Activation of Bovine Monocytes and Neutrophils by the Bb Fragment of Complement Factor B: Demonstration by the Uptake of ^{3}H-Deoxyglucose

Manjeet S. Sethi, Henry Tabel and Vikram Misra

ABSTRACT

The Bb fragment is the enzymatically active split product of bovine complement factor B. The Bb fragment was obtained after zymosan treatment of fresh bovine serum and fractionation of the treated serum, first over diethylaminoethyl-Sephacel and then over an affinity column made up of monoclonal antibody to bovine Bb, coupled to cyanogen-bromide-activated Sepharose. Purified Bb has a molecular weight of 64,000, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The ability of purified Bb to activate phagocytes was assessed. The activation assay was based on the principle that the primary source of energy for the phagocytes is obtained from glucose. ^{3}H-deoxyglucose, a nonmetabolizable analogue of glucose, was used to obtain the quantitative measurement of the activation process. The activation by Bb was shown by the uptake of the labelled deoxyglucose in the phagocytic cells and was comparable to the activation caused by phorbol myristate acetate and N-formyl-L-methionyl-L-leucyl-L-phenylalanine, run in parallel. These data showed that fragment Bb activates bovine monocytes and neutrophils and also suggested that, when generated after complement activation, Bb may stimulate monocytes and neutrophils for enhanced phagocytosis.

RÉSUMÉ

La portion du facteur B du complément chez le bovin qui en contient l'activité enzymatique est le fragment Bb. Ce fragment a été obtenu de sérum frais de bovin suite à un traitement au zymosan. Ce sérum a par la suite été fractionné sur, premièrement, une colonne Séphacel diéthyle-amino-éthyle et deuxièmement, sur une colonne Sépharose activée au bromure cyanogène contenant des anticorps monoclonaux dirigés contre le fragment Bb. Le poids moléculaire de ce fragment obtenu suite à une électrophorèse sur gel de polyacrylamide de dodécyle sodique sulfaté était de 64 K. Par la suite, l'effet activateur de ce fragment a été vérifié sur des phagocytes. Ce test est basé sur le principe que la première source d'énergie pour la cellule phagocytaire est le glucose.

Le ^{3}H-désoxyglucose, un analogue non métabolisable du glucose, était employé comme marqueur quantitatif de l'activité phagocytaire. L'activation par le fragment Bb était démontrée par l'incorporation du désoxyglucose tritié par le phagocyte et cette activation était similaire à celle obtenue par l'acétate de phorbol myristate et le N-formyle-L-méthionyle-L-leucyle-L-phenylalanine.

Les résultats démontrent que le fragment Bb peut stimuler l'activité des monocytes et des neutrophiles de bovins et que, lorsque généré suite à une activation du complément, le fragment Bb peut accroître la fonction phagocytaire des monocytes et des neutrophiles.

INTRODUCTION

Monocytes/macrophages and neutrophils are the major phagocytes of infectious agents and play a pivotal role in host resistance, inflammation and immunoregulation (1-5). Macrophage products have great influence on almost every aspect of the immune function (6). Activated macrophages obtained from animals during a certain stage of infection are more spread out on glass, more heavily endowed with mitochondria and lysosomes, more phagocytic than their normal counterparts and have enhanced microbicidal activity (7). Activated neutrophils, like other secretory cells, also undergo a number of biochemical changes which enable the cells to respond to surface stimulants (8).

The alternative complement pathway (ACP) is a natural defence mechanism which operates independently of antibody against infectious agents. The human serum protein factor B is a single chain glycoprotein of the ACP with an approximate molecular weight of 90,000 (9). In association with C3b and Mg^{2+} it is involved in the generation of the ACP

Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0.

Reprint requests to Dr. H. Tabel.

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C3-convertase, C3b.Bb (10, 11). This molecule is the precursor of a serine protease, the active site of which resides in the Bb subunit (12), a cleavage product of factor B. The C3-convertase cleaves native C3 to produce C3b, which in turn forms an additional C3-convertase with a second molecule of factor B. This positive feedback process results in the deposition of large numbers of C3b molecules on the surface of activating particles such as zymosan and gram negative bacteria (13, 14) which then may lead to phagocytosis of the target cells or to lysis by the terminal complement cascade. The complete primary structure of human factor B has been established from protein and cDNA sequencing (15) and the gene coding for factor B has been mapped to the major histocompatibility complex (16).

It has been demonstrated that human Bb expresses residual hemolytic and proteolytic activity after it dissociates from the ACP C3-convertase (C3b.Bb) (17). In an earlier study human Bb was shown to activate monocytes as demonstrated by their ability to spread on glass coverslips (18). Human Bb has also been shown to stimulate intracellular killing of *Staphylococcus aureus* by monocytes (19) and induce lymphocyte blastogenesis (20).

Bovine factor B has previously been purified by conventional chromatographic procedures (21). The split product Bb dissociated from the C3-convertase (C3b.Bb) has been measured after activation of the ACP by zymosan (22) and by *Trypanosoma congolense* (23).

In the present study, the activation of bovine monocytes and neutrophils was demonstrated by factor Bb of the bovine ACP. The activation assay was based on the principal that the primary source of energy for the phagocytes is obtained from the metabolism of glucose (24-27). ^{3}H-2-deoxyglucose, a nonmetabolizable analogue of glucose, was used to obtain a quantitative measurement of the activation process. The activation by the Bb fragment of phagocytic cells, as measured by the accumulation of the radio-labelled 2-deoxyglucose, was comparable to the activation caused by known activators such as phorbol-12-myristate-acetate (PMA) and N-formyl-L-methionyl-L-leucyl-L phenylalanine (FMLP).

MATERIALS AND METHODS

REAGENTS

2-Deoxy-D-(1-^{3}H) glucose was purchased from Amersham Canada Limited. It had a specific activity of 17.0 Ci/mmole. The radiochemical concentration was 1.0 mCi/mL. Aqueous counting scintillant, ACS II was also obtained from the same source. Sodium azide (SA), sodium fluoride (SF), PMA, FMLP, alpha-naphthyl acetate esterase staining kit and phenylmethylsulphonylfluoride (PMSF) were purchased from Sigma Chemical Company, St. Louis, Missouri. FMLP was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10^{-2}M, and stored in aliquots at -70°C. Dilutions were made in glucose-free Hanks' balanced salt solution (HBSS), just prior to use. Dimethyl sulfoxide, at a concentration of 0.1%, had no effect on cell viability. The 'Pyrogent' limulus amebocyte lysate kit to detect endotoxin with a sensitivity of 0.06 endotoxin units (EU)/mL and *E. coli* O111:B4 endotoxin were purchased from Whittaker M.A. Bioproducts Inc., Walkersville, Maryland. Polymyxin B sulfate (500,000 units) was obtained from Wellcome Medical Division, Kirkland, Quebec. Ficoll-Hypaque was purchased from Pharmacia, Quebec.

LIMULUS AMEBOCYTE LYSATE ASSAY FOR ENDOTOXIN DETECTION

The test was performed according to the directions of the manufacturer. The results were expressed in endotoxin units/mL (EU/mL), where 1 EU/mL = 100 pg/mL.

ANTISERA

Antiserum to bovine factor B was produced by immunizing rabbits as described previously (22). All rabbits immunized produced antibodies to bovine factor B and the split products Ba and Bb. This antiserum was used to monitor the purification of Bb.

IMMUNOELECTROPHORESIS (IE)

Immunoelectrophoresis was carried out in a 1% agarose gel in veronal buffer, pH 8.6, ionic strength 0.05, plus 5 mM disodium ethylenediaminetetracetic acid (EDTA). The electrophoresis chamber contained veronal buffer, pH 8.6, ionic strength 0.1, plus 10 mM EDTA.

MONOCLONAL ANTIBODY PRODUCTION

Bb was purified as described previously (22) and emulsified in an equal volume of Freund's complete adjuvant. Balb/c mice (Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 0.1 mL (33 μg) of Bb. Two weeks later, the mice were given a booster dose of purified Bb in Freund's incomplete adjuvant. Two weeks later, the mice were bled and the serum tested for anti-Bb reactivity by double immunodiffusion. Mice with high titers against Bb were selected for preparation of hybridomas. Three days after a final intravenous injection of 33 μg of Bb in 0.1 mL saline, spleen cells from the immunized mice were fused with cells of the mouse myeloma cell line NS-1 (28). The procedures followed the guidelines of the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The supernatants of growing hybridomas were screened for production of Bb specific antibodies in a solid phase immunoassay using Bb adsorbed to nitrocellulose membrane. The membrane was placed in a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Mississauga, Ontario) and was incubated with the antigen Bb for 45 min. It was then blocked with 1% bovine serum albumin for 30 min. After one washing, supernatants from the hybridomas were added and incubated for 45 min with the antigen. The membrane was then washed twice and incubated with horseradish peroxidase-conjugated goat antimouse IgG (Bio Rad Laboratories, Mississauga, Ontario) and the bound antibody was visualized with 4-chloro-1-naphthol (Bio Rad Laboratories, Mississauga, Ontario). Clones secreting antibody against Bb were subcloned by the limiting dilution method. Ascites fluid was obtained by intraperitoneal injection of 10^6 to 10^7 specific antibody producing hybridoma cells into mice previously primed (2-6 wk) with Pristane

(2,6,10,14 tetramethyl pentadecane, Aldrich Chemicals, Montreal, Quebec). The immunogobulin isotype of the positive clones was determined by an enzyme-linked immunosorbent assay kit (Zymed-Dimension Laboratories, Mississauga, Ontario). One positive clone producing monoclonal antibody against Bb was designated TS-1.

PURIFICATION OF FACTOR Bb

For the affinity purification of Bb, bovine serum was first treated with zymosan (10 mg/mL) at 37°C for 30 min. It was then centrifuged at 1000 x *g* for 10 min to remove the zymosan. The supernatant, containing the zymosan activated serum, was dialyzed against 0.01 M sodium phosphate buffer, pH 8.0, and then passed over DEAE-Sephacel column (Pharmacia, (Canada) Inc., Quebec), equilibrated with the same buffer. The unretained first peak was concentrated in a Collodion bag ultrafiltration apparatus (Schleicher & Schuell). This concentrate was then applied to a CNBr-Sepharose-TS-1 column. The coupling of monoclonal TS-1 to CNBr-activated Sepharose was accomplished according to the instructions of the manufacturer (Pharmacia). Prior to the above application, the column was equilibrated with phosphate-buffered saline (PBS), pH 7.2 containing 10 mM EDTA, 0.5 M NaCl, 1% Tween-20 and 2 mM PMSF. Bovine factor Bb was eluted from the column with 0.2 M glycine-HCl pH 2.5 into Tris-HCl pH 8.0. The sample was desalted on Biogel P6DG (Bio Rad, Mississauga, Ontario). Precautions were taken throughout the purification processes to minimize endotoxin contamination. Pyrogen-free water (Abbott Laboratories, Montreal, Québec) was used to make all buffers and solutions. All glassware was wrapped in aluminum foil and baked for 4 h at 180°C. Columns and plastic tubing were washed with methanol and rinsed thoroughly with pyrogen-free water. The purified preparation of bovine factor Bb contained approximately 25 pg/mL of endotoxin as determined by the Limulus amebocyte lysate assay (Whittaker M.A. Bioproducts, Inc., Maryland). Bovine factor Bb was then analyzed by immunoelectrophoresis (IE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

Affinity purified bovine factor Bb was solubilized in electrophoresis sample buffer and analyzed on 7.5% SDS-PAGE using the discontinuous buffer system of Laemmli (29). Electrophoresis was carried out under nonreducing conditions. In each experiment molecular weight markers (Bio Rad Laboratories, Mississauga, Ontario) were electrophoresed in parallel and visualized by staining with Coomassie blue.

ISOLATION AND PURIFICATION OF BOVINE MONOCYTES AND NEUTROPHILS

For the purification of bovine monocytes, the method of Goddeeris *et al* (30) was followed. Briefly, blood was collected into an equal volume of Alsever's solution, layered onto Ficoll-hypaque (1.077 g/mL) and centrifuged at 900 x *g* for 35 min at 23°C. Mononuclear cells were aspirated from the interphase and resuspended in Alsever's solution and centrifuged at 450 x *g* for 10 min. The mononuclear cells were further washed three times for 10 min at 180 x *g* at 23°C. The cells were then suspended in medium RPMI 1640 containing 2 mM L-glutamine and 50 μg/mL gentamycin. Viability was determined by trypan blue dye exclusion test.

SEPARATION OF MONOCYTES BY THE ADHERANCE TO GELATIN/PLASMA

Gelatin (2%) was used to coat 150 cm^2 culture flask (Costar) at 38°C for 2 h. After removing the gelatin by suction and drying the flasks, autologous plasma was added and incubated at 38°C for 1 h. The plasma was removed and the flasks were washed with unsupplemented RPMI 1640. Cell suspension (30 mL of 5×10^6/mL) was then added to the flasks and incubated for 90 min at 38°C. Nonadherent cells were removed with gentle washing with medium warmed to 38°C. Monocytes were then obtained by incubating the adherent cells with ice-cold HBSS without Ca^{2+} and Mg^{2+} containing 10 mM ethylenediaminetetraacetate (EDTA). The adherent cells were pelleted at 4°C at 180 x *g* for 10 min and washed three times in ice-cold glucose-free HBBS and suspended in the same medium. Cell concentration and cell viability were then determined. Cytospin smears were stained with the alpha-naphthyl acetate esterase staining kit to identify the monocytes. Diffuse black cytoplasmic dots were attributed to the alpha-naphthyl acetate esterase activity of monocytes (31). Monocytes of $>90\%$ purity and $>95\%$ viability were used for all assays.

PURIFICATION OF NEUTROPHILS

After the removal of mononuclear cells from the interphase, the intervening layer of Ficoll-hypaque was removed. The pellet containing the erythrocytes and the neutrophils was then treated with 0.89% ammonium chloride to lyse the erythrocytes and centrifuged at 250 x *g* for 10 min. The neutrophils were washed twice in glucose-free HBSS and suspended in the same medium, and cell concentration and viability were determined. Giemsa staining was done to determine the purity of the neutrophil population obtained. Neutrophils of $>96\%$ purity and $>95\%$ viability were used for the assay.

2-DEOXYGLUCOSE UPTAKE ASSAY

The 2-deoxyglucose uptake assay has been used earlier to study the activation of macrophages of guinea pigs and other animal species (32-35). It has also been modified to study the activation of human monocytes and neutrophils (36). Preliminary experiments had indicated that a population of 5×10^5 cells per well and a concentration of 16 Ci/well of the ^{3}H-deoxyglucose were optimum. The cells in glucose-free HBSS were delivered into a 96-well round bottom microtiter plate (Linbro, Flow Laboratories) and incubated for 30 min at 37°C in 5% CO_2 in a humidified incubator to deplete endogenous glucose. Fixed concentrations of the stimulators PMA, FMLP, metabolic inhibitors

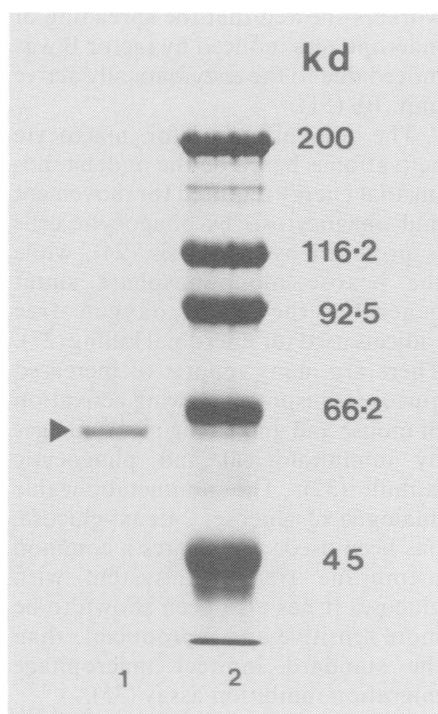

Fig. 1. Analysis of affinity-purified factor Bb on 7.5% SDS-PAGE under nonreducing conditions and stained with Coomassie blue. Lane 1, affinity-purified Bb. Lane 2, molecular weight markers.

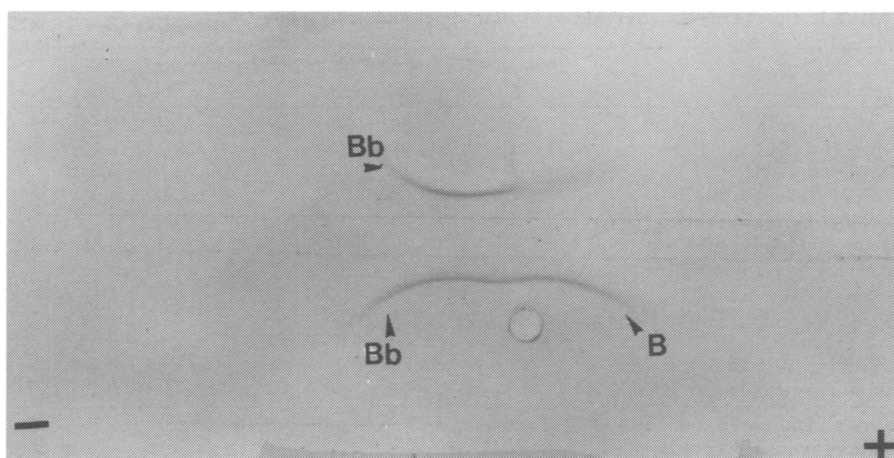

Fig. 2. Immunoelectrophoretic analysis of affinity-purified Bb in 1% agarose. Wells: Affinity-purified bovine Bb in upper well. Zymosan-activated serum in lower well. Trough: Rabbit antibovine Bb.

SA and SF along with factor Bb were added to the wells immediately after the addition of ^{3}H-deoxyglucose to the cells. The final dilutions were made in glucose-free HBSS. The total volume per well was 200 μL. The microtiter plate was then incubated at 37°C in a humidified incubator containing 5% CO_2 for 70 min. The plates were then centrifuged at 800 x g for 5 min at 4°C. Volumes of 50 μL of the supernatants from each well were removed and added to scintillation vials. Aqueous counting scintillant II (5 mL) was added to each vial and the radioactivity determined in a Beckman liquid scintillation counter. The absolute uptake of the ^{3}H-deoxyglucose was calculated using the formula:

$$\text{Deoxyglucose uptake} = \text{Total dpm added} - \text{dpm in supernatant}$$

$$\%\ \text{Deoxyglucose uptake} = \frac{\text{Total dpm added} - \text{dpm in supernatant}}{\text{Total dpm}} \times 100$$

STATISTICAL ANALYSIS

The percent uptake of ^{3}H-deoxyglucose was transformed using arcsine and a one-way analysis of variance was then performed on the data.

RESULTS

MONOCLONAL ANTIBODY PRODUCTION

Although several anti-Bb producing clones were initially detected by the dot-blot technique, all except one, designated TS-1, eventually lost the ability to produce Bb specific antibody. Antibody produced by TS-1 was of the IgG1, kappa isotype.

SDS-PAGE

Bb purified by affinity chromatography exhibited a single band under nonreducing conditions and had an apparent molecular weight of 64,000 (Fig. 1).

IMMUNOELECTROPHORESIS

Immunoelectrophoresis of the affinity-purified Bb demonstrated a single precipitin arc when reacted against rabbit antibovine Bb (Fig. 2).

LIMULUS AMEBOCYTE LYSATE ASSAY

The preparation of affinity-purified Bb was contaminated with endotoxin at a concentration of 0.25 EU/mL. This concentration of endotoxin was used in all experiments performed, and served as a control in the activation assay.

DEOXYGLUCOSE UPTAKE: EFFECT OF Bb ON MONOCYTES AND NEUTROPHILS

Phorbol-12-myristate-acetate and FMLP are known activators of monocytes and neutrophils (36) and were used as positive controls. Sodium azide and SF are inhibitors of the oxidative and the glycolytic pathways respectively (36), and were used as negative controls along with untreated/unstimulated phagocytic cells. Phenylmethylsulphonylfluoride is a serine protease inhibitor and was used to inactivate Bb, which is a serine protease. Polymyxin B was used to show that the activation of the phagocytes was indeed due to Bb and that any effect of the contaminating endotoxin in the Bb preparation could be abrogated, as polymyxin B has been shown to bind endotoxin and neutralize its effect (37, 38).

Table I shows the effects of the various agents on monocytes and neutrophils. Phorbol-12-myristate-acetate and FMLP indeed had a stimulating effect on both monocytes and neutrophils. The metabolic poisons showed an inhibitory effect, with SF showing a more profound effect than SA. The deoxyglucose uptake of the fragment Bb-stimulated cells was also significantly higher than that of unstimulated control cells.

The presence of the contaminating endotoxin in the Bb preparation did not contribute to the stimulation of the phagocytes since polymyxin B did not reduce the stimulating effect of Bb. Phenylmethylsulphonylfluoride, when added to the Bb fragment, was able to abolish the activating effect of Bb on the phagocytes indicating that the enzymatic activity of Bb is

TABLE I. Uptake of ^{3}H-deoxyglucose by monocytes and neutrophils

Treatments	% Uptake of ^{3}H-deoxyglucose: Monocytes	Neutrophils
Nonstimulated	5.50 ± 0.30[a]	7.30 ± 0.55
PMA[b] (1 μg/mL)	19.96 ± 0.63*	22.20 ± 0.99*
FMLP[c] (10^{-5} M)	28.13 ± 1.30*	24.60 ± 0.49*
Sodium azide (1 mM)	3.90 ± 1.00	6.00 ± 1.00
Sodium fluoride (5 mM)	1.47 ± 0.03*	3.60 ± 1.10*
Factor Bb (100 μg/mL)	18.20 ± 0.36*	18.40 ± 0.85*
Bb + Polymyxin B (5 μg/mL)	18.16 ± 1.00*	19.40 ± 0.26*
Polymyxin B (5 μg/mL)	6.63 ± 0.72	8.50 ± 1.10
Bb + PMSF[d] (2 mM)	3.63 ± 0.38	8.20 ± 1.30
PMSF(2 mM)	6.40 ± 0.45	8.10 ± 0.63
Endotoxin (0.25 EU/mL)	8.80 ± 1.20*	9.60 ± 0.40
Endotoxin + Polymyxin B (5 μg/mL)	6.27 ± 0.52	7.40 ± 0.50

*Shows significant differences ($p < 0.05$) compared to nonstimulated cells
[a]Results expressed as mean ± SEM
[b]Phorbol myristate-acetate
[c]N-formyl-L-methionyl-L-leucyl-L-phenylalanine
[d]Phenylmethylsulphonylfluoride

required for its activating effect. Phenylmethylsulphonylfluoride by itself did not show any significant effect on the phagocytes.

DISCUSSION

The ACP C3-convertase (C3b.Bb) is formed when factor B, in a Mg^{2+} dependent complex with C3b, is cleaved by factor D. Bb is the catalytic site-bearing subunit of the C3-convertase which is dissociated from the complex upon decay of the enzyme (39). It has been demonstrated that Bb expresses residual hemolytic and proteolytic activity after decay dissociation from the ACP C3-convertase (C3b.Bb) (17). Other physiological effects of human Bb on cells have also been documented. Bb has been shown to cause macrophage spread (40), stimulate intracellular killing of *Staphylococcus aureus* by monocytes (19) and induce lymphocyte blastogenesis (20).

Affinity purified bovine Bb had a molecular weight of 64,000 as determined by SDS-PAGE. This is in accordance with that determined for humans (17) and for guinea pigs (41). An earlier report (22) calculated the molecular weight of bovine factor Bb at 40,000, which is lower than expected when compared to human and guinea pig Bb. The probable reason for this low molecular weight could be explained by the degradation of the protein during the purification process.

Activation of macrophages and neutrophils, their responses to various stimuli, their destruction of microorganisms or tumor cells *in vivo* reflects a complex sequence of inter-related events. These events eventually lead to homeostasis or give rise to some form of injury. Many soluble secretagogues such as immune complexes (42, 43), C5a (42, 44), N-formylated peptides (45, 46), and leukotrienes (47) elicit transient responses by these phagocytic cells. Phorbol-12-myristate-acetate has proven to be the most potent.

Although the molecular mechanisms of phagocyte activation remain to be clearly defined, the identification of activating molecules and the enzymes synthesizing effector molecules would be useful in understanding the underlying mechanisms. Bianco *et al* (18, 48) demonstrated the regulation of macrophage migration by products of the complement system using the capillary tube assay. The same group further analyzed the complement-dependent spreading of mouse peritoneal macrophages and showed that it was induced by the enzymatically active Bb fragment of factor B of the ACP (40, 49). Bb produced a dose-dependent inhibition of migration and a dose correlation existed between enhanced spreading and inhibited migration under both *in vitro* and *in vivo* conditions. In another study, it was shown that human monocytes could be made to spread by factor B (50). In the subsequent study the workers showed that the spreading of macrophages induced by factor B was indeed due to the enzymatically active unit, Bb (51).

The present assay for phagocyte activation is based on the understanding that energy required for movement and phagocytosis by phagocytic cells is provided by glycolysis (24), while the hexose monophosphate shunt generates the toxic oxygen free radicals used for microbial killing (27). There are many reports of increased glucose transport following activation of mouse and guinea pig macrophages by immunological and phagocytic stimuli (32). The nonmetabolizable analogue of glucose, 2-deoxyglucose, has been used, as it shares a common membrane transport system with glucose. It has also been shown to be more sensitive and reproducible than the standard indirect macrophage migration inhibition assay (35).

The experimental design called for the preparation of fragment Bb as the initial step. This was done by making monoclonal antibody TS-1 against Bb which was then used to purify Bb by affinity chromatography. Purified Bb displayed a single band on SDS-PAGE (Fig. 1). Immunoelectrophoresis was also done to demonstrate the antigenicity and purity of the affinity-purified Bb.

To demonstrate the functional effect of the fragment Bb, the contaminating effect of endotoxin had to be ruled out. To do this, appropriate precautions were taken during the purification process. A limulus amebocyte lysate assay, having a sensitivity of 0.06 EU/mL, was performed on the purified Bb. It was found that the Bb was contaminated with 0.25 EU of endotoxin per mL. There are studies which report that to activate phagocytes, as determined by production of reactive oxygen species, at least 1 EU/mL (100 pg) is required (51). There have been reports (37, 38) that polymyxin B binds to endotoxin and abrogates its effect. Polymyxin B (5 μg/mL), as can be seen from Table I, removed the activating effect of endotoxin. When polymyxin B was incubated with Bb and then used in the activation assay, there was no significant decrease in the activating effect of Bb. Polymyxin B, by itself did not affect the cells. This clearly demon-

strates that the activation of bovine monocytes and neutrophils was entirely due to the Bb fragment and not due to endotoxin.

Fragment Bb is a serine protease and contains the enzymatically active site of factor B (12). In an earlier study, the protease inhibitor diisopropylfluorophosphate (DFP) had been shown to completely abrogate the inducing effect of factor Bb (40). In our case, PMSF was used to determine if the enzymatic activity of bovine Bb could be abrogated or reduced. It was found that the activation induced by Bb as demonstrated by the uptake of ^{3}H-deoxyglucose was also totally removed on incubation of Bb with PMSF. By itself, PMSF had no deleterious effect on the phagocytic cells. This also supports the conclusion that the activation of bovine phagocytes is due to Bb alone.

The significant point to note here is that factor B, along with other complement proteins, is synthesized and secreted by macrophages (6, 53, 54). Thus, at the site of inflammation, factor B would not only play a fundamental role in the formation of the C3-convertase and amplify the ACP, but it is postulated that, by activating the monocytes, it might cause its own synthesis, thereby promoting a positive feedback. This would make sufficient factor B available at the site of inflammation, as long as there is a means to cleave factor B to Bb. This cleavage is possible directly by macrophage proteinases or indirectly by plasmin (55).

Our study is the first to report the activation of bovine neutrophils as seen by the uptake of deoxyglucose. It is postulated that such activation of neutrophils might lead to enhanced phagocytic ability of these cells. Studies in this regard are presently underway.

In summary, a simple procedure has been developed by using monoclonal antibody for affinity purification of bovine factor Bb from serum. The availability of affinity-purified factor Bb made it possible to study the interaction of this important complement protein with phagocytic cells. One of the functional responses manifested is the activation of the phagocytes as demonstrated by the uptake of the radio-labeled deoxyglucose. Other functional aspects are under study and may provide further insights into the phenomenon of inflammation and natural defence mechanisms of the mammalian body.

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REFERENCES

1. **NATHAN CF, MURRAY HW, COHN ZA.** The macrophage as an effector cell. N Engl J Med 1980; 303: 622-626.
2. **WEISSMAN G, SMOLEN JE, KORCHAK HM.** Release of inflammatory mediators from stimulated neutrophils. N Engl J Med 1980; 303: 27-34.
3. **JOHNSTON RB.** Defects of neutrophil function. N Engl J Med 1982; 307: 434-436.
4. **COHEN MS, BRITIGAN BE, HASSETT DJ, ROSEN GM.** Phagocytes, O_2 reduction and hydroxyl radicals. Rev Infect Dis 1988; 10: 1088-1096.
5. **THOMAS EL, LEHRER RI, REST RF.** Human neutrophil antimicrobial activity. Rev Infect Dis 1988; 10: 5450-5456.
6. **NATHAN CF.** Secretory products of macrophages. J Clin Invest 1988; 79: 319-326.
7. **MACKENESS GB.** The mechanisms of macrophage activators. In: Mudd S, ed. Infectious Agents and Host Reactions. Philadelphia: WB Saunders Company, 1970: 62-67.
8. **SANDBORG RR, SMOLEN JE.** Early biochemical events in leukocyte activation. Lab Invest 1988; 59: 300-320.
9. **CURMAN B, SANDBERG-TRAGARDH L, PETERSON EA.** Chemical characterisation of human factor B of the alternative pathway of activation. Biochemistry 1977; 16: 5368-5375.
10. **FISHELSON Z, PANGBURN MK, MÜLLER-EBERHARD HJ.** C3 convertase of the alternative complement pathway. Demonstration of an active, stable C3b.B (Ni) complex. J Biol Chem 1983; 258: 7411-7415.
11. **GÖTZE O, MÜLLER-EBERHARD HJ.** The C3-activator system: an alternative pathway of complement activation. J Exp Med 1971; 134: 90s-108s.
12. **MEDICUS RG, GÖTZE O, MÜLLER-EBERHARD HJ.** The serine protease nature of the C3 and C5 convertases of the classical and alternative pathways. Scand J Immunol 1976; 5: 1049-1055.
13. **MÜLLER-EBERHARD HJ, GÖTZE O.** C3 proactivator convertase and its mode of action. J Exp Med 1972; 135: 1003-1008.
14. **PANGBURN MK, MÜLLER-EBERHARD HJ.** The alternative pathway of complement. Spring Semin Immunopathol 1984; 7: 163-192.
15. **MOLE JE, ANDERSON JK, DAVISON EA, WOODS DE.** Complete primary structure for the zymogen of human complement factor B. J Biol Chem 1984; 259: 3407-3412.
16. **CAROLL MC, CAMPBELL RD, BENTLEY DR, PORTER RR.** A molecular map of the major histocompatibility complex class III region linking complement genes C4, C2 and factor B. Nature 1984; 307: 237-241.
17. **FISHELSON Z, MÜLLER-EBERHARD HJ.** Residual hemolytic and proteolytic activity expressed by Bb after decay-dissociation of C3b. J Immunol 1984; 132: 1425-1429.
18. **BIANCO C, EDEN A, COHN ZA.** The induction of macrophage spreading: Role of coagulation factors and the complement system. J Exp Med 1976; 144: 1531-1544.
19. **LEIJH PCJ, TH VAN DEN BARSELAAR, DAHA MR, VAN FURTH R.** Stimulation of the intracellular killing of *Staphyloccocus aureus* by monocytes: regulation by immunoglobulin G and complement components C3/C3b and B/Bb. J Immunol 1982; 129: 332-337.
20. **SUNDSMO JS.** Leukocyte complement: a possible role for C5 in lymphocyte stimulation. J Immunol 1983; 13: 886-891.
21. **PANG ASD, ASTON WP.** The alternative complement pathway in bovine serum: the isolation of a serum protein with factor B-like activity. Immunochemistry 1978; 15: 529-534.
22. **TABEL H.** Alternative pathway of bovine complement. Immunochemical studies on factor B-like serum protein and its conversion product Bγ2. Can J Comp Med 1981; 45: 291-298.
23. **TABEL H.** Activation of the alternative pathway of bovine complement by *Trypanosoma congolense*. Parasite Immunol 1982; 4: 329-335.
24. **BORREGAARD N, HERLIN T.** Energy and metabolism of human neutrophils during phagocytosis. J Clin Invest 1982; 70: 550-557.
25. **BAEHNER RL.** Respiration and glucose oxidation in human and guinea pig leukocytes: Comparative studies. J Clin Invest 1970; 49: 692-700.
26. **KLEBANOFF SJ.** Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin Hematol 1975; 12: 117-142.
27. **BABIOR BM.** Oxygen-dependent microbial killing by phagocytes (first of two parts). N Engl J Med 1978; 298: 659-668.
28. **KÖHLER G, HOWE SC, MILSTEIN C.** Fusion between immunoglobulin secreting and non-secreting myeloma cell lines. Eur J Immunol 1976; 6: 292-295.
29. **LAEMMLI UK.** Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature (London) 1970; 227: 680-685.
30. **GODDEERIS BM, BALDWIN CL, MOIYOI O, MORRISON WI.** Improved methods for the purification and depletion of monocytes from bovine peripheral blood mononuclear cells. J Immunol Methods 1986; 89: 165-173.

31. **YAM LT, LI CY, CROSBY WH.** Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 1971; 55: 283-291.
32. **BOVENTRE PF, MUKKADA AJ.** Augmentation of glucose transport in macrophages after particle ingestion. Infect Immun 1974; 10: 1391-1396.
33. **MUKKADA AJ, BOVENTRE PF.** Membrane transport by mouse and guinea pig macrophages: Characteristics of the glucose transport system. J Reticuloendothel Soc 1975; 27: 20-29.
34. **BOVENTRE PF, STRAUSS D, BAUGHN RE, IMHOFF J.** Enhancement of carrier-mediated transport after immunological activation of peritoneal macrophages. J Immunol 1977; 118: 1827-1835.
35. **McCORMACK JG, MOORE AL, JOHNSON JC, PHILIP JR.** Amplified lymphokine production phenomenon confirmed by a macrophage 2-D-3H-deoxyglucose transport assay. Infect Immun 1981; 32: 707-715.
36. **SEOW WK, SMITH SE, McCORMACK JG, THONG YH.** Uptake of 3H-deoxyglucose as a microassay of human neutrophil and monocyte activation. J Immunol Methods 1987; 98: 113-118.
37. **DUFF GW, ATKINS E.** The inhibitory effect of polymyxin B on endotoxin-induced endogenous pyrogen production. J Immunol Methods 1982 52: 333-340.
38. **ISSEKUTZ AC.** Removal of gram-negative endotoxin from solutions by affinity chromatography. J Immunol Methods 1983; 61: 275-281.
39. **FISHELSON Z, MÜLLER-EBERHARD HJ.** C3 convertase of the human complement: enhanced formation and stability of the enzyme generated with nickel instead of magnesium. J Immunol 1982; 129: 2603-2607.
40. **GÖTZE O, BIANCO C, COHN ZA.** The induction of macrophage spreading by factor B of the properdin system. J Exp Med 1979; 149: 372-386.
41. **HAMURA J, HADDING U, BITTER-SUERMANN D.** Fragments Ba and Bb derived from guinea-pig factor B of the properdin system: Purification, characterization and biologic activities. J Immunol 1978; 120: 438-444.
42. **GOLDSTEIN IM, ROOS D, KAPLAN HB, WEISSMANN G.** Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. J Clin Invest 1975; 56: 1155-1163.
43. **JOHNSTON RB Jr, LEHMEYER JE.** Elaboration of toxic oxygen by-products by neutrophils in a model of immune complex disease. J Clin Invest 1976; 57: 836-841.
44. **WEBSTER RO, HONG SR, JOHNSTON RB Jr, HENSON PM.** Biological effectors of the human complement fragments C5a and C5a desarg on neutrophil function. Immunopharmacology 1980; 2: 201-219.
45. **SIMCHOWITZ L, SILBER I.** Chemotactic factor-induced generaton of superoxide radicals by human neutrophils: Evidence for the role of sodium. J Immunol 1979; 123: 2428-2435.
46. **BECKER EL, SIGMAN M, OLIVER JM.** Superoxide production induced in rabbit polymorphonuclear leukocytes by synthetic chemotactic peptides and A23187. The nature of the receptor and the requirement for Ca^{2+}. Am J Pathol 1979; 95: 81-98.
47. **SUMIMOTO H, TAKESHIGE K, MINAKAMI S.** Superoxide production of human polymorphonuclear leukocytes stimulated by leukotriene B4. Biochim Biophys Acta 1984; 803: 271-277.
48. **BIANCO C, GÖTZE O, COHN ZA.** Regulation of macrophage migration by products of the complement system. Proc Natl Acad Sci USA 1979; 76: 888-891.
49. **GÖTZE O, BIANCO C, COHN ZA.** The induction of macrophage spreading by Factor B of the properdin system. J Immunol 1978; 120: 1776 (Abst).
50. **SUNDSMO JS, GÖTZE O.** Human monocyte spreading induced by factor B of the alternative pathway of complement activation. Cell Immunol 1980; 52: 1-17.
51. **SUNDSMO JS, GÖTZE O.** Human monocyte spreading induced by factor Bb of the alternative pathway of complement activation. J Exp Med 1981; 154: 763-777.
52. **GUTHRIE LA, McPHAIL LC, HENSON PM, JOHNSTON RB Jr.** Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. Evidence for increased activity of the superoxide-producing enzyme. J Exp Med 1984; 160: 1656-1671.
53. **SUNDSMO JS, CHIN JR, PAPIN RA, FAIR DS, WERB Z.** Factor B; the complement alternative pathway serine proteinase, is a major constitutive protein synthesized and secreted by resident and elicited mouse macrophages. J Exp Med 1985; 161: 306-322.
54. **MINTA JO.** Synthesizis of complement factor B by the human monocyte U-937 cell line. J Immunol 1988; 141: 1636-1341.
55. **SUNDSMO JS, WOOD LM.** Activated factor B (Bb) of the alternative pathway of complement cleaves and activates plasminogen. J Immunol 1981; 127: 877-880.