

THE INDUCTION OF MACROPHAGE SPREADING BY FACTOR B OF THE PROPERDIN SYSTEM*

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Mouse peritoneal macrophages obtained after the injection of substances which produce a local inflammatory response in the peritoneal cavity such as Brewer's thioglycollate, differ in a number of functional and biochemical aspects from resident cells. These elicited or stimulated macrophages have been shown to exhibit changes in their endocytic capacity (1, 2), their expression of several membrane-associated enzymes (3, 4), their synthesis and secretion of neutral proteinases, including plasminogen activator (5-8), their motility and their ability to rapidly spread on surfaces to which they have become attached (9). It has been noted that in vitro spreading may be a consequence and correlate of in vivo macrophage activation by immunological mechanisms as it is a property of cells activated in vivo by Bacille Calmette-Guérin injections or by other means of lymphocyte stimulation (10-12). Thus, rapid spreading of freshly explanted macrophages has been taken as an early expression of the morphological, functional and biochemical changes occurring upon cell activation (13). Previous studies have demonstrated that the rapid spreading observed with activated macrophages can be induced in nonstimulated, resident cells by factors generated in plasma upon activation of the contact phase of blood coagulation or independently by activation of the complement system (14).

In this report we further analyze the complement-dependent spreading reaction of mouse peritoneal macrophages and show that it is induced by the enzymatically active b-fragment of factor B of the properdin system, i.e., the alternative pathway of complement activation.

The results have in part been presented at the Seventh International Complement Workshop held in St. Petersburg, Florida in November 1977 (15).

Materials and Methods

Reagents. EDTA, kaolin, inulin, fetal calf serum (FCS),¹ Dulbecco's modified Eagle's medium, H-21, (DMEM) were obtained as before (14). Medium RPMI-1640 was from Grand

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¹ *Abbreviations used in this paper:* A, anti-sheep E serum; AG LMe, acetyl-glycyl-lysyl-methyl ester; C3, third complement component; CVF, cobra venom factor; DFP, di-isopropylphosphorfluoridate; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; E, sheep erythrocytes; FCS, fetal calf serum; ³H-AG LMe, ³H-methyl acetyl glycl-L-lysine methyl ester-HCl; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VB, veronal buffer; Z, effective molecules of B/cell.

Island Biological Co., Grand Island, N. Y. Sheep erythrocytes (E) were from Colorado Serum Co., Denver, Colo. Anti-sheep E serum (A) and its IgM fraction, respectively, were obtained from Cordis Laboratories, Inc., Miami, Fla. Antisera against whole mouse serum, mouse C3, mouse albumin, mouse transferrin, and mouse immunoglobulins were obtained from Cappel Laboratories, Inc., Cochranville, Pa. Human blood was bought from the New York Blood Center, New York.

Di-isopropylphosphofluoridate (DFP), from Aldrich Chemical Co., Inc., Milwaukee, Wis., was diluted in propylene glycol. 1 mCi of (^3H)methyl acetyl glycl-L-lysine methyl ester-HCl (^3H -AG LMe, 195 mCi/mmol) was obtained from Biochemical and Nuclear Corporation, Burbank, Calif. It was dissolved in 1 ml water, further diluted 1/100 with water, and frozen in small aliquots at -70°C . Unlabeled AG LMe was from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol (DTT) was from Calbiochem, La Jolla, Calif. and reduced glutathione was from Boehringer, Mannheim Biochemicals, Indianapolis, Ind. Ion exchange celluloses, DE 32 and CM 32 were from Whatman, Inc., Clifton, N. J. QAE-Sephadex A50 and Sephadex G150 were obtained from Pharmacia Fine Chemicals Inc., Piscataway, N. J. and Bio Gel A-0.5 m was from Bio-Rad Laboratories, Richmond, Calif.

Purified Proteins. Human C3 (16), C3b (17), factor B (18), factor D (19) and cobra venom factor (CVF) (20) were purified according to published methods. Their purity was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 7% gels and by immunochemical means. Mouse factor B was partially purified from mouse serum (Pel-Freez Farms Inc., Rogers, Ark.) by column chromatography using in succession QAE-Sephadex A50, CM32-cellulose, and Biogel A-0.5 m. The final product was subjected to 6% alkaline PAGE. The gels were sliced into 1.5-mm segments which were analyzed for the presence of factor B. Gel segments containing the active protein were emulsified with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and were used to raise antisera in rabbits. The two antisera obtained were found to be monospecific for mouse factor B when analyzed by double diffusion-in-gel and by immunoelectrophoresis using mouse serum and various concentrated fractions prepared from mouse serum by column chromatography. The antisera did not react with antigens present in human serum. The IgG fractions of the anti-mouse B sera were obtained as the proteins not absorbed by DE32-cellulose at pH 7.3 and 0.005 M sodium phosphate. F(ab')₂ fragments were prepared from the purified IgG by pepsin digestion at pH 4 at a ratio of 3 mg pepsin (Worthington Biochemical Corp., Freehold, N. J.) per 100 mg of protein as described (21). The F(ab')₂ fragments were separated from other digestion products and from undigested IgG by gel filtration on Sephadex G150 and were stored at 4°C after their concentration to 0.20 of the original antiserum volume and the addition of 0.2% sodium azide. Before its use with macrophages the anti-B F(ab')₂ was extensively dialyzed at 4°C against DMEM.

Macrophage Spreading Assay. This was performed as previously described (14) in RPMI-1640 supplemented with 10% heat inactivated (1 h, 56°C) FCS. Cells were adjusted to 2×10^6 /ml and were plated (0.1 ml) on 13-mm round cover slips (Clay Adams, Inc., Parsippany, N. J.). After incubation for 30 min at 37°C in a 5% CO_2 , 100% humidity atmosphere, they were washed three times with medium to remove nonadherent cells. The cover slips were then transferred to dry Petri dishes, were covered with 0.1 ml of medium or test solution and were further incubated for 1–2 h at 37°C . The cells were then fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and were left under water at 4°C until cell spreading was evaluated by phase-contrast microscopy by using the criteria described previously (14).

Functional Assay for Factor B. Factor B was assayed through its ability to form a stable C3, Bb complex when incubated with purified CVF and factor D. The complex which was formed during a first incubation step was then titrated by its ability to induce lysis of glutathione-treated human erythrocytes in the presence of human EDTA-serum serving as a source of C3-9 (22). Factor B (0.2–10 μg factor D in a total vol of 0.21 ml GVBS-Mg for 30 min at 37°C). 0.1 ml of 5×10^8 /ml glutathione-treated erythrocytes in GVBS-E and 0.2 ml human EDTA-serum (one part 0.2 M EDTA, pH 7.4 + nine parts normal human serum) were then added and the incubation continued for 30–60 min. The reaction was stopped by the addition of 2 ml of cold 0.15 M NaCl and the fraction of unlysed cells was determined spectrophotometrically at 541 nm. Lysis was expressed in effective molecules of B/cell (Z).

Radiochemical Assay for Factor B. This was performed according to Imanari et al. (23) as based on the work of Roffman et al. (24). The assay is based on the fact that methanol generated by

proteinase from [^3H]methyl ester-containing substrates can be extracted into a toluene-based scintillation cocktail and can then be measured in a liquid scintillation spectrometer. Unhydrolyzed substrate largely remains in the aqueous phase and, because very small amounts of substrate are used, gives rise to only low background counts. 100 μl Veronal buffer (VB) containing purified B or Bb was incubated at 37°C in tightly capped plastic tubes with 20 μl [^3H]AG LMe diluted with water such that 75 μl of this mixture contained a total of 95,000–98,000 cpm. The reaction was stopped by pipetting 75 μl of the reaction mixture into scintillation vials containing 10 ml scintillation fluid and 50 μl stop solution (nine parts 0.01 M AG LMe in water + one part glacial acetic acid). The scintillation vials were tightly capped, vigorously shaken for 10 s, and were counted in a refrigerated liquid scintillation spectrometer. Total counts were obtained by using dioxane-based Aquafluor (New England Nuclear Corp., Boston, Mass.) as the scintillation fluid. Liberated [^3H]methanol was determined by using the toluene based scintillation fluid liquifluor (New England Nuclear Corp.). Kinetic experiments established that hydrolysis of AG LMe by Bb proceeds very slowly. Therefore, 16 h of incubation at 37°C was routinely used for this assay. Controls for unspecific hydrolysis of substrate were carried through in all experiments and their results, 2.8–4.6% were subtracted appropriately. To keep unspecific hydrolysis low it was found necessary to filter-sterilize and boil all buffer solutions before use.

PAGE. Electrophoresis in 6% alkaline PAGE at pH 8.9 was performed as described (25). Gels from which protein was to be eluted were first subjected to electrophoresis for 2 h at 4°C. After replacing the electrode buffers, the samples (50–100 μl) in 10% sucrose were layered on top of the gels. Electrophoresis was continued until the tracking dye (bromphenyl blue) had reached the anodal end of the gels. For elution the gels were sliced into 1-mm segments which were eluted overnight at 4°C with 0.1 ml PBS. Selected eluates were dialyzed against DMEM for 15 h at 4°C before their use in cell spreading assays. SDS-PAGE in 7% gels was performed as described by Weber and Osborn (26). All samples were boiled before electrophoresis for 5 min in 1% SDS, 7×10^{-3} M DTT, 5 M urea. The proteins used as molecular weight markers were human fibrinogen (kindly provided by Dr. P. C. Harpel, Cornell Medical College, New York) and soybean trypsin inhibitor (Worthington Biochemical Corp.).

Radiolabeling. Purified human factor B was radiolabeled with ^{125}I using the lactoperoxidase method of David and Reisfeld (27) with Sepharose-bound lactoperoxidase (Worthington Biochemical Corp.).

Buffers

PHOSPHATE-BUFFERED SALINE (PBS). 0.14 M NaCl containing 0.01 M sodium phosphate, pH 7.2. **VBS-Mg.** Isotonic veronal-buffered saline containing 10^{-3} M MgCl_2 . **Gvbs-Mg.** VBS-Mg containing 0.1% gelatin. **Gvbs.** GVBS-Mg containing in addition 1.5×10^4 M CaCl_2 . **Gvbs-E.** GVBS lacking divalent cations but containing 0.02 M EDTA.

Results

Lack of Spreading Activity Generated from Components of the Classical Pathway. Previous experiments using CVF-activated serum had suggested that the induction of macrophage spreading might be due to activation products of the third complement component (C3) and/or components of the alternative pathway because C1, C2, and C4 are not grossly affected by the addition of CVF to plasma or serum and because a role for C5 appeared to be excluded on the basis of results using serum from mice genetically deficient in this component. To substantiate these findings 10^9 EA prepared with IgM antibodies were incubated with (a) C1, (b) C1 and C4, (c) C1, C2, C3, and C4 and (d) C1, C2, C3, C4, and C5 in a total vol of 1 ml. After 30 min at 37°C the cells were removed by centrifugation and the supernates were tested for spreading activity. No activity was detected (Table I).

Induction of Macrophage Spreading by Activated Components of the Human Properdin System. Because of the failure to generate macrophage spreading activity from components of the classical pathway experiments with highly purified proteins of the

TABLE I
Lack of Induction of Macrophage Spreading by Components of the Classical
Complement Pathway

Supernate from*	Macrophages spread‡ %
E IgM + C1	7
E IgM C1 + C4	8
E IgM C14 + C2 + C3	6
E IgM C1423 + C5	7

* Supernates were prepared by incubation of 10^9 E IgM for 30 min at 37°C with 10,000 U of guinea pig C1 and 1,000 U of C4, C2, and C3 in PBS containing Ca^{++} and Mg^{++} .

‡ Determined after 1 h at 37°C in RPMI-1640 containing 10% heat inactivated FCS.

alternative pathway were performed. Mixtures of human factor B, D, C3b, and C3 were incubated in VBS-Mg for 30 min at 37°C . Consumption of factor B and conversion of C3 were determined by titration (factor B) and immunoelectrophoresis (C3) in 1% agar. Both were found to be virtually complete in those mixtures which contained all essential factors, i.e., C3b, factor B and factor D. Residual factor B was 0.1–1.18% Z of the controls. C3 was completely converted to the more anodally migrating C3b. Aliquots of all mixtures were tested for spreading activity in duplicate. Each glass cover slip with attached macrophages received 0.1 ml of the reaction mixtures diluted in DMEM and containing various combinations of $7.5\ \mu\text{g}$ B, $0.15\ \mu\text{g}$ D, $7.5\ \mu\text{g}$ C3b, and $15.1\ \mu\text{g}$ C3. The results, presented in Table II, indicated that macrophage spreading activity is generated only in mixtures which contain factor B together with cofactors necessary for the engagement of the positive feedback mechanism of the properdin system. This mechanism involves first the Mg^{++} -dependent complex formation of C3b and factor B and secondly the cleavage of the complexed factor B by factor D into the two fragments, Ba and Bb. The resulting complex C3b, Bb represents the labile C3 convertase of the properdin system. The data in Table II also suggests that C3a, the anaphylatoxin cleaved from C3 by the action of C3b, Bb, is not required for the generation of spreading activity and that C3b by itself is also inactive.

Correlation of Spreading Activity with Activated Factor B (Bb). The next series of experiments was designed to establish which of the proteins or combinations of proteins present in the active mixtures were able to initiate rapid spreading of freshly explanted macrophages. Fresh frozen human serum (230 ml) was thawed and incubated for 30 min at 22°C with kaolin (1 mg/ml) to completely remove spreading activity associated with the contact phase of blood coagulation. To the absorbed serum was then added 0.32 mg of ^{125}I -labeled factor B ($37.6\ \mu\text{Ci}$) and 2.5 g packed, PBS-washed inulin particles. The inulin was dispersed in the serum and the mixture was incubated for 60 min at 37°C . After removal of the inulin particles by centrifugation (30 min, 10,000 g), the supernate was dialyzed against 0.005 M sodium phosphate, pH 7.3 (starting buffer) and applied to a 5.8×35 cm column packed with DE 32-cellulose equilibrated in the same buffer. The column was washed with 3 liters of this buffer and was then eluted with a continuous 3.2L sodium chloride gradient made in starting buffer. 20-ml fractions were collected at a flow rate of 60

TABLE II
*Macrophage Spreading Induced by Purified Components of the Alternative
 Complement Pathway*

Components added*	Spreading‡
	%
B \bar{D} C3bC3	72
B \bar{D} C3b	63
B \bar{D}	18
\bar{D} C3b	17
C3bC3	9
B	22
\bar{D}	20

* Human purified components preincubated for 30 min at 37°C. Amounts used per assay: B, 7.5 μ g; \bar{D} , 0.15 μ g; C3b, 7.5 μ g; C3, 15 μ g. All dilutions were made in PBS containing Ca⁺⁺ and Mg⁺⁺.

‡ Determined after 1 h at 37°C in RPMI-1640 containing 10% heat inactivated FCS.

ml/h. The distribution of ¹²⁵I was determined by counting 0.5-ml aliquots of every second fraction in a scintillation spectrometer. The results are shown in Fig. 1 (top). Three areas of radioactivity were found. Accordingly, three pools, A, B, and C were made as indicated. They were each concentrated to 24 ml by pressure filtration in stirred Amicon cells using UM 10 membranes for pool A and B and a UM 2 membrane for pool C and were then dialyzed against PBS. Analysis of the pools by double-diffusion in gel and immunoelectrophoresis (both in 1% agarose) revealed the presence of Bb in pool A, of native B and Bb (in about equal amounts) in pool B and of Ba in pool C. Both pools B and C also contained C3b as identified by immunoelectrophoresis (in 1% agar) whereas pool A did not react with antiserum to C3. When tested for the induction of macrophage spreading both pools A and B contained activity which was dose-related, with inhibition at higher concentrations, while pool C was inactive (Fig. 1, bottom). These results, together with the data in Table II, strongly suggested that spreading activity was related to the b-fragment of factor B and that the presence of C3b was not required for its expression.

Next, 10 ml of pool A was chromatographed on a 2 × 21 cm CM 32-cellulose column equilibrated in 0.02 M sodium phosphate, pH 6.0 (starting buffer). After a 500-ml wash with the starting buffer 1 L NaCl gradient made in starting buffer was applied. 10-ml Fractions were collected at a flow rate of 40 ml/h. 1 ml of each fraction was assayed for ¹²⁵I. Aliquots of selected fractions were dialyzed against PBS and were then tested for the induction of macrophage spreading. The results of this experiment can be seen in Fig. 2, which shows a good correlation between the distribution of ¹²⁵I, i.e., Bb, and spreading activity. Fractions 59–66 were pooled, concentrated to 4 ml and subjected to molecular sieve chromatography on a 3.8 × 102 cm column of Sephadex G 150 equilibrated in PBS. 5.8-ml Fractions were collected at a flow rate of 13 ml/h. The peak of ¹²⁵I eluted at 764 ml together with the last of four protein peaks. When tested for macrophage spreading activity very good correlation was again obtained between the distribution of ¹²⁵I and the presence of spreading activity in the column fractions (now shown).

The active fractions from the gel filtration column were pooled and concentrated to 2 ml. 0.1-ml Aliquots of this material were subjected to alkaline PAGE. At the end

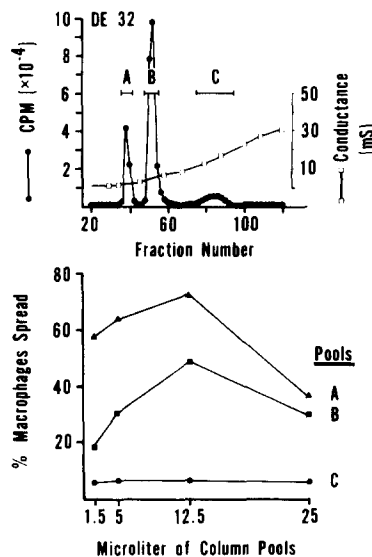


FIG. 1. DEAE-cellulose chromatography of ^{125}I -B containing serum after inulin activation and evaluation of macrophage spreading activity of the ^{125}I -containing column pools. Upper panel: 230 ml fresh frozen human serum containing $37.6 \mu\text{Ci } ^{125}\text{I}$ -labeled factor B were activated with inulin and fractionated on DEAE-32 cellulose. Three separate ^{125}I -containing areas were identified at a conductance of 1.5, 5 and 15 mS pools A, B, and C. Lower panel: dose-response of the cell spreading activities of the 10-fold concentrated column pools. Pool A contained Bb, pool B contained factor B and Bb, and pool C contained Ba.

of the electrophoretic run the gels were sliced into 1-mm segments and 0.1 ml PBS was added to each of the slices before they were assayed for ^{125}I . The contents of selected tubes were then dialyzed against DMEM and were tested for the ability to induce macrophage spreading. The results, graphed in Fig. 3, show an excellent correlation of radioactivity (^{125}I) and macrophage spreading activity. Using an antiserum to factor B only Bb was detected in this material by immunoelectrophoresis. The molecular weight of the ^{125}I -labeled protein in the Sephadex G-150 pool was estimated by SDS-PAGE in 7.0% gels. 50- μl aliquots of the pooled material were boiled in SDS, urea, and DTT and were then applied to the gels in 25% glycerol. After the run the gels were sliced into 1-mm segments which were assayed for ^{125}I . Replicate gels with marker proteins were stained with Coomassie Brilliant Blue G250 and used for the construction of a molecular weight-calibration curve. The mol wt of the ^{125}I -labeled product of factor B was estimated to be 64,000 (Fig. 4), in excellent agreement with previous determinations which, using the same method, had indicated a mol wt of 63,000 (28).

Analysis of Macrophage Spreading Activity Generated from Highly Purified Human Factor B. All the evidence accumulated so far pointed at the b-fragment of factor B as the inducer of macrophage spreading generated in complement-activated serum. To rule out the possibility that a contaminating protein which might copurify with Bb was responsible for the induction of cell spreading, rather than Bb itself, the following purification procedure for Bb was adopted. Bb was generated in mixtures of purified B, D, and C3b and was then isolated by DE 32-cellulose chromatography which separates the more basic Bb molecule from the more acidic native B as well as from Ba, D, and C3b all of which also elute later than Bb. 4 mg B, 10 $\mu\text{g } ^{125}\text{I}$ B (1.18 μCi)

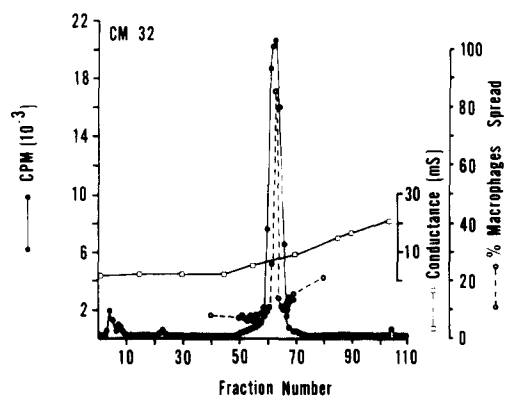


FIG. 2. Correlation of macrophage spreading activity and distribution of Bb upon CM-cellulose chromatography. Pool A from Fig. 1 was fractionated on CM-32 cellulose and the distribution of radioactivity (^{125}I) in the fractions determined. After dialysis against PBS, selected fractions were tested for macrophage spreading activity. Both activities eluted at a conductance of 7.5 mS.

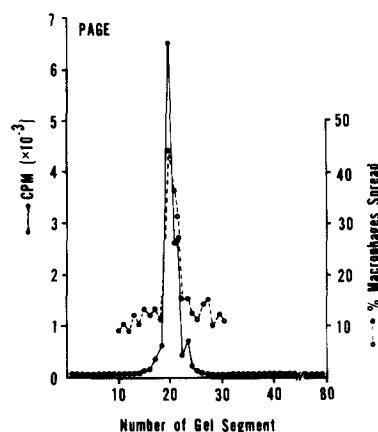


FIG. 3. Correlation between macrophage spreading activity and distribution of Bb upon PAGE. ^{125}I -Bb containing Bb was purified from inulin activated serum by DE-32, CM-32, and Sephadex G-150 chromatography. 0.1-ml Aliquots of the active material were electrophoresed on 6% alkaline gels which were sliced into 1-mm segments. All gel segments were assayed for ^{125}I . Selected segments were eluted and dialyzed against PBS before their macrophage spreading activity was determined

50 μg D and 1 mg C3b were incubated for 30 min at 37°C in VBS-Mg. The mixture was then dialyzed against 0.005 M sodium phosphate, pH 7.3 and was applied to a 1 \times 15 cm DE 32-cellulose column equilibrated in the same buffer. The column was washed and Bb was then eluted with a NaCl gradient. The position of Bb was determined by the distribution of ^{125}I in the column eluates. The first ^{125}I -containing peak contained Bb. It was pooled and concentrated to 0.8–1.25 mg/ml and dialyzed against PBS before storage at -70°C . Fig. 5 shows SDS-PAGE gels of the purified Bb obtained by this procedure.

Dose-response experiments with such preparations of purified Bb yielded a reasonably straight line, 1.6 μg Bb being necessary to induce spreading of 50% of 5×10^4 glass-attached macrophages (Fig. 6).

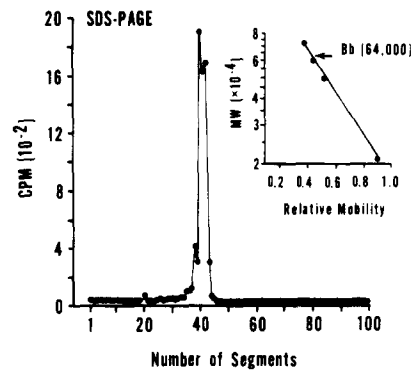


FIG. 4. Molecular weight estimation of Bb containing ^{125}I -Bb purified from inulin activated serum. SDS-PAGE was performed in 7% acrylamide. After the run the gel was sliced in 1-mm segments which were assayed for ^{125}I . The inset shows the position of the ^{125}I peak in relation to the mobilities and mol wt of the α , β , and γ -chains of fibrinogen (70,900, 60,400, and 49,400) and of soybean trypsin inhibitor (21,000).

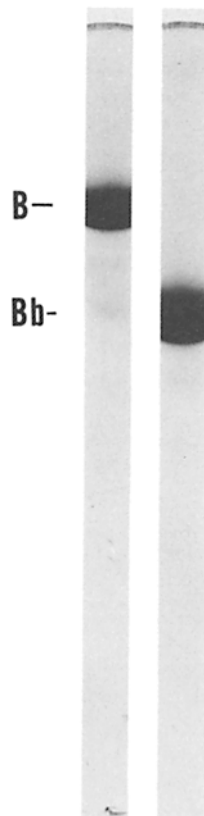


FIG. 5. SDS-PAGE of Bb and its precursor factor B. Purified factor B and its b-fragment (Bb) prepared from incubation mixtures of isolated C3b, factor B and factor D were analysed by SDS-PAGE in 7% acrylamide. 65 μg of the isolated proteins were applied to the gels which were stained with Coomassie Brilliant Blue G-250. The cathode was at the top.

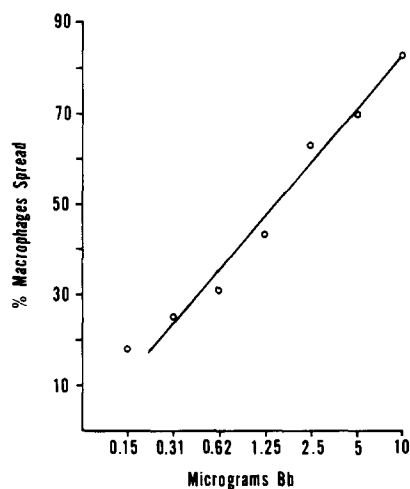


FIG. 6. Dose-response of isolated Bb as an inducer of macrophage spreading.

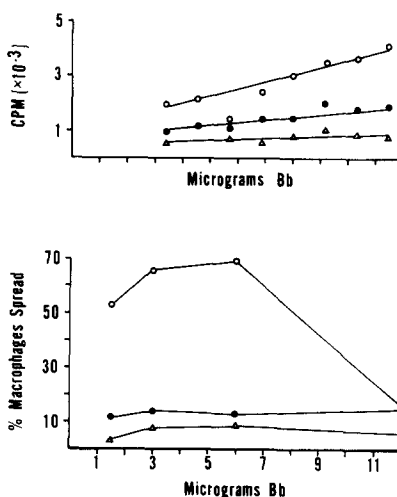


FIG. 7. Abrogation of the macrophage spreading activity of isolated Bb by its prior treatment with DFP. Isolated Bb was treated with DFP in propylene glycol at two different concentrations or with propylene glycol alone and was dialyzed against PBS. (^3H -methyl) AG LMe hydrolyzing activity and macrophage spreading activity were determined in parallel. ○, Bb; ●, Bb + 5×10^{-4} DFP; △, Bb + 2×10^{-3} DFP.

Requirement of the Intact Catalytic Site on Bb for Spreading Activity. Two modes of action of Bb on macrophages were considered, enzymatic action of the serine protease Bb on a cell surface substrate and/or binding of Bb to a specific cell surface structure (receptor). While in screening experiments unequivocal evidence for specific binding of ^{125}I -Bb to macrophages could not be obtained, other experiments suggested that Bb acted as an enzyme. It was observed that purified Bb which had spontaneously become inactive with respect to its ability to hydrolyze AG LMe had also lost its cell-spreading capacity. More direct evidence came from experiments in which Bb-treated DFP was used. Bb generated and purified from isolated B as described was treated

TABLE III
Effect of Anti-Mouse B Fab'2 on Complement Induced Macrophage Spreading

Microliter of Fab'2*	Percent macrophages spread‡
0	67
10	56
25	35
80	15
100	11
150	10

* Anti-B Fab'2 incubated for 15 min at 37°C with 0.1 ml mouse serum and 1 U CVF and then added to macrophage monolayers. The mouse serum had been pretreated with 10 mg kaolin/ml.

‡ Determined after 1 h at 37°C. Controls with Fab'2 and CVF in the absence of serum showed spreading of 10% of the cells.

with the serine esterase inhibitor DFP (diluted in propylene glycol) for 1–2 h at 22°C. The treated material and a control treated only with propylene glycol were dialyzed against PBS at 4°C and were then tested for cell spreading and enzyme activity using ³H-AG LMe as a substrate. (In some experiments in which only ³H-AG LMe hydrolysis was tested, Bb was added to the substrate without prior dialysis.) As shown in Fig. 7, a parallel decrease in ³H-AG LMe-hydrolysis and macrophage-spreading activity was evident in samples treated with 5×10^4 M or 2×10^{-3} M DFP.

Macrophage Spreading Activity Derived from Mouse Factor B. In all previous experiments described here human complement components and mouse macrophages had been used. In fact, because of the difficulties preparing sufficient amounts of purified mouse factor B and mouse cofactors it would have been quite laborious to work with both mouse cells and mouse proteins. That mouse factor B is involved in the spreading of mouse macrophages by complement-activated mouse serum was, therefore, demonstrated indirectly. In these experiments F(ab')₂ fragments prepared from the immunoglobulins of rabbit antiserum to mouse factor B were used to block spreading activity. 0.1 ml of fresh, kaolin-adsorbed mouse serum was incubated with 5 μg CVF and increasing amounts of F(ab')₂ anti-mouse B for 15 min at 37°C. The mixtures were then diluted and added to macrophage monolayers. Cell spreading was determined after 60 min at 37°C. The results, presented in Table III, indicated that specific anti-mouse B antibody is able to abrogate the spreading activity generated in mouse serum by CVF.

Discussion

The experiments described here show that the enzymatically active b-fragment of the proteinase factor B of the properdin system, i.e., Bb, has the activity to induce rapid spreading of freshly explanted, nonstimulated, glass-attached mouse peritoneal macrophages. The findings confirm and expand previous observations which demonstrated the presence in plasma of two distinct effector systems which, upon activation, induced rapid spreading of adherent mouse macrophages (14). One of these systems was found to be associated with the contact phase of blood coagulation, the other one was triggered by agents which activate the complement reaction sequence. The complement-dependent spreading activity could be generated by a variety of substances which are known to activate either the classical, or the alternative,

or both pathways. Thus, sheep erythrocytes sensitized with antibodies of the IgM class (EA), CVF, inulin particles, or zymosan were all efficient in generating cell-spreading activity in mouse plasma or serum obtained from either normal or genetically C5-deficient animals. These results can now be explained by the production of Bb through the properdin system's positive feedback mechanism which is triggered by C3b (activated C3) regardless of its mode of generation. Once generated, C3b can associate with native factor B to form a complex, C3b, B in which B is susceptible to activation by limited proteolysis by the properdin system enzyme factor D or by other proteinases. Of the two cleavage products thus generated, Ba and Bb, the a-fragment is released whereas the b-fragment remains associated with C3b. The C3b, Bb complex, which constitutes the labile C3 convertase of the properdin pathway, decays quickly due to the dissociation of C3b and Bb. Free Bb, although still able to act on small substrates such as acetyl-glycyl-lysyl-methyl ester (AG LMe) (29), retains very little or no C3-cleaving activity (18, 30).

The finding that Bb which had spontaneously lost its AG LMe cleaving activity and DFP treated Bb do not possess cell spreading activity, together with the lack of evidence for ^{125}I -Bb binding to adherent macrophages (unpublished observation) strongly suggested that Bb exerts its action on the cells through its enzyme activity. This proposed mode of action of Bb immediately raises the question as to the cellular substrate responsible for the observed phenomenon. In view of the limited specificity of the Bb enzyme the possibility has to be considered that one of the known substrates, i.e., C3 or C5 is expressed on the macrophage surface and is activated in typical fashion. Such a hypothesis appears to be at variance with the fact that free Bb does neither act on C3 nor on C5 (18, 30, 31). However, membrane-expressed C3 or C5 could conceivably be cleaved by free Bb because the conformation of the membrane-associated complement components might differ from that of the components in solution. Thus, native C5 passively adsorbed to Raji lymphoblastoid cells has been shown by Dierich and Landen to be activated by erythrocytes carrying the classical complement pathway analogue of C3b, Bb, the C4b, 2a enzyme, although fluid phase C5 does also not serve as a substrate for the C4b, 2a complex (32). The possible involvement of membrane-associated C3 or C5 in the macrophage spreading reaction is presently being explored.

The cell-directed actions of factor B are not confined to its b-fragment. The smaller, more acidic a-fragment of guinea pig factor B has recently been shown by Hamuro et al. to stimulate the directed migration of guinea pig peritoneal polymorphonuclear leukocytes in Boyden chambers although in these experiments relatively large amounts (10–21 μg) of Ba were required (33).

Whereas macrophage spreading activity was consistently generated by purified Bb and could be observed under conditions which allowed the production of Bb, such activity could not be generated from the classical complement pathway analogue of factor B, the second component of complement (Table I). This is surprising because the structure, mode of activation, and substrate specificity of C2 and its active cleavage product C2a are very similar or identical to that of factor B. Both C2 and factor B consist of one polypeptide chain of approximately 100,000 daltons, both are proteolytically activated by complement enzymes, and both their major cleavage products C2a and Bb, which carry the active sites of the enzymes, have to be in complex with a second activated complement protein, C4b or C3b respectively, to be able to act on their natural substrates, the complement components C3 and C5. In

addition, the structural genes for both C2 and factor B are present on the same chromosome (chromosome 6 in man) and are linked to the major histocompatibility complex, the HLA region (34–36). The unresponsiveness of mouse macrophages to activated guinea pig C2 (Table I) remains unexplained at present. Conceivably, the difference between the effects of C2a and Bb with respect to macrophage spreading reflects the susceptibility of the membrane associated substrate to these specialized proteinases.

Almost all of the results presented here have been obtained with mouse macrophages and human complement proteins. In fact, this study was greatly facilitated by the finding that human Bb, which can be prepared in milligram amounts in highly purified form, is able to interact with mouse cells. Yet the involvement of mouse factor B in the previously studied spreading of mouse macrophages by complement-activated mouse serum (14) could be demonstrated indirectly through the use of a rabbit antiserum specific for mouse factor B (Table III).

The effects of Bb on macrophages have recently been confirmed with purified human peripheral mononuclear phagocytes (monocytes) (37). In contrast to mouse peritoneal macrophages which responded immediately to Bb, human peripheral monocytes required 3–4 d of culture before they spread maximally in response to human Bb, suggesting the requirement for an *in vitro* maturation process.

Taken together, these results have possible implications for hypotheses of the cooperation between monocytes and other cells of the immune system. Human monocytes and animal macrophages have been shown to synthesize several complement proteins, notably C2, C3, C4 and factor B and factor D (38–41) and thus have themselves the potential to furnish all components required for the generation of Bb (19) and their stimulation by this effector molecule (37). Furthermore, Littman and Ruddy have shown that human peripheral blood mononuclear cells respond with an earlier onset of C2 synthesis and an increase in the amount of C2 produced if the cultures were set up in the presence of antigen or if adherent monocytes were cultured in the presence of lymphokine-rich medium harvested from antigen-stimulated mononuclear cells (42).

It is conceivable that the secretion of factor B is mediated by similar mechanisms, and the Bb is an active mediator of lymphocyte-macrophage interactions. Bb can be generated by proteinases derived from cells or contained and activated in blood plasma either acting on C3, to generate C3b, or acting directly on Factor B, initiating locally the complement feedback system. Proteinases derived from activated cells or contained and activated in the blood plasma may act on C3 to generate C3b, or act directly on factor B, to locally initiate the complement feedback system, resulting in the production of complement cleavage products including Bb. Such local complement activation has a profound influence on macrophage motility. Studies presented separately, indicate that Bb has migration inhibitory activity similar to that of the macrophage migration inhibitory factor in a capillary migration assay, and that migration inhibition or enhancement of migration are regulated by the local balance between Bb and C5a, the chemotactic factor produced upon activation of C5 (43).

In unpublished experiments it was found that pretreatment of mice with intravenous injections of C56B such that their complement levels as detected by radial immunodiffusion were below 5% of normal, reduced the number of cells recovered from the peritoneal cavity after thioglycollate injection, and abrogated the rapid spreading of these cells making them appear similar to normal, nonstimulated

peritoneal macrophages. This suggests that Bb is one of several factors regulating the focussing and emigration of macrophages in inflammatory lesions and either promoting or facilitating their differentiation into an activated cell with microbicidal and cytotoxic properties.

Summary

Unstimulated mouse peritoneal macrophages attached to a glass substratum responded to activated human factor B (Bb) of the properdin system but not to native factor B with rapid spreading and a concomitant increase in their apparent surface area. Excellent correlation of the distribution of Bb protein and cell-spreading activity was found upon purification of Bb by ion-exchange and molecular sieve chromatography and alkaline polyacrylamide gel electrophoresis. 1.6 μg of purified Bb was sufficient to induce spreading in 50% of 5×10^4 glass attached macrophages within 1–2 h at 37°C. Treatment of Bb with di-isopropyl-fluorophosphate indicated that the intact catalytic site of the serine-proteinase Bb was required for the initiation of macrophage spreading. The involvement of factor B in the induction of rapid cell spreading could also be indirectly demonstrated in an autologous system in which $\text{F(ab}')_2$ fragments of an antiserum to mouse B prevented mouse macrophages from spreading in response to complement-activated mouse serum. These experiments suggest a role for factor B and the alternative pathway of complement fixation in the localization of mononuclear phagocytes to areas of inflammation.

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