Hemolytically Inactive C5b67 Complex: An Agonist of Polymorphonuclear Leukocytes

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The activity of hemolytically inactive C5b67, designated iC5b67, was evaluated as an agonist for functional responses of human polymorphonuclear leukocytes (PMN). C5b67 was formed from purified human complement components and decayed in phosphate-buffered saline (PBS) until it had no lytic activity for sheep erythrocytes in a standard assay. iC5b67, at nanomolar concentrations, stimulated PMN chemotaxis and Ca2+ fluxes, but inhibited superoxide production and failed to upregulate CR1 and CR3. There was no significant contamination of the iC5b67 with C5a to explain these results. Neither isolated C5b6 nor C7 alone exhibited the activities of iC5b67, while insolubilized anti-C7 could remove the PMN agonist activity from the iC5b67 preparation.

The formation of C5b67 is initiated by the limited proteolysis of complement C5 to form C5b, which binds C6 to form a stable C5b6 complex. The addition of C7 to C5b6 forms the C5b67 complex, which transiently rearranges to expose the hydrophobic domains necessary for membrane insertion.1,2 There are three possible fates for the nascent C5b67: (1) insert into membrane lipids and act as a signaling ligand3,4; (2) insert into membrane lipids and, by providing binding sites for C8 and C9, become part of a transmembrane channel (hemolytically active); or (3) decay to a hemolytically inactive complex in the fluid phase (iC5b67).5 The iC5b67 complex can react with components C8 and C9 and with vitronectin (S-protein) and clusterin, but no biologic functions have been described for any of these nonhemolytic terminal complement complexes.

In retrospect, over 2 decades ago a fluid phase complex comprised of C5, C6, and C7, likely what we refer to as iC5b67, was reported to have chemotactic activity.6,7 There were at least two reasons why this work was not pursued: first, critics at that time held that fluid phase C5b67 could not be important as a chemotactic factor if C6 deficient rabbits had normal chemotactic function.8 We now have a growing appreciation of the redundancy of crucial mediators, even a deficiency of C5, which precludes the generation of both C5a and iC5b67, does not lead to a global immune defect. The second reason that studies of the chemotactic activity of iC5b67 were not pursued related to the discovery of C5a as a potent chemotaxin.9 With the chromatographic and immunologic reagents available at the time, it would have been difficult to rule out the possibility of C5a contamination of iC5b67. With newer chromatographic methods, specific antisera, and better assays, it is now possible to define the composition of this ligand. Therefore, we reexamined the signaling potential of iC5b67 for human polymorphonuclear leukocytes (PMN). Our findings indicate that iC5b67 is potent in nanomolar concentrations for inhibiting superoxide production, while stimulating chemotaxis and Ca2+ fluxes. The ability of iC5b67 to deactivate the chemotactic activity of C5a and FMLP, as well as the demonstration of pertussis toxin (PTX) inhibition of iC5b67 activity, are consistent with iC5b67 sharing elements of the signaling pathway used by C5a and FMLP. When complement is activated in the fluid phase, such as would happen in the presence of soluble lipopolysaccharide (LPS) or immune complexes, a large fraction of the C5b67 formed may decay to iC5b67, and iC5b67 need not insert in the PMN membrane to induce signaling. Two lines of evidence suggest that iC5b67 and C5a and FMLP share common steps in intracellular signaling (1) pretreatment of PMN with iC5b67 deactivates PMN for C5a- and FMLP-induced chemotaxis; and (2) pretreatment of PMN with pertussis toxin inhibits iC5b67-induced chemotaxis. Thus, iC5b67 has important effects on the activity of PMN and G-proteins and Ca2+ are involved in the signaling.

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Materials and Methods

Complement Complexes and Assays

C5b6 was made from purified C5, C6, cobra venom factor (CVF), factor B (all from Quidel, San Diego, CA), and recombinant mouse factor D (adipsin; gift of Dr Ty White, Scios, Mountain View, CA) with modifications10 of the original method.11 The C5b6 was fractionated by high performance liquid chromatography (HPLC) on a DEAE column (AP-1 Protein Pak 8HR; Waters Associates, Milford, MA) as described.10 The peak C5b6 containing fractions were pooled, and the pool titered using human erythrocytes. One unit of C5b6 was defined as the amount of C5b6 required to produce 50% lysis of 2.5 × 107 human E when incubated in a total volume of 300 μl with C7 (0.1 μg), C8 (0.5 μg), and C9 (0.5 μg) (all from Quidel).11 To form C5b67, 30 μg of C5b6 and a twofold molar excess of C7 (20 μg) were mixed and incubated at 37°C for 15 minutes, then 4°C overnight. Although iC5b67 may aggregate,12 we have used the monomeric molecular weight (mw) of 400 kD in the calculation of molarity.

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Antibody Methods

For the iC5b7 enzyme-linked immunosorbent assay (ELISA), wells (Immulon 2; Dynatech, Chantilly, VA) were coated with polyclonal goat antihuman C7 (1/200 dilution; Quidel) overnight at 4°C. All subsequent steps were performed at room temperature. Phosphate-buffered saline (PBS) 0.1% Tween was used for all washes and dilutions, except as noted; blocking was performed with Superblock buffer (Pierce, Rockford, IL). Dilutions of sample, as well as dilutions of an iC5b7 standard (16–130 ng), were added to wells for 30 minutes. Indications monoclonal antibody (MoAb) anti-C6 (Quidel; 1/1,000 dilution) for 30 minutes followed by protein A-Sepharose (Pierce) for 30 minutes, and finally 100 μL of the substrate solution (TMB Microwell Peroxidase Substrate System; Kirkegaard & Perry). After blue color developed, the reaction was terminated by addition of 100 μL of 250 mM/L phosphoric acid. The absorbance unit (AU) at 450 nm of the wells were read within 1 hour using a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA). The assay was linear over a range of 16 to 130 ng.

Protein Methods

Protein was assayed using a modified Folin assay (BCA, enhanced, Pierce), using BSA as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 5% to 10% gradient slab gels with discontinuous buffers. To radioiodinate C5b6, approximately 40 μg of C5b6 was mixed with 40 μCi carrier free sodium 125I (New England Nuclear, Boston, MA) in iodogen (Pierce)-coated tubes according to the manufacturer’s specifications. A PD-10 sizing column (Pharmacia) was used to remove free 125I. Ninety-six percent of the cpm of the 125I-C5b6 pool were precipitable with 10% trichloroacetic acid (TCA), yielding a specific activity of 5 × 107 cpm/μg. The 125I-C5b6 was fully functional in terms of forming C5b6 and iC5b7.

Preparation of PMN

PMN were isolated at room temperature from 20 mL of venous blood from normal volunteers. Blood was anticoagulated with 5 mM/L EDTA (Pheannacia) and centrifuged for 20 minutes at 500g. The PMN anduffy coat were separated with a 3-pm pore polycarbonate filter (Neuro Probe #PFD3). An incubation time of 45 minutes was sufficient time to allow cells responding to chemoattractants to migrate through the filter. The PMN were washed 2X, quantified by hemocytometer, and reabsorbed to protein (P-7700; Sigma, St Louis, MO) for use in the iC5b7 absorption experiments.

Chemotaxis

Three different chemotactic assay procedures were used. Freshly isolated PMN (2.5 × 107) were placed in the upper wells and dilutions of chemoattractant solutions (28 μL), or control buffer (HBSS+ with 0.1% ovalbumin) were added to the bottom wells of a microchemotoxin chamber (Neuro Probe, Cabin John, MD). The apparatus was placed in a 37°C, humidified, 5% CO2 incubator for a specified time to allow migration. The first assay to assess PMN chemotaxis utilized 5 μm pore polivinylpyrrolidone-free polycarbonate filters (Nucleopore, Neuro Probe #NFBS). Each dilution of chemoattractant was set up in quadruplicate. After a 60-minute incubation, the filters were stained with Wright-Giemsa (Diff Quik; Baxter Scientific, McGaw Park, IL) and the cells on the bottom surface of the filter were enumerated by an individual blinded to the protocol. For each well, four to five fields on the filter were analyzed using a 63X objective. The second chemotactic assay utilized 5 μm pore nitrocellulose mesh filters (Tyo, Neuro Probe #TCBS). Each dilution of chemoattractant was added to triplicate wells. After a 60-minute incubation, the filters were fixed and stained with Congo red. An Optomax V image analyzing system (Analytical Instruments, Shaffton Walden, Essex, England) enumerated the cells in four 25×-fields at 20-μm intervals, beginning 20 μm from the upper surface. The third chemotactic assay measured the ability of PMN that were loaded with BCECF-AM (Molecular Probes, Eugene, OR) (2 μmol/L for 20 minutes) to migrate through a 3-μm pore polivinylpyrrolidone-free polycarbonate filter (Neuro Probe #PF3D). An incubation time of 45 minutes was sufficient time to allow cells responding to chemoattractants to migrate through the filter, and minimized the chance the PMN would fall off the filter, as detected by the fluorescein in the lower chamber. Fluorescence was quantified with a fluorescence plate reader (Cytoscan, Millipore, Bedford, MA) using excitation at 485 nm and emission at 530 nm. The ability of PTX (Sigma) (2 μg/mL for an hour at 37°C) to inhibit the chemotactic response was assessed using the first chemotactic assay.

Superoxide Release Assay

Superoxide release was measured as the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c. In the routine assay, PMN suspensions (8 × 107 cells/mL) were incubated with ferricytochrome c (1 mg/mL; Sigma) and either buffer or buffer plus agonist. Replicate tubes contained SOD (21 μg/mL; Sigma) in addition. In the experiment depicted (see Fig 3), the reaction tubes were preincubated with a 0.5% gelatin solution, which eliminated the release of superoxide by resting PMN. After 15 minutes at 37°C, the reaction was stopped by chilling to 4°C and centrifugation. The optical density (OD) 550 nm of the supernatants was determined in a spectrophotometer (DU series 60; Beckman Instruments, Fullerton, CA). In the microassay, each component was reduced to 1/4 volume of the regular assay, and 210 μL of the supernatant was transferred to a microtiter plate well and the OD 550 nm quantified with a ThermoMax microtiter plate reader (Molecular Devices, Menlo Park, CA). All reactions were performed in duplicate or triplicate for the regular assay and in triplicate for the microassay. Results are expressed as the mean nanomoles superoxide/number of cells/15 minutes.

To test if iC5b7 might itself inhibit the detection of superoxide anion, superoxide was generated in a cell free system. Purine (10 mmol/L Sigma), cytochrome c (1 mg/mL Sigma) and either iC5b7 (3 × 10−3 mol/L), or buffer, or SOD were mixed, followed by the addition of xanthine oxidase 15 μL (0.05 U; Worthington Biochemical Corp, Freehold, NJ). The reaction mixtures were incubated for 15 minutes at 37°C then immediately put on ice. Cold PBS (750 μL) was added to each tube and the OD 550 nm was determined.

Ca2+ Flux Assay

PMN (1 × 107/mL in HBSS−) were incubated with Indo-1 AM (5 μmol/L; Molecular Probes, Eugene, OR) at 37°C for 7 minutes. The cell suspension was diluted 5X with HBSS+, and incubated a further 10 minutes at 37°C. The Indo-loaded cells were pelleted at 500g for 10 minutes, and resuspended in HBSSA at 107/mL at room
was added to the tubes sequentially at the indicated times for incubation at 37°C. At zero time, 100 μL human E (2.5 × 10⁷ cells) diluted with 400 μL prewarmed HBSA⁺, and 50 μL 4°C were expressed.

Assay for CR1 and CR3 Expression

PMN (200 μL of 2 × 10⁶/mL HBSA⁺) were incubated at 37°C for 30 minutes with iC5b67 (10⁻⁴ mol/L, buffer alone (negative control)), and either C5a (10⁻⁷ mol/L) or FMLP (10⁻⁷ mol/L), as positive controls. Fifty microliters of the above reaction suspensions was aliquoted into four tubes, and further manipulations were done at 4°C. Each aliquot of cells was reacted with saturating doses of one of the following MoAb: anti-HLA class I (W6/32), anti-CR3 (OKM-1), or an equivalent amount of an irrelevant MoAb (TIB157, anti-human Ig λ chain). After addition of FITC–goat-anti-mouse IgG (Jackson Labs, West Grove, PA), the fluorescence intensity of washed cells was measured by FACS (BD FacSort; Becton Dickinson).

Statistical Analysis

Data are presented as mean ± SEM. The Student’s t-test was used to compare two samples.

RESULTS

Confirmation That C5b67 Decays to a Hemolytically Inactive Form

Although the decay of C5b67 to a hemolytically inactive form was previously experimentally documented, the purification and characterization of the components of the complex and the method of forming the complex at that time differed from our procedures. Because the characterization of iC5b67 is central to the thesis of this work, we reevaluated the time course of the decay of C5b67 to iC5b67. One hundred microliters of diluted C5b6 (9 ng) was aliquoted to tubes and a twofold molar excess of C7 (6 ng) was added to different tubes at -30, -15, -5, -2, and -1 minutes for incubation at 37°C. At zero time, 100 μL human E was added to each tube with incubation at 37°C for 20 minutes followed by addition of 0.5 μg C8 and 0.5 μg C9 and a further incubation at 37°C for 60 minutes. The maximal hemolytic potential of the C5b6 was assessed by adding C5b6 and C7 to a tube already containing E, and continuation of the reaction as noted above. All reactions were stopped by the addition of 2 mL 0.15 mol/L saline-EDTA to each tube, centrifugation of the samples, and determination of the hemoglobin in the supernatant (OD 541 nm). The initial half-life of this second order decay reaction was approximately 30 seconds (Fig 1). Although this value is longer than the < 0.1 second half-life previously reported when different experimental conditions were used, our results do confirm
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addition of trypsin caused the time-dependent loss of cell associated cpm that differed for C5b67 and iC5b67. Consistent with its known insertion in membranes after 20 minutes of trypsin treatment, only 34% of the nonbuffer elutable cpm associated with EC5b67 were susceptible to trypsin cleavage; whereas for EC5b6, which is known not to insert

that there is a relatively rapid decay of C5b67 hemolytic function.

Accessibility of Erythrocyte-Bound 125I-iC5b67 to Trypsin

Although iC5b67 was not active in lysing erythrocytes, it was possible that it inserted into the plasma membrane. Recently published data indicate that hemolytically competent terminal complement complexes, including C5b67 and C5b-8, as well as C5b-9, can directly interact with G proteins and thereby mediate signaling by a receptor independent mechanism. In the early studies of the biology of the terminal complement complexes, evidence that hemolytically competent C5b67 was physically inserted into the plasma membrane of target E came from measuring the accessibility of radiiodinated components of the complex to trypsin digestion. When EC5b6 was formed using radiolabeled C6, virtually all the cpm were solubilized when the target cells were treated with trypsin. In contrast, when EC5b67 was formed using either radiolabeled C6 or radiolabeled C7, approximately 50% of the original cpm remained cell associated after trypsin treatment. We used a similar experimental design to test the relative trypsin susceptibility of bound EC5b67 and hemolytically competent EC5b6, which were formed from 125I-C5b6 and C7 (Fig 2). Only about 10% of the cpm associated with either erythrocyte-complement complex eluted in buffer during the 1-hour incubation. The

Fig 3. iC5b67 inhibits C5a- and FMLP-induced superoxide production by PMN. Additions of buffer, or iC5b67 (10⁻⁶ mol/L) were made to PMN (2 x 10⁵), which were suspended in ferricytochrome c (1 mg/mL), and the reactants were incubated at 37°C. After 15 minutes, C5a (10⁻⁶ mol/L) or FMLP (10⁻⁶ mol/L) were added for an additional incubation of 15 minutes at 37°C. Reactions were set up in sextuplicate with half the samples containing SOD (21 μg/mL). The reaction tubes had been precoated with gelatin 10.50101 for 1 hour at room temperature before the addition of PMN to decrease contact activation of the cells. A microassay was performed as described in Materials and Methods. The nanomoles of superoxide produced per 2 x 10⁵ PMN per 15 minutes were calculated from the differences in the OD₅=₅₀ of the supernatants from samples with SOD and without SOD. The results are the mean ± SEM of triplicate values. * Compared with C5a or FMLP alone, P < .05. This experiment is representative of two performed.

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Fig 4. The specificity of the iC5b67 ligand. (A) iC5b67 activity is not reproduced by isolated C5b6 or C7. C5b6 and C7 used for making iC5b67 were assayed for superoxide production by PMN as described in Materials and Methods. The reaction tubes were not precoated with gelatin, which explains the high constitutive release of resting cells. C5b6 (0.18 pg) and C7 (0.8 pg) did not affect the superoxide production of PMN. The results represent the mean ± SEM of triplicate values. Compared with resting, * P > .05; ** P < .05. The supernatant from anti C7-beads had no reactivity by ELSA and failed to inhibit superoxide production by PMN.
in the membrane, 75% of the cpm were trypsin susceptible (Fig 2 inset). Eighty-one percent of the Eic5b67 associated cpm were trypsin sensitive, indicating that iC5b67, like C5b6, was extracellular.

**iC5b67 Inhibits C5a- and FMLP-Induced Superoxide Production**

Superoxide production is a well-characterized response of PMN to agonists such as C5a and FMLP. We used a standard assay based on the reduction of cytochrome c to test whether iC5b67 might have an effect on the generation of superoxide. Unexpectedly, iC5b67 not only failed to stimulate superoxide production (data not shown), it inhibited superoxide production elicited by C5a (10^{-8} mol/L) and FMLP (10^{-9} mol/L) (Fig 3).

To rule out the possibility that iC5b67 might be inhibiting the assay for superoxide, superoxide was generated in a cell free system using purine as the substrate for xanthine oxidase and cytochrome c as the electronic acceptor and indicator of color change. When additions of PBS, iC5b67 (3 \times 10^{-9} mol/L), or SOD (21 \mu g/mL) were made to the reaction, the mean OD 550 was 0.012, 0.011, and 0.0, respectively (n = 5). Thus, the iC5b67 must be inhibiting the ability of PMN to generate superoxide, because the iC5b67 has no significant effect on the detection of chemically generated superoxide by this assay.

Additional experiments were performed to confirm that the biologic activity we were noting was really caused by the iC5b67 and was not caused by one of its constituents, or a contaminant. In these experiments the reaction tubes were not precoated with gelatin, and there is substantial superoxide produced by the resting cells, presumably because of contact activation. First, C5b6 (0.18 \mu g) alone and C7 (0.8 \mu g) alone were tested and neither affected the ability of PMN to produce superoxide (Fig 4A). Second, the ability of insolubilized anti-C7 to remove the biologic activity of the iC5b67 was tested. iC5b67 complex was mixed with anti-C7 IgG- (or, anticomplement factor H IgG-) protein G-beads for an indicated time. After the beads were pelleted, the supernatants were assayed for the biologic activity of the iC5b67 by ELISA. The supernatant from anti-C7 protein G-beads were ELISA-negative and failed to inhibit PMN superoxide production, whereas the supernatants from anti-H protein G-beads retained inhibitory biologic activity (Fig 4B).

**iC5b67 Complex Does Not Upregulate the Expression of CR1 and CR3**

iC5b67 was compared with FMLP and C5a for its ability to affect the expression of class I MHC, CR1 (CD35), and CR3 (CD11b, CD18). None of the agonists significantly affected the expression of class I MHC. FMLP and C5a both upregulated CR1 and CR3, as previously noted. By contrast, iC5b67 (10^{-9} mol/L) did not affect the expression of these complement receptors (Fig 5). In another experiment, seven twofold dilutions of iC5b67 from 6.4 \times 10^{-8} mol/L to 10^{-9} mol/L had no effect on class I, CR1, or CR3 expression (data not shown).

**Chemotaxis and Chemokinesis**

To screen for an effect of iC5b67 on PMN mobility, dilutions of iC5b67 (10^{-12} to 10^{-7} mol/L) were tested for chemotactic activity using a Boyden chamber with a polyclinylpyrolidone-free polycarbonate filter. There was distinct chemotactic activity, maximal activity at 10^{-9} mol/L, with apparent high dose inhibition at 10^{-8} and 10^{-7} mol/L (Fig 6). Effects of iC5b67 on random migration were evaluated. To perform this analysis, three chambers with nitrocellulose filters were used: one chamber contained dilutions of iC5b67 in the lower chamber to test chemotaxis; the second contained dilutions of iC5b67 in the upper chamber to assess chemokinetic activity; and the third contained dilutions of iC5b67 in both the upper and lower, which is also a test of chemokinesis. Each chamber also contained positive controls for the migratory capacity of the PMN: PMN in the upper wells, with FMLP (10^{-8} mol/L) in the lower wells. The results (Table 1) again show a peak of iC5b67 chemotactic activity at 10^{-9} mol/L, and no dose-related effect on chemokinesis. In fact, when iC5b67 was in the upper wells with the cells, there was frequently less migration into the filter than when the cells were in medium alone, which is indicated by negative numbers in Table 1. In this assay, cells were counted at multiple depths within the filter, which specifically would allow the identification of distinct subpopulations of migrating cells. The distribution of cells migrating to iC5b67 was...
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Fig 6. iC5b67 stimulates chemotaxis of PMN. Different doses of iC5b67 (10^{-12} - 10^{-7} mol/L, 28 μL) were tested in chemotaxis assays using polycarbonate filters, as described in Materials and Methods. The filter was fixed and stained by Diff Quick. The mean PMN number per field was calculated. iC5b67 significantly stimulated chemotaxis of PMN and the effects were dose-dependent. The error bars indicate standard errors of quadruplicate samples. This experiment is representative of four performed.

Deactivation of C5a- or FMLP-Induced Chemotaxis by iC5b67

Both C5a and FMLP are well characterized chemotactic factors for PMN. Although each has a distinct G protein coupled receptor, exposure to one factor diminishes the response to the second, by a process known as cross-deactivation. Experimental evidence indicates that cross-deactivation results from the fact that both the C5a receptor and the FMLP receptor share the same signaling pathway. To test if iC5b67 might also share the same pathway, BCECF-loaded PMN that had been pretreated with iC5b67 (10^{-7} mol/L at 37°C X 1 hour), were compared with control cells incubated with buffer alone, for their migratory response to C5a (10^{-7} mol/L) or FMLP (10^{-8} mol/L) in the bottom wells. After a 45-minute incubation, the fluorescence of the cells that reached the bottom of the filter was quantified. The results (Fig 7) indicate that previous exposure to iC5b67 inhibited the chemotactic response of PMN to both C5a and FMLP.

PTX Inhibition of iC5b67 Stimulated Chemotaxis

The receptors for both C5a and FMLP signal through PTX inhibitible G_{i} proteins. We tested the PTX susceptibility of iC5b67 signaling by preincubating the PMN with the toxin (2 μg/mL for 1 hour at 37°C). Subsequently, the PMN were assayed for their chemotactic response to iC5b67 (10^{-8} mol/L) and FMLP (10^{-9} mol/L). Compared with PMN that had been preincubated in buffer, PMN that were preincubated with PTX had a modest inhibition of random motility (Fig 8). PTX completely inhibited the PMN chemotactic response
We performed ligand binding assays using $^{125}$I-iC5b67 to establish if there were saturable binding, which would provide evidence for a specific receptor on the PMN. Replicate samples did not yield reproducible binding (data not shown), a fact we attribute to aggregation of this hydrophobic ligand. Aggregation of iC5b67 has been noted previously.  

To determine if iC5b67 were acting on the surface of the cell, the accessibility of erythrocyte bound $^{125}$I-iC5b67 to trypsin was assessed. We used erythrocytes for these studies because they had been used to compare the trypsin accessibility of EC5b6 and EC5b6. Additionally, complement-induced release of intracellular proteases is less of a consideration with erythrocytes than with PMN. In the published study, 90% of the cpm associated with bound $^{125}$I-C5b6 were released by trypsin digestion, while only about 50% the cpm associated with bound $^{125}$I-C5b6 were released by trypsin. These data were interpreted as evidence that the C5b6 was on the surface of the cell, while the C5b6 was inserted in the plasma membrane and partially shielded from proteolysis by trypsin. In terms of its susceptibility to trypsin digestion, the iC5b67 behaved like C5b6 (Fig 2), which is outside the cell. Thus, our results strongly suggest that iC5b67 binds to the cell surface, perhaps to a specific cell receptor. The degree of proteolysis of C5b67 was comparable with that previously reported. In retrospect, the reported cpm released by trypsin from E-$^{125}$I-C5b67, as well as our current result, may overestimate the accessibility of the complex. We now know that when the C5b67 is formed, some may insert, and the iC5b67 to iC5b67, and significantly inhibited the PMN response to FMLP. Thus, iC5b67, like C5a and FMLP must use a G protein for signaling.

**iC5b67 Induces the Ca$^{2+}$ Flux of PMN**

Ca$^{2+}$ fluxes are an integral part of many signaling pathways used by the PMN and other cells. Indo-1-loaded PMN were tested for their ability to generate a Ca$^{2+}$ flux when challenged with iC5b67. The Ca$^{2+}$ flux was determined by the ratio of fluorescent intensity (405/485) as measured by flow cytometry (Fig 9). Over the $1.4 \times 10^{-11}$ to $2.8 \times 10^{-9}$ mol/L range tested, iC5b67 induced a dose-dependent increase in the Ca$^{2+}$ flux of PMN.

**DISCUSSION**

We have confirmed that newly formed C5b67 decays rapidly to a hemolytically inactive form, designated iC5b67 (Fig 1). Using a different experimental method, the half-life previously reported was calculated to be less than 0.1 second. Because the formation of C5b67 is rate-limiting, experimental conditions that would favor the formation of C5b67 would shorten the apparent half-life of nascent C5b67. The important consideration for the interpretation of our results is the confirmation that the C5b67 complex we formed using a twofold molar excess of C7 is labile with respect to its hemolytic activity. Although the decay rate we measured was at 37°C and we routinely stored our C5b67 at 4°C, we confirmed that ligand, which was active for PMN, had no hemolytic activity.
which is also formed may associate with the cell surface and be accessible to trypsin degradation.

The iC5b67 ligand exhibited divergent biologic activities. iC5b67 did not stimulate PMN superoxide production, rather it inhibited it. This was surprising because C5b-9 has been reported to stimulate superoxide production.25 By contrast, we found that a preparation of C5b-9 made from purified C5b6 and terminal components also inhibited superoxide formation, but the active moiety was iC5b67 (C. Wang and A. Nicholson-Weller, unpublished results, July 1994). We propose that the differences in activity may be due to our use of purified components, compared with the use of terminal complexes made from deficient sera. Many ligands can lead to the stimulation of superoxide production, including the chemotactic factors C5a and FMLP, which act through specific receptors, as reviewed.22,23 iC5b67 was able to inhibit C5a- and FMLP-induced superoxide production (Fig 3). Neither C5b6, nor C7 could reproduce the activity of iC5b67 (Fig 4A), while anti-C7 could remove the activity (Fig 4B). All these data support the conclusion that the active ligand is iC5b67. Receptor pathways, which inhibit superoxide are not known, although the intracellular accumulation of cAMP is known to inhibit PMN superoxide production.27 iC5b67, unlike C5a and FMLP,22,23 did not cause the upregulation of CR1 and CR3 (Fig 5), which is a second example of this complement complex evoking a different response than C5a and FMLP.

iC5b67 did stimulate chemotaxis (Fig 6) with maximal activity at $10^{-9}$ mol/L, and displayed inhibition at higher doses. An assay to test for directed versus random migration confirmed that iC5b67 primarily stimulated directed migration (Table 1). These results are important for two reasons: first, they confirm the original reports of iC5b67 chemotactic activity42; second, they indicate that the ability of iC5b67 to inhibit superoxide production and its failure to stimulate complement receptor upregulation are not the result of a general suppression of all cell functions. The ability of iC5b67 to inhibit C5a and FMLP directed chemotaxis (Fig 7) may be via activation of the same pathway that inhibited C5a and FMLP-induced superoxide production, or it may reflect cross-deactivation. Cross-deactivation occurs when the same signaling machinery is used by two receptors, and the first receptor activated preempts the ability of the second ligand to signal, as reviewed.26 The PTX susceptibility of iC5b67 induced chemotaxis (Fig 8) indicates that this ligand uses G-proteins for signaling. However, the finding of iC5b67 chemotactic activity in vitro may not mean that this is the principal function of this ligand in vivo. For example, c-kit is chemotactic for mast cells, but its primary function is apparently as a growth/differentiation factor.28

Indo-1-loaded PMN showed a iC5b67 dose-dependent Ca$^{2+}$ flux (Fig 9). Ca$^{2+}$ fluxes are common to many signaling pathways,29 and further studies will be necessary to define if the Ca$^{2+}$ is coming from intracellular or extracellular stores.

We have shown in these studies that iC5b67 is active in signaling PMN. Further studies will be necessary to define if iC5b67 occurs in vivo, and if the binding of vitronectin (S-protein) or the later complement components affects iC5b67 signaling. The fact that iC5b67 is potent at nanomolar concentrations suggests that it, like other potent complement ligands including C3a and C5a, may have a short half-life.

To date, active terminal complement complexes have included membrane inserted C5b67, C5b-8, and C5b-9 complexes. Now we have also reconfirmed that iC5b67 has biologic activity, and that unlike the other terminal complement complexes, it apparently signals from outside the cell, rather than by insertion through the bilayer. We do not know if it is acting through a specific receptor. It is interesting that the changes in light polarization could be inhibited by PTX, making iC5b67 similar to signaling by C5a and FMLP, yet in the assays of superoxide and CR1/CR3 upregulation, iC5b67 evoked different responses. Preliminary data indicate that multiple signaling pathways are triggered by this ligand (C. Wang and A. Nicholson-Weller, manuscript in preparation). Finally, the fact that iC5b67 is large, hydrophobic, and has some proinflammatory and some antiinflammatory activities, while C5a is small, hydrophilic, and is only proinflammatory, suggests that these two ligands have differing roles in the initiation and regulation of inflammation.

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