyden chemotaxis chambers. In four independent experiments, the hemocytes present in the upper chamber migrated toward the lower chamber containing undiluted CFH. The percentage of migrating cells ranged from 13.0 to 15.5% (mean value, 14.05%). No hemocyte migration was observed in control experiments in which MS replaced CFH in the lower chamber. This result suggests the presence in *Ciona* CFH of endogenous factors that can stimulate the migration of hemocytes.

Chemoattractant activity released from LPS-treated hemocytes

To determine whether LPS-activated hemocytes are able to produce and secrete the chemoattractant in vitro, hemocytes recovered from the hemolymph were incubated (8 × 10^6/ml) for 3 h in MS containing LPS. The resultant CFM, used undiluted in chemotaxis experiments, promoted 21.8% of directional cell migration. No hemocyte migration was observed in control experiments in which the CFM was added to the upper chamber or when an equivalent amount of MS containing LPS was substituted for CFM in the lower well.

Expression of rCiC3-1a in E. coli

To assess the presence of an inflammatory pathway in *C. intestinalis*, we subcloned and expressed the putative *Ciona* C3-1a fragment, corresponding to the human C3a (Fig. 1), in *E. coli*. When the purity and identity of the expressed CiC3-1a were checked by SDS and mass spectrometry, the preparation was found to be >95% pure.
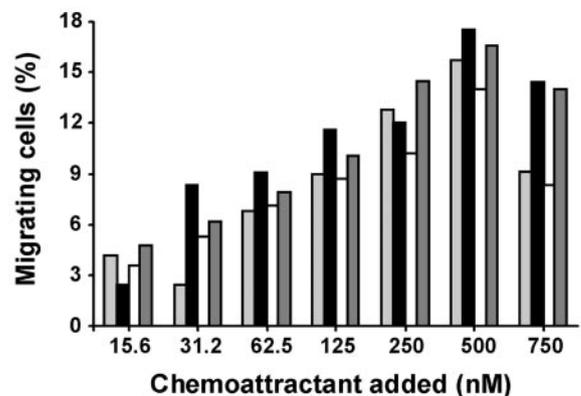
Hemocyte migration toward rCiC3-1a

To verify that CiC3-1a has C3a-like chemotactic activity and can stimulate hemocyte chemotaxis, serial dilutions of rCiC3-1a

(15.6–750 nM) were used in chemotaxis experiments (Fig. 2). Hemocyte migration was stimulated in a dose-dependent manner and peaked at 500 nM, with 17.5% of the cells recovered in the lower chamber. When the highest concentration of rCiC3-1a (750 nM) was used, the extent of cell migration decreased, suggesting that cells could be desensitized. No spontaneous migration of cells was observed in control experiments in which the lower chamber contained no chemoattractant. Furthermore, we did not observe any migration when rCiC3-1a was added to the upper chamber alone, indicating that cell migration is not the effect of a random increase in cell motility. These findings strongly suggest that rCiC3-1a can promote the true chemotaxis of *Ciona* hemocytes.

In other systems studied to date, the C3a C terminus is the binding and effector site of the anaphylatoxins (6). To determine whether the C-terminal region of CiC3-1a plays a similar role, a synthetic peptide representing the 18 C-terminal amino acids (CiC3-1a$^{59-76}$) was prepared and used in chemotaxis experiments. Seven independent experiments were conducted using serial dilutions of the peptide from 0.62 to 30 μM (Fig. 3A). The results consistently indicated that hemocyte migration was dose dependent. The highest percentage of cell migration obtained (16.1%) was similar to that observed in the experiments performed with rCiC3-1a, but it was achieved using a higher peptide concentration, thereby indicating a lower specific potency of the peptide with respect to the entire molecule.

To extend these observations, we examined whether the synthetic peptide CiC3-1a$^{59-75}$, lacking the terminal arginine, would induce hemocyte chemotaxis. In six independent experiments, the

FIGURE 2. Chemotactic response of hemocytes to rCiC3-1a. The *Ciona* C3-1a fragment, corresponding to amino acid residues 675–750 of the CiC3-1a sequence, was expressed in *E. coli* and used in chemotaxis experiments. Cell migration toward increasing concentrations of the chemoattractant in MS is shown. Four of the 12 independent experiments conducted are presented in the figure. Migration is expressed as the percentage of the total number of cells added to the upper well of the chemotaxis chamber.
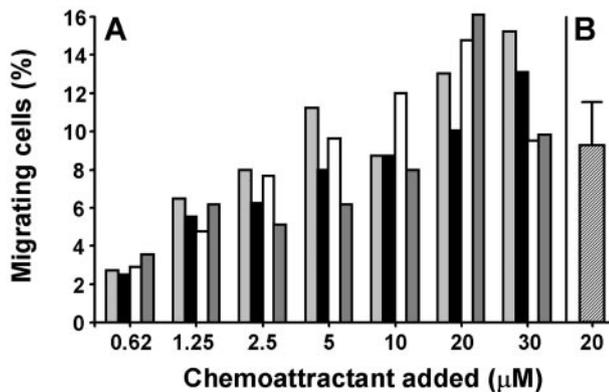
FIGURE 3. Chemotactic response of hemocytes to the synthetic peptides. The 18-aa peptide, CiC3-1a$^{59-76}$ (IALARLNSGTRRQRVQGR), representing the C terminus of the CiC3-1a fragment, was synthesized and used in chemotaxis assays. Four representative experiments are shown in *A*. The hemocyte migratory response toward 20 μM desArg peptide, CiC3-1a$^{59-75}$, is shown in *B*, in which the mean value $\pm$ SD for six experiments is reported. The chemotactic effect was calculated as the percentage of the total number of cells added to the upper chamber.

desArg peptide promoted concentration-dependent chemotaxis, retaining most of the activity of the CiC3-1a$^{59-76}$ peptide. Fig. 3*B* shows the hemocyte migratory response toward the desArg peptide at 20 μM, which is the concentration of CiC3-1a$^{59-76}$ giving the maximal chemotaxis activity.

No hemocyte migration was observed in control experiments in which the peptides were added to the upper chamber at the highest concentration tested.

Inhibition of chemotaxis by specific Ab

To determine the specificity of CFH-mediated hemocyte migration, and CFM or rCiC3-1a-induced chemotaxis of hemocytes, we prepared a rabbit polyclonal antiserum against the *Ciona* rC3-1a. Purified IgG from the immune serum and the corresponding preimmune serum were used to inhibit cell migration induced by *Ciona* CFH, CFM, and rCiC3-1a.

The CFH-induced hemocyte migration was considerably reduced (72%) when anti-rCiC3-1a IgG were added to the lower chamber at 0.23 mg/ml. Control experiments performed with preimmune IgG at the same concentration promoted hemocyte migration comparable to the control without IgG (Fig. 4*A*).

The presence of C3-1a in the CFM was demonstrated by inhibiting the chemotaxis by the specific Ab. The anti-rCiC3-1a IgG inhibited the chemotaxis by 78%, while the preimmune IgG did not affect the activity (Fig. 4*B*).

Inhibition of rCiC3-1a-induced chemotaxis was performed by adding the anti-rCiC3-1a rabbit IgG to the recombinant protein, used at 125 nM, the concentration giving ~50% of the maximum directional cell migration. As shown in Fig. 4*C*, the specific Ab considerably reduced hemocyte migration. The chemotaxis was reduced to a minor extent when the recombinant protein was used at 250 nM (data not shown). In parallel experiments conducted with preimmune IgG, chemotaxis was not influenced.

Pertussis toxin sensitivity of CiC3-1a-induced chemotaxis

To ascertain whether CiC3-1a-induced chemotaxis is mediated through G$_i$ protein-coupled transmembrane receptors as in mammals, hemocytes were incubated with pertussis toxin and then challenged in assays of rCiC3-1a-induced chemotaxis. Pretreatment of hemocytes with pertussis toxin at 0.5 μg/ml, in five independent experiments, reduced the chemotactic response to the
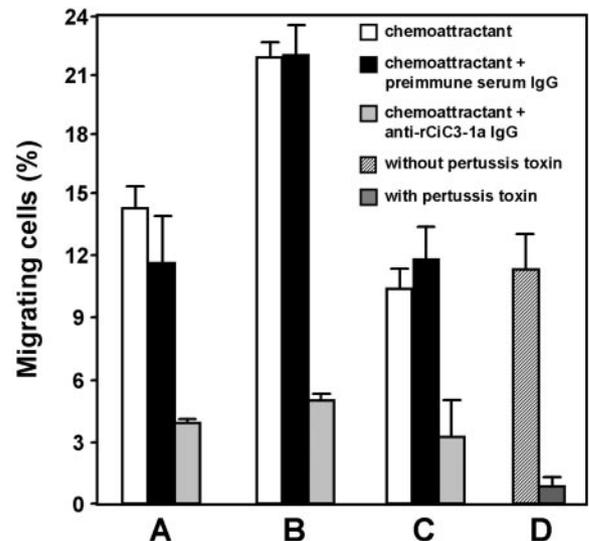

FIGURE 4. Inhibition of hemocyte migration by specific Abs and pertussis toxin. The chemotactic activity of undiluted CFH (*A*), undiluted CFM (*B*), or 125 nM rCiC3-1a in MS (*C*) was inhibited by anti-rCiC3-1a IgG at 0.23 mg/ml. Control experiments were run in parallel using the IgG purified from the corresponding preimmune rabbit serum at the same concentration. Pertussis toxin at 0.5 μg/ml in MS was used to inhibit the chemoattractant activity of 500 nM rCiC3-1a (*D*). Control experiments, in which pertussis toxin was substituted by an equivalent amount of MS, were performed under the same conditions. In all inhibition experiments, cell migration was expressed as the percentage of the total number of the cells added. Results are expressed as mean $\pm$ SD of five independent experiments.

recombinant protein by 93% (Fig. 4*D*). Hemocyte viability, after pertussis toxin treatment, was assessed by trypan blue staining. Cell viability was always >98%. These results suggest that *Ciona* C3a, like mammalian C3a, activates a signal transduction pathway through a G$_i$ protein-coupled receptor.

In vivo hemocyte migration toward the site of inflammation

To verify whether the hemocytes involved in local inflammatory reactions are actively engaged in C3 production, tissue sections of tunic obtained from animals injected with LPS were compared with control sections of animals injected with PBS. The hemocytes were examined and counted after the immunocytochemical staining with the anti-CiC3-1a Ab. In control sections, neither the total number of cells nor the ratio among the hemocyte types was found to change from 1 to 48 h after the injection. In addition, no staining was detected in any hemocytes after Ab treatment at various observation times, as shown in Fig. 5*A*, in which a granular amebocyte can be seen. The majority of the granular amebocytes between 4 and 48 h exhibited a clear staining after LPS injection (Fig. 5*B*). At the same time, the number of these cells increased considerably. In compartment cells, characterized by large vacuoles, the staining was absent until 12 h, whereas the CiC3-1a Ag was detectable between 24 and 48 h after the LPS injection (Fig. 5*C*), with the labeling confined to the bristles of cytoplasm that surround the large vacuoles. Univacuolar refractile granulocytes, which are characterized by the presence of a large refractile granule, were not stained even at 48 h after the injection. Some of these cells did show a labeling that was limited to the cytoplasmic peripheral ring (Fig. 5*D*). Morula cells, which are characterized by large granules that occupy the cytoplasm, and produce a typical morular aspect, were always unstained (Fig. 5*E*). Table I summarizes the results of cell counts in the histological sections of tunic samples at 48 h
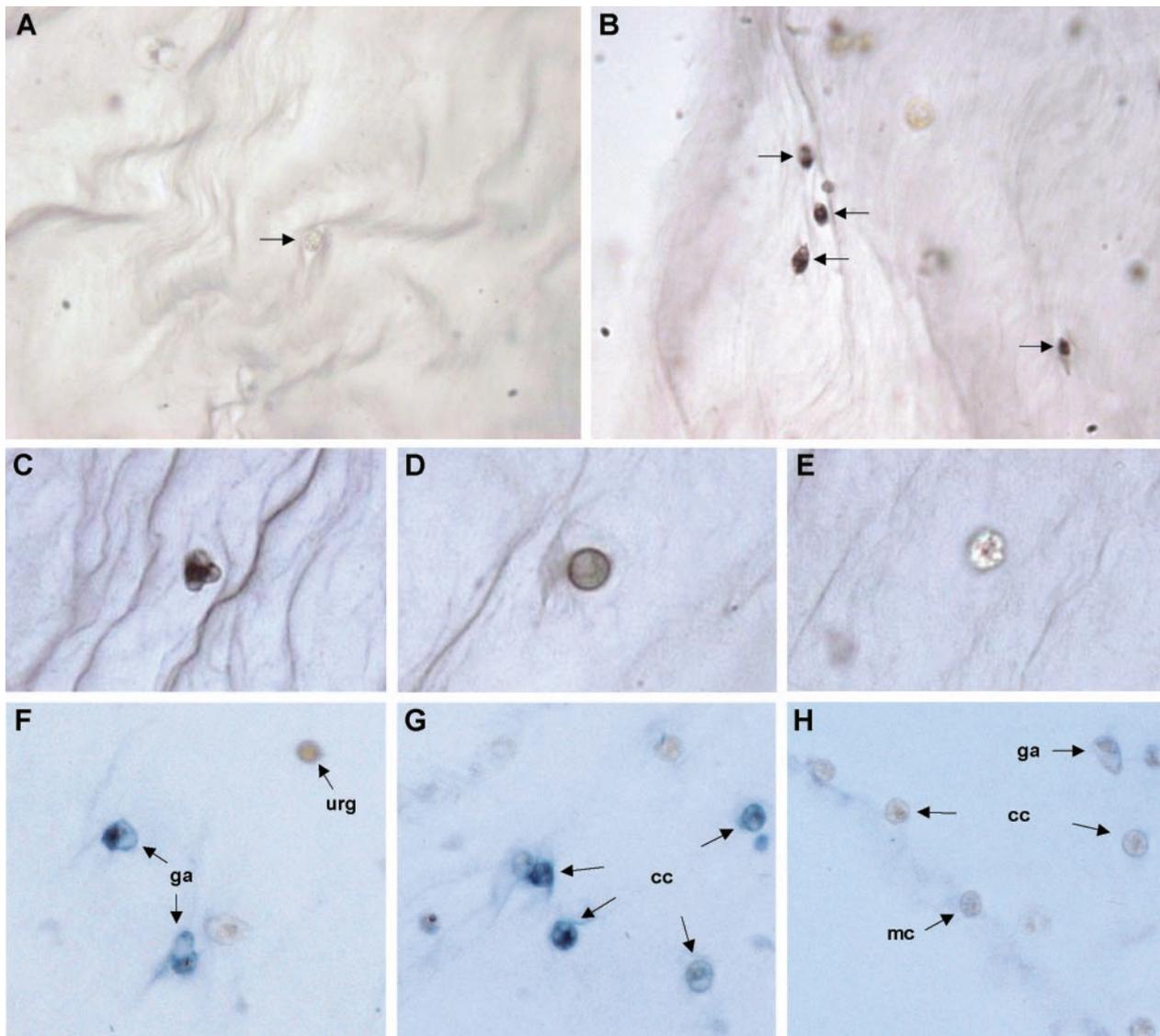
FIGURE 5. Immunohistochemical staining and in situ hybridization of transverse sections of tunic at 48 h after injection with LPS. Hemocytes present in the tunic area near the injection site were tested for the presence of CiC3-1 by incubation with anti-rCiC3-1a rabbit IgG at 2.4 μg/ml. No staining can be detected in control sections from PBS-injected animals (*A*): a granular amebocyte is indicated by the arrow. Immunohistochemical staining of tunic sections from LPS-injected animals (*B–E*) revealed the intracellular localization of CiC3-1a Ags in granular amebocytes (*B*) and compartment cells (*C*). Univacuolar refractile granulocytes were occasionally stained in the area of the cytoplasmic peripheral ring (*D*). No labeling was detectable in morula cells (*E*). Hemocytes present in the injured area of the tunic were also tested for the presence of CiC3-1 transcripts using an antisense riboprobe (*F* and *G*) or the corresponding sense probe (*H*). In *F* are indicated labeled granular amebocytes (ga) and an unstained univacuolar refractile granulocyte (urg). In *G*, several labeled compartment cells (cc) can be recognized. No staining was detected in control sections: in *H* are indicated compartment cells (cc), a morula cell (mc), and a granular amebocyte (ga).

Table I. *Immunohistochemical analysis of blood cell types in tunic sections of animals injected with PBS or LPS*

| | Cell Number after Injection with | | | |
| | PBS | | LPS[b] | |
Cell Type	Total	Positive	Total	Positive
Granular amoebocytes	5	0	73	60
Compartment cells	3	0	17	17
Morula cells	11	0	13	0
Univascuolar refractile granulocytes	4	0	15	0

[a] Average number of cells in five randomly selected tunic sections of 48 h after the injection. Counting was performed on 5-mm^2 fields on the borders of the injected area.

[b] A total of 500 μg of LPS in 0.2 ml of PBS was used in each injection.

after the injection. Five areas of 5 mm^2 were examined for both the control and LPS-treated samples. Hemocytes were 5 times as numerous in the injured tissue sections as in the control sections, with granular amebocytes ~15 times more numerous. No staining was observed when control immunocytochemical reactions were performed on PBS- or LPS-treated samples using the preimmune IgG.

To determine whether blood cell types immunostained by the anti-CiC3-1a Ab do also have CiC3-1 transcripts, in situ hybridization experiments were conducted on tunic sections of LPS-injected animals. CiC3-1 gene seemed to be expressed by only two cell types. Hybridization signals appeared in compartment cells and granular amebocytes (Fig. 5, *F* and *G*), while morula cells and univacuolar refractile granulocytes did not show any expression of the gene. This expression pattern corresponds to the anti-rCiC3-1a Ab reaction pattern, thus indicating that the two cell types, namely

compartment cells and granular amebocytes, are engaged in C3 production. No labeling was detected in control experiments conducted in parallel with the corresponding sense probe (Fig. 5*H*).

Discussion

A variety of immune defense responses such as phagocytosis, cytotoxic reaction, and encapsulation have been described in invertebrate species (26). In ascidians, these reactions have been analyzed mainly at morphological level. In particular, in *C. intestinalis*, an inflammatory reaction elicited by erythrocytes or soluble Ags injected into the tunic has been reported (27–29). Challenge to the tunic leads to the infiltration of the granular amebocytes, univacuolar refractile granulocytes, and vacuolated hemocytes. The migration of these cells from the hemopoietic nodules present in the connective tissue, which underlies the epidermis (30), to the site of injury is mediated by an array of endogenous and exogenous chemoattractant molecules whose nature is largely unknown. Indeed, most of the available data deal with the effects of cellular extracts or only partially purified hemolymph components. A true chemotactic activity, contributed by two 14- to 18-kDa hemolymph proteins (tunIL1-α and tunIL1-β), has been reported in the ascidian *Styela*, whose hemocytes also respond to LPS exposure by increasing their random, nondirected migration. The hemocyte migration in *Styela* seems to be affected by multiple factors, enhancing both hemocyte chemokinesis and chemotaxis, under the control of different molecular mechanisms whose relative contributions have not been established (31). In *Pyura stolonifera*, a partially purified 8.5-kDa peptide enhances hemocyte migration (21), but no evidence has been presented to account for specific chemotaxis rather than cell migration due to a random nondirected motility (1).

Recent reports of multiple complement components in ascidians (14, 19, 20) had prompted us to investigate the presence of complement-mediated chemotaxis in the ascidian *C. intestinalis*, as a means of verifying the presence of the inflammatory pathway of the complement system in the deuterostome invertebrates.

In a first series of in vitro experiments, we found that *C. intestinalis* CFH contained factors that promote hemocyte migration. Then we demonstrated that the source of the chemoattractant in the hemolymph are the hemocytes, which, when isolated and LPS activated, release factors mediating true chemotaxis in the medium. The ability of the specific anti-rCiC3-1a IgG to inhibit this activity pointed to CiC3-1a as the molecule responsible for the hemocyte directional migration. To better characterize this activity, we performed chemotaxis experiments using the protein rCiC3-1a, representing the C3a fragment of *Ciona* C3-1 molecule. These experiments indicated that rCiC3-1a promotes true chemotaxis, because the cells migrated only when the chemoattractant was added in the lower well, creating a concentration gradient between the lower and upper chemotaxis wells. The rCiC3-1a chemotactic activity was dose dependent and specific, in that the anti-rCiC3-1a Ab was able to inhibit the rCiC3-1a-mediated chemotaxis. Similarly, the anti-rCiC3-1a Ab significantly inhibited migration of hemocytes toward CFH, showing that CiC3-1a is constitutively present in the hemolymph apart from the LPS activation of the hemocytes. The concentration range of activity of rCiC3-1a toward *Ciona* hemocytes and of human C3a toward eosinophils, respectively, is almost completely superimposable, but the percentage of attracted hemocytes was 17.5% in ascidians and 50% in humans (7). This discrepancy may be accounted for by the fact that in humans the effect has been demonstrated on a homogeneous cell type (7). Furthermore, minor differences in experimental procedure, as well as different methods of cell counting, make direct comparisons difficult (8, 9).

Previous studies have indicated that in mammals the C-terminal domain of the anaphylatoxin C3a is the effector site of the molecule (6, 32, 33). Likewise, we found that the chemotactic activity of *Ciona* C3-1a is localized to the C terminus of CiC3-1a, because a synthetic peptide representing the 18 C-terminal amino acids of CiC3-1a (CiC3-1a$^{59-76}$) promoted dose-dependent hemocyte chemotaxis. This activity was only slightly reduced when we substituted the desArg peptide, CiC3-1a$^{59-75}$. Furthermore, pretreatment with pertussis toxin abolished almost completely the rCiC3-1a-mediated activation, indicating the involvement of a G_i protein in complement-activated signal transduction in *C. intestinalis* hemocytes.

Analysis of C3a molecules from mammalian species has identified two regions, the anaphylatoxin domain and the C-terminal portion, as relevant from a structural and functional viewpoint (6). In particular, the anaphylatoxin domain is characterized by six cysteines in conserved positions, and structural studies have indicated that three disulfide bonds stabilize a tightly packed core consisting of four antiparallel helical regions. CiC3-1a does not possess a canonical anaphylatoxin domain. In fact, the CiC3-1a sequence exhibits five cysteine residues, and only four of them are in conserved positions, suggesting the presence of two disulfide bonds. Other highly conserved amino acid residues of the anaphylatoxin domain are present in CiC3-1a, together with a series of cationic side chains (Fig. 1).

The mammalian C3a C-terminal region is ~10 residues long, in a random coil conformation, with the conserved terminal pentapeptide sequence L-G-L-A-R. Although there is a general consensus that in all C3a anaphylatoxins the C-terminal portion represents the essential effector site for the recognition and binding to the specific membrane receptors, both the extent and the primary structure of the C-terminal sequence contributing to the anaphylatoxin function are the subject of intense investigations. In a recent study, the use of synthetic C3a analogues that mimic the sequences of this region has allowed researchers to establish that the tripeptide LAR is essential for the binding to eosinophils and crucial for the induction of biological effects, such as changes of intracellular Ca^{2+} concentration and the release of reactive oxygen species (33). More recently, it has been demonstrated that C3a desArg also retains some of the immunological functions of C3a through its binding to the FcϵRI. According to these findings, cells express two kinds of receptors: C3aR, which probably binds only C3a, and FcϵRI, which binds C3a and C3a desArg (34). The *Ciona* C-terminal region differs in length and composition from the complement mammalian anaphylatoxins, being 1 and 2 aa residues shorter than mammalian C3a and C5a, respectively. In addition, more cationic side chains are localized to this region. The *Ciona* C-terminal pentapeptide sequence R-V-Q-G-R does not fit with any of the anaphylatoxin sequences thus far identified, including that of C4a. Our demonstration of chemoattractant activity in both CiC3-1a$^{59-76}$ and CiC3-1a$^{59-75}$ indicates that in *Ciona* the effector site is also localized to the C-terminal end of CiC3-1a molecule. Our results further suggest that the mammalian consensus sequence of the last amino acid residues cannot be extended to invertebrate species (Fig. 1). Furthermore, these results could account for the interaction either with an anaphylatoxin receptor similar to the FcϵRI, which binds both C3a and C3a(desArg), or, as in mammals, with receptors exhibiting different ranges of specificities.

The lower activity of the C-terminal peptide with respect to the entire molecule could indicate that other parts of the *Ciona* anaphylatoxin contribute to the functional activity. Indeed, in humans, a two-site C3a/C3aR interaction model has been suggested in

which the C-terminal sequence G-L-A-R engages a primary effector site in the receptor, while a secondary binding interaction involves the positively charged C-terminal helical region of C3a and the two negatively charged regions of the large extracellular loop of the receptor (6, 35). The presence of positively charged amino acid residues in the *Ciona* sequence corresponding to the C-terminal helical region of human C3a (36) may point to a two-site interaction model in this species as well. The results of current studies aimed at identifying the C3-1a receptor should allow us to elucidate the molecular mechanisms underlying the C3a/C3a-receptor interaction in deuterostome invertebrates.

In the tunic of *C. intestinalis*, ameboid hemocytes with cytoplasmic granules were identified within 48 h of the injection of an inflammatory agent. The distribution and number of ameboid granulocytes varied depending on the observed tunic sections and individual variability. However, a significantly increased density in the granulocyte population has consistently been seen in comparison with PBS-treated animals (28, 29, 37). Our immunohistochemical analysis of tunic sections from LPS-injected animals showed that the total hemocyte number in the injured area was 5 times higher than in the control, and a 15-fold increase in granular amebocytes was observed in the injured area. At the same time, granular amebocytes and compartment cells were actively engaged in the production of C3, reaching the highest level of expression at 48 h after LPS injection.

To discriminate the cells producing from those internalizing the Ags, we performed in situ hybridization experiments. This analysis, conducted using a riboprobe corresponding to the 3′-terminal sequence of CiC3-1, revealed the specific expression of this gene in only two types of blood cells, which we were able to identify as granular amebocytes and compartment cells. These results, while confirming the engagement of granular amebocytes and compartment cells in CiC3-1 synthesis, do not allow establishing whether they are also able to bind and internalize the protein. Univacuolar refractile granulocytes, occasionally exhibiting a weak immunostaining in the area of the cytoplasmic peripheral ring, did not show any hybridization signal, thus suggesting that these cells could only internalize CiC3-1a through a specific membrane receptor.

Taken together, these findings indicate that C3-1a functions as a chemotaxin for *Ciona* hemocytes and that the C3-1a-mediated hemocyte recruitment to sites of injury may play an important role in the inflammatory process. To our knowledge, this is the first report clearly demonstrating an inflammatory effector mechanism as part of the complement system in deuterostome invertebrates, in which until now only pathogen opsonization had been demonstrated (18). Our findings have particular relevance because of the unique evolutionary position of the ascidian *C. intestinalis*, which as an invertebrate chordate is phylogenetically located at the base of the vertebrate lineage. This species, which predates the emergence of the adaptive immune system, already possesses elements of an advanced innate immune response, in the form of the alternative and lectin-mediated complement pathways. It has been suggested that gene duplications and/or genome-wide duplication events, which are believed to have occurred twice in the early stages of vertebrate evolution (38), have been important in the evolution of the complement system. This seems to be the case for C4 and C5 molecules, which originated through duplication events that occurred in the jawed vertebrates (39, 40). Hence, the immune system in *C. intestinalis* offers the opportunity to study specific physiological functions that can be attributed solely to C3a, in an immunological context far removed from the complexity of the mammalian system.

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