

HUMAN MONOCYTE SPREADING INDUCED BY  
FACTOR Bb OF THE ALTERNATIVE PATHWAY  
OF COMPLEMENT ACTIVATION\*

A Possible Role for C5 in Monocyte Spreading

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Resistance to facultative intracellular pathogens is mediated through the activities of immunologically committed lymphocytes and the bactericidal activities of macrophages (1-3). Mononuclear phagocytes from infected animals show characteristic changes in "marker" enzymes (4), exhibit increased phagocytic activity (1, 3), and spread more rapidly and more extensively in vitro than macrophages from normal animals (5-7). The spreading of mononuclear phagocytes in vitro has been correlated kinetically with increased bactericidal activity in vivo (8); for this reason, the macrophage spreading reaction has been viewed as one in vitro manifestation of a state of increased macrophage activity in vivo. A wide variety of agents have been reported to induce macrophage spreading in vitro. These include proteases, metal ions, and cationic anesthetics (7). Recently it has been shown that activated factor B (Bb),<sup>1</sup> the central serine esterase of the alternative pathway of complement activation (APC) (9), induces murine macrophages (10) and human monocytes (11) to spread. Expression of monocyte spreading activity by Bb requires that the catalytic site be structurally intact because treatment with heat (56°C for 30 min) or diisopropyl fluorophosphate (10<sup>-3</sup> M) destroys both enzymatic and spreading activities (9-11).

Native factor B (B) is a protein of 93,000 mol wt that enters into a Mg<sup>++</sup>-dependent complex with C3b (12) and is cleaved by factor D (13), yielding fragments of 60,000 and 33,000 mol wt, termed Bb and Ba, respectively (9, 12, 14). The C3b,Bb complex serves as the convertase of the APC, which cleaves C3 and C5 (9, 12, 14). Enzymatic activity of the C3b,Bb complex resides in the Bb fragment, which retains limited esterolytic activity after it is released from the complex by decay dissociation (9, 13, 15). Considering the restricted substrate specificity of C3b,Bb in the complement system, the question arose as to whether either of the natural substrates of Bb, namely

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<sup>1</sup> *Abbreviations used in this paper:* ABS, acetate-buffered saline; APC, alternative pathway of complement activation; B, factor B; Bb, activated enzymatically active factor B; FITC, fluorescein isothiocyanate; GBS, glycine-buffered sodium chloride; NHS, normal human serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

C3 or C5, might serve as a monocyte-surface substrate of Bb. Activation of C5 by C3b,Bb is the central event initiating assembly by the APC of the C5b,6,7,8,9 (C5b-9) membrane attack complex in serum (16).

### Materials and Methods

*Media.* Medium 199 and RPMI 1640 were purchased from Grand Island Biological Co., Grand Island, N.Y. After supplementing these media with glutamine (100 µg/ml), nonessential amino acids, pyruvate (1 mM) and penicillin, streptomycin, and fungizone, the media were mixed 1:1 for use in monocyte cultures (17).

*Complement Proteins and Antiserum to Complement.* Complement proteins C1q (18), C3 (19), C4 (20), C5 (21), and factor B (12), were prepared as described previously. The b fragment of factor B (Bb) was prepared as described previously (9, 10, 12) by cleaving factor B with factor D in the presence of C3b (22). Bb was separated from the other reactants by chromatography at low ionic strength on DE-32 (Whatman, Inc., Clifton, N. J.) (12).

Antisera was raised in goats to C1q, C3, C4, C5. The specificity of these antisera was established by two-directional radial immunodiffusion and immunoelectrophoresis in agarose using normal human serum (NHS), purified serum proteins, and purified complement proteins as antigens. In certain experiments, described in the text, the specificity of anti-C5 Fab' antibody fragments was verified by absorbing with C5, which was purified by conventional procedures (21) and then subjected to additional purification by electrophoresis on and elution from alkaline 7.5% polyacrylamide gels (23).

*Antibody Fragments Directed toward Complement Proteins.* An immunoglobulin (Ig) fraction was prepared from goat antiserum by precipitating Ig at 33% ammonium sulfate saturation twice. F(ab')<sub>2</sub> fragments of antibody were prepared by digesting Ig with 4% (wt:wt) pepsin (Sigma Chemical Co., St. Louis, Mo.) for 16–36 h at 37°C, pH 3.8, in 0.12 M acetate-buffered 0.15 M saline (ABS). F(ab')<sub>2</sub> was separated from nondigested Ig by molecular-sieve chromatography on Ultrogel Aca 34 (LKB Instruments, Rockville, Md.) in 0.1 M glycine-NaOH buffer, pH 8.0, containing 0.15 M sodium chloride (GBS), and Fab' fragments of goat antibody were prepared by reducing F(ab')<sub>2</sub> fragments for 60 min at 37°C with 0.04 M cysteine in GBS, alkylating with 0.025 M iodacetamide for 60 min at 37°C, and separating Fab' from nonreduced F(ab')<sub>2</sub> by molecular-sieve chromatography on Ultrogel Aca 44 (LKB Instruments). Fab were prepared by digesting goat Ig with 1% papain (wt:wt) (activated in 0.01 M cysteine and 0.002 M EDTA) for 18 h at 37°C in 0.15 M phosphate buffer, pH 7.0. Fab was separated from nondigested Ig and Fc by molecular-sieve chromatography on Ultrogel Aca 44 in GBS.

Antibody fragments were prepared for use in studies with human monocytes by dialyzing for 16 h against phosphate-buffered (0.01 M) saline (0.14 M), (PBS), pH 7.2, and for 16 h against tissue culture medium. The molecular size homogeneity of goat Ig, F(ab')<sub>2</sub>, Fab', and Fab fragments used in these experiments was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (24).

*Monocytes.* To prevent complement activation, human blood was collected directly into EDTA (final concentration of 20 mM EDTA) and monocytes were prepared by a four-step procedure described previously (11). Briefly, leukocytes were separated by sedimentation, first, on density 1.09 g/ml Ficoll-Hypaque; second, on density 1.077 g/ml Ficoll-Hypaque; third, at low speed to remove platelets; and, fourth, on linear 2.7–5.5% Ficoll gradients. These procedures yielded a cell preparation that was composed of >90% monocytes and <10% lymphocytes by several criteria (11). Monocytes were separated from lymphocytes by adherence to glass coverslips (13-mm Diam; Clay Adams, Parsippany, N. J.) in microtiter wells (16-mm Diam; Falcon Labware, Oxnard, Calif.), for 16 h at 37°C in medium containing 20% heat-inactivated (54°C for 60 min) autologous serum. Nonadherent lymphocytes were removed by washing with medium, serum-containing medium was replaced with serum-free medium, and adherent monocytes (~2–4 × 10<sup>5</sup>/coverslip) were incubated for 3 d in serum-free medium to effect their maturation to macrophages and to increase their responsiveness to factor Bb (11). Monocytes used for spreading assays were >95% monocytes by morphology after Wright staining, >80% phagocytized carbonyl iron latex particles, >75 and >85% possessed receptors for Fc or C3b, respectively (11).

*Detection of C3 and C5 on Monocytes by the Direct Immunofluorescent Antibody Technique.* Anti-C3 F(ab')<sub>2</sub> and anti-C5 F(ab')<sub>2</sub> were conjugated with fluorescein isothiocyanate (FITC) for use in immunofluorescence microscopic studies as described previously (25). Immunofluorescence staining of cells was accomplished by treating adherent monocytes on glass coverslips with fluorescent antibody fragments at a concentration of 0.6 mg/ml protein in medium for 30 min at 37°C. Cultures were then washed three times with medium, and examined as a wet mount preparation using a Zeiss photomicroscope III (Carl Zeiss, Inc., New York) equipped for epifluorescent illumination with a BG 450-490 excitation filter, FT510 beam splitter, and BP 520 barrier filter.

*Assay for Factor Bb-induced Monocyte Spreading.* Spreading of human monocytes was assayed as described previously (11) by adding 0.3 ml of 3 µg factor Bb/ml to 3-d human monocyte cultures on glass coverslips. Monocytes treated with factor Bb for 3 h were spread two to three times wider and up to six to eight times longer than control untreated monocytes (11). Measurements of cell surface area and circumference were performed using planimetry and phase-contrast photomicrographs of monocytes at a final magnification of × 2,500. Adherent human monocytes were found to have a cell-surface area of 400 ± 100 µm<sup>2</sup> (SD) and a circumference of 56 ± 10 µm (SD); in contrast, monocytes treated with factor Bb for 3 h were found to have a cell surface area of 2,100 ± 400 µm<sup>2</sup> (SD) and a circumference of 230 ± 80 µm (SD). For routine analysis, spreading was assessed microscopically on viable cultures at × 400 magnification using an inverted microscope fitted with a 0.5-mm ocular grid. A monocyte was considered to be spread if at least one of its dimensions was more than twice that of "unspread" monocytes in control cultures. The percentage of spread monocytes was determined by counting two to three microscopic fields (containing 200–300 cells) for each coverslip culture. The mean percentage ± standard deviation was calculated from the results of duplicate or triplicate samples.

*Assays for Inhibition of Monocyte Spreading.* Inhibition of monocyte spreading by Fab' or Fab antibody fragments was assayed by adding 0.3 ml of dilutions of Fab' or Fab in medium to 3-d cultures of glass-adherent monocytes for 15 min at 37°C. The responsiveness of treated monocytes was tested by adding 10 µl of a stock solution of 90 µg factor Bb/ml, and by counting the number of spread and unspread cells after 3 h, as described above.

*Radiolabeling of C5 in Monocyte Extracts with [<sup>14</sup>C]- and [<sup>3</sup>H]Leucine.* Biosynthesis of C5 by monocytes was investigated by preparing monocytes from 1–2 liters of fresh human blood by a modification of the four-step procedure described above. Briefly, monocytes were prepared by centrifugation (2,000 rpm for 20 min) on 80 ml of Ficoll-Hypaque 1.09 g/ml and then 1.077 g/ml in 250-ml centrifuge bottles using an International PR-2 centrifuge (International Equipment Co., Needham Heights, Mass.). The remaining preparative steps were performed as described previously (11). Conditions for radiolabeling with [<sup>3</sup>H]- or [<sup>14</sup>C]leucine (120 or 325 mCi/mM, respectively; New England Nuclear, Boston, Mass.) are described in the text. After labeling, adherent monocytes were washed three times with medium and twice with PBS, and then extracted with 100–200 ml of 1% Triton X-100 in 2 mM sodium carbonate buffer, pH 7.5, containing 1.0 mM phenylmethylsulfonylfluoride. Extracts were centrifuged 12,000 g for 30 min to remove cellular debris, and concentrated by ultrafiltration on Amicon PM30 membranes (Amicon Corp., Lexington, Mass.) to a final volume of 1–2 ml. Samples were analyzed for radiolabeled C5 by adding goat anti-C5 Ig and 10% NHS (as a carrier antigen); immunoprecipitates were collected by centrifugation, washed, and dissociated in ABS, pH 3.0, containing 1% SDS; solubilized precipitates were analyzed by SDS-PAGE (24). Radioactivity was determined by slicing the acrylamide gels, solubilizing them in Protosol (New England Nuclear), and by counting in a Beckman LS 230 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). In other experiments, monocytes were labeled with [<sup>14</sup>C]leucine and C5 was precipitated with goat anti-C5 Ig by double immunodiffusion in 2% agarose containing 1% Triton X-100. Nonspecifically trapped radioactivity was removed by washing twice a day for 5 d with veronal-buffered saline containing 1% Triton; then for 1 d with PBS; and finally for 1 d with distilled water. Specifically bound radioactivity was determined by autoradiography for 20 d with X-ray film (Eastman Kodak Co., Rochester, N. Y.).

### Results

Experiments were performed to investigate whether C3 or C5 were expressed on the monocyte surface, and whether these proteins play a role in the spreading reaction induced by Bb.

*Demonstration of C3 and C5 Antigens on Monocytes by the Direct Fluorescent Antibody Technique.* The expression of C3 and C5 on monocytes was investigated by the direct immunofluorescent antibody technique employing monospecific FITC-conjugated goat anti-C3 and anti-C5 F(ab')<sub>2</sub> antibody fragments. Positive immunofluorescence staining was observed with FITC-conjugated anti-C5 F(ab')<sub>2</sub> on  $6 \pm 7\%$  of freshly prepared monocytes and on  $70 \pm 6\%$  of monocytes incubated for 3 d in vitro in serum-free medium. The fluorescence micrographs presented in Fig. 1 show patching and capping of cell-surface determinants, which were stained by anti-C5 F(ab')<sub>2</sub> at 37°C.

In parallel studies, the expression of C3 on monocytes was investigated and weak fluorescence staining was observed with FITC-conjugated anti-C3 F(ab')<sub>2</sub> on  $6 \pm 5\%$  of freshly prepared monocytes. The percentage of cells stained positively for C3 did not increase significantly during in vitro incubation.

*Detection of Radiolabeled C5 in Extracts of Monocytes Incubated with [<sup>14</sup>C]- and [<sup>3</sup>H]-Leucine.* Experiments were conducted to investigate whether monocytes biosynthetically incorporate [<sup>3</sup>H]leucine into C5 during 7 d of in vitro incubation in serum-free medium. The results presented in Fig. 2 indicate that anti-C5 Ig precipitates a tritium-labeled monocyte protein that has the same mobility in SDS-PAGE as purified C5 run in a replicate gel. In other experiments, the kinetics of C5 synthesis was investigated by incubating monocytes with [<sup>14</sup>C]leucine, extracting cells with Triton X-100,

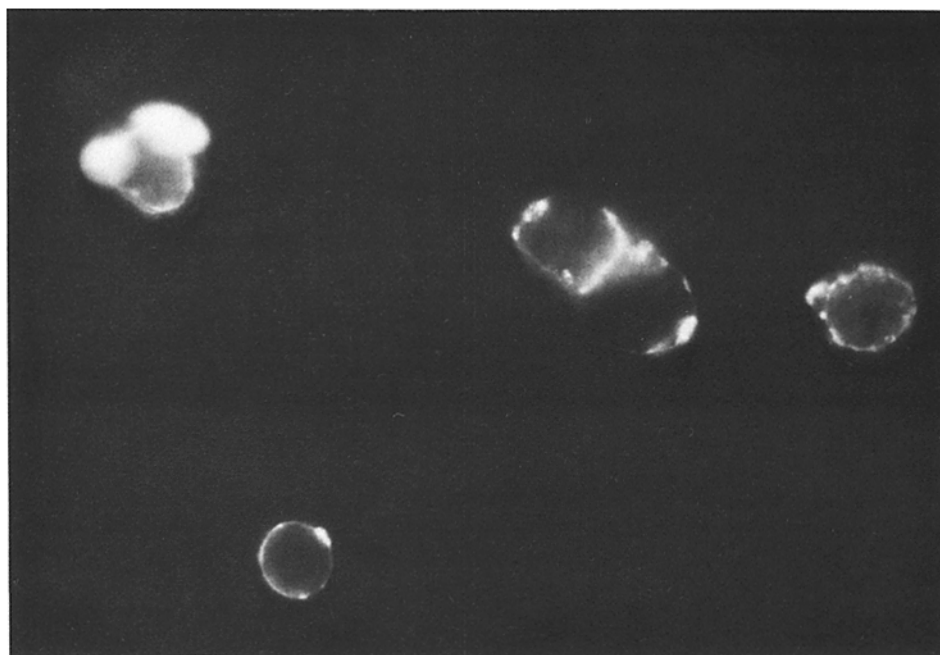


FIG. 1. Positive immunofluorescence staining at 37°C of human peripheral blood monocytes by FITC-conjugated goat anti-C5 F(ab')<sub>2</sub>; monocyte cultures maintained for 3 d in vitro in serum-free medium before staining; original magnification  $\times 660$ .

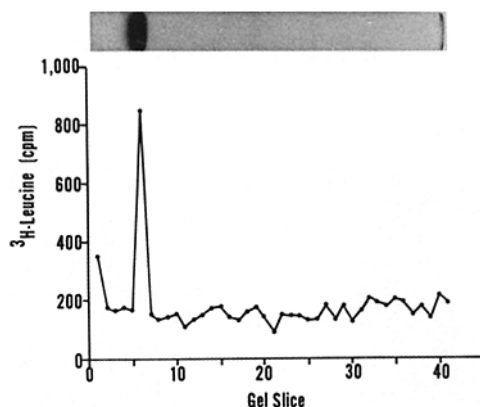


FIG. 2. Incorporation of [ $^3\text{H}$ ]leucine by human peripheral blood monocytes into immunoprecipitable C5-antigen, which was extracted from cells with Triton X-100 and detected on SDS-PAGE by scintillation counting of gel slices: the upper panel is purified C5; the lower panel is immunoprecipitable monocyte C5 run on a replicate gel.

and precipitating radiolabeled C5 antigen by double immunodiffusion in agarose with goat anti-C5 Ig and 10% NHS as a carrier antigen. After washing to remove free radiolabeled proteins, the immunoprecipitates were analyzed for precipitated  $^{14}\text{C}$ -labeled C5 antigen by autoradiography with X-ray film. Radiolabeled C5 antigen was detected in five out of five immunoprecipitation tests performed with extracts of monocytes at days 4, 7, and 11 of in vitro serum-free culture; however, no radiolabeled C5 antigen was detected (0/9 tests) on days 1, 2, or 3. In other experiments, an attempt was made to promote C5 synthesis by monocytes on days 1–3 of culture by adding 2–5% autologous serum to the culture media; however, no effect was noted. C5 antigen was detected in extracts of monocytes maintained in serum-containing cultures only after day 3. It is significant that monocytes incubated under these conditions do not acquire responsiveness to Bb-induced spreading until after day 3 (11).

*Inhibition of Bb-induced Monocytes Spreading by Anti-C5 Fab and Fab' Antibody Fragments.* The possible role of C3 and C5 in the factor Bb-induced monocyte spreading reaction was investigated by treating monocytes with Fab antibody fragments directed toward these proteins. Monocytes were treated at  $37^\circ\text{C}$  for 30 min with dilutions of anti-C3 or anti-C5 Fab and Fab'; Bb was then added to the assays and the percentage of cells spread was determined 3 h later. The results presented in Fig. 3 indicate that anti-C5 Fab or Fab' inhibited monocyte spreading by up to 95%. Less than 10% of monocytes treated with anti-C5 Fab' were observed to be spread compared with 60–90% spread in control cultures receiving Bb alone. Anti-C3 Fab or Fab' did not inhibit monocyte spreading induced by Bb (Fig. 3). We reasoned that anti-C5 Fab' might exert its inhibitory effect on monocyte spreading by binding to cell surface C5 antigen. To test whether such binding of anti-C5 Fab' was a sufficient condition for the expression of its inhibitory activity, monocytes were treated with anti-C5 Fab', washed, and then Bb was added. As a control, monocytes were treated in a similar manner with anti-C3 Fab'. The results presented in Table I indicate that a brief exposure to anti-C5 Fab' was sufficient to inhibit a significant percentage of monocytes from spreading when they were treated with factor Bb: spreading induced

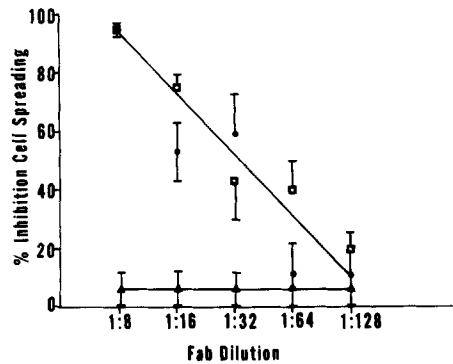


FIG. 3. Inhibition by goat anti-C5 Fab and Fab' antibody fragments of the spreading reaction induced by factor Bb (Fab and Fab' protein concentration 10–12 mg/ml before dilution).  $\Delta$ , anti-C3 Fab' or Fab;  $\square$ , anti-C5 Fab;  $\bullet$ , anti-C5 Fab'.

TABLE I  
Inhibition of Bb-induced Monocyte Spreading by Pretreatment of Monocytes with Anti-C5 Fab' Antibody Fragments\*

Designation	Treatment	Condi- tions wash $\ddagger$	Addition	Spreading $\S$	Inhibi- tion of spread- ing $\parallel$
				%	%
Negative control	None	—	None	7 $\pm$ 7	—
Positive control	None	—	Bb	43 $\pm$ 9	0
Experimental	Anti-C3 Fab'	—	Bb	41 $\pm$ 4	6
Experimental	Anti-C3 Fab'	+	Bb	59 $\pm$ 9	0
Experimental	Anti-C5 Fab'	—	Bb	16 $\pm$ 3	75
Experimental	Anti-C5 Fab'	+	Bb	24 $\pm$ 7	53

\* Assay with 3-d human monocyte cultures.

$\ddagger$  Monocytes were incubated for 20 min at 22°C with a 1:4 dilution of Fab', washed (as indicated) with medium three times, and then tested for their ability to become spread when Bb (3  $\mu$ g/ml) was added.

$\S$  Percentage of cells  $\pm$  SD after 3 h at 37°C.

$\parallel$  Inhibition of spreading = 1.0 -

$$\frac{(\text{spreading experimental}) - (\text{spreading negative control})}{(\text{spreading positive control}) - (\text{spreading negative control})} \times 100.$$

by Bb was inhibited by 53% when monocytes were pretreated for 20 min at 20°C. In contrast, pretreatment with anti-C3 Fab' was without effect (Table I). (Anti-C5 Fab' did not cause cells that were already spread to become unspread). Experiments were next conducted to quantitatively compare the inhibitory activity of anti-C5 Fab' in monocyte spreading assays with the inhibitory activity in complement hemolytic assays. (Anti-C5 Fab' used in these experiments was the same as that used in the experiments presented in Fig. 3 and Table I.) It was found that anti-C5 Fab' (a) inhibited by 50% the monocyte spreading reaction of  $1-2 \times 10^5$  cells at a dilution of

1:30 (corresponding to 30–90  $\mu\text{g}/\text{ml}$  Fab<sup>†</sup>); and (b) inhibited 1.5 Z of C5 (0.2–0.5  $\mu\text{g}$ ) hemolytic activity at a 1:300 dilution.

The specificity of the anti-C5 Fab' inhibitory effect on monocyte spreading was investigated by absorbing anti-C5 Fab' with highly purified C5. For these studies, C5 was purified by conventional techniques (12), and then subjected to further purification by electrophoresis on the elution from alkaline polyacrylamide gels (23). Anti-C5 Fab was titered to determine the lowest dilution giving 95–100% inhibition of monocyte spreading, this dilution was then absorbed with 1–10  $\mu\text{g}$  of highly purified C5 for 15 min at 22°C. The results presented in Table II indicate that 1–10  $\mu\text{g}$  of C5 absorbed 70–100% of the inhibitory activity of a 1:24 dilution of anti-C5 Fab'. Monocytes in control cultures treated with <10  $\mu\text{g}$  C5 per assay and no anti-C5 Fab' spread in a normal manner (Table II).

We reasoned that if factor Bb exerts its effects on monocytes by interacting with C5 on the cell-surface, the addition of relatively large amounts of C5 to the reaction

TABLE II  
*Inhibition of Bb-induced Monocyte Spreading by Anti-C5 Fab': Addition of C5  
Antigen Reverses the Inhibitory Effect\**

Experiment	Addition‡	Fab'§	C5	Spreading	Inhibition
		$\mu\text{g}/\text{assay}$	$\mu\text{g}/\text{assay}$	%	%
1	None	0	0	16 ± 1	—
	Bb	0	0	65 ± 9	0
	Bb	0	10	66 ± 5	0
	Bb	280	0	6 ± 4	100
	Bb	280	1	38 ± 4	55
	Bb	280	2.5	44 ± 4	43
	Bb	280	5	59 ± 7	12
	Bb	280	10	59 ± 12	12
2	None	0	0	10 ± 1	—
	Bb	0	0	53 ± 10	0
	Bb	0	10	52 ± 5	0
	Bb	170	0	9 ± 1	100
	Bb	170	1	17 ± 3	84
	Bb	170	2.5	7 ± 0	100
	Bb	170	5	58 ± 13	0
	Bb	170	10	44 ± 7	21
3	None	0	0	10 ± 4	—
	Bb	0	0	43 ± 4	0
	Bb	0	0	44 ± 3	0
	Bb	250	0	11 ± 1	97
	Bb	250	2.5	33 ± 13	30
	Bb	250	5	32 ± 18	33
	Bb	250	7.5	41 ± 7	6

\* Assay with 3-d human monocyte cultures.

‡ 3  $\mu\text{g}$  Bb/ml.

§ The minimum amount of anti-C5 Fab' needed to give 90–100% inhibition of Bb-dependent spreading.

|| Percent inhibition =  $\frac{(\text{spreading Bb} + \text{Fab}') - (\text{spreading none})}{(\text{spreading Bb}) - (\text{spreading none})} \times 100$ .

mixture might competitively inhibit the action of Bb. The results presented in Table III confirm this hypothesis and indicate that 25–50  $\mu\text{g}$  of C5 inhibit the monocyte spreading reaction induced by 1  $\mu\text{g}$  of factor Bb. In data not presented here, control experiments were performed in which up to 200  $\mu\text{g}$  of human IgG (Cohn fraction II), human albumin, or ovalbumin were added to the monocyte spreading assay. The addition of these proteins to the assay did not inhibit monocyte spreading induced by factor Bb. The addition of C5 to monocytes that had already been induced by Bb to become spread did not cause these cells to become unspread.

### Discussion

The spreading of mononuclear phagocytes has been viewed as one *in vitro* manifestation of a state of increased macrophage activity *in vivo*. Recently, it has been shown that the central serine esterase of the alternative pathway of complement activation, Bb, induces murine macrophages (10) and human monocytes (11) to become spread on a glass substrata. It has also been established that to induce the spreading reaction, the catalytic site of the Bb enzyme must be structurally intact since treatment of Bb with heat (56°C for 30 min.) or diisopropyl fluorophosphate ( $10^{-3}$  M) destroyed both enzymatic and spreading activities (9–12).

Native factor B is a single chain polypeptide of 93,000 mol wt, which enters into an  $\text{Mg}^{++}$ -dependent complex with C3b (C3b,B) and is cleaved by factor D, yielding fragments of 60,000 and 33,000 mol wt (14), termed Bb and Ba, respectively (9, 12, 14). Binding and cleavage of B initiate formation of the C3b,Bb complex, which is the C3/C5 convertase of the APC. The serine esterase activity of this complex is associated with Bb (9, 12–15), whereas the localization of the complex on biological membranes is a property of the C3b molecule, which transiently expresses an active thioester group that can form covalent bonds with molecules in the surface of particles that activate the APC (26–32). Cleavage of C3 by cell-bound C3b,Bb leads to the deposition of additional C3b molecules on the surface that are required to express C5 convertase activity (33). It has recently become apparent that cell-bound C3b or fluid-phase C3b (34) binds to C5 with a low affinity; and that such cell-bound (33) or fluid-phase-complexed (34) C5 is susceptible to cleavage by the APC C5 convertase.

TABLE III  
*Inhibition by C5 of Bb-induced Monocyte Spreading*

Addition*	C5 $\mu\text{g}/\text{assay}$	Spreading %	Inhibition‡
None	0	10 $\pm$ 4	—
Bb	0	43 $\pm$ 4	0
Bb	50	12 $\pm$ 5	94
Bb	35	9 $\pm$ 3	100
Bb	25	9 $\pm$ 5	100
Bb	20	52 $\pm$ 7	0
Bb	10	59 $\pm$ 12	0

Assay with 3-d monocyte cultures;  $1-3 \times 10^5$  monocytes per assay.

\* 3  $\mu\text{g}$  Bb/ml.

‡ Percent inhibition =  $\frac{(\text{spreading Bb} + \text{C5}) - (\text{spreading none})}{(\text{spreading Bb}) - (\text{spreading none})} \times 100$ .



In molecular terms, it is probable that expression of C5 convertase activity involves binding of C5 to multiple C3b molecules where one or more of the C3b molecules also contains bound Bb enzyme. Bb released from the C3b,Bb complex by decay dissociation fails to bind to C3b, causing it to lose its potential as a significant C3 or C5 convertase (9). However, free Bb retains esterolytic activity on synthetic substrates (9, 12-15) and a limited ability to cleave plasminogen (35) and prothrombin (36). It is possible that under appropriate conditions, free Bb may also cleave C3 and C5 or that Bb may to a limited extent be able to reform a C3b,Bb complex on the cell surface.

The restricted substrate specificity of factor Bb in the complement system raised a question about whether either of the natural substrates of Bb might serve as a monocyte surface substrate for Bb in inducing the monocyte spreading reaction. In preparation for the present study, experiments were conducted to define the optimum conditions for stimulation of monocyte spreading by factor Bb, and the results of these experiments have been published previously (11). Studies have also been initiated to investigate whether Bb stimulates changes in monocyte "marker-enzyme" systems and it has been found that Bb stimulates (a) a chemilluminescence response in 3-d serum-free cultures of human peripheral blood monocytes and (b) plasminogen activator secretion (J. S. Sundsmo, unpublished data). Significantly, it was found that human peripheral blood monocytes require 3 d incubation in vitro to acquire responsiveness to factor Bb-induced spreading (11). This finding caused us to investigate in the present study whether the surface expression of C3 or C5 on monocytes changed during in vitro culture in serum-free medium and it was found that the percentage of monocytes staining positively for C3 or C5 was  $6 \pm 7\%$  at the initiation of in vitro culture, and that anti-C5 staining increased to  $70 \pm 6\%$  after 3 d incubation in serum-free medium, whereas C3 staining remained constant (Fig. 1). Biosynthesis of C5 by monocytes was investigated using [ $^{14}\text{C}$ ]- and [ $^3\text{H}$ ]leucine, immunoprecipitation techniques, and SDS-PAGE to evaluate C5 antigens in Triton X-100 extracts of monocytes. It was found that monocytes incorporate radiolabeled leucine into a protein with an apparent mol wt of 180,000, which is antigenically similar to serum C5 (Fig. 2); and that the radiolabeled C5 antigen was only detected in cell extracts after 3 d of in vitro incubation. It is perhaps significant that C5 may be acquired by human monocytes during a time in which monocyte maturation into macrophages has been recognized to take place in vitro (37-39). The finding of biosynthetically radiolabeled C5 confirms and extends the observations of Stecher and Thorbecke (40), Levy et al. (41), and Ooi and Colten (42, 43), and Ooi et al. (44), who have reported that murine macrophages synthesize C5.

Involvement of cell-surface C3 or C5 in the monocyte spreading reaction induced by factor Bb was investigated in the present study by testing for the ability of anti-C3 and anti-C5 Fab' fragments to inhibit the spreading reaction induced by factor Bb. Anti-C5 Fab' (but not anti-C3 Fab') was found to block by up to 100% the spreading induced by factor Bb in a serum-free system (Fig. 3). Inhibition by anti-C5 Fab' was found to be exerted in a dose-dependent fashion (Fig. 3) and the inhibitory effect was exerted when monocytes were treated with anti-C5 Fab' and washed before the addition of factor Bb (Table I), suggesting that anti-C5 Fab' exerts its inhibitory effect on the monocyte spreading reaction by binding to monocytes rather than by interfering with some presumptive fluid-phase reaction involving factor Bb. The antigenic

specificity of this inhibitory effect was established by absorbing anti-C5 Fab' with PAGE-purified C5 and this treatment caused the inhibitory activity of anti-C5 Fab' to be quantitatively decreased. We reasoned that if factor Bb exerts its effect on monocytes by interacting with cell-surface C5, then the addition of purified C5 to the reaction mixture might competitively inhibit the action of Bb on monocyte surface C5. This hypothesis was confirmed when it was found that fluid-phase C5 inhibited the action of Bb if it was added in 20–100-fold molar excess of the amount of factor Bb needed to induce the monocyte spreading reaction (Table III). These results suggest a previously unrecognized property of Bb, namely, that purified Bb interacts with C5 on the monocyte surface or in fluid phase in the absence of C3b.

The results presented here are analogous to those we have reported previously (45) in a study designed to investigate expression of complement proteins on lymphocytes and the possible functional role of C5 in lymphocyte activation. In that study, human peripheral blood lymphocytes were found to synthesize C5, which had an apparent mol wt of 180,000 on SDS-PAGE; lymphocytes stimulated with phytohemagglutinin incorporated [<sup>14</sup>C]leucine into C5, C6, C7, and C8 antigens as detected by immunoprecipitation in two-directional immunodiffusion and autoradiography (45); and anti-C5 Fab' antibody fragments inhibited lymphocyte stimulation by phytohemagglutinin-mitogen or allogeneic mixed lymphocyte culture as determined by inhibition of [<sup>3</sup>H]thymidine incorporation (46).

It is established that many agents induce monocyte spreading *in vitro* (7). What is unique about factor Bb of the complement system is that mononuclear phagocytes have been reported to synthesize factor B (47–49), factor D (48, 50), C3 (40, 51–53) and C3b inactivator (50, 54), giving these cells the potential to activate complement through the alternative pathway and to regulate complement activation. In addition, elastase, which is associated with mononuclear phagocytes (55, 56), has been shown to cleave C3 generating a "C3b-like" fragment, which is functionally active in consuming factor B (56); and, plasmin, which is also capable of cleaving factor B (14) and C3 (57), has been shown to be formed following secretion of plasminogen activator from "activated" macrophages (58–60). Leukocytes and lymphoblastoid cells activate complement (25, 61–70); cellular receptors have been identified that bind complement activation fragments (71), and the Bb complement activation fragment has been implicated as a potential mediator of macrophage migration inhibition (72), and C5a also exerts profound effects on neutrophils (73).

Complement has been considered a mediator of humoral immunity, active in antibody-mediated lysis of bacteria and cells, and in anaphylactic and Arthus reactions. The recognition that lymphocytes and monocytes synthesize and activate complement, and that factor Bb induces significant changes in the physiology of mononuclear phagocytes, raises the possibility that proteins of the leukocyte complement system may also serve as mediators of cellular immunity.

### Summary

The central serine esterase of the alternative pathway of complement (APC) activation, activated factor B (Bb), has been shown recently to induce murine macrophages and human monocytes to become spread on a glass substrata. It has also been established that to induce the spreading reaction, the catalytic site of the Bb enzyme must be structurally intact since treatment of Bb with heat (56°C for 30 min)

or diisopropylfluorophosphate ( $10^{-3}$  M) destroyed both enzymatic and spreading activities. In the C3b,Bb complex, Bb exhibits restricted substrate specificity for C3 and C5. With this in mind, the role of C3 and C5 in the monocyte spreading reaction was explored in the present study. Expression of C3 and C5 on the surface of human peripheral blood monocytes was investigated by the direct fluorescent antibody technique employing fluorescein isothiocyanate-conjugated anti-C3 or C5 F(ab')<sub>2</sub> antibody fragments. It was found that C3 and C5 were present on  $6 \pm 7\%$  of freshly prepared monocytes and that expression of C5, but not C3, increased to  $70 \pm 6\%$  when monocytes were incubated for 3 d in serum-free medium. Biosynthesis of C5 was indicated when it was found that under serum-free conditions, monocytes incorporated [<sup>3</sup>H]leucine into immunoprecipitable C5 with an apparent mol wt of 180,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The role of C3 and C5 in the monocyte spreading reaction induced by factor Bb was explored by testing for the ability of anti-C3 and anti-C5 Fab' antibody fragments to block monocyte spreading. It was found that anti-C5 Fab' inhibited by up to 100% the 3-h human monocyte spreading reaction induced by Bb; in contrast, anti-C3 Fab' or anti-C4 Fab' inhibited by <10%. That the inhibitory effect of anti-C5 Fab' was exerted directly on the monocyte was established when it was found that the 3-h monocyte spreading reaction was significantly inhibited by pretreating monocytes with anti-C5 Fab' for 20 min and then washing before the addition of Bb. The specificity of the inhibitory effect of anti-C5 Fab' was established by quantitatively absorbing the antibody fragments with polyacrylamide gel-purified C5 antigen: >4  $\mu$ g of C5 absorbed by 100% the inhibitory activity of 10–20  $\mu$ g of anti-C5 Fab'. That factor Bb exerted its effect on monocytes by interacting directly with cell surface C5 was indicated when it was found that purified C5 inhibited the monocyte spreading reaction induced by Bb; >25  $\mu$ g of C5 inhibited by 100% the spreading reaction induced by 3  $\mu$ g factor Bb.

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