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rC5a Directs the In Vitro Migration of Human Memory and Naive Tonsillar B Lymphocytes: Implications for B Cell Trafficking in Secondary Lymphoid Tissues

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Human C5a is a potent chemoattractant for granulocytes, monocytes, and dendritic cells. In mice C5a has been shown to bechemotactic for germinal center (GC) B cells. To date, no information is available on the effects of C5a on human B cell locomotion. Here we demonstrate that rC5a increases polarization and migration of human tonsillar B cells. The locomotory response was due to both chemokinetic and chemotactic activities of rC5a. Moreover, memory and, at a lesser extent, naive B cell fractions from purified tonsillar populations displayed rC5a-enhanced migratory properties, whereas GC cells did not. Flow cytometry revealed C5aR (CD88) on approximately 40% memory and 10% naive cells, respectively, whereas GC cells were negative. Immunohistochemistry showed that a few CD88+ cells were of the B cell lineage and localized in tonsillar subepithelial areas, where the majority of memory B cells settle. Pretreatment of memory B cells with the CD88 mAb abolished their migratory responsiveness to rC5a.

Finally, the C5 gene was found to be expressed in naive, GC, and memory B lymphocytes at both the mRNA and the protein level. This study delineates a novel role for C5a as a regulator of the trafficking of human memory and naive B lymphocytes and supports the hypothesis that the B cells themselves may serve as source of C5 in secondary lymphoid tissues. The Journal of Immunology, 1999, 162: 6510–6517.

The immune response to foreign Ags is operated by innate and Ag-specific effector mechanisms that act in concert to eliminate noxious agents. A well-regulated trafficking of immunocompetent cells, particularly B and T lymphocytes, is crucial for a fast and efficient immune response to take place (1).

Leukocyte migration depends on the expression of specific adhesion molecules that allow selective cell homing to different anatomical districts and on the delivery of chemotactic signals that trigger cell locomotion (1, 2). Chemoattractants are heterogeneous in nature and include bacterial components, cytokines, and complement fragments (3). The role of cytokines in the control of human T and B lymphocyte migration is well established (4–16); in contrast, little information is available on the chemotactic activity of complement components vs human lymphocytes. In this study we have investigated the in vitro migratory response of human tonsillar B lymphocytes to rC5a based upon the following rationale: 1) murine germinal center (GC) B cells isolated during an ongoing immune response migrate in vitro upon exposure to C5a (17); and 2) mice with targeted disruption of the C3 and C4 genes display defects in humoral immunity and in the formation of GC (18). The latter findings suggest that C3 or C4 or complement components downstream that are not generated in these knockout animals are involved in the control of B cell trafficking.

Together with C3a and C4a, C5a is one of three low molecular mass peptides referred to as anaphylatoxins (19). The classic and alternate C5 convertases cleave C5a from the 190-kDa C5a disulfide-linked heterodimer; thereafter, C5a is released in fluid phase (20). C5a binds to a specific surface receptor known as CD88 (21, 22). CD88 is a 40-kDa molecule belonging to the G protein-linked transmembrane segment superfamily and expressed on mast cells, basophils, endothelial cells, neutrophils, monocytes-macrophages, lung bronchial and epithelial cells, vascular smooth muscle cells, and hepatocytes (23). The binding of C5a to CD88 is followed by signal transduction and initiates a wide range of proinflammatory effects (23). For example, C5a promotes chemotaxis and chemokinesis of neutrophils (24) and increases their adhesiveness by up-regulating CD11b (25). Recently, it has been shown that human dendritic cells (26) and mast cells (27) also migrate in response to rC5a. To date, there has been no demonstration of the expression of CD88 on human B lymphocytes or of a chemotactic activity of C5a toward these cells.

Here we show that human tonsillar B cells, in particular the memory and naive cell populations, express CD88, polarize, and migrate in vitro upon incubation with rC5a. The essential role of CD88 in rC5a-triggered B cell locomotion was demonstrated by the abrogation of the phenomenon upon pretreatment of B cell suspensions with a CD88 mAb.

Materials and Methods
Cell separation
Mononuclear cells were isolated from surgically removed tonsils on Ficoll-Hypaque density gradients and were depleted of T cells by rosetting with neuraminidase-treated SRBC. Non-T cells were subsequently deprived of...
macrophages and NK cells by incubation with the CD68 and CD56 mAbs (see below) followed by rosetting with ox-E coated with a goat anti-mouse Ig antiserum (28). The resulting cell preparations contained consistently >98% B cells, as assessed by staining with a CD19 mAb.

Fractionation of total tonsillar B lymphocytes into GC, naive, and memory cells was performed as follows. Naïve B lymphocytes were isolated as IgD− cells from total B lymphocyte suspensions by immune rosetting (29). The IgD− B cell fractions were further separated into CD38+ (GC) cells and CD38− (memory) cells by immune rosetting (29). All the above separation procedures were performed at 4°C to prevent spontaneous apoptosis of GC B cells.

In some experiments, tonsillar B lymphocytes or their purified cell subsets were cultured for the indicated times with or without a CD40 mAb (1 µg/ml; see below) and 10 ng/ml IL-4 (Genzyme, Milan, Italy).

Immunophenotypic analysis

The murine mAbs used in this study were CD3, CD10, CD19, CD23, CD56, CD68, and anti-human IgD (Dako, Glostrup, Denmark); CD39 and CD40 from Immunotech (Marseillels, France); and CD88 from SeroLogic (Oxford, U.K.). The CD38 mAb used throughout this study (clone IB4) was produced by one of us (F.M.) (30). Goat anti-human IgM and IgG mAbs and the FITC-conjugated goat anti-mouse Ig antiserum were obtained from Dako. In brief, B cells (5 × 10^5) were treated at 4°C for 30 min with optimal concentrations of mAbs, washed twice in PBS supplemented with 2% FCS, and further incubated with FITC-conjugated goat anti-mouse IgG. B cells were fixed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA). Controls were untreated cells, cells treated with FITC-second reagent alone, and cells treated with irrelevant isotype-matched mAb followed by the FITC-second reagent.

Polarization assay

The B cell fractions were resuspended in RPMI 1640 medium supplemented with 0.1% BSA (Sigma, St. Louis, MO) and mixed with human rC5a (Sigma) at concentrations ranging from 0.1–10,000 nM. Experiments were conducted using polystyrene round-bottom tubes (LP, Milan, Italy). The tubes, containing 200 µl of cell suspension at a concentration of 2 × 10^5/ml, were incubated for 20 min at 37°C; thereafter, 200 µl of 10% formaldehyde was added. The fixed cell suspensions were examined at ×400 magnification. At least 200 cells were counted in each preparation. Cells deviating from a spherical outline were scored as polarized and expressed as a percentage of the total number of cells counted (31).

Migration assay

Cell locomotion was studied using the leading front method (32). Tests were conducted in duplicate using blind well chambers (Nucleopore, Cambridge, MA) with an 8-µm pore size cellulose ester filter (SCWP0 1300, lot R4 MMS8776, Millipore, Milan, Italy) separating the cells (4 × 10^5) from the chemoattractant tested at different concentrations or from medium alone (control). After incubation at 37°C for 2 h, the filters were removed, fixed in ethanol, stained with Harris hematoxylin, dehydrated, cleared with xylene, and mounted in Eukitt (Kindler, Freiburg, Germany). Duplicate chambers were run in each case, and the distance (microns) travelled by the leading front of cells was measured at ×400 magnification. Five randomly chosen fields were read for each filter. In some experiments, B cell fractions were preincubated with the CD88 mAb (10 µg/ml) or an isotype-matched mAb of irrelevant specificity for 30 min at 4°C and washed. Thereafter, the migration assays were conducted as described above.

The filters were also scored by counting the number of cells per high power field at intervals of 10 µm into the depth of the filter. Five measurements were performed at each focal plane, and the mean of the determinations from the duplicate filters was calculated. The results obtained were analyzed, plotting the log_2 of the number of cells per high power field vs the square of the distance travelled by the cells, according to the method of Zigmond and Hirsch (33).

Collagen invasion assay

Type I collagen solution was purchased from Sigma (catalogue no. C4580, lot 126H4667). Gels were prepared according to the Sigma protocol. Briefly, 800 µl of collagen solution was mixed with 100 µl of 10× Earle’s buffered saline and 100 µl of reconstitution buffer (2.2% sodium bicarbonate in 0.8 N sodium hydroxide) to restore collagen to physiological pH and osmolality. Thereafter, collagen solution (final gel concentration, 0.88 mg/ml) was allowed to gel in 24-well plates in the absence or the presence of the chemoattractants, 10 nM rC5a or 1 ng/ml rTNF (BioSource International, Camarillo, CA), in duplicate. After gelification, cells (8 × 10^3/well) were overlaid on the gel surface and incubated at 37°C for 10 h. At the end of incubation, gels were fixed for 30 min with 2.5% glutaraldehyde, and cell migration was measured as the distance between the top of the gel and the plane in which the two faster cells invading the gel were in focus (×100 original magnification) (10).

Checkerboard analysis

Assays of cell migration with different doses of rC5a (0.1, 1, and 10 nM) on both sides of the filter were also performed (33). The results of these experiments were collected in checkerboard form by which chemokinesis (i.e., change in the intensity of random locomotion) and chemotaxis (i.e., change in the directional response to the stimulus) were calculated according to the method of Zigmond and Hirsch (33).

Western blot

Purified B lymphocytes (2.5 × 10^6) were washed twice with ice-cold PBS and lysed in 100 µl of a solution containing 1% Triton X-100, 50 mM Tris–HCl (pH 7.5), 1 mM DTT, 0.5 M EDTA (pH 8), 1 mM PMSF, and 5 mM benzamidine on ice for 15 min. After centrifugation (10,000 × g, 10 min, 4°C) supernatants were recovered. Cell lysates were separated in 8% SDS-PAGE and transferred onto nitrocellulose filters (Hybond-C Extra, Amersham, Aylesbury, U.K.). The filters were pretreated with 5% skim milk, 0.5% BSA, and 0.05% Tween-20 in Tris-buffered saline (TBS) and reacted with goat anti-human C5 antiserum (Quidel, San Diego, CA) or with goat nonimmune serum as a control at room temperature for 1 h. After washing, the filter was incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG (Dako) at room temperature for 1 h. The protein bands were subsequently visualized by using SuperSignal ULTRA chemiluminescentsubstrate (Pierce, Rockford, IL) in accordance with the manufacturer’s manual.

Immunohistochemistry

Cryostat tonsillar tissue sections were air-dried at room temperature for 6 h and were fixed with cold acetone for 5 min. Endogeneous peroxidases were blocked with 0.3% H_2O_2 in TBS. The CD88 mAb was diluted 1/100 in TBS and layered onto tissue sections overnight at 4°C. After three washings, an rabbit anti-mouse Ig antiserum was incubated by flow cytometry with a FACSscan (Becton Dickinson, Mountain View, CA). Controls were untreated cells, cells treated with FITC-second reagent alone, and cells treated with irrelevant isotype-matched mAb followed by the FITC-second reagent.

RNA analysis by RT-PCR

Total RNA was extracted from B cells according to the method of Chomczynski and Sacchi (35) and were reverse transcribed into cDNA by means of a commercial kit (Clontech, Palo Alto, CA) using oligo(dT) as primer. Each cDNA was then diluted to a final volume of 100 µl, and the efficiency of each primer was evaluated by amplifying a 550-bp fragment with the primers supplied with the kit. Afterward for each PCR analysis 5 µl of cDNA was amplified in a 50-µl reaction by adding 25 pmol of each specific primer and 2 U of Taq polymerase (Clontech). The primers used were the following: C5 forward primer, TCT CAG GTT CAT CTG TCT CC; and C5 reverse primer, GAA TTT CTG GCT TGC TTA CTG G. The amplification protocol was 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The amplification was conducted for 35 cycles, and the amplified product was visualized on a 2% agarose gel stained with ethidium bromide. Preliminary control experiments showed that these primers did not generate any band when tested with genomic DNA. The specificity of the amplification product was checked by Southern blot hybridization of the amplified band with the following internal oligonucleotide: GCC AAT GTG 6511TTT CAC CTA GCT GGA CTT ACC. The amplified band was checked by Southern blot hybridization of the amplified band with the following:

Statistical analysis

The results are expressed as the mean ± 1 SD. Differences among groups were determined by the nonparametric Mann-Whitney test. Differences among groups in dose-response experiments were determined using
which was recently described as a potent B cell chemoattractant. Based upon these results, all subsequent experiments show the typical bell-shaped curve obtained with most chemoattractants. Thus, the dose-response experiments were conducted using checkerboard analysis. As shown in Table I, rC5a augmented significantly by 1 and 10 nM rC5a (p < 0.01, respectively), with a decrease to the baseline values at higher rC5a concentrations (Fig. 1A). Similar results were obtained when B cells were tested for their ability to migrate through a nitrocellulose filter. The spontaneous locomotion of B lymphocytes was augmented significantly by 1 and 10 nM rC5a (p < 0.05 and p < 0.01, respectively), with a decrease to the baseline values at higher rC5a concentrations (Fig. 1B). Thus, the dose-response experiments show the typical bell-shaped curve obtained with most chemoattractants. Based upon these results, all subsequent experiments were conducted using 10 nM rC5a. Next, the B cell chemoattractant activity of rC5a was tested in the collagen invasion assay, which mimics the events occurring in vivo during cell locomotion. In these experiments rC5a was compared with rTNF, which was recently described as a potent B cell chemoattractant.

Results

**RC5a is a chemoattractant for human tonsillar B lymphocytes**

B lymphocytes were purified (>98%) from human tonsillar mononuclear cells by sequential depletion of T cells, NK cells, and macrophages and were tested for their ability to undergo polarization and locomotion in the presence or the absence of various rC5a concentrations. As shown in Fig. 1A, tonsillar B lymphocytes displayed a spontaneous polarization that was increased in a dose-dependent manner by rC5a, with a statistically significant difference at concentrations of 1 and 10 nM (p < 0.05 and p < 0.01, respectively). With increasing rC5a concentrations, a return of polarization to the background values was observed, although with some fluctuations (Fig. 1A). Similar results were obtained when B cells were tested for their ability to migrate through a nitrocellulose filter. The spontaneous locomotion of B lymphocytes was augmented significantly by 1 and 10 nM rC5a (p < 0.05 and p < 0.01, respectively), with a decrease to the baseline values at higher rC5a concentrations (Fig. 1B). Thus, the dose-response experiments show the typical bell-shaped curve obtained with most chemoattractants. Based upon these results, all subsequent experiments were conducted using 10 nM rC5a.

**Purified tonsillar B lymphocytes are a cell population with heterogeneous migratory competence**

An analysis of the distribution of purified tonsillar B lymphocytes through the filter in response to 10 nM rC5a is shown in Fig. 3. This representation allows assessment of whether the distance travelled by the leading front is representative of the movement of the whole migrating population or, rather, of only a faster cell subgroup (33). The logarithm of the number of cells at a given distance was plotted vs the square of the distance (33). The data expressed in this way were found to fit better with the logarithmic relationship (r = 0.98882, F = 219.85969, p < 0.0001), than with the linear relationship (r = 0.93639, F = 35.59577, p < 0.0019).

### Table I. Checkerboard assay of purified tonsillar B cells in response to rC5a

<table>
<thead>
<tr>
<th>rC5a (nM) Above the Filter</th>
<th>rC5a (nM) Below the Filter</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>0</td>
<td>40.1 ± 5.4</td>
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<tr>
<td>1</td>
<td>42.6 ± 3.7 (45.9 ± 2.6)</td>
</tr>
<tr>
<td>10</td>
<td>42.2 ± 6.2 (49.7 ± 4.6)</td>
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*Results are expressed as distance migrated (µm in 2 h) by the leading front of cells (mean ± SD, n = 3). Figures in parentheses show the calculated absolute concentration.

**FIGURE 1.** Dose-response curves of purified tonsillar B cells to rC5a in polarization and locomotion assays. B lymphocytes were purified as described in Materials and Methods and were tested in polarization (A) and locomotion (B) assays. Results are the mean ± 1 SD from four different experiments. *, p < 0.05; **, p < 0.01 (by Kruskal-Wallis nonparametric ANOVA analysis).

**FIGURE 2.** Collagen gel invasion by purified tonsillar B cells in the absence or the presence of rC5a or rTNF. Results are expressed as microns travelled from the top of the gel by the two fastest cells. rC5a, 10 nM; rTNF, 1 ng/ml. The incubation time was 10 h. Results are the mean ± 1 SD from four experiments. *, p < 0.05 (by nonparametric Mann-Whitney U test).

As shown in Fig. 2, both rTNF and rC5a significantly enhanced the spontaneous penetration of tonsillar B cells into the collagen matrix (p < 0.05). To investigate whether the rC5a-dependent increase in B cell locomotion was attributable to true chemotaxis, experiments were conducted using checkerboard analysis. As shown in Table I, rC5a stimulated both the rate of cell locomotion and the true chemotaxis. In particular, taking into account spontaneous migration (i.e., in the absence of rC5a above and below the filter), 10 nM rC5a above and below the filter (chemokinetic conditions) enhanced cell migration by 34.8 ± 20.9% (mean ± 1 SD; n = 3). On the other hand, compared with B cell migration in the absence of rC5a above and below the filter (i.e., spontaneous migration), 10 nM rC5a placed only below the filter (chemotactic conditions) augmented cell migration by 46.2 ± 5.0% (mean ± 1 SD; n = 3). On the contrary, cell migration in negative gradients was consistently lower than that calculated on the basis of the expected response to absolute concentration alone.
characteristic of a uniformly migrating population (Fig. 3) (33). These findings suggested that the B cell fractions tested contained at least two cell subsets with different migratory capabilities.

**rC5a enhances the locomotion of memory and naive, but not GC, B cells**

Tonsillar B lymphocytes are comprised of three major cell populations, i.e., GC, naive, and memory B lymphocytes (29, 38–42). These cell fractions differ as for immunophenotype, anatomical location, and functional features (38–43). To investigate what B cell subset(s) were the targets of the rC5a-mediated chemotactic enhancement, purified tonsillar B lymphocytes were separated into naive (IgD⁺, CD38⁻), GC (CD38⁻, CD10⁻) and memory (CD38⁺, IgD⁻) cells (29, 38–42, 44) before being tested for locomotion in the presence or the absence of rC5a. Consistent with previous reports (29, 43, 44) most IgD⁺ naive B cells were CD39⁺, IgM⁺, IgG⁺, CD38⁻, CD10⁻, whereas approximately one-half of them expressed the CD23 marker (not shown). CD38⁺, IgD⁻ GC B cells were CD10⁻, CD39⁺, CD23⁻ (not shown). IgD⁻, CD38⁺ memory B lymphocytes were predominantly CD39⁺, IgG⁺, CD23⁻, CD10⁻ (not shown) (29, 43, 44). As shown in Fig. 4, memory B lymphocytes displayed a high degree of spontaneous migration that was increased significantly by the exposure to rC5a (p < 0.05; Fig. 4). Naive B cells exhibited a lower spontaneous locomotion that was augmented significantly by rC5a (p < 0.05; Fig. 4). Finally, the spontaneous migration of GC B cells was unaffected by treatment with rC5a (Fig. 4). Control experiments ruled out that the failure of GC B lymphocytes to migrate in vitro in response to rC5a was related to their propensity to undergo apoptosis (41). Thus, all the separation procedures were conducted at 4°C to prevent spontaneous apoptosis, and freshly isolated GC B cells contained consistently >90% viable lymphocytes, as assessed by trypan blue staining. An equivalent proportion of viable GC B lymphocytes was detected after 2-h incubation for the locomotory assays.

Previously it was shown that human tonsillar GC B cells can be induced to migrate in vitro upon incubation with CD40 mAb and IL-4 (45). In addition, GC B lymphocytes exposed to the latter stimuli displayed a further increase in locomotion in response to anti-Ig or CD32 Abs (45). We therefore investigated whether purified GC B cells that had been cultured for 6–24 h with CD40 mAb and IL-4 migrated upon subsequent exposure to rC5a. In three different experiments GC B lymphocytes incubated with CD40 mAb and IL-4 migrated significantly faster than control cells cultured in medium alone for the same time interval (45). However, incubation of the former cells with rC5a over a wide range of concentrations did not change their baseline locomotion in vitro.

**CD88 is expressed by tonsillar B lymphocytes and is involved in their migratory response to rC5a**

The binding of C5a to the cell surface is mediated by a single class of receptors (CD88) that are widely distributed among different cell types (23). In subsequent experiments, the expression of CD88 on freshly isolated memory, naive, and GC B cells was investigated. Flow cytometric analysis of the three B cell fractions showed that memory and naive B lymphocytes expressed CD88 (ranges from four different experiments: 33–47% and 10–14%, respectively), whereas GC B cells did not (Fig. 5). Notably, no CD88 expression was detected on GC B cells that had been cultured for 6–24 h with CD40 mAb and IL-4. In subsequent experiments the in vivo localization of CD88⁺ cells was investigated by...
double staining of frozen tonsillar tissue sections with CD88 and CD20 mAbs. The majority of CD88+ cells were CD20+ macrophages that stained blue (Fig. 6, A and B, APAAP staining) and localized predominantly to the interfollicular and subepithelial areas below the cripts (C), whereas CD20+ cells were concentrated in the secondary lymphoid follicles where GC are evident. Nuclear counterstaining was performed with methyl green. Arrows point to the area that is shown at higher magnification in C. B, Staining for CD88 (APAAP, blue staining) and CD20 (peroxidase, brown staining; ×300). Nuclear counterstaining was performed with methyl green. CD20+ cells are found predominantly in the GC and, to a lesser extent, in the adjacent follicular mantle of a secondary lymphoid follicle. In the latter area, two typical CD88+ macrophages are shown (arrows) together with a high endothelial venule (HEV). C, Staining for CD88 (APAAP, blue staining) and CD20 (peroxidase, brown staining) at high magnification (×1250) of the subepithelial area pointed out by arrows in A. Nuclear counterstaining was performed with methyl green. Double-positive (CD88+, CD20+) cells display a violet staining (arrows) resulting from the overlapping of a blue granular staining with a brown uniform staining at the surface of the same cells.

FIGURE 6. Immunohistochemical analysis of the localization of CD88+ cells in human tonsillar tissue sections. A, Staining for CD88 (APAAP, blue staining) and CD20 (peroxidase, brown staining) detects at low magnification (×125) two discrete populations of single-positive cells. CD88+ cells localize in the interfollicular and subepithelial areas below the cripts (C), whereas CD20+ cells are concentrated in the secondary lymphoid follicles where GC are evident. Nuclear counterstaining was performed with methyl green. Arrows point to the area that is shown at higher magnification in C. B, Staining for CD88 (APAAP, blue staining) and CD20 (peroxidase, brown staining; ×300). Nuclear counterstaining was performed with methyl green. CD20+ cells are found predominantly in the GC and, to a lesser extent, in the adjacent follicular mantle of a secondary lymphoid follicle. In the latter area, two typical CD88+ macrophages are shown (arrows) together with a high endothelial venule (HEV). C, Staining for CD88 (APAAP, blue staining) and CD20 (peroxidase, brown staining) at high magnification (×1250) of the subepithelial area pointed out by arrows in A. Nuclear counterstaining was performed with methyl green. Double-positive (CD88+, CD20+) cells display a violet staining (arrows) resulting from the overlapping of a blue granular staining with a brown uniform staining at the surface of the same cells.

In the following experiments the hypothesis that the C5 gene could be expressed in tonsillar B cell subsets was investigated. To this end, RNA was extracted from freshly isolated naive, GC, and memory B lymphocytes; reverse transcribed; and subjected to RT-PCR. As shown in Fig. 8A, C5 transcripts were detected in the three B cell subsets; the specificity of the amplification products was confirmed by Southern blot hybridization of the same filters with an internal oligonucleotide (not shown). Next, Western blot experiments with freshly isolated memory, naive, and GC B cells were conducted using an anti-C5 goat antiserum to demonstrate the presence of the C5 protein. As shown in Fig. 8B, two bands of the expected molecular masses, i.e., 115 and 75 kDa, respectively, were detected in the cell lysates from the three B cell fractions under reducing conditions. Such bands correspond to the α- and β-chains of the C5 molecule, respectively (20). These experiments suggest that C5 produced in vivo by freshly isolated memory, naive, and GC tonsillar B lymphocytes may serve as substrate for the generation of biologically active C5α in secondary lymphoid tissues.
lymphoid organs, where, in the absence of interaction with Ag, B lymphocytes to rC5a may relate to their migratory properties in response to rC5a. Untreated and irrelevant mAb-treated memory B cells migration in the absence of rC5a vs in the presence of 10 nM rC5a, p < 0.05; CD88 mAb-treated memory B cells migration in the absence of rC5a vs in the presence of 10 nM rC5a, p > 0.05 (by nonparametric Mann-Whitney U test).

Discussion

The complement system may be considered an effector mechanism of innate immunity that signals to the acquired immune system how to discriminate between dangerous and innocuous Ags (46). This contention has been elegantly proven by the demonstration that hen egg lysozyme fused to murine C3d is a better inducer of Ab production than hen egg lysozyme alone and that immunogenicity increases with the copies of C3d present in the fusion protein (47). Thus, C3d has been defined as a “molecular adjuvant of innate immunity that profoundly influences an acquired immune response” (47). Additional evidence for the pivotal influence of the complement system in B cell differentiation and function comes from studies conducted with mice rendered deficient in the genes for CD21 and CD35 complement receptors (48, 49) or C3 and C4 complement components (18). These knockout animals display consistent abnormalities in B cell responses to T cell-dependent Ags due to defects in the B cell compartment and a reduction in the number and the size of GC (18). The latter observation suggests that CD21 and CD35 complement receptors as well as C3 and C4 complement components are involved in the control of B cell trafficking. On the other hand, although a C5aR genetically deficient mouse has been generated, its lymphoid tissue morphology and lymphocyte trafficking have not been studied (50, 51).

Here we have investigated the in vitro locomotory response of human tonsillar B lymphocytes to rC5a. rC5a significantly enhanced their spontaneous polarisation and migration through an increased rate of locomotion, i.e., chemokinesis, and an increased directional migration, i.e., chemotaxis. When tonsillar B cells were fractionated into the naive, GC, and memory cell subsets it was found that memory and, to a lesser extent, naive B lymphocytes migrated in vitro in response to rC5a, whereas GC B cells did not. Accordingly, memory and naive B lymphocytes expressed CD88, as assessed by flow cytometry, and preincubation of memory B cells with a CD88 mAb abrogated their migratory responsiveness to rC5a.

The observed hierarchical responses of memory, naive, and GC B lymphocytes to rC5a may relate to their migratory properties in vivo. Naive B cells migrate from the bone marrow to the secondary lymphoid organs, where, in the absence of interaction with Ag, either die or are recruited into a pool of cells that recirculate through blood and secondary lymphoid organs (52, 53). Here, following encounter with Ag in the T zone, naive B cells undergo clonal expansion and colonize the primary lymphoid follicles, where they differentiate into GC cells. A wave of cell proliferation is followed by somatic hypermutation of Ig variable region genes and positive selection of GC B cells according to the affinity of surface Ig for Ag displayed on follicular dendritic cells (FDC) (38–41, 52, 53). Positively selected GC B cells differentiate into memory or plasma cells, whereas most nonselected cells undergo apoptosis without leaving the lymphoid follicle (38–41, 52, 53).

GC B lymphocytes freshly isolated from human tonsils have little propensity to locomotion (Refs. 11, 25, and 45 and this study), perhaps due to the lack of expression of CD44 (29, 54) or L-selectin (29), which allows cell binding to specialized postcapillary high endothelial venules (55). Upon short term culture with CD40 mAb and IL-4, GC B cells acquire migratory competence, and their baseline locomotion can be further enhanced by the exposure to some stimuli, such as anti-Ig and CD32 Abs (45), but not to others, as demonstrated for C5a in this study. Kupp and co-workers have previously shown that GC B cells isolated from murine lymph nodes 2–4 days after antigenic stimulation migrated in vitro upon exposure to C5a generated from zymosan-treated serum (17). The human counterpart of these cells may be a small GC B

FIGURE 7. Involvement of CD88 in the rC5a-induced enhancement of memory B cell locomotion. Freshly purified memory B lymphocytes were treated with medium alone (Nil), with 10 µg/ml CD88 mAb (CD88 mAb), or with an isotype-matched mAb of irrelevant specificity (control mAb); washed; and tested in the nitrocellulose filter assay in the absence (■) or the presence of 10 nM rC5a (▲). Untreated and irrelevant mAb-treated memory B cells migration in the absence of rC5a vs in the presence of 10 nM rC5a, p < 0.05; CD88 mAb-treated memory B cells migration in the absence of rC5a vs in the presence of 10 nM rC5a, p > 0.05 (by nonparametric Mann-Whitney U test).

FIGURE 8. C5 gene expression in freshly isolated naive, GC, and memory tonsillar B lymphocytes. A, RNA was extracted from freshly isolated naive, GC, and memory B cells; reverse transcribed; and amplified by PCR with primers specific for the C5 gene. In control experiments, not shown, RT-PCR was carried in the absence of added template. No band was detected under these conditions. B, Western blot analysis of C5 protein expression in freshly isolated naive, GC, and memory B cells. Cell lysates were prepared and processed as detailed in Materials and Methods. From left to right, negative control (K562 erythroleukemia cells); positive controls (HepG2 hepatoma cells and EBV-infected lymphoblastoid B cells, respectively); and naive, GC, and memory B cell fractions. One representative experiment of the three performed is shown. Arrows point to the two bands detected (i.e., 115 kDa, corresponding to the C5 α-chain, and 75 kDa, corresponding to the C5 β-chain).
cell fraction (56) that displays an IgD⁺, IgM⁻, CD38⁻ immunophenotype and, in part, germline Ig genes (56). These cells have been defined as GC founder, since they may represent the "trait-d’union" between naive and GC B lymphocytes (56). Alternatively, murine GC B cells that were isolated during an ongoing immune response could have received in vivo signals that primed them for a migratory response to C5a.

In immunized mice splenic memory B cells have been localized predominantly to the marginal zone (57); shortly after antigenic challenge, specific B lymphocytes disappear from the marginal zone due to their migration into the follicles and subsequently to the T cell zones. Thereafter, memory B cells either move to the splenic red pulp where they differentiate to plasma cells or return to the marginal zone 3 days after immunization. Thus, the loco-
motion of memory B lymphocytes depends on their Ag-activated state (57, 58). In human tonsils, IgD⁺, CD38⁻ memory B cells are found within and underneath the epithelium lining the crypts (44). In this study the findings that CD88⁺ B lymphocytes were detected in the subepithelial areas where the bulk of memory B cells settle (44) and that freshly purified memory B lymphocytes were the most motile cells in response to rC5a support the hypothesis that C5a is an important chemoattractant for memory B lymphocytes in vivo.

C5 gene expression was here demonstrated in freshly isolated naive, GC, and memory B lymphocytes at both the mRNA and the protein level. This finding is in line with a previous report showing that human T and B lymphoblastoid cell lines can synthesize C5 (59) and raises the possibility that not only macrophages, but also the B cells themselves, serve as sources of C5 in the secondary lymphoid tissues. Since FDC retain Ag-antibody complexes on their surface (41), it is tempting to speculate that C5a is generated in the lymphoid follicles via classic complement activation trig-
gerized by FDC-bound immune complexes. It is also conceivable that follicular macrophages handling immune complexes contrib-
ute to C5a production. Locally produced C5a, in turn, might be involved in the control of short range migration of memory and naive B lymphocytes.

The B cell-directed chemotactic activity of C5a may help un-
derstand the mechanisms of B lymphocyte recruitment to inflamed tissues, such as, for example, the synovial membrane in rheuma-
toid arthritis (60) and the salivary glands in Sjögren’s syndrome (61). C5a generated in these sites by classic and/or alternate C5 convertases would increase endothelial permeability and promote the extravasation of B lymphocytes to the tissues, where GC for-
mation is frequently observed (60, 61). In this connection, C3 gene expression can be up-regulated by T cell-derived IFN-γ released in the microenvironment of secondary lymphoid follicles (62). The increased availability of C3 in the course of acute or chronic inflammation might lead to augmented generation of C5a with con-
sequent enhancement of B cell locomotion.

In conclusion, this study delineates a novel role for C5a in B lymphocyte-dependent immune responses and provides clues that may provide a better understanding of the pathophysiology of auto-immune and lymphoproliferative disorders.

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