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Human T Cells Express the C5a Receptor and Are Chemoattracted to C5a

Serge Nataf,* Nathalie Davoust,* Robert S. Ames,† and Scott R. Barnum2*

The anaphylatoxin C5a is a potent mediator of inflammation that exerts a broad range of activity on cells of the myeloid lineage. In this study, we present the first evidence that human T cells express the C5a receptor (C5aR) and are chemoattract to C5a. Using FACS analysis, we found that the C5αR was expressed at a low basal level on unstimulated T cells and was strikingly up-regulated upon PHA stimulation in a time- and dose-dependent manner. CD3+ sorted T cells as well as Jurkat T cells were shown to express C5αR mRNA as assessed by RT-PCR. Moreover, semiquantitative RT-PCR analysis demonstrated that C5αR mRNA was down-regulated in purified T cells upon long-term PHA stimulation. To demonstrate that C5α was biologically active on T cells, we investigated the chemotactic activity of C5α and observed that purified CD3+ T cells are chemoattractive to C5α at nanomolar concentrations. Finally, using a combination of in situ hybridization and immunohistochemistry, we showed that the T cells infiltrating the central nervous system during experimental allergic encephalomyelitis express the C5αR mRNA. In summary, these results suggest that C5α exerts direct effects on T cells and could be involved in the trafficking of T cells under physiological and pathological conditions, including inflammatory diseases of the central nervous system. The Journal of Immunology, 1999, 162: 4018–4023.

Activation of the complement system generates the anaphylatoxins C3a, C4a, and C5a. Of these mediators, C5α elicits the broadest range of activity, including the degranulation of mast cells and basophils (1), increased vascular permeability (2), and the induction of cytokine synthesis by leukocytes (3, 4). C5α is also known as a powerful chemotactic factor for all human myeloid lineages, especially eosinophils (5, 6). Interestingly, studies have suggested that C5α exerts biological effects on lymphoid cells as well as myeloid cells. C5α was shown to be chemotactically active for a subset of murine B cells (7) and for a murine pre-B cell line transduced with the human C5α receptor (C5αR)3 (8). C5α also enhances Ag- and alloantigen-induced T cell proliferation as well as autologous T cell responses in vitro (9, 10).

In addition, recent studies indicate that C5α could be an important mediator of CD4+ T cell-mediated delayed-type hypersensitivity in vivo (11, 12). The biological activities of C5α are mediated through its binding to the C5αR, a protein G-coupled seven transmembrane domain receptor that is widely expressed on myeloid cells, hepatocytes, astrocytes, and neurons (13–17). The C5αR has a high affinity for its ligand (~1 nM) and a molecular mass ranging from ~40 to 65 kDa depending upon the cell type expressing the receptor (6). Like most chemoattractant receptors, C5α-induced chemotaxis requires C5αR coupling to the pertussis toxin-sensitive α subunit of Gi proteins (Giα) (18).

The trafficking of T cells under physiological or pathological conditions involves numerous chemoattractant receptors, the majority of which have been cloned only recently (19). However, until now there was no direct proof of C5αR expression in human T cells. In this report, we provide the first evidence that the C5αR is expressed by human T cells at the protein and mRNA level. Moreover, we demonstrate that human rC5α is a chemotactic factor for unstimulated and PHA-stimulated T cells, and that C5αR+ T cells are present in abundance in the central nervous system (CNS) of rats with experimental allergic encephalomyelitis (EAE). These data suggest that C5αR may play an important role in the trafficking of disease-causing T cells into the CNS.

Materials and Methods

Reagents

Human rC5α was prepared as described previously (20). Mouse anti-human CD3, CD4, and CD8 Abs were purchased from Serotec (Washington, DC). FITC-labeled goat anti-rabbit IgG, phycoerythrin-labeled goat anti-mouse IgG, and unlabeled mouse control IgG were supplied by Southern Biotechnology Associates (Birmingham, AL). Unlabeled rabbit control IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Preparation and characterization of rabbit anti-human C5αR Ab

Rabbit anti-human C5αR Ab was prepared by immunizing animals with a peptide corresponding to amino acids 7–24 (TPPDYGHYDDKDTLD LNT) of the extracellular amino-terminus of C5αR, using the multiple antigenic peptide resin technology (Research Genetics, Huntsville, AL). The antisera were characterized by ELISA using the immunizing peptide and by flow cytometry Ab staining in which the Ab bound to the rat basophilic leukemia cell line RBL-2H3 (American Type Culture Collection, Manassas, VA) stably transfected with human C5αR cDNA but not with cells transfected with vector alone.

Cells, cell lines, and cell culture conditions

RBL-2H3 cells stably transfected with either human C3α receptor (C3αR) cDNA or human C5αR cDNA were grown as described previously (21).
Jurkat T cells (kindly provided by Dr. Ed Blalock, Department of Physiology and Biophysics, University of Alabama at Birmingham) were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. In most experiments, human lymphocytes were isolated by elutriation from PBLs of healthy donors. Otherwise, mononuclear cells were isolated by density gradient centrifugation on LymphoH (Atlanta Biologicals, Norcross, GA) and T cell enrichment was performed using nylon wool columns. Both of these procedures yielded lymphocytes of 85–95% purity as assessed by flow cytometry using anti-CD3 Ab staining. When needed for the assessment of C5αR mRNA by RT-PCR, highly purified T cell populations were obtained by cell sorting CD3+ cells on a cell sorter (FACSCalibur, Becton Dickinson, Heidelberg, Germany) under sterile conditions. T cells maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 50 μM 2-ME, 100 IU/ml penicillin, and 100 μg/ml streptomycin were cultivated with or without 2.5 or 10 μg/ml PHA (Sigma) for 1–60 h. In some experiments, T cells were treated with cycloheximide (CHX) (10 μg/ml) (Sigma, St. Louis, MO) without PHA stimulation or starting at 2 h before PHA stimulation.

Flow cytometric analysis

In each experiment, 0.5 × 10^6 to 1 × 10^6 cells of the enriched T cell preparation were incubated on ice for 30 min with polyclonal rabbit anti-C5αR IgG alone or in combination with anti-CD3, anti-CD4, or anti-CD8 mAbs. Single staining experiments with the CD markers mentioned above were performed in parallel. As a control, cells were incubated with the same concentration of nonimmune polyclonal rabbit IgG or unlabeled control mouse IgG. After two washes, cells were stained with FITC-conjugated anti-rabbit IgG and/or phycoerythrin-conjugated anti-mouse IgG or PBS containing 2% FCS to evaluate nonspecific binding of the secondary antibody. In all cases, Abs were used at saturating concentrations. Next, cells were washed twice in PBS, assessed in a FACSscan flow cytometer, and analyzed by CellQuest software (Becton Dickinson).

Standard and semiquantitative RT-PCR analyses

Total RNA was isolated from unstimulated and PHA-stimulated enriched T cell preparations, unstimulated and PHA-stimulated cell–sorted T cells, Jurkat T cells, C5αR-transfected RBL cells (RBL-C5αR), and C3αR-transfected RBL cells (RBL-C3αR). RNA was extracted using RNA-STAT (Tel-Test B, Friendswood, TX) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed as described previously (15). The reverse-transcribed products were amplified using primers for C5αR (based on sequences 400–421 and 919–940) (obtained from Stratagene, La Jolla, CA) and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (based on sequences 363–386 and 731–755) (kindly provided by Meg Mosteller-Barnum, Department of Gastroenterology, University of Alabama at Birmingham). Samples were amplified for 35 cycles for C5αR and 25 cycles for GAPDH. In semiquantitative RT-PCR experiments, various numbers of PCR cycles were performed followed by extension for 10 min as described previously (22). In all cases, products were separated on 1.5% agarose gels containing ethidium bromide for visualization and transferred to Nylon filter paper (Micron Separations, Westborough, MA) for Southern blot analysis as described previously using 32P-labeled C5αR and GAPDH cDNA fragments labeled by the random prime method with a kit (Promega), and probes were labeled with digoxigenin-UTP (Boehringer Mannheim). To identify T cells expressing C5αR mRNA, ISH was performed using anti-TCR mAb (Serotec) specific for T cells. Infiltrating macrophages or activated microglia were identified using the ED1 mAb (Serotec), which binds to a specific moiety expressed on these cells. Immunohistochemistry was initially performed according to an immunoenzymatic staining method supplied by the manufacturer (Vectastain ABC kit, Vector, Burlingame, CA) and as described elsewhere (24). The use of RNase free reagents including diethyl pyrocarbonate-treated phosphate buffer allowed good preservation of the mRNA before ISH was performed. As a control, and to verify that immunohistochemistry procedures did not interfere with ISH, an isotype-matched mouse mAb was used for immunohistochemistry; ISH was performed using both a sense and an antisense C5αR probe.

Migration assay

The ability of human rC5α to chemoattract T cells was evaluated using transwell plates (Costar, Cambridge, MA) with polycarbonate inserts with 8-μm pores (Costar 3422) as described previously (25). Wells with inserts were loaded with 200 μl of migration media (MM) consisting of RPMI 1640 and 0.5% BSA (fraction V; Sigma) and were incubated for 1 h at 37°C, 5% CO2. Next, lower wells were aspirated and loaded with 200 μl of MM alone or containing rC5α at concentrations of 1 or 10 nM. For each condition, experiments were conducted in triplicate. To avoid any possibility of T cell stimulation through the cross-linking of CD3, we purified T cells by negative isolation. Unstimulated T cell preparations were stained with an anti-CD11b Ab (Boehringer Mannheim) recognizing monocyte/macrophages and with an anti-CD19 Ab (Becton Dickinson) recognizing B cells. Cells negative for both markers were sorted (FACSCalibur, Becton Dickinson) under sterile conditions. The purity of the T cell population isolated by this procedure was analyzed by FACS analysis and was 98–99% CD3+.

Results

C5αR is expressed on human T cells and up-regulated upon PHA stimulation

Using a rabbit polyclonal anti-C5αR Ab, we observed that a subset of PBL-derived unstimulated T cells were labeled by FACS analysis (Figs. 1 and 2; Table I). The percentage of C5αR and CD3 double-positive cells in fresh preparations of T cells varied between individuals, ranging from 1 to 8.6%, and could be up-regulated after 24 h of stimulation with either 2.5 or 10 μg/ml PHA, T cells isolated by this procedure consistently demonstrated a striking up-regulation of C5αR expression on both CD4 and CD8 subsets compared with cells cultivated for 60 h without PHA (Table I). Moreover, in kinetic studies, PHA-induced C5αR up-regulation was clearly time-dependent and could be observed as early as 1 h after PHA stimulation and continued to increase for the remaining timepoints (Fig. 2). A PHA-induced up-regulation of C5αR appears to be, in large part, independent of protein synthesis, as treatment with CHX inhibited increased C5αR expression by only 16% and 29% after 4 and 12 h of exposure, respectively (Fig. 3). Moreover, T cells treated with CHX for 2 h and then stimulated with PHA for 1 h showed a striking increase in C5αR expression that was comparable with the level observed in the absence of CHX (Fig. 3). It is also worth noting that CHX treatment for ≥ 24 h did not alter the constitutive expression of C5αR on T cells (data not shown). To confirm C5αR expression on the T cell lineage, we also examined the Jurkat T cell line and found a low constitutive level of C5αR expression by FACScan analysis (data not shown). However, in contrast to PBL-derived T cells, C5αR expression on Jurkat cells was not reproducibly up-regulated upon PHA stimulation (data not shown).
C5aR mRNA is expressed in PBL-derived T cells and Jurkat T cells

Using Northern blot analysis, we were not able to readily detect C5aR mRNA in enriched preparations of human T cells. Therefore, to further document C5aR expression on T cells, C5aR mRNA expression was assessed by RT-PCR analysis of total RNA extracted from unstimulated and PHA-stimulated T cell preparations as well as Jurkat T cells. Total RNA from RBL-C5aR and RBL-C3aR was also analyzed as a positive and a negative control, respectively. In all samples except RBL-C3aR, an appropriately sized band of 550 bp was amplified (Fig. 4A). In parallel, samples were amplified using the GAPDH primers as an internal control (data not shown). To ensure that the amplified C5aR mRNA was actually derived from T cells, RT-PCR experiments were also performed on cell-sorted CD3⁺ cells. As shown in Fig. 4B, the same results were obtained, thus formally demonstrating that C5aR mRNA is expressed in human T cells, although at a low level.

C5aR mRNA is down-regulated in PHA-stimulated T cells

To assess C5aR mRNA regulation in PHA-stimulated T cells, semiquantitative RT-PCR experiments were performed using GAPDH amplification products as a measure of input RNA. Based on our flow cytometry data, we chose to analyze C5aR mRNA expression in T cells stimulated with 10 μg of PHA for 60 h, a timepoint for which C5aR expression is maximal. In two independent experiments, enriched T cell preparations were cultured for 60 h with or without PHA, sorted for CD3 expression, and analyzed for C5aR expression by semiquantitative RT-PCR. Surprisingly, we found that C5aR mRNA decreased by >50% in PHA-stimulated T cells compared with unstimulated T cells (Fig. 4C).

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FIGURE 1. Flow cytometric analyses of PBL-derived T cells using C5aR Ab. PBLs obtained from healthy donors were cultured for 24 h with or without stimulation with 2.5 μg/ml PHA and stained with a control nonimmune rabbit IgG (A and D) or rabbit polyclonal anti-C5aR Ab (B and E). In parallel, PBLs were double-stained using C5aR and CD3 Abs (C and F). The fraction of PBLs positive for C5aR (B and E) or C5aR and CD3 (C and F) is indicated. One representative experiment of three is shown.

FIGURE 2. Kinetics of C5aR expression on PHA-stimulated, PBL-derived T cells. Flow cytometric analyses were performed on PBLs obtained from healthy donors and grown without PHA (A) or with 10 μg/ml PHA for 1 h (B), 24 h (C), or 48 h (D). PBLs were double-stained using C5aR and CD3 Abs, and quadrant markers were based on the staining of control rabbit IgG and mouse IgG, respectively. For each timepoint, the fraction of PBLs positive for C5aR and CD3 is indicated. One representative experiment of three is shown.
Human T cells are chemotactic to C5a

To determine whether the C5aR on T cells was functionally active, we decided to evaluate the ability of C5a to chemotactically purify T cells. Three independent experiments were performed using fresh PBL-derived T cells that had been isolated by negative selection as described in Materials and Methods. T cells were then examined for their ability to migrate in response to human rC5a. The results presented in Fig. 5 demonstrate that C5a at concentrations as low as 1 nM reproducibly and significantly induced chemotaxis of unstimulated T cells. In one set of experiments, fresh T cells were stimulated with PHA (10 μg/ml) for 1 h, cell-sorted, and used in a chemotactic assay. As shown in Fig. 5, the results of these experiments demonstrate a dramatic increase in the number of T cells that migrated in response to C5a when compared with MM alone. Interestingly, with knowledge of the total number of loaded cells and the total number of migrated cells, we were able to evaluate the percentage of loaded cells that migrated in response to C5a. We found that ~7% of loaded cells migrated in the experiments using freshly isolated T cells, whereas 15% of loaded cells migrated in the experiments using PHA-stimulated T cells.

C5aR is expressed on T cells infiltrating the CNS of rats with EAE

Recently, we analyzed the kinetics of C5aR expression during EAE in the Lewis rat (24). Using ISH and immunohistochemistry, we observed that numerous blood-derived infiltrating cells expressed C5aR mRNA but did not belong to the monocyte/macrophage lineage based on staining experiments using the ED1 Ab (24). To verify whether some of the C5aR+ cells were T cells, we performed a combination of immunohistochemistry using an anti-TCR Ab and ISH for the C5aR on spinal cord sections from animals sacrificed at the clinical peak of the disease (Fig. 6). Staining with the anti-TCR Ab alone identified a number of infiltrating T cells in the spinal cords of animals with EAE (Fig. 6A). An isotype-matched control Ab did not show any immunoreactivity (Fig. 6B). The combination of immunohistochemistry and ISH demonstrated that a large proportion of the cells recognized by the anti-TCR Ab strongly expressed the C5aR mRNA (Fig. 6C). These cells were located in the parenchyma in or around blood vessels as well as in the meninges. Using a C5aR sense probe, we did not observe any double-staining, although T cells could be easily recognized by immunohistochemistry (Fig. 6D). In parallel experiments, we were also able to demonstrate C5aR mRNA by ISH on cytosin preparations of PBL-derived human T cells (data not shown).

Discussion

In this study, we examined the expression of C5aR on human T cells at the protein and mRNA level. We observed that C5aR as...
assessed by flow cytometry was expressed by a small subset of unstimulated T cells and was strikingly up-regulated upon PHA stimulation in a dose- and time-dependent manner. These findings are in accordance with previous studies showing that C5aR expression is up-regulated on different cell types upon LPS or phorbol ester stimulation (14, 26). The fact that the C5aR was strongly induced on T cells after only 1 h of PHA stimulation suggests that this regulatory process takes place at the posttranscriptional level. Indeed, C5aR turnover at the cell surface of polymorphonuclear leukocytes was shown to be independent of protein synthesis (27). A similar result was observed for T cells, because CHX did not dramatically alter the PHA-induced up-regulation of C5aR (Fig. 3). These data suggest that the increased C5aR expression induced by PHA is not due, at least at early timepoints, to de novo synthesis. In addition, these data are consistent with the hypothesis that T cells have significant intracellular pools of the C5aR. Experiments are currently in progress to investigate the intracellular trafficking of C5aR in T cells and to determine whether PHA stimulation alters the turnover of C5aR from the cell surface to intracellular pools of preformed C5aR proteins.

Using RT-PCR, we were able to detect C5aR mRNA in both human T cells and Jurkat T cells. Indeed, other cell types such as astrocytes and endothelial cells also express low amounts of C5aR mRNA despite readily detectable expression of C5aR protein at the cell surface (15, 28). Surprisingly, in semiquantitative RT-PCR experiments, we found that PHA-stimulated T cells express a lower amount of C5aR mRNA compared with unstimulated T cells. This result further supports the hypothesis that C5aR up-regulation on the cell surface is regulated posttranscriptionally in PHA-stimulated T cells.

To investigate the function of C5aR on human T cells, we performed chemotaxis assays and found that human rC5a was able to chemoattract unstimulated and PHA-stimulated T cells. This result

![FIGURE 5](image-url)  
**FIGURE 5.** Human rC5a is chemotactic for T cells. Three independent experiments were performed in which a pure population of T cells was obtained by negative selection cell sorting. In one set of experiments, T cells were stimulated for 1 h with PHA 10 μg/ml before use in the chemotactic assay. The chemotactic activity of human rC5a (1 and 10 nM) was evaluated in triplicate using transwell plates as described in Materials and Methods. As a control, lower chambers were loaded with MM alone. In each well, the same number of T cells was loaded in the upper chamber; plates were incubated for 3 h at 37°C. The values on the y-axis represent the mean number of T cells that migrated into the bottom well. The results obtained from three different unstimulated T cell preparations were pooled, and error bars are the SD of nine experiments for each condition. One experiment was performed using PHA-stimulated T cells, and error bars are the SD of triplicate experiments. *, p < 0.03; **, p < 0.015 (Student’s t test).

![FIGURE 6](image-url)  
**FIGURE 6.** Expression of the C5aR by T cells in EAE. Spinal cords from EAE rats that had been sacrificed on day 12 postimmunization were analyzed by a combination of ISH for the C5aR and immunohistochemistry using an anti-TCR Ab to identify T cells, as described in Materials and Methods. A, Immunohistochemistry staining for the TCR using an anti-TCR mAb (dark brown staining) in EAE rat spinal cord parenchyma. B, Control immunohistochemistry using an isotype-matched IgG control Ab in place of the anti-TCR mAb. C, ISH using a C5aR antisense riboprobe (purple color) combined with immunohistochemistry staining using the anti-TCR Ab to identify T cells. The inset shows a higher magnification of TCR/C5aR+ cells. D, ISH using a C5aR sense riboprobe combined with immunohistochemistry staining using the anti-TCR Ab in EAE rat spinal cord parenchyma. The inset shows a higher magnification of TCR+ cells, with no background staining using the C5aR sense riboprobe. Original magnifications: A and B, ×100; C and D, ×200.
is in agreement with previous work showing chemotaxis of enriched preparations of human T cells toward C5a (29). It is worth noting that the percentage of migrating cells was evaluated at 7% of loaded cells, which is in the range of unstimulated T cells expressing C5aR (between 1 and 8.6%). Interestingly, the percentage of migrating cells reached ~15% of loaded cells when T cells were stimulated for 1 h with 10 μg/ml PHA before the chemotactic assay. Thus, in both unstimulated and PHA-stimulated T cells, the ability of C5a to chemotact human T cells appears to correlate with the number of C5aR-expressing T cells. This finding, along with the fact that C5a chemoattracts T cells at nanomolar concentrations, argues for a C5aR-mediated mechanism. However, we cannot exclude the possibility that C5a binds to another receptor that is not recognized by our Ab and/or that C5a induces the synthesis of soluble mediators that, in turn, exert chemotactic activities. Previous studies have described different C5aR subtypes with different biochemical characteristics and various affinities for their ligands (6, 17). Similarly, the cell-specific effects of C5a may occur through a differential regulation of C5aR protein G coupling. Thus, it is conceivable that C5a may elicit different types of responses from activated T cells depending upon the input stimuli, the T cell subtype (CD4 vs CD8), and/or the profile of cytokine secretion (Th1 vs Th2).

Interestingly, using a combination of ISH and immunohistochemistry, we were able to detect C5aR mRNA in T cells infiltrating the CNS of rats with EAE. This is of particular interest because C5aR-expressing cells invade the CNS during EAE far before the onset of clinical symptoms (24). This finding raises the possibility that at the earliest stages of the disease, activated T cells could be chemotacted in the CNS through an interaction between the C5aR and its ligand.

The experiments reported in the present study clearly demonstrate that human T cells express the C5aR at the protein and mRNA level and are chemotactic toward human rC5a. These results are in accordance with recent findings showing that C5a plays a major role in vivo in the recruitment of delayed-type hypersensitivity effector CD4+ T cells during the elicitation of contact sensitivity (11, 12). Similarly, anti-C5 mAbs were shown to inhibit collagen-induced arthritis, a T cell mediated autoimmune disorder, when administered before or during the effector phase of the disease (30). Additional studies are needed to determine whether an Ag-driven stimulation can up-regulate C5aR expression on human T cells. In addition to chemotaxis, C5a elicits a broad range of activities on cells of the myeloid lineage and may exert similar effects on T cells, including cytokine synthesis and an augmentation of adhesion to endothelial cells and/or the extracellular matrix (4, 28). The demonstration of C5aR-expressing T cells in the CNS of EAE rats also suggests that C5a could be involved in T cell trafficking in the CNS. This hypothesis is currently being tested using C5aR-deficient mice.

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